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Palladin, a novel microfilament protein

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ACADEMIC DISSERTATION

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Abstract

Palladin is a novel actin microfilament associated protein, which together with myotilin and myopalladin forms a novel cytoskeletal IgC2 domain protein family. Whereas the expression of myotilin and myopalladin is limited mainly to striated muscle, palladin is widely expressed in both epithelial and mesenchymal tissues, including heart and the nervous system. Palladin has a complex genetic structure and it is expressed as several different sized and structured splice variants, which also display differences in their expression pattern and interactions. In muscle cells, all the family members localize to the sarcomeric Z-disc, and in non-muscle cells palladin also localizes to the stress-fiber-dense regions, lamellipodia, podosomes and focal adhesions. A common feature of this protein family is the binding to α-actinin, but other interactions are mostly unique to each member. Palladin has been shown to interact with several proteins, including VASP, profilin, Eps8, LASP-1 and LPP. Its domain structure, lack of enzymatic activity and multiple interactions define it as a molecular scaffolding protein, which links together proteins with different functional modalities into large complexes. Palladin has an important role in cytoskeletal regulation, particularly in stress fiber formation and stabilization. This assumption is supported by several experimental results. First, over-expression of palladin in non-muscle cells results in rapid reorganization of the actin cytoskeleton and formation of thick actin bundles. Second, the knock-down of palladin with anti-sense and siRNA techniques or knock-out by genetic methods leads to defective stress fiber formation. Furthermore, palladin is usually up-regulated in situations requiring a highly organized cytoskeleton, such as differentiation of dendritic cells, trophoblasts and myofibroblasts, and activation of astrocytes during glial scar formation. The protein family members have also direct disease linkages; myotilin missense mutations are the cause of LGMD1A and myofibrillar myopathy. Palladin mutations and polymorphisms, on the other hand, have been linked to hereditary pancreatic cancer and myocardial infarction, respectively.

In this study we set out to characterize human palladin. We identified several palladin isoforms, studied their tissue distribution and sub-cellular localization. Four novel interaction partners were identified; ezrin, ArgBP2, SPIN90 and Src-kinase.The previously identified interaction between palladin and α-actinin was also characterized in detail. All the identified new binding partners are actin cytoskeleton associated proteins; ezrin links the plasma membrane to the cytoskeleton, ArgBP2 and SPIN90 localize, among other structures, to the lamellipodia and in cardiomyocytes to the Z-disc. Src is a transforming tyrosine kinase, which besides its role in oncogenesis has also important cytoskeletal associations. We also studied palladin in myofibroblasts, which are specialized cells involved in diverse physiological and pathological processes, such as wound healing and tissue fibrosis. We demonstrated that palladin is up-regulated during the differentiation of myofibroblasts in an isoform specific manner, and that this up-regulation is induced by TGF-β via activation of both the SMAD and MAPK signalling cascades.

In summary, the results presented here describe the initial characterization of human palladin and offer a basis for further studies.

Contents

List of original publications

This thesis is based on the following publications:

I
O.M. Mykkänen, M. Grönholm, M. Rönty, M. Lalowski, P. Salmikangas, H. Suila and O. Carpen. (2001). Characterization of human palladin, a microfilament-associated protein, *Mol. Biol. Cell* **12**, 3060–3073.

II
M. Rönty, A*, Taivainen*, M. Moza, C.A. Otey and O. Carpen. (2004). Molecular analysis of the interaction between palladin and alpha-actinin, *FEBS Lett.* **566**, 30–34. (*equal contribution)

III
M. Rönty, A. Taivainen, M. Moza, G.D. Kruh, E. Ehler and O. Carpen. (2005). Involvement of palladin and alpha-actinin in targeting of the Abl/Arg kinase adaptor ArgBP2 to the actin cytoskeleton, *Exp. Cell Res.* **310**, 88–98.

IV
M. Rönty, A.Taivainen, L. Heiska, C.A. Otey, E. Ehler, W.K. Song and O.Carpen. (2007).Palladin interacts with SH3 domains of SPIN90 and Src and is required for Src-induced cytoskeletal remodeling.*Exp Cell Res*. **313**, 2575-85.

V
M. Rönty, S. Leivonen, B. Hinz, A. Rachlin, C.A. Otey, V.M. Kähäri and O. Carpen. (2006). Isoform-specific regulation of the actin-organizing protein palladin during TGF-beta1-induced myofibroblast differentiation, *J. Invest. Dermatol.* **126**, 2387–2396.

The publications are referred to in the text by their roman numerals.

Abbreviations

aa amino acid
ab antibody
ABD actin-binding domain
Abl Abelson tyrosine kinase
ABP actin-binding protein
ADP adenoside diphosphate
ALP actinin-associated LIM protein
AMI acute myocardial infarction
Ankrd2 ankyrin repeat domain 2 protein
Arg Abl-related gene
ArgBP2 Arg-binding protein 2
Arp2/3 actin-related protein 2/3
ATP adenosine triphosphate
ATRA all-trans retinoic acid
Bp base pair
CaM calmodulin-like domain
CARP cardiac ankyrin repeat protein
cDNA complementary deoxyribonucleic acid
Clik1 CLP-36 interacting kinase 1
CLP-36 C-terminal LIM protein
C-terminal carboxy-terminal
DARP diabetes-related ankyrin repeat protein
DC dendritic cell
DIP diaphanous-interacting protein
ECM extra-cellular matrix
EGFR epidermal growth factor receptor
EMT epithelial-mesenchymal-transition
Eps8 epidermal growth factor receptor pathway substrate 8
ERK1 extracellular signal-regulated kinase 1
ERM ezrin/radixin/moesin
EST expressed sequence tag
F-actin filamentous actin
FAK focal adhesion kinase
FATZ filamin-, actinin- and telethonin-binding protein of the Z-disc
FL full-length
G-actin globular actin
GFP green fluorescent protein
GST glutathione S-transferase
GTP guanosine triphosphate
HA hemagglutinin antigen
ICAM intercellular adhesion molecule
IgC2 immunoglobulin C2 (domain)

IRSp53 insulin receptor tyrosine kinase substrate p53
IVT *in vitro* translation
kb kilobase
kDa kilodalton
LASP-1 LIM and SH3 protein
LGMD limb girdle muscular dystrophy
LPA lysophosphatidic acid
LPP lipoma preferred partner
LIM lin-11, isl-1, mec-3
LIMK LIM-kinase
MAPK mitogen-activated protein kinase
mDia mammalian homologue of *Drosophila diaphanous*
MEF murine embryonic fibroblast
MEKK1 MAPK/ERK kinase kinase 1
MFM myofibrillar myopathy
MLC myosin light chain
MLCK myosin light chain kinase
MMP matrix metallo-protease
MOM mitochondrial outer membrane
mRNA messenger ribonucleic acid
MuRF muscle-specific ring finger protein
NF2 neurofibromatosis 2
NRC neonatal rat cardiomyocyte
PAK p21-activated kinase
PCR polymerase chain reaction
PDGF platelet-derived growth factor
PSD-95 postsynaptic density 95
Rho Ras homology gene family
Rig-K retinoic-acid-induced gene-K
RNA ribonucleic acid
ROCK rho-kinase
RT-PCR reverse transcriptase PCR
SH3 Src homology region 3
siRNA short interfering RNA
SMA smooth muscle actin
SNP single nucleotide polymorphism
SoHo sorbin homology (domain)
SPIN90 SH3 protein interacting with nck, 90 kDa
TGF-ß transforming growth factor ß
VASP vasodilator-stimulated phosphoprotein
WASP Wiskott-Aldrich syndrome protein
WAVE WASP family Verprolin-homologous protein
ZASP Z-band alternatively spliced PDZ-motif protein
ZO-1 zona occludens 1

Review of the literature

1. The cytoskeleton

The cytoskeleton of eukaryotic cells is a complex meshwork of three major components – microtubules, intermediate filaments and actin microfilaments. These structures are assembled from soluble precursors under precise spatial and temporal control during multiple cellular processes. The filament systems are cross- and interlinked by binding proteins, which also link other proteins with different functional modalities to the cytoskeleton. The cytoskeleton is required for achieving/maintaining cellular structure and polarity, and is also indispensable for cell movement and cell division.
Of the three components of the cytoskeleton, actin microfilaments are the smallest, ca. 8 nm in diameter. Microtubules are the largest, having a diameter of about 25 nm, and as the name indicates, intermediate filaments have a diameter between that of microfilaments and microtubules, approx. 10 nm (reviewed in Insall and Machesky, 2001).

1.1. Microtubules and intermediate filaments

Microtubules are built from dimers of two 50-kDa proteins, α- and β-tubulin. The dimers polymerize end to end, giving the filament polarity. The actual tubules are composed of 13 protofilaments encircling a hollow core, resulting in a rigid structure, contrary to the other filament systems. Microtubules mostly occupy the perinuclear region of the cell. They originate from a central structure called the centrosome and radiate outward, only occasionally extending to the edge of the cell. When the cells divide, the mitotic spindle is mainly composed of microtubules, the role of which in the process is high-lighted by the cytotoxic drugs, the taxanes, which prevent cell division by stabilizing the microtubules and preventing their normal function. Microtubules form also the core structure of the cilia and participate in the movement of these structures (reviewed in Amos, 2005) (Joshi, 1998).

In intracellular trafficking, vesicles and organelles move along microtubules, and therefore use specific motor proteins such as kinesin. For example in neuronal axons, which can be as long as 1m, microtubule motors shuttle material between the cell body and the axonal terminus. This movement is directed by the polarity of the tubule and the specific orientation of the motor protein; for instance most kinesins move only from the central minus end of the tubule to the peripheral plus end (reviewed in Baas and Karabay, 2005).

Intermediate filaments are also composed of monomers originating from a large family of related genes (reviewed in Foisner, 2001). The monomers are not uniform but have a rather large range of sizes from ca 40 kDa to more than 100 kDa (Strelkov et al., 2003). The largest group of these genes consists of keratins, which are mostly expressed in epithelial cells in a tissue-specific manner. The family also includes neuron-specific neurofilaments, muscle-restricted desmin, as well as vimentin, which is widely expressed

in mesenchymal cells and tissues. In the filament assembly the monomers organize to a rope-like structure, with several monomers intertwined. The resulting structure has considerable tensile strength but lacks obvious polarity (Kreplak and Fudge, 2007). A specific group of intermediate filaments, called lamins, make up the nuclear lamina during cell division. The filaments usually extend throughout the cell, forming a complex meshwork. The individual filaments are rather stable structures which do not go through constant remodelling like the actin microfilaments and microtubules. This notion has led to the assumption that their main function is to retain cell structure, particularly under physical stress.

1.2. Actin cytoskeleton

1.2.1 Organization of actin cytoskeleton

The core structures of actin cytoskeleton, microfilaments, are composed of only a single subunit, i.e. actin. Actin monomeres are referred to as G-actin (for globular) and the filamentous form F-actin. Humans have six actin isoforms coded by separate genes, which are divided into three groups: alpha (α), beta (β) and gamma (γ). α-actins are expressed mainly in muscle cells (α-skeletal, α-smooth muscle, α-cardiac), whereas β- and γ-isoforms are found in non-muscle cells (β- and γ1-cytoplasmic). Each actin monomer has a molecular weight of 42 kDa and contains a bound adenine nucleotide, either adenosine triphosphate (ATP) or adenosine diphosphate (ADP). During polymerization, actin sub-units assemble head-to-tail, and in the process ATP is usually hydrolyzed to ADP. F-actin forms a simple helix and has a defined polarity which is important for its cellular function (reviewed in Insall and Machesky, 2001; Ampe and Vandekerckhove, 2005). The individual actin filaments are rather flexible and do not possess much tensile strength. In living cells they are therefore linked together into multiple higher order structures by actin-binding proteins (ABPs, see section 2) (Ayscough, 1998). Several types of actin structures with different functional properties have been described.

The lamellipodium is defined as the thin (~0.2 μm) membrane protrusion at the front of spreading and migrating cells (a.k.a. leading edge) (Small et al., 2002) (Figure 1). The membrane is remodelled and relocated by the force generated by the actin polymerization underneath it. Lamellipodia contain a highly dynamic mesh of actin filaments joined to each other at a precise angle. One of the most important ABPs involved in the organization of the actin network is the Arp2/3 complex (Ayscough, 1998; Small, 1994; Small et al., 2002). The complex binds to the sides of existing filaments and initiates growth (nucleates) of new filaments at a distinctive 70 degree angle, thus creating a branched actin network. Other actin-associated proteins localizing to lamellipodia are for instance cortactin, filamin, SPIN90, α-actinin, and palladin (Goicoechea et al., 2006; Kim et al., 2006; Small et al., 2002). The lamellipodium is a very dynamic structure in which constant tread-milling (polymerization/de-polymerization cycles) of actin filaments takes

place during cell movement. Both endo- and exocytosis also takes place in the lamellipodia.

Another actin-based cellular structure is the *stress fiber* (reviewed in Vallenius;2004 academic dissertation). Stress fibers are thick bundles traversing the cell (Figure 1).In these structures individual actin filaments are bundled together through cross-linking proteins. They have the ability to contract, and via adhesions transmit the generated energy to the ECM; they can thus be considered to be the non-muscle equivalent of the sarcomere (Small and Gimona, 1998). The structure of the fibers is not uniform and they contain regularly spaced thickenings called dense bodies. These structures resemble the Z-discs of the sarcomere (Small and Gimona, 1998). Dense bodies are composed of several ABPs (e.g. α-actinin), scaffolding proteins and probably also transiently include proteins involved in signalling, such as kinases.

According to an elegant study by Hotulainen and Lappalainen (2006), there are two distinct ways in which stress fibers are formed. The authors divided stress fibers into two categories, i.e., dorsal stress fibers and transverse arcs. They showed that dorsal fibers are connected to the ECM at one end by focal adhesions and are formed by formin (mDia1/DRF1) -mediated actin polymerization at the contact sites. The transverse arcs which are not directly attached to the ECM are, on the other hand, generated by endwise annealing of myosin bundles and Arp2/3-nucleated actin bundles at the lamella. Both of these fiber types can be converted to ventral stress fibers, which are connected to focal adhesions at both ends.

The formation of stress fibers also requires, as the name implies, physical stress applied to the cells. Under normal conditions *in vivo* most cells are protected against such forces by the three-dimensional ECM, and only a few cell types, endothelial cells for instance, are under constant direct physical stress. When the support of the ECM is compromised, e.g. by a wound in the dermis, the cells react rapidly to this new situation by forming stress fibers. This is also true for cells that have to adapt to a two-dimensional growth environment on tissue culture dishes (reviewed in Vallenius; 2004 academic dissertation). Once formed, the stress fibers are able to contract as shown directly in *in vitro* experiments and also indirectly in *in vivo* studies on wound healing. *In vitro* fibroblasts grown in collagen lattices attached to the bottom of culture dishes develop stress fibers and contract rapidly once the tension is relieved upon detachment. On the other hand, fibroblasts grown on floating lattices do not form stress fibers and therefore fail to contract (Tomasek et al., 2002). *In vivo* specialized fibroblastic cells called myofibroblasts are responsible for generating the force needed to contract granulation tissue during wound healing (Hinz et al., 2001; Hinz and Gabbiani, 2003b, see Chapter 4). Myofibroblasts have highly developed focal adhesions and stress fibers which, in addition to other actin isoforms, contain also α-SMA (Hinz et al., 2001; Hinz and Gabbiani, 2003a; Hinz et al., 2003). SMA has been shown to enhance the contraction of these cells by a yet unknown mechanism (Hinz et al., 2001).

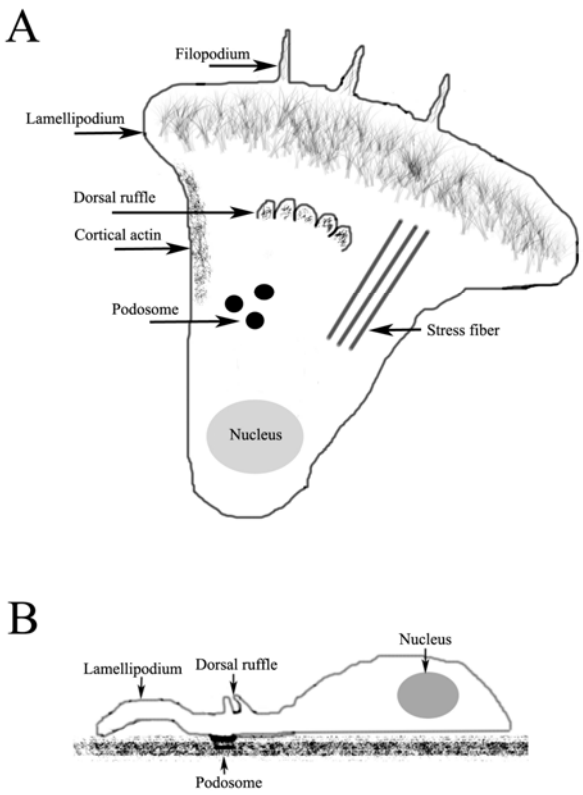

Figure 1. **A)** A simplified illustration of different actin-based cellular structures.**B)** In a lateral view, the orientation of some of these strucutres is shown. As the name implies the dorsal ruffles are located on the dorsal surface of the cell and project up-ward. On the contrary, the podosomes are found on the ventral surface where they attach the cell to the extra-cellular matrix.

1.2.2. Actin cytoskeleton of striated muscle

The actin cytoskeleton of striated muscle cells, myocytes and cardiomyocytes, is organized in repeated ordered arrays of thin and thick filaments which form the contractile units, sarcomeres. Different zones of the sarcomere are named as either bands or lines: the A-band is composed of myosin and myosin-binding proteins, whereas the major component of the I-band is actin. The M-line is located in the middle of the A-band, and the Z-disc forms the centre of the I-band. One sarcomere unit is defined as the area between two Z-discs (Clark et al., 2002) (Figure 2).

The adjacent sarcomeres are connected by the Z-discs, composed of several proteins with multiple functional modalities (reviewed in Salminkangas, P; 2001 academic dissertation, Klaavuniemi, T; 2006 academic dissertation). In the cell periphery, Z-discs are connected to the plasma membrane via specialized multiprotein complexes called costameres, which resemble the focal adhesions of non-muscle cells (Ervasti, 2003). Due

to these multiple connections the Z-disc is a primary conduit of the force generated by sarcomere contraction. One of the major components of the Z-discs is α-actinin, which crosslinks the thin actin filaments. The giant proteins, titin and nebulin, are also partially located in the Z-discs (Clark et al., 2002). In addition to its structural function the Z-disc is nowadays also considered to be a platform for multiple signaling cascades, and it can act as a direct stretch sensor (Epstein and Davis, 2003) (Frank et al., 2006). Some of the Z-disc components can shuttle between the sarcoplasm and the nucleus, transmitting signals and probably regulating gene transcription (Clark et al., 2002; Frank et al., 2006).

Although skeletal and cardiac muscles are closely related, there are also some differences between them. Cardiac muscle is composed of branched muscle fibers which are connected to one another through *intercalated discs*. This structure is unique to cardiomyocytes and is not found in skeletal myocytes. There are three types of membrane junctions within an intercalated disc: fascia adherens, macula adherens, and gap junctions. Actin filaments, and hence the sarcomere, are anchored to the membrane via fascia adherens. Macula adherens (a.k.a desmosome) provides structural stability during contraction by linking together the intermediate filaments of adjoining cells. Gap junctions pass ions between the cells and thus allow the spread of action potentials causing depolarization of the heart muscle (Perriard et al, 2003).

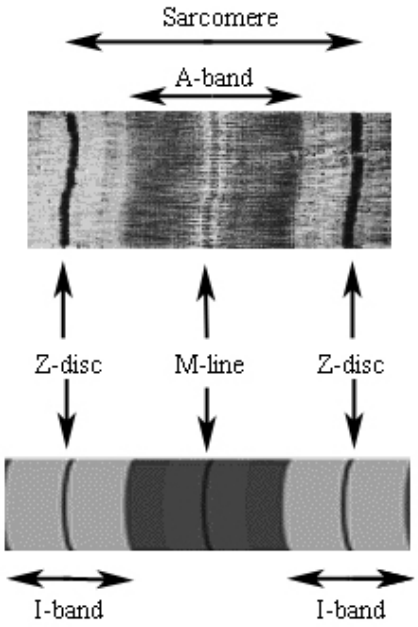

Figure 2. Above is an electron micrograph of a sarcomere and below is a schematic model of the structure.

2. Actin-associated proteins

The structure and functions of the actin cytoskeleton are regulated by a large number of actin-binding proteins (ABP). These proteins do not form a single uniform protein family, since both proteins with and without enzymatic activity can bind directly to actin. In a recent review Dos Remedios et al. (2003) suggested that ABPs could be divided into seven groups. 1) Proteins binding to monomeric G-actin and preventing polymerization (thymosin β4). 2) Depolymerizing proteins which convert F- to G-actin (cofilin). 3) Filament end-binding proteins; these proteins bind either to the pointed end (tropomodulin) or the barbed end (CapZ) capping the actin filament and preventing the exchange of monomers. 4) Filament severing proteins which cut pre-existing actin filaments into shorter fragments (gelsolin). 5) Actin cross-linking proteins, which contain at least two F-actin binding sites; these proteins can bundle actin filaments or form branched filament networks (the Arp2/3 complex). 6) Stabilizing proteins which bind to filaments and prevent depolymerization (tropomyosin). 7) Motor proteins which move along actin filaments (myosin). Actin nucleating proteins such as the Arp2/3 complex form in fact an additional 8th group not included in the review (Ampe and Vandekerckhove, 2005), Dos Remedios et al counted that at least 162 ABPs had been identified in the literature by the year 2003 (Dos Remedios et al, 2003). The sheer amount and diversity of the functions of ABPs allows the formation of complex, yet highly dynamic and effective structures needed to fulfill the various cellular requirements. For instance, these structures include the lamellipodium and stress fibers (reviewed in section 1.2.1).

2.1. The myotilin/myopalladin/palladin protein family

Myotilin, myopalladin and palladin are recently characterized actin-cytoskeleton-associated proteins, which due to their structural homology are considered to form a novel protein family (Otey et al., 2005). They all seem to have an important role in the regulation of the actin cytoskeleton.

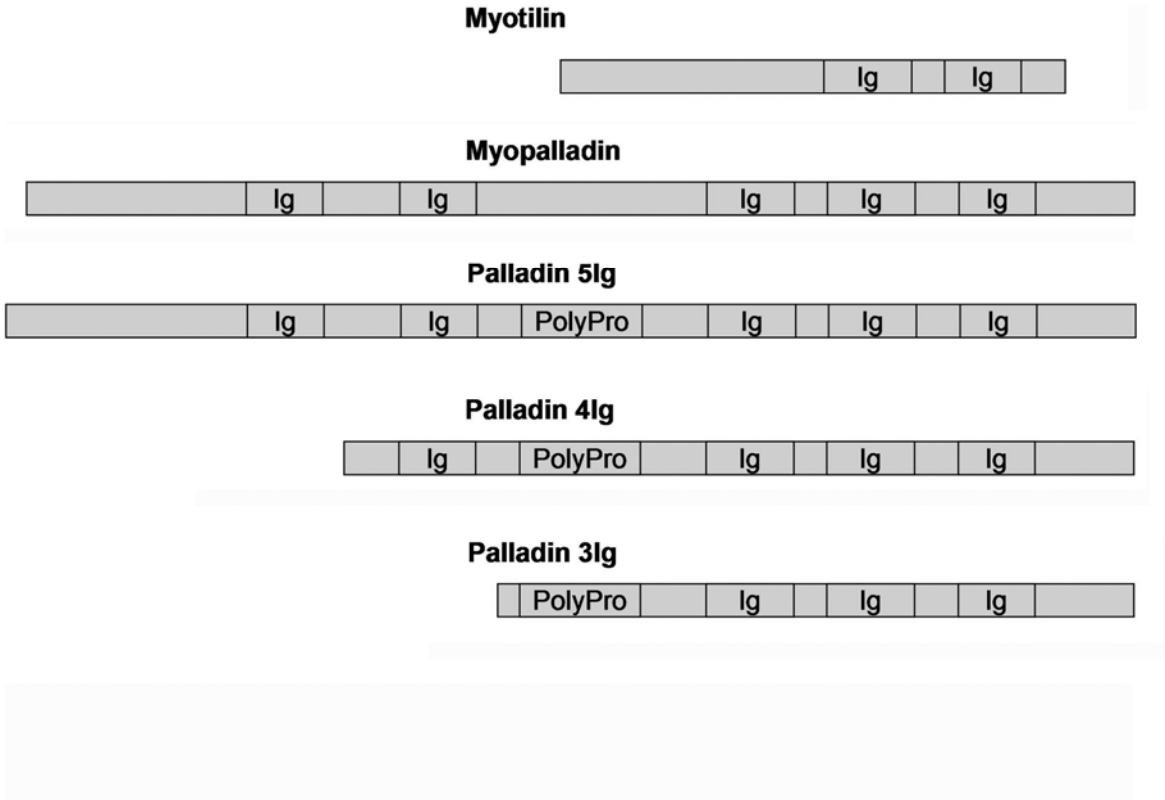

Figure 3. A schematic domain structure illustration of the myotilin/myopalladin/palladin protein family members. Only the three major palladin isoforms are included (see section 2.1.3.2. on palladin genomic structure). Ig = IgC2 domain, PolyPro = poly proline sequence

2.1.1. Myotilin

The first characterized member of the protein family was myotilin. It was identified in a yeast two-hybrid screen in which α-actinin was used as bait (Salmikangas et al., 1999). The myotilin cDNA encodes a 498 amino-acid protein composed of a serine-rich amino-terminus followed by two carboxy-terminal IgC2 domains. The IgC2 domains are most homologous to palladin and myopalladin (Otey et al., 2005). In adult humans and mice myotilin is expressed mainly in skeletal muscle; lower expression levels are detected in heart and peripheral nerves (Mologni et al., 2001; Salmikangas et al., 1999). Myotilin plays a role in the late stages of myofibrillogenesis, and when it is overexpressed prematurely in myoblasts it prevents normal sarcomere assembly. In the differentiated muscle cells myotilin localizes to the Z-disc where it probably provides structural stability to the sarcomere by several interactions with other Z-disc proteins. So far myotilin has been shown to interact with at least 7 proteins: α-actinin, filamin C, actin, FATZ, ZASP, telethonin and MuRF (Salmikangas, P. 2001, academic dissertation) (Gontier et al.,

2005;Witt et al., 2005) (Von Nandelstadh, P. 2006 ASCB annual meeting B191; Suila, H. 2006 ASCB annual meeting B190).

The interaction between α-actinin and myotilin is mediated by the C-terminal EF hand domains 3 and 4 of α-actinin and a short amino-terminal segment of myotilin located between amino-acids 79-125. The interaction with filamin C links the sarcomere laterally to the sarcolemma at the costamere, since filamins can also interact with adhesion protein integrins. Filamins also cross-link actin filaments, and thus the interaction with myotilin can enhance the stability of the sarcomere structure (van der Ven et al., 2000).

Myotilin binds directly to F-actin and bundles actin filaments (Salmikangas et al., 2003). The binding is mediated by the second Ig domain together with a short C-terminal sequence (von Nandelstadh et al., 2005). Furthermore, both the Ig domains and short flanking regions within the amino- and carboxy-terminus are needed for actin-bundling activity, indicating that the mere actin-binding sequence is insufficient for the actin-regulating activity. The bundling may also be enhanced by homo-dimerization of myotilin (Salmikangas et al., 2003). The interaction with FATZ links myotilin also indirectly to costameres, since FATZ can also interact with filamins (Gontier et al., 2005). MuRF, on the other hand, is involved in transcriptional regulation initiated by mechanical load. The actual role of the interaction with myotilin, however, is still mostly unclear, but it may involve ubiqutination of myotilin, as MuRF is also a known ubiquitin ligase (Witt et al., 2005). The interactions with ZASP and telethonin are also interesting but still need to be further characterized.

Probably the most interesting charateristic of myotilin was revealed at the same time as its genetic locus was identified. The myotilin gene is located in chromosome 5q31 which also contained an unidentified autosomal dominant limb-girdle muscular dystrophy gene (Salmikangas et al., 1999). Subsequently, mutations in the myotilin gene were identified as the cause of limb-girdle muscular dystrophy 1A (LGMD1A) (Hauser et al., 2000) and later of another myopathy called myofibrillar myopathy (MFM, a.k.a. desmin storage disease) (Selcen and Engel, 2004). These diseases have a rather mild phenotype; the affected persons manifest with proximal muscle weakness of the extremities, usually later in adulthood. MFM patients may also have related cardiomyopathy and peripheral neuropathy, fitting well with the above-mentioned adult expression pattern of the myotilin gene. The microscopic findings in both of the disorders include Z-disc streaming and intracellular aggregates of fibrillar material originating from thin (actin) filaments. The causative mutations lead to single amino-acid substitutions, all of which have so far been located in the amino-terminal serine-rich part of myotilin. The exact mechanism of how these mutations lead to a disease phenotype remains unknown, but they nevertheless seem to have a dominant negative effect on the protein function. This assumption is supported by the fact that while myotilin knock-out mice lack an obvious phenotype and seem to have normal muscle function, a transgenic mouse strain expressing a mutated form of myotilin (T57I) recapitulates the disease phenotype (Garvey et al., 2006; Moza et al., 2007). These mice develop progressive myofibrillar pathology that includes Z-disc streaming, myofibrillar vacuolization and myofibrillar aggregation, and progresses with age. The mutations do not seem to have any effect on the interaction of myotilin with either actin or other binding partners. One possibilty is that the mutations lead to defective

signaling/function of the protein via impaired phosphorylation, since the serine residues replaced by mutations are potential phosphorylation sites.

2.1.2. Myopalladin

Like myotilin, myopalladin was first identified in a yeast two-hybrid screen, in which nebulin was used as bait. Myopalladin is a 145-kD protein which has five IgC2 domains highly homologous to those of palladin and myotilin (Bang et al., 2001). However, it lacks the serine-rich part unique to myotilin and the amino-terminal proline-rich region of palladin. Like myotilin, myopalladin is mainly expressed in skeletal and cardiac muscle where it also localizes to the Z-disc. It interacts with the SH3 domains of nebulin and nebulette, proteins which are thought to play a role in sarcomere assembly (Bang et al., 2001). Although myopalladin doesn't have a conventional SH3 ligand PXXP sequence, it was shown to bind with micromolar affinity to nebulin SH3 (Ma and Wang, 2002). A similar segment in palladin 4Ig isoform was later shown to bind to the LASP-1 SH3 domain which is highly similar to nebulin (Rachlin and Otey, 2006). Like other members of the palladin/myotilin/myopalladin family, myopalladin also binds to α-actinin EF hand domains, suggesting that they all may have a somewhat similar role in Z-disc assembly (Bang et al., 2001).

The amino-terminal part of myopalladin binds to the cardiac ankyrin repeat protein (CARP), a nuclear protein involved in the control of muscle gene expression. The CARP protein family also includes Ankrd2 (a.k.a. Arpp) and diabetes-related ankyrin repeat protein DARP. These proteins are possibly involved in relaying signals from the cytoskeleton/sarcomere to the nucleus (Kojic et al., 2004; Miller et al., 2003).

2.1.3. Palladin

2.1.3.1. Characterization

Palladin is the third member of the myotilin/myopalladin/palladin family and the main subject of this dissertation. Palladin was characterized independently by three groups, one group cloned the mouse palladin gene and the other two focused on human palladin. Mouse palladin was identified in Carol Otey's laboratory by the use of an uncharacterized monoclonal antibody called C10 (Parast and Otey, 2000). This antibody stained focal adhesions and stress-fiber-dense regions, resembling the staining pattern observed for α-actinin. Futher studies demonstrated that the antibody did not recognize α-actinin, but rather a new 90-92 kDa cytoskeletal protein which was named palladin after an Italian Renaissance architect, Andrea Palladio. The cDNA sequence was determined by an

antibody screen of a phage expression library and data-mining of the mouse sequence databases.

Human palladin was identified in 2000 by Liu et al., who described a series of novel genes up-regulated by all-trans retinoic acid (ATRA) in the NB4 cell line. One of them, Rig-K (Retinoic-acid-induced gene-K), was the human homolog of palladin (Liu et al., 2000). Almost simultaneously, Mykkänen et al. identified human palladin in a yeast-two-hybrid screen using a part of the ERM family protein ezrin as bait.

2.1.3.2. Genomic structure

Beyond this dissertation, most of the published studies deal with mouse palladin, with only a few exceptions so far. The genomic structure of palladin is very complex, and is best characterized in mouse – therefore used here as the reference (Otey et al., 2005; Rachlin and Otey, 2006). The murine palladin gene is located in chromosome 8 (human palladin is located in 4q32.2), spanning 400kb and consisting of at least 24 exons. Human palladin has a very similar exon structure. The size of the gene and its structure with at least three different promoter regions allows for a very complex transcription process resulting in at least 9-10 different products. At the protein level the most common products/isoforms have molecular weights of 200, 140 and 90-92kDa. They can also be logically named according to their molecular structure, that is, the number of the IgC2 type domains. The largest 200kDa isoform has two amino-terminal and three carboxy-terminal IgC2 domains, and is thus called the 5Ig isoform. The 140kDa variant has four domains, one amino-terminal domain and three carboxy-terminal domains, and is named 4Ig isoform. The smallest, and most common, 90-92kDa isoform has only three carboxy-terminal domains and is therefore called the 3Ig isoform. Located between the second and third Ig domains of the 5Ig isoform is a proline-rich sequence that is also contained in the 4Ig isoform and partly in the amino-terminus of the 3Ig isoform. This sequence contains ligands for VASP family member proteins, profilin and SH3 domains. At least in humans, additional isoforms exist, which lack the entire proline-rich sequence, but their exact identities and sequences are still not known (Rönty, M., unpublished observation).

2.1.3.3. Interactions

Besides structural differences, the palladin isoforms have different expression patterns and, most likely, partly different interaction partners. The most common 3Ig isoform is ubiquitously expressed, at least in developing rodent tissues (Parast and Otey, 2000). The 140kDa 4Ig isoform seems to be more restricted in its expression, and highest levels of this isoform are detected in smooth muscle (Rachlin and Otey, 2006). The largest 5Ig isoform seems to be mainly expressed in heart where it may, due to its high sequence homology, have functions similar to those of myopalladin (Rachlin and Otey, 2006).

Several binding partners for mouse palladin have been described so far (summarized in Table 1, Results and discussion), the first one being α-actinin (see above, and a separate

section below) (Parast and Otey, 2000; Ronty et al., 2004). The second identified partner was *Va*sodilator-*S*timulated *P*hosphoprotein (VASP). The VASP family proteins contain a specific motif called the EVH1 domain which binds directly to a consensus sequence, (D/E)-FPPPP-X(D/E)(D/E), and plays an essential role in targeting these proteins to focal adhesions (Krause et al., 2003). Palladin 4Ig isoform contains two and the 3Ig isoform one conserved FPPPP sequence(s), and thus palladin was a good candidate for a VASP interactor. This was verified by several methods including immunoprecipitation, blot overlay and co-localization studies. The proteins were shown to co-localize partially in the focal adhesions and stress-fiber-dense regions (Boukhelifa et al., 2004). VASP proteins are involved in the regulation of actin assembly and bind directly to both G and F actin (Krause et al., 2003). At the lamellipodia, VASP regulates cell motility but its role in stress fibers is mostly unknown. It is possible that VASP promotes actin polymerization in dense regions, and palladin targets it to these specific sites, but the precise function of the interaction remains to be seen.

The proline-rich sequences found in palladin can also bind profilin (Boukhelifa et al., 2006), which is a small actin-binding protein that forms a 1 : 1 complex with monomeric actin. Profilin promotes the formation of ATP-actin and interacts with the fast-polymerizing (+)-ends of actin filaments, targeting actin sub-units for growing filaments (Korenbaum et al., 1998). Profilin binds also to polyphosphoinositide lipids, the ARP2/3 complex, members of the VASP, WASP (Wiskott-Aldrich Syndrome Protein) and formin families of proteins, all involved in actin dynamics (Witke, 2004). Since palladin also plays an important role in the regulation of the actin cytoskeleton, its interaction with profilin is likely to have a similar function as with other profilin-binding proteins.Palladin also interacts with integrin αvβ5 at the focal adhesions and probably links it to the cytoskeleton, but this interaction has not been well characterized (Lai et al., 2006).

One of the most interesting interactions of palladin is with Lasp-1 (Rachlin and Otey, 2006). Lasp-1 is an actin-associated protein that contains several conserved domains functioning as protein-interaction sites: an amino-terminal LIM domain, followed by an actin-binding nebulin repeat domain and a carboxy-terminal SH3 domain (Schreiber et al., 1998). Lasp-1 binds directly to actin and localizes (together with palladin) in actin-rich structures, including focal adhesions and lamellipodia (Chew et al., 2002). The SH3 domain of Lasp-1 is 80% identical and 86% conserved to that of nebulin, a previously characterized binding partner for myopalladin (Bang et al., 2001; Li et al., 2004). Moreover, palladin 4Ig isoform contains a myopalladin homologous proline triplet which in myopalladin mediates its interaction to nebulin. These facts led Rachlin et al. to test whether LASP-1 would bind to palladin 4Ig, and this turned out to be the case. The interaction could be shown with several methods including yeast-two-hybrid, GST-pulldown and co-localization (Rachlin and Otey, 2006). Since the part mediating the binding to LASP-1 is not included in the 3Ig isoform sequence, and the expression of the 5Ig isoform is mostly limited to heart, the interaction seems to be a rather unique feature of the 4Ig isoform. It also seems to have a distinct overexpression phenotype, compared to the 3Ig isoform: compact star-like actin arrays vs. robust actin-cables, respectively. Together, these findings suggest that the different palladin isoforms may also have different functional properties (Rachlin and Otey, 2006). The function of this interaction,

again, remains unknown, but LASP-1 has been implicated to play a role in cytoskeletal reorganization in several cell types and localizations. It is overexpressed in some breast carcinomas, and possibly plays a role in metastasis through its regulation of cell migration (Grunewald et al., 2006). It also localizes to motile growth cones of cultured neurons and is involved in chemotaxis (Phillips et al., 2004).

Eps8 was recently identified as a palladin-interacting protein (Goicoechea et al., 2006). Eps8 is a cytoskeletal protein that links growth factor stimulation to actin dynamics and participates in the transduction of signals from Ras to Rac (Offenhauser et al., 2004; Scita et al., 1999). It binds to several proteins, such as F-actin, EGFR and IRSp53 (Disanza et al., 2006; Funato et al., 2004). Eps8 acts as an actin-filament barbed-end capping protein and it localizes to membrane ruffles and podosomes (Disanza et al., 2004). Palladin was shown to localize both to the PDGF-induced membrane ruffles and phorbol ester-induced podosomes in A7r5 cells, together with Eps8. The knock-down of palladin with a siRNA resulted in a decrease in Rac activity (possibly via the Eps8 interaction) and also prevented the formation of podosomes (Goicoechea et al., 2006). These actin-based membrane protrusions have been detetcted in several cell lines, both benign and malignant, and are thought to play a role in adhesion and particularly in invasion (Linder and Aepfelbacher, 2003). Recently, Eps8 expression was shown to be increased in pancreatic cancer (Welsch et al., 2007). This is particularly interesting since a palladin mutation has been suggested to be one of the causes of familial pancreatic cancer (Pogue-Geile et al., 2006).

Recently Jin et al (2007) identified palladin as a Lipoma-preferred partner (LPP) interacting protein. LPP is a LIM domain family protein that localizes in focal adhesions. The palladin-interacting region of LPP was mapped, and both LPP and palladin were shown to enhance cell migration and spreading. In FAK-/- cells the expression of LPP and palladin was down-regulated, but could be restored by the expression of paired-related homeobox gene-1 protein. Also Angiotensin II enhanced expression of both proteins (Jin et al., 2007).

2.1.3.4. Disease associations

Like myotilin, palladin mutations have been shown to have disease linkages. Probably due to their wider expression in tissues, palladin mutations have been shown to play a part in such diverse disease states as acute myocardial infarction and familial pancreatic cancer. However, unlike myotilin, the described palladin mutations are not concentrated at any particular site in the gene. In fact, the SNP suggested to be associated with AMI is not in the coding region but is located in the intronic sequence. In one study this SNP lead to an increased risk of myocardial infarction (odds ratio OR 1.40, for carriers of two vs. zero risk alleles) via an unknown mechanism (Shiffman et al., 2005). In a recent study by Home et al (2007) this association, however, could not be validated.

The other association is even more intriguing. Already in 1995, a large family in which pancreatic cancer was inherited in an autosomal dominant fashion was described (Evans et al., 1995). Diabetes and exocrine insufficiency were shown to precede pancreatic cancer in the affected family members. Subsequently, the genetic locus was mapped to chromosome

4q32-34 (Eberle et al., 2002) and finally a mutation in the palladin gene was tracked in all the affected family members, while it was absent in the non-affected members (Pogue-Geile et al., 2006). The mutation causes a proline (hydrophobic) to serine (hydrophilic) amino acid change (P239S). Palladin RNA was found to be overexpressed in the precancerous dysplastic tissue. The discovered mutation was also shown to have a phenotypic effect when a mutated form of the protein was expressed in cultured cells: the cells displayed abnormal actin bundle assembly and increased migration. However, the study did not answer the question whether this mutation is actually involved in the transformation of the cells and, if so, what is the mechanism. In fact, besides the simple migration assay and the over-expression phenotype, no function was shown for the mutation. These findings prompted other investigators to test whether this mutation could be the causative factor for other pedigrees with similar traits. To date, at least three independent materials have been screened for this particular mutation, but they have turned out negative (Salaria et al., 2007; Slater et al., 2007; Zogopoulous et al., 2007). In fact, even the methods used in the primary report were questioned in a recent study. It was shown that palladin is not overexpressed in most pancreatic cancer cells, but instead in the desmoplastic stroma (note: the stroma also harbours SMA-positive myofibroblasts). The authors thus concluded that overexpression of palladin is not likely to be responsible for the invasive and migratory capacity of pancreatic cancer cells (Salaria et al., 2007). Obviously further studies are needed to settle the issue.

A suggestive linkage at chromosome 4q32 in pre-eclampsia was recently identified, but so far the responsible gene has not been mapped. Interestingly, very recently Jarvenpaa et al. (2007) reported from an independent material that palladin is down-regulated in pre-eclampsia. Together these studies suggest that palladin might play a role in the pathogenesis of pre-eclampsia, but this result still needs to be verified.

2.1.3.5. Palladin in the nervous system

The role of palladin in tissues has been studied mostly in the rodent central nervous system. Otey et al. first showed that palladin is widely, though not uniformly, expressed in rat brain and spinal cord. The highest expression was detected in the olfactory bulb, cerebral and cerebellar cortex, hippocampus, amygdala, superior colliculus, and superficial laminae of the spinal dorsal horn. In these locations palladin co-localized at the sub-cellular level with synaptophysin, a presynaptic vesicle-associated protein. More precisely, palladin was shown to be selectively expressed in excitatory synapses (Hwang et al., 2001).

In cultured neuronal cells, palladin was shown to localize to the developing axons but not to the dendrites. Palladin was highy concentrated in the axonal growth cone, a place where constant and rapid re-organization of the cytoskeleton takes place. When palladin expression was down-regulated by antisense technology, the neurites didn't develop normally, suggesting a role for palladin in the axonal extension (Boukhelifa et al., 2001).

Palladin is highly expressed, not only in neurons, but also in astrocytes. These cells respond to injury to the nervous system and form the so-called glial scar. When astrocyte

cultures were wounded, thus simulating an injury, they showed rapid up-regulation of palladin in the cells at the wound edge. Simultaneously, the morphology of the cells changed from the inactive stellate to the active polygonal shape. *In vivo* palladin was similarly up-regulated at the edges of an experimantal stab wound to the cerebral cortex of rats (Boukhelifa et al., 2003). These results suggest that the up-regulation of palladin might be an important step in the acquisition of the reactive astrocyte morphology and thus formation of the glial scar.The results obtained with palladin knock-out mice (see next section) also suggest that palladin plays an important role in the nervous system (Luo et al., 2005).

2.1.3.6. Genetically modified palladin-negative (knock-out) mice

In 2005, the first construction of a genetically modified palladin-negative (knock-out) mouse strain was reported (Luo et al., 2005). The investigators showed that the genetic inactivation of palladin leads to embryonic lethality due to defects of cranial neural tube closure and herniation of liver and intestine. The palladin$^{-/-}$ mouse embryos had neural tube closure defects and died already at E15.5 (Luo et al., 2005). Palladin was shown to be expressed in the cranial neural folds of the wild-type embryos. Also, an idependent study focusing on chicken neural crest development showed a similar expression pattern (Gammill and Bronner-Fraser, 2002). Since the closure of the neural tube requires active migration of the cells, it is likely that the resulting closure defect in palladin-/- embryos was caused by disturbed cell migration. This notion was further supported by the findings obtained *in vitro* with palladin-/- murine embryonic fibroblasts (MEF). The cells displayed defects in the formation of stress fibers, lamellipodia and focal adhesions, adhesion to the ECM, and migration (Luo et al., 2005). G- to F-actin ration was elevated and β1-integrin was down-regulated, reflecting the disorganization of the cytoskeleton and diminished adhesion (Liu et al., 2007b).Furthermore, palladin-/- embryos have defects in erythropoiesis due to the abnormal function of macrophages, and a consequently compromised erythropoietic microenvironment (Liu et al., 2007a).

2.2. α-actinin

The first characterized interaction partner of palladin was α-actinin (Parast and Otey, 2000). In humans, there are four α-actinin genes encoding proteins, α-actinin1-4. α-actinins 2 and 3 are restricted to striated muscle, where they localize to the Z-disc and sarcolemma. Nonmuscle α-actinins 1 and 4 are located in focal contacts, cell-cell contacts, stress-fiber-dense regions and cortical actin networks. All the α-actinins have a similar structure: an amino-terminal actin-binding domain (ABD) composed of two calponin homology domains followed by a central rod-domain consisting of four spectrin-like repeats, and a carboxy-terminus consisting of a calmodulin-like (CaM) domain with two EF-hand motifs. *In vivo* the protein forms an antiparallel dimer which efficiently cross-

links actin fibers. Besides its function in stabilizing the actin bundles α-actinin also connects the cytoskeleton to transmembrane proteins in both cell-cell and cell-matrix contacts. It regulates both directly and indirectly different receptor activities and links the cytoskeleton to signaling pathways (reviewed in Vallenius(2004); academic dissertation) (Otey and Carpen, 2004).

α-actinin has a plethora of binding partners both in muscle and non-muscle cells; so far >40 proteins have been identified as interactors (reviewed in Klaavuniemi(2006); academic dissertation, Vallenius(2004); academic dissertation and Otey and Carpen, 2004). Since the list of binding partners is too elaborate to be discussed in detail here, only a few examples of each of the specific cell types and sub-cellular compartments are mentioned.In non-muscle cell adhesion sites, such as, focal adhesions, hemidesmosomes and neuronal synpases, α-actinin interacts with several transmembrane proteins, linking cells either to extracellular matrix or to neighbouring cells. In focal adhesions it binds to the ß1 integrin subunit that anchors the cytoplasmic actin filaments to the plasma membrane (Otey et al., 1990). Another plasma-membrane-associated interactor is syndecan-4, a transmembrane proteoglycan, which also binds to the extracellular matrix (Greene et al., 2003). Intercellular adhesion molecules (ICAMs) and adherent junction protein ADIP and α-catenin bind also to α-actinin. One of the most important binding partners on the cytoplasmic part of the focal adhesions is vinculin, one of the most studied focal adhesion proteins. The role of α-actinin in the adhesions is to provide stability for theses structures and thus maintain cell shape. It can also enhance the adhesion by clustering transmembrane proteins to these sites, as is known for ICAM-2 (Carpen et al., 1992). α-actinin also participates in the signaling cascades which converge in the adhesions. This is particularly highlighted by the fact that the integrin-activated tyrosine kinase, Focal Adhesion Kinase (pp125FAK) phosphorylates α-actinin and reduces its affinity towards actin (Izaguirre et al., 2001).

The major function of α-actinin in actin stress fibers appears to be its cross-linking of actin filaments. It localizes to specific structures called dense regions, which are considered to be the non-muscle equivalent of the sarcomeric Z-discs (Small and Gimona, 1998). In this localization α-actinin also associates with several proteins, such as zyxin, CLP-36 and palladin (Crawford et al., 1992; Parast and Otey, 2000; Vallenius et al., 2000). Although the precise function of these α-actinin-associated molecules in the stress fibers is mostly unknown, they possibly facilitate the actin cross-linking role or α-actinin and regulate stress fiber dynamics. In addition to this structural role, α-actinin can participate in signaling in the stress fibers simply by regulating the distribution of proteins within the cell. This is true at least for zyxin and the CLP-36 -associated kinase Clik1, which are known to shuttle between the cytoplasm and nucleus (Reinhard et al., 1999; Vallenius and Makela, 2002).

In the muscle cells α-actinin localizes to the sarcomeric Z-disc and the costameres, specific structures linking the sarcomere to the extracellular matrix via adhesion proteins. The Z-disc is a highly organized structure within the muscle cell where the thin actin filaments of opposing sarcomeres are crosslinked together. The structure of the Z-disc contributes to the force transmission between sarcomeres and is involved in the regulation of contractile and elastic properties of muscles. It is also considered to be a converging

point of several signaling cascades within the muscle cells (reviewed in Salmikangas (2001); academic dissertation and Klaavuniemi(2006); academic dissertation, Frank et al., 2006). α-actinin is probably the most important component of the Z-disc and binds most of the known Z-disc proteins, including ALP, ZASP, FATZ, myopalladin, palladin, ArgBP2 and titin (Klaavuniemi(2006); academic dissertation).

Studies on *Drosophila* mutants have shown that α-actinin plays a crucial role in maintaining the sarcomeric integrity and muscle function. α-actinin-deficient embryos die early at the larval stage due to degeneration of myofibrils characterized by the disruption of Z-disks (Fyrberg et al., 1998).

In the costamere, located at the Z-disc/sarcolemmal junction, α-actinin connects the contractile apparatus to the extracellular matrix via several interactions to both sarcoplasmic and transmembrane proteins, such as vinculin, dystrophin and integrins. These interactions enable the force generated by the contraction of the sarcomere to be mediated to the extracellular matrix.

2.3. The ERM (Ezrin/Radixin/Moesin) protein family

The ERM (*ez*rin/*r*adixin/*m*oesin) family consists of cytoskeletal proteins that are concentrated in actin-rich cell-surface structures where they link the actin cytoskeleton with the plasma membrane. They share a homologous amino-terminal domain, referred to as *F*ERM (protein *4*.1 ERM), followed by an α-helical region and a carboxy-terminal domain (Bretscher et al., 2002). ERMs remain in an inactive conformation in the cytoplasm through an intramolecular interaction between their amino- and carboxy-terminal domains. Once they are activated, either by binding to phospholipids or by phosphorylation, they are able to bind to the cytoplasmic part of transmembrane proteins, including CD43, CD44, CD95, ICAM-1, -2, -3, and syndecan-2 (Bretscher et al., 2002). ERM family proteins also bind actin and thus provide a link between the cytoskeleton and transmembrane proteins in cell-cell and cell-matrix adhesions, similar to α-actinin. They localize beneath the plasma membrane in the subcortical actin network, in specialized cellular structures such as microvilli and lamellipodia (Bretscher et al., 1997; Bretscher, 1999; Bretscher et al., 2002). They also maintain cell shape and polarity, and participate in cell motility and membrane trafficking. Besides this structural role, they are directly linked to signaling pathways, and can for instance activate members of the rho protein family. The neurofibromatosis 2 (NF2) tumour-suppressor protein merlin (a.k.a schwannomin), is highly homologous with the ERM proteins and is now considered to be a member of this protein family. It has some overlapping functions with other ERMs but also some distinctly unique features, most importantly, the tumor-suppressor function (Bretscher et al., 2002).

2.3.1. Ezrin

Ezrin was first described to be a cytoskeletal component of the intestinal-brush border (Bretscher, 1986; Bretscher et al., 2002). *In vivo,* it is mainly expressed in epithelium but has a wider distribution in cultured cells. Besides adhesion molecules (see above) ezrin interacts with signaling proteins of the phosphatidylinostiol-3-kinase (PI3K), protein kinase A and Rho pathways (Ivetic and Ridley, 2004). It thus works as a molecular scaffold in targeting intracellular signals. Ezrin is also phosphorylated *in vivo* both on serine/threonine by protein kinase C and Rho kinase, and on tyrosine by Src family kinases (Autero et al., 2003; Gautreau et al., 2000; Heiska and Carpen, 2005).

Studies on ezrin knockout mice have shown that, while ezrin is not crucial for the formation of brush border microvilli or for the maintenance of epithelial cell polarity, it plays a critical role in the lumen formation and expansion during villus morphogenesis (Saotome et al., 2004). The up-regulation of ezrin expression has morphogenic effects and is associated with tumor metastasis (Curto and McClatchey, 2004; McClatchey, 2003). In a mouse model of osteosarcoma, ezrin has been shown to be necessary for metastasis (Khanna et al., 2001; Khanna et al., 2004; Wan et al., 2005; Yu et al., 2004). Its expression provided a survival advantage for cancer cells that reached the lung, partially dependent on the activation of MAPK. These experimental results are supported further by the finding that high ezrin expression correlates with poor outcome in pediatric osteosarcoma patients (Kim et al., 2007).

The findings described above suggest that ezrin (and the ERM proteins) plays an important role in various cellular processes including adhesion, apoptosis, cell movement and tumor metastasis.

2.4. The ArgBP2 protein family

ArgBP2 forms, together with Vinexin and CAP/Ponsin, an adaptor protein family (Kioka et al., 2002). From the structural point of view, all the family members share a SoHo (sorbin homology domain) in their amino-terminal part, followed by three carboxy-terminal SH3 domains. They also contain several proline-rich sequences that can serve as ligands for SH3 domains. The sequence identity within the domains is high among the family members, ranging from 34% (SoHo domains) to 73% (SH3 domains). Since the SH3 domains are known to have a broad specificity towards their ligands, it is not surprising that all the ArgBP2 family proteins have several binding partners, some of which are shared between the members (e.g. vinculin) (Kioka et al., 2002). Specific binding partners have also been identified, including Sos and flotillin for Vinexin; and Grb4, ataxin 7, c-Cbl, the insulin receptor and Paxillin for CAP/Ponsin (Kioka et al., 2002). Besides their structural similarities, the proteins share other properties as well. They all have a ubiquitous tissue distribution but show the highest expression in heart. They also have a partly overlapping sub-cellular localization; all of them localize to focal

adhesions, and CAP/Ponsin and ArgBP2 also localize partly to the nucleus. However, unlike its relatives, ArgBP2 also localizes to the stress-fiber-dense regions in non-muscle cells and in sarcomeric Z-discs in cardiomyocytes (Kioka et al., 2002).

ArgBP2 was initially isolated as a binding partner for the non-receptor tyrosine kinase c-Arg (c-Abl2) in a yeast-two-hybrid screen (Wang et al., 1997). It co-localizes with Abl in cells, and is phosphorylated on tyrosine by Abl both *in vitro* and *in vivo* (Wang et al., 1997). Although the physiological function of the Abl family kinases is not entirely clear, they do have several connections to the cytoskeleton: both Abl and Arg can directly bind actin and they have several cytoskeletal targets, such as p130CAS and cortactin (Hernandez et al., 2004; Lanier and Gertler, 2000; Wang et al., 2001). ArgBP2 can also regulate indirectly the activity of Abl by linking it to the ubiquitin ligase Cbl. Phosphorylation of Cbl and ArgBP2 by Abl results in the stabilization of their interactions, thus facilitating Cbl-induced ubiquitination and subsequent degradation of Abl and ArgBP2 (Soubeyran et al., 2003). ArgBP2 links also to cell survival/apoptosis via its interactions with Akt and PAK1 (Yuan et al., 2005). ArgBP2 binds to both of these kinases and is phosphorylated on serine/threonine by Akt. This phosphorylation induces PAK1 activation which subsequently prevents DNA-damage-induced apoptosis (Yuan et al., 2005). Yet another ArgBP2 interacting kinase is Pyk2, whose activation, among other things, has been linked to dilatation-induced cardiac remodelling (Haglund et al., 2004). Since it has no enzymatic activity itself, ArgBP2 probably functions as a molecular adaptor which via several potentially simultaneous protein-protein interactions brings together effectors and their targets. This is also probably true in cardiomyocytes in which several signalling cascades have been shown to converge at the Z-disc (Frank et al., 2006).

In neuronal tissue, ArgBP2 is expressed as a specific isoform, nArgBP2, which contains a 606 amino-acid zinc-finger motif insertion. It localizes to the postsynaptic density where it interacts with SAPAP (SAP90/PSD95-associated protein) and afadin-1 (Kawabe et al., 1999). nArgBP2 also interacts with several cytoskeletal proteins, including spectrin, dynamin, synaptojanin, and WAVE isoforms, as well as WAVE regulatory proteins (Cestra et al., 2005). At least two of the ArgBP2/nArgBP2 binding partners, synaptojanin 2B and WAVE2, undergo ubiquitination and Abl-dependent tyrosine phosphorylation. The knockdown of nArgBP2 with a specific siRNA induces redistribution of focal adhesion proteins and an increase in peripheral actin ruffles in astrocytes (Cestra et al., 2005). The multitude and nature of nArgBP2 interactions suggests that, although it might have additional functions mediated by the zinc-finger insertion, it definitely acts as scaffolding protein, like ArgBP2 in other cell types.

2.5. SPIN90

SPIN90 (SH3 Protein Interacting with Nck, 90 kDa) cDNA was first isolated from a yeast two-hybrid screen where Nck Src homology 3 (SH3) domains were used as bait (Lim et al., 2001). Later, an independent group characterized the same protein which they named diaphanous-interacting protein (DIP) (Satoh and Tominaga, 2001). For the sake of simplicity, the results dealing with DIP are discussed here under the name SPIN90.

SPIN90 is a 722 residue protein, containing an SH3 domain, proline-rich motifs, a serine/threonine-rich region, and a long C-terminal hydrophobic region. The amino acid sequence of the SH3 domain has the highest homology with Src family kinases (Src, Fyn,Yes). It shows high sequence similarity to VIP54 and WISH (Lim et al., 2001). SPIN90 is ubiquitously expressed in human tissues; the highest expression is seen in heart, brain, and skeletal muscle. It localizes to the sarcomere Z-discs in cardiomyocytes (Lim et al., 2001), to filopodia and dendritic spines in neuronal cells, and to actin-based structures such as membrane ruffles in non-muscle cells (Kim et al., 2006; Kim et al., 2005). It plays a role in myofibril and sarcomere assembly, since both depletion and over-expression of SPIN90 in cardiomyocytes results in sarcomere disruption (Lim et al., 2001) (E.Ehler, unpublished observation). SPIN90 probably acts as a scaffolding protein, since it interacts with several actin-regulating proteins including Nck, N-WASP, βPIX, the Arp2/3 complex, dynamin, PSD-95 and mDia (Kim et al., 2006; Kim et al., 2005; Lee et al., 2006).

During cell adhesion and PDGF stimulation, SPIN90 is phosphorylated by ERK1 and subsequently forms a complex with betaPIX and WASP. Furthermore, this complex interacts with Nck at focal adhesions. These interactions probably play a role in establishing stable cell adhesion and can be dynamically modulated by SPIN90 phosphorylation by ERK1 (Lim et al., 2003).

Several studies have suggested that SPIN90 is involved in regulating the cortical actin cytoskeleton during lamellipodia formation and cell movement. When cells are stimulated by growth factors such as EGF and PDGF, they rapidly form membrane protrusions (called lamellipodia) at the leading edge preceding cell movement. Upon such stimulation SPIN90 re-localizes from its normal rather diffuse cytoplasmic position to the cortical actin network. There SPIN90 binds to several actin-associated and signaling proteins, such as, p190RhoGAP, Vav2, the Arp2/3 complex, palladin and actin itself (Eisenmann et al., 2007; Kim et al., 2006; Meng et al., 2004). SPIN90 is phosphorylated by Src and subsequenlty mediates the phosphorylation of p190RhoGAP and Vav2. This leads to inactivation of Rho and activation of Rac, which in turn results in a loss of actin stress fibers and formation of lamellipodia. Binding of SPIN90 to the Arp2/3 complex mediates its activation and and formation of branched actin networks at the leading edge of cells. These processes are inhibited when the expression of SPIN90 is down-regulated with a specific siRNA.

In the neuronal cells SPIN90 is involved in diverse processes, such as synaptic vesicle endocytosis and dendritic spinogenesis and synaptogenesis. SPIN90 is expressed in both pre- and postsynaptic compartments (Kim et al., 2005). In the presynaptic position it interacts which dynamin I, a membrane-associated protein involved in endocytosis. Both overexpression and siRNA-mediated knock-down of SPIN90 interfered with synaptic vesicle endocytosis, possibly by modulating the interaction of dynamin I with other endocytic proteins (Kim et al., 2005).

On the postsynaptic side, SPIN90 interacts with PSD-95 and is involved in dendritic spinogenesis by modulating the actin dynamics of the spines (Lee et al., 2006). When SPIN90 is overexpressed in neuronal cells it increases the number and size of the dendritic spines and, *vice versa,* knock-down of SPIN90 reduces the density of the spines. A neuronal isoform of ArgBP2, i.e. nArgBP2, has been also shown to localize to the postsynaptic density where it interacts with SAPAP (SAP90/PSD-95-associated protein) (Kawabe et al., 1999). This raises the possibility that ArgBP2 and SPIN90 could be involved in the same processes, particularly since they both associate with palladin.

3. Regulation of the actin cytoskeleton

The actin cytoskeleton is involved in several cellular processes including regulation of cell shape and polarity, motility and adhesion. In order to perform all these functions, the cytoskeleton must be able to form stable and durable structures but also, at the same time, be highly dynamic and flexible. This duality is achieved by formation of divergent complexes with actin-binding proteins (ABP) resulting in actin-based structures with different physical and functional properties. Various cellular cues regulate the actin cytoskeleton via signaling proteins and small molecules which mainly act by either activating or inhibiting ABPs. For example, during cell migration, actin filaments are assembled into a complex network at the protruding leading edge, while they must be disassembled simultaneously in the cell body to facilitate movement.

Several signaling pathways are involved in actin cytoskeleton regulation; one of the most extensivley studied is the Rho family of small GTPases. This family includes RhoA, Rac1 and Cdc42, each of which have different functions. Activation of Cdc42 and Rac1 control the formation of filopodia and lamellipodia, respectively. RhoA, on the other hand, induces the formation of stress fibers. These proteins cycle between an active GTP form and an inactive GDP form. The activation status is regulated by guanine nucleotide exchange factors (GEF) that promote GDP-GTP exchange, GTPase activating proteins (GAP) that stimulate inactivation, and Rho guanine nucleotide dissociation inhibitors (Rho-GDI) that stabilize the inactive protein form.

RhoA can be activated by several pathways which include both chemical mediators (LPA) and physical stress (shear stress). Once activated, RhoA induces stress fiber and focal adhesion formation by interacting with several effector proteins including Rho-Kinase (ROCK), PI 5-kinase and Formins (Maekawa et al., 1999; Matsui et al., 1996). ROCK subsequently activates LIM-kinase (LIMK) and phosphorylates Myosin Light Chain (MLC) (Maekawa et al., 1999). LIMK in turn phosphorylates and thus inactivates

cofilin (an actin filament depolymerizing factor) leading to accumulation of actin stress fibers (Ohashi et al., 2000). The phosphorylation of MLC also increases the contraction and assembly of stress fibers (Kureishi et al., 1997). Although this pathway is indeed quite complex and can be regulated at each step by additional factors, it is still very swift and efficient.

Rac1 is active at the leading edge of cells, and its inhibition reduces lamellipodium extension. Rac1 targets WAVE and N-WASP also localize in the lamellipodia, where they promote actin nucleation by the Arp2/3 complex (Small et al., 2002). Interestingly, palladin is also involved in lamellipodia organization by interacting with Eps8 and by indirectly regulating Rac activity (Goicoechea et al., 2006).

Besides the Rho family, actin cytoskeleton is also regulated by both receptor and non-receptor tyrosine kinases. These include for instance the PDGF receptor (PDGFR) and the Src and Abl kinase families. There is considerable cross-talk between these kinase cascades, and in fact PDGF mediates its cytoskeletal effects partly via Src, which subsequently can activate Abl kinases (Boyle et al., 2007).

3.1. The Src-kinase family

The Src family of tyrosine kinases (SFK) has nine members: Src, Lck, Hck, Fyn, Blk, Lyn, Fgr, Yes and Yrk (Brown and Cooper, 1996). The kinases are 52-62 kD proteins, which have a similar structure with conserved domain regions called SH (Src homology) domains 1-4. The SH4 domain is myristylated and targets the kinases to the cell membrane. The SH3 and SH2 domains mediate both intra- and intermolecular interactions to proteins affecting catalytic activity and sub-cellular localization; they also link the kinases with their targets (Brown and Cooper, 1996). SH3 domains bind to proline-rich target sequences which have a PxxP core motif (Kay et al., 2000). SH2 domains, on the other hand, bind to phospho-tyrosine residues within longer amino-acid sequences. The kinase domain, SH1, displays kinase activity and also binds to substrates.

As the name implies, Src is the founding member of the Src family protein tyrosine kinases. It was originally identified as the transforming protein (v-Src) of the oncogenic retrovirus, Rous Sarcoma Virus (RSV) (Brown and Cooper, 1996). v-Src is a mutated and thus constantly activated version of the normal cellular protein (c-Src). Because its uncontrollable activity can lead to cellular transformation and tumor formation, v-Src is an oncogene, whereas c-Src is a proto-oncogene. c-Src is held in an inactive state by an auto-inhibitory mechanism involving the SH3 and SH2 domains. The SH2 domain binds to a phosphorylated inhibitory tyrosine (Y527) near the carboxy-terminus of the protein, thus facilitating the binding of the SH3 domain to a polyproline site within a linker region between the SH2 and Kinase domains. This leads to a conformational change in the kinase domain's active site, thus inactivating the enzyme. Src can be activated by several

mechanisms including integrin engagment and receptor tyrosine kinases, for instance PDGFR (platelet-derived growth factor receptor)(Brunton et al., 2004). Activation of Src leads to tyrosine phosphorylation of several cytoskeletal/adhesion target proteins including vinculin, cortactin, paxillin, ezrin, p130cas, β - and γ -catenin, ZO-1 and connexin 43 (Brown and Cooper, 1996; Heiska and Carpen, 2005). It also phosphorylates signalling proteins such as FAK, Vav, p190rhoGAP and Eps8 (Brown and Cooper, 1996).

Src activation results in alterations of the cell structure, particularly in the actin cytoskeleton and the adhesion networks controlling cell migration. It also transmits signals that regulate proliferation and cell survival. The cytoskeletal effects induced by Src include disruption of cell-cell adhesions and stress fibers, and formation of membrane ruffles and podosomes (Brunton et al., 2004; Frame et al., 2002; Frame, 2004). Podosomes are actin-rich membrane protrusions which are involved in both adhesion and invasion (Linder and Aepfelbacher, 2003). Interestingly, palladin and a known Src substrate Eps8 have recently been shown to localize to these structures (Goicoechea et al., 2006). As a result of these events, the transformed cells are more motile and thus probably more prone to invade tissues and metastasize to remote sites (Frame, 2002).

4. The myofibroblast

Myofibroblasts are specialized cells that originate from normal fibroblasts, circulating progenitor cells called fibrocytes and epithelial cells through a process termed epithelial-mesenchymal-transition (EMT) (Hinz et al., 2007). They are found *in vivo* in both physiological and pathological processes (Powell et al., 1999). The main function of myofibroblasts is to modulate the extracellular matrix (ECM) by actively secreting variable extracellular proteins, such as structural matrix proteins (fibronectin, collagen), proteases (Matrix Metallo Proteases, MMP), cytokines (Monocyte Chemotactic Protein - 1) and growth factors (TGF and PDGF) (Tomasek et al., 2002; Tomasek et al., 2002). The most studied and therefore the best known function of myofibroblasts is in the formation of granulation tissue during dermal wound healing. In granulation tissue, the cells generate the contractile force which ensures wound closure. After the wound has healed, myofibroblasts normally disappear by apoptosis (Desmouliere et al., 1995). In fibrocontractive diseases, such as idiopathic lung fibrosis, glomerulosclerosis and contractures, myofibroblasts avoid apoptosis and remain in the tissue. They continue to contract actively and to produce the ECM components leading to tissue deformation and fibrosis (Desmouliere et al., 2003; Desmouliere et al., 2005). This process represents “over-healing” due to repeated bouts of tissue damage followed by continuing myofibroblast activation.

The formation of myofibroblasts from normal fibroblasts, fibrocytes and epithelial cells involves a mechanical stimulus to the cells by tension in the ECM, as well as chemical stimuli by growth factors and cytokines (Hinz and Gabbiani, 2003a; O'Kane and Ferguson, 1997). Under normal physiological condition, fibroblasts reciding in the ECM, for example in dermis, do not have cytoplasmic stress fibers. Once an injury, such as a wound, is inflicted the mechnical tension and the numerous chemical mediators secreted

by the inflammatory cells activate the cells, leading first to the formation of so-called proto-myofibroblasts (Tomasek et al., 2002). These cells differ from normal fibroblasts by containing stress fibers composed of cytoplasmic β and γ actins. Upon further maturation the cells acquire some smooth muscle characteristics, including neo-expression of actin-associated proteins and *α*-smooth muscle actin (α-SMA)(Tomasek et al., 2002). To date, α-SMA is considered to be the most reliable marker of the differentiated myofibroblast (Darby et al., 1990; Skalli et al., 1986). The proto-myofibroblast/myofibroblast sequence also explains the discrepancies between the initial and more recent characterization of these cells. Since myofibroblasts were initially studied by electron microscopy, they were identified by the the presence of stress fibers and focal ahdesions (fibronexus). Electron microscopy cannot, however, distinguish the α-SMA-negative proto-myofibroblasts from the fully differentiated α-SMA-positive myofibroblasts. This lead to misinterpretations after the charazterization of the α-SMA antibody (so-called α-SMA-negative myofibroblasts). Besides expressing α-SMA, myofibroblasts can express other smooth muscle proteins including smooth muscle myosin heavy chains, calponin, SM-22α and calgizzarin (Malmstrom et al., 2004). Usually they do not attain all the characteristics of smooth muscle, for instance, they only rarely express desmin and smoothelin (Tomasek et al., 2002). The process of epithelial-to-mesenchymal transition (EMT) has recently received much attention. This process has been shown to be an important way of producing myofibroblasts at the site of tissue injury, for example in lung fibrosis and glomerulosclerosis (Desmouliere et al., 2003; Hinz et al., 2007). In the former, differentiated epithelial cells lose their epithelial phenotype (expression of cytokeratin isoforms and cell-cell adhesion molecules such as E-cadherin) and start to express mesenchymal markers, most importantly vimentin and α-SMA.

The best characterized chemical mediator of myofibroblast differentiation is transforming growth factor-β1 (TGF-β1). TGF-β1 is produced in dermal wounds by keratinocytes, leukocytes and fibroblasts, and it is also released by platelets (O'Kane and Ferguson, 1997). Inflammatory leukocytes, especially macrophages, are a source of TGF-β1 also in lung and hepatic fibrosis. TGF-β1 binds to its receptor at the cell membrane leading to receptor autophosphorylation. The active receptor is a serine/threonine kinase which subsequently phosphorylates Smad2 and Smad3 members of the SMAD signaling pathway. Both Smad 2 and 3 bind to Smad4 and translocate to the nucleus to regulate gene transcription together with transcription factors. This pathway can be inhibited by the action of Smad7, an endogenous inhibitory member of the Smad family (Massague, 2000). TGF-β1 also activates the mitogen-activated protein kinase (MAPK) signaling cascade (Leivonen et al., 2002). Most importantly, it activates ERK1/2 (extracellular signal-regulated kinase) and p38. Both of these proteins are serine/threonine kinases which regulate various cellular activities, such as gene expression, differentiation and apoptosis. There is also cross-talk between the Smad and MAPK signaling cascades, and MAPKs can influence Smad activation either positively or negatively (Leivonen et al., 2005).

Aims of the study

This study was initiated by Dr. Olli-Matti Mykkänen together with Prof. Olli Carpen. They were looking for novel binding partners for the ERM-family protein ezrin and identified a novel protein, subsequently named palladin. Characterization of this novel protein became the focus of further studies which eventually led to this dissertation.

The initial aims included:

1) determination of the structure of the human palladin gene and protein
2) characterization of the tissue distribution of palladin and its sub-cellular localization
3) characterization of the interaction between palladin and ezrin and the regulation of palladin expression during dendritic cell differentiation

After this preliminary work the subsequent studies aimed to:

1) characterize the interaction between palladin and α-actinin in detail
2) identify novel binding partners for palladin
3) study the expression of palladin during myofibroblast differentiation and in myofibroblastic lesions

Methods

The materials and methods are described in detail in the original publications, and only summarized in the table below.

Method	Used in study
Northern blot analysis	I
Cell cultures and transfections	I-V
Polyclonal antibody production	I, IV
Immunofluorescence microscopy	I-V
Immunohistochemistry	I, IV
Production of recombinant DNA constructs	I-III
Yeast two-hybrid analysis - library screen - mating assay	 I I-III, V
Protein affinity precipitation	I-III, V
Protein blot overlay	I
Western blot analysis and immunoblotting	I, IV
Co-immunoprecipitation	III, V
In vitro protein translation	II, III, V
Flow cytometry	I
Production of recombinant proteins	I-V
RNA interference by siRNA	V
Primary culture of neo-natal rat cardiomyocytes	III
RNA isolation and RT-PCR	IV
Mitochondrial outer membrane (MOM) targeting assay	II, III, V
Recombinant adenovirus transduction	IV
Experimental rat cutaneous wound model	IV

RESULTS AND DISCUSSION

1. Characterization of human palladin (I)

1.1. Cloning and sequence analysis of human palladin cDNA

In order to find novel molecules interacting with ezrin, we performed a yeast two-hybrid screen with a bait construct containing the α-helical and carboxy-terminal domains (amino acids 278-585) of ezrin. With this approach, two identical 899-bp clones encoding for the last 254 amino acids of a novel gene product were isolated from a HeLa cell cDNA library. Sequence comparisons in the BLAST database identified a 4347-bp cDNA clone (accession no. AB023209, protein accession no. KIAA0992), which included the isolated sequence. This cDNA was predicted to encode the C-terminal 772 amino acids of a novel protein. Because the mouse ortholog was identified during the progress of this work and named palladin (Parast and Otey, 2000), we decided to comply with the nomenclature, and name also the human protein palladin (the sequences across these species are highly homologous, showing ~90% similarity). Since the AB023209 cDNA did not contain an in frame stop codon or a Kozak consensus sequence before the first ATG codon, it was considered to be a partial cDNA. More recent studies with the mouse palladin gene, however, suggested that this cDNA indeed contains the full-length human palladin 3Ig isoform sequence and has an additional 5' part from the 4Ig isoform (Otey et al., 2005; Rachlin and Otey, 2006)

By structural prediction, the identified human palladin cDNA contains three Ig-domains in the carboxy-terminal part of the molecule. These Ig-domains are most homologous to those found in myopalladin and myotilin, two Z-disk-associated sarcomeric proteins (~63% and ~49% identity with palladin, respectively). Together these three proteins form a new Ig domain protein family. The Ig domains of palladin are also homologous to Ig domains Z6-8 present in the Z-disc-associated region of the sarcomeric protein titin (31% identity). Part of the amino-terminal region is homologous to myotilin (34% identity and 44% similarity for residues 118-327 of palladin and 2-191 of myotilin). However, palladin has two polyproline stretches not found in myotilin.

1.2. Expression pattern of palladin

Northern blot analysis indicated that palladin is expressed in a wide variety of tissues. A strong signal was seen in prostate, testis, ovary, small intestine and colon. A weaker or nondetectable signal was present in hematopoietic tissues: thymus, spleen, and peripheral blood lymphocytes. The size of the major transcript was ~4.5 kb. Western blot analysis of the tissue lysates was performed with polyclonal antibodies raised against a synthetic 17-mer peptide. The analysis showed strong reactivity in prostate, ovary, and colon, tissues

positive in mRNA analysis, and in kidney, whereas hematopoietic tissues expressed little palladin.

1.3. Localization of palladin

The subcellular localization of palladin was studied by immunofluorescence microscopy of cultured cells. In HISM (human intestinal smooth muscle), U251mg glioblastoma and HeLa cervical squamous carcinoma cells, palladin was detected in the stress-fiber-dense regions and to some extent in focal adhesions. In transient transfection experiments, a myc-tagged carboxy-terminal palladin construct was expressed in HeLa cells and detected with an anti-myc mAb. The transfected protein localized along actin microfilaments in a pattern resembling that of the endogenous protein. The transfection results confirmed the immunolocalization of the endogenous protein and indicated that the Ig-domain-containing carboxy-terminus can be targeted to microfilaments. Palladin distribution was also analyzed in frozen sections of a human stomach carcinoma specimen. Strong staining was observed in the smooth muscle layer of the stomach, and the serosal vessel walls (tunica media). Reactivity was also detected in the adenocarcinoma cells invading the muscular layer.

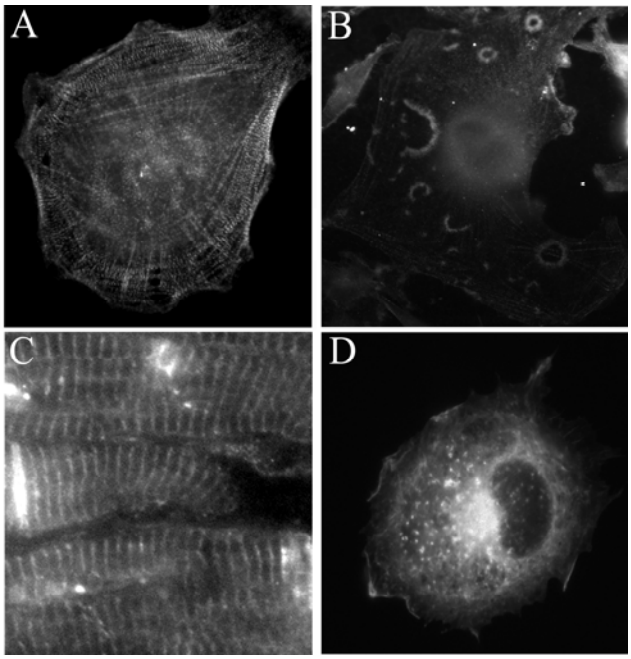

Figure 4. Palladin localizes to different actin-based cellular structures. A) U251 cells stained with the palladin 3Ig antibody display the typical pattern of the stress-fiber-dense region, B) palladin also localizes to membrane ruffles and wave-like structures. C) In cardiomyocytes, a prominent Z-disc staining pattern is seen, also intercalated discs between adjacent cells are high-lighted. D) In vascular smooth muscle (A7r5) cells palladin localizes to the phorbol-ester-induced dot-like podosomes

1.4. Interaction between palladin and ezrin

Since palladin was found in a two-hybrid screen with a carboxy-terminal ezrin bait, it was of interest to find out whether these proteins co-localize in cells. Ezrin usually localizes to plasma membrane associated structures such as microvilli and lamellipodia, while palladin localizes mostly to the stress fibers. When ezrin and palladin were double-stained in HISM cells, ezrin demonstrated a filamentous staining pattern and partial co-localization with palladin. This localization for ezrin is quite different from that in epithelial cells but is in line with another ERM protein, merlin, which has previously been shown to localize in the microfilaments. These results suggest that ezrin and palladin may interact in smooth muscle cells. In additional unpublished experiments, palladin and ezrin have been shown to co-localize also in the lamellipodia of migrating cells (Rönty et al., unpublished results).

The regions mediating interaction between palladin and ezrin were mapped with the use of the yeast two-hybrid system. A carboxy-terminal construct of ezrin (aa 278-585) interacted with palladin (Ig1-3) and with a carboxy-terminal construct containing the second and third Ig-domains (Ig2-3), but not with a construct containing only the first Ig-domain (Ig1). Ezrin 1-585 (wt), ezrin 1-339, and ezrin 479-585 did not interact with any of the constructs. The palladin binding site is thus probably masked in the dormant wild-type ezrin. Further evidence for the interaction was obtained by additional experimental approaches. The α-helical (278-531) and C-terminal (477-585) domains of ezrin were expressed as GST-fusion proteins and bound to glutathione beads. Lysates of yeast cells expressing HA-tagged palladin Ig1, Ig2-3, and the N-terminal part of ezrin (1-309) were incubated with beads, and bound proteins were detected with HA-antibody. Palladin Ig2-3 bound to the α-helical ezrin construct but not to the carboxy-terminus, whereas palladin Ig1 bound to neither ezrin construct. The two-hybrid and affinity precipitation results indicate that the α-helical region of ezrin mediates the interaction with the carboxy-terminal Ig-domains of palladin.

The blot overlay method was used to identify whether ezrin is a major palladin-binding protein in cell lysates. Lysates of HISM and U251 cells, full-length ezrin, and purified recombinant N-terminal half of ezrin were run in SDS-PAGE, blotted on nitrocellulose filters, and probed with biotinylated GST-palladin Ig1-3 and GST-palladin Ig2-3. The palladin probes bound to denatured full-length ezrin but not to the amino-terminus; this is in line with the idea that the interaction site resides in the α-helical region of ezrin. In cell lysates, both probes bound to protein bands migrating at ~63 and 75 kDa and in U251 to an additional band migrating at 85 kDa. Reprobing of the filters by ezrin antibody identified the 75 kDa as ezrin. The identity of the two other bands is not known to date.

1.5. Expression of palladin during dendritic cell differentiation

The differentiation of monocytes into dendritic cells (DC) is accompanied among other things by changes in cellular morphology. During the process, round monocytes are transformed into immature DCs containing filopodial projections and actin-rich podosomes. After final differentiation, mature DCs acquire an elongated morphology with dendrite-like processes. The maturation is accompanied by up-regulation of cell surface molecules, such as CD1a in immature DCs and CD1a and CD83 in mature DCs. We studied the expression and localization of palladin during the maturation process. Western blotting revealed that peripheral blood monocytes were devoid of palladin, whereas immature and mature DCs showed an immunoreactive doublet migrating at ~90-92 kDa. As a control, the lysates were blotted for moesin, a member of the ERM-family, which is expressed in the hematopoietic lineage. Unlike palladin, no differences were detected in the moesin expression level. Immunostaining demonstrated that in immature cells, palladin was concentrated in the actin-containing podosomes. In mature DCs, palladin decorated the thin long actin filaments.

2. Analysis of the interaction between palladin and alpha-actinin (II)

2.1. Identification of the interaction sites between palladin and α-actinin

Palladin was shown to co-localize and co-immunoprecipitate with α-actinin by Parast and Otey (2000) but the interaction was not characterized in detail. We therefore set out to study the interaction with both *in vitro* and *in vivo* methods. Demonstration of a direct interaction and mapping the interaction sites was initially done with the yeast two-hydrid system. Several palladin inserts were cloned in the bait vector and co-transformed with α-actinin prey constructs. In these experiments, a short segment, amino acids 222–280, mediated an interaction with α-actinin. None of the constructs lacking this sequence bound to α-actinin. The palladin binding site in α-actinin was located in the carboxy-terminal domain, within EF-hand domains 3–4.

To confirm the results by another method, we performed an affinity precipitation assay with *in vitro translated* (IVT) palladin and GST-α-actinin constructs. Palladin polypeptides 8–772, 101–280 and 389–772 were tested with several α-actinin constructs. The selection of the constructs 101–280 and 389-772 was based on previous results: in our yeast two-hybrid analysis the binding site was mapped between amino acids 222–280, whereas in a previous report by Bang et al. (2001) the Ig-domains of myopalladin were shown to mediate the interaction, and this particular segment is homologous to palladin sequence 389–772.. In line with the yeast two-hybrid results, IVT products 8–772 and 101–280 bound α-actinin, whereas the carboxy-terminal palladin construct did not show any binding.

The experiments thus identified a sequence within residues 222–280 of palladin that was sufficient and necessary for binding of the carboxy-terminal EF-hand domains 3–4 of α-actinin. In myotilin, a homologous segment is located between amino acids 80-125 and it also mediates the interaction with α-actinin (Hauser et al., 2000). The highest homology between the proteins is within this sequence – particularly in a short 17 residue segment (64% identity and 94% similarity). This sequence does not have features of any known structural domain, neither do homology searches identify related sequences in myopalladin or any other known protein. Interestingly, this segment does seem to have an affinity towards calcium-binding sequences such as the EF-hand domain, since in our unpublished experiments we have identified other interaction partners for palladin sharing this motif. Also, the amino-acid substitution caused by the mutation identified by Pogue-Geile et al. (2006) in a familiar form of pancreatic cancer maps to this segment. It is noteworthy that the region indicated in α-actinin binding in myopalladin (three carboxy-terminal Ig-domains) did not mediate the interaction in palladin, although the sequences are 63% identical. The binding region in the EF-hand domains 3–4 of α-actinin's carboxy-terminus is common to myopalladin, palladin and myotilin, and also titin has been shown to bind to the same region.

2.2. Role of interaction sites in targeting of α-actinin and palladin

The interaction was studied *in vivo* by transient transfection assays. First the subcellular localization of palladin constructs was analyzed. Three of these constructs (8–772, 8–387 and 101–387) contained the α-actinin binding site identified in the biochemical analyses. The fourth one is a cDNA variant that contains altogether 5Ig domains but lacks the poly-proline sequences and also the mapped α-actinin binding site. This construct is part of the NM_016081 sequence accession and was cloned from a human heart cDNA library. It contains the two additional amino-terminal Ig-domains and is fused to AB023209 sequence at residue 269 (in the original article this construct was called N-Alt). All four constructs localized to stress fibers, but their localization displayed interesting differences. Only the constructs which contained the identified α-actinin binding site localized in dense regions, whereas N-Alt construct decorated the fibers in an even manner. While the reason for this difference is probably due to the fact that N-Alt did not bind α-actinin, the mechanism by which this construct is targeted to stress fibers remains so far unknown. It is possible that this construct binds directly to F-actin, since it contains the Ig-domains which in the homologous protein myotilin are responsible for this function (von Nandelstadh et al., 2005). Palladin's ability to target endogenous and transfected α-actinin was also studied. For this purpose we used COS cells that have a poorly arranged cytoskeleton devoid of stress fibers. Transfection of palladin GFP-8–772 resulted in the formation of thick actin bundles, a phenomenon seen also in astrocytes. Endogenous α-actinin was partly relocated to these bundles, implying that palladin can also target α-actinin. The transfected palladin N-alt construct formed cytoplasmic aggregates which contained F-actin, but α-actinin was not targeted to these structures. Interestingly, when a palladin construct containing the mutation identified as the cause of a form of familial

pancreatic cancer was introduced into the cells it also formed aggregates. The mutation is in the region that mediates the interaction with α-actinin and substitutes a single residue (P239S) (Pogue-Geile et al., 2006). It is possible that this mutation disrupts the interaction with α-actinin, but this has not been studied. Although the results described above suggest that the targeting of α-actinin by transfected palladin is not, at least entirely, due to re-organization of actin, the possibility of another indirect mechanism was ruled out by additional experiments. This was done by cloning parts of palladin into MOM (*M*itochondria *O*uter *M*embrane)-targeting vector (Kaufmann et al., 2000) and co-transfecting it into cells with GFP–α-actinin lacking the actin-binding domain (R1–R4EF). This α-actinin construct has been shown to disrupt stress fibers and remain diffusely in the cytoplasm (Edlund et al., 2001). Co-transfection of R1–R4EF together with palladin MOM-8–387 or MOM-222–280 resulted in recruitment of the α-actinin construct from diffuse localization to the mitochondria, whereas the empty MOM vector failed to do so.

All in all, these results show that palladin interacts directly with α-actinin, and that the proteins can target each other to the specific subcellular localization.

3. Palladin and α-actinin interact with Abl/Arg kinase adaptor ArgBP2 and link it to the actin cytoskeleton (III)

3.1. ArgBP2 interacts with palladin

In order to identify novel interaction partners for palladin, we searched the literature for proteins fulfilling three requirements: (1) the protein ought to be expressed in several cell types including cardiomyocytes, (2) it should localize to silmilar subcellular stuctures as palladin, i.e., sarcomeric Z-discs and stress-fiber-dense regions and (3) it should contain an SH3 domain or domains, which could mediate the binding of palladin's amino-terminal proline-rich regions. Since ArgBP2 met all three requirements it was considered to be a potential interaction partner (Wang et al., 1997).

This hypothetical interaction was first tested in a yeast two-hybrid analysis with several palladin and ArgBP2 constructs. In this analysis, the full-length and carboxy-terminal SH3 domain sequences of ArgBP2 were shown to interact with the proline-rich amino-terminal segment of palladin. The amino-terminal ArgBP2 prey construct lacking the SH3 domains, did not interact with any of the palladin baits.

3.2. Localization of ArgBP2 and palladin constructs in non-muscle cell lines and neonatal rat cardiomyocytes (NRC)

In transient transfection experiments with different ArgBP2 constructs we noted an unexpected localization pattern. The full-length protein localized like the endogenous

protein, i.e., to stress-fiber-dense regions and focal adhesions. This was also true for the carboxy-terminal part of the protein, with the exception that it localized more prominently to the adhesions. However, the amino-terminus of ArgBP2 without the SH3 domains also localized to stress fibers and adhesions. This was unexpected, since it only contains one domain, namely the SoHo domain, which has been shown to interact only with flotillin (a membrane raft associated protein) (Kimura et al., 2001). The amino-terminus also displayed nuclear localization in some cells, possibly due to the fact that it has previously been shown to contain a nuclear localization signal. As expected, double staining of co-transfected palladin and full-length ArgBP2 demonstrated a high degree of co-localization.

In neonatal rat cardiomyocytes, both endogenous ArgBP2 and trasfected palladin constructs localized to the Z-discs and intercalated discs (Figure 5). The carboxy-terminal ArgBP2 construct with the SH3 domains was mostly targeted to intercalated discs and less prominently to Z-discs. The amino-terminal construct, on the other hand, resembled the distribution of endogenous protein.

Although we did not use neuronal cells in the experiments, it is interesting to note that both palladin and a specific ArgBP2 isoform, nArgBP2, are highly expressed in the nervous system where they both regulate astrocyte cytoarchitecture (Boukhelifa et al., 2003; Cestra et al., 2005).

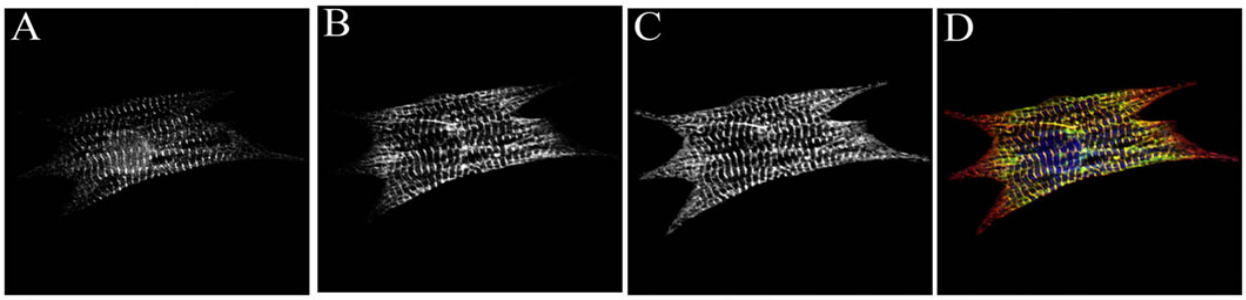

Figure 5. Palladin and ArgBP2 co-transfection in cardiomyocytes. Neonatal rat cardiomyocytes were co-transfected with ArgBP2 and palladin constrcuts and stained for A) M-band Titin (blue), B) palladin (GFP-green), C) ArgBP2 (Ha-red). The combined figure D shows that ArgBP2 and palladin co-localize in the Z-disc (yellow).

3.3. ArgBP2 interacts with α-actinin

The results of the transfection experiments and the previous studies, which have shown that Z-disc proteins often exist in multi-protein complexes, led us to investigate whether ArgBP2 would also interact with α-actinin. We tested this possibility with a yeast two-hybrid analysis, in which an ArgBP2 bait construct encoding residues 25–630 was tested with several α-actinin prey constructs. In this assay, ArgBP2 interacted specifically with the amino-terminal actin-binding domain (ABD), but not with the spectrin repeat-containing rod domain or with the carboxy-terminal EF-hand region. In addition, amino- and carboxy-terminal ArgBP2 prey constructs were tested with an α-actinin ABD-R1 bait sequence. The amino terminus of ArgBP2 showed a clear interaction, whereas the carboxy terminus, which interacted with palladin, did not bind α-actinin.

3.4. Verification of the identified interactions

Since both of the above-described interactions were novel, we needed to verify them with additional methods. First we used an affinity precipitation assay. In the assay *in vitro* translated (IVT) palladin was allowed to bind to individual ArgBP2 SH3 domains fused with GST. This assay showed that palladin binds specifically to the SH3-1 domain of ArgBP2, while no interaction was detected between palladin and SH3-2 or SH3-3 domains.

The interaction between α-actinin and ArgBP2 was verified by a similar method, except that α-actinin was produced in trasfected cells rather than as an IVT product. In the assays α-actinin bound to both ArgBP2 FL (full length) and amino-terminal constructs.

Next, the interaction between palladin and ArgBP2 was studied *in vivo* with a mitochondrial targeting assay. Palladin MOM8-387 construct (described in the α-actinin article) was co-transfected with GFP constructs containing the amino- or carboxy-terminus of ArgBP2 to COS cells. MOM3-387 was able to target ArgBP2 carboxy terminus but not the amino terminus to the mitochondria.

3.5. Palladin, α-actinin and ArgBP2 form a trimeric complex

Since both palladin and ArgBP2 can bind to different regions of α-actinin and also to one another, we tested whether they can form a three-way complex. This was tested *in vivo* with an immunoprecipitation assay. COS cells were transiently co-transfected with HA-tagged palladin and GFP-tagged FL (full length) ArgBP2. Palladin was precipitated from cell lysates with an anti-HA antibody and the precipitates were immunoblotted for α-actinin and GFP. Both α-actinin and ArgBP2 could be detected in the precipitate, suggesting formation of a trimeric complex. The complex was also studied with a transfection-based targeting assay. As shown above, over-expression of palladin in COS cells results in re-organization of the cytoskeleton and induction of thick actin bundles; endogenous α-actinin is also recruited to these structures. This time we analyzed the

behavior of the amino-terminal ArgBP2 construct co-transfected with palladin. The amino-terminal ArgBP2 fragment was also targeted to the bundles in analogy with α-actinin, although this fragment does not bind to palladin. Since this ArgBP2 fragment binds to α-actinin, the result implies that a complex of the three proteins might exist *in vivo* in line with the results of the precipitation assay.

Taken together, our results identified ArgBP2 as a palladin-interacting protein, and showed that ArgBP2 also binds to α-actinin. Furthermore, a complex of the three proteins was identified *in vivo*.

4. Palladin interacts with SPIN90 and Src and is involved in Src-induced cytoskeletal remodeling (IV)

4.1. Palladin interacts with the SH3 domains of SPIN90 and Src

SPIN90 was identified as a potential palladin-binding protein with the same rationale that was used for ArgBP2: it is an actin-cytoskeleton-associated protein with SH3 domain(s) and a wide tissue distribution including cardiomyocytes. In the original publication characterizing SPIN90, its SH3 domain was shown to have highest homology (35% identity and 50% similarity) with the SH3 domains of Fyn, Yes, and c-Src (Lim et al., 2001). Since Src is known to regulate actin cytoskeleton and has been shown to phosphorylate SPIN90 we also included it in these analyses. Furthermore, in the first paper characterizing palladin it was proposed to be a phosphoprotein, although no experimental evidence was shown in order to verify this (Parast and Otey, 2000).

The interactions were characterized essentially in a similar way as with ArgBP2, and are therefore described only briefly here. First, we used the yeast two-hybrid system, and found that both SPIN90 and Src SH3 domains interacted with the amino-terminal proline-rich segment of palladin (3Ig isoform). The preliminary results were then verified with an affinity precipitation assay. The results showed that the segments identified in the yeast two-hybrid assay indeed mediated the interactions. Also, palladin and SPIN90 could be co-immunoprecipitated from lysates of co-transfected cells. *In vivo*, the interaction was shown by targeting assays. Trasfected SPIN90 co-localized to palladin-induced actin bundles in COS cells and also to the outer mitochondrial membrane in cells transfected with the previously described palladin MOM-construct (8-228).

4.2. The effect of PDGF treatment and activation of the Src-kinase on palladin and SPIN90 localization

Both palladin and SPIN90 have been shown to localize to PDGF-induced membrane ruffles and lamellipodia (Goicoechea et al., 2006; Kim et al., 2006). Since the effects of PDGF are at least partly mediated by Src, we studied how PDGF and Src activation would

affect palladin and SPIN90 distribution in U251 glioma cells. Under basal conditions palladin localized, as previously described, to stress-fiber-dense regions, while SPIN90 showed a diffuse cytoplasmic distribution. PDGF treatment led to a rapid re-distribution of both proteins to the newly formed membrane ruffles and wave-like actin-based structures. These effects were abolished by addition of the selective Src-kinase family inhibitor PP2.

Although we did not study the localization of palladin and SPIN90 in neural cells, it is of interest that both proteins have been shown to localize predominantly in the presynaptic compartment of neurons. In the synapses, palladin co-localizes with vesicle protein synaptophysin (Hwang et al., 2001), and SPIN90 has been shown to be involved in synaptic vesicle endocytosis (Kim et al., 2005). These findings point to the possibility that the proteins might interact also in the synapses, but this hypothesis needs to be verified experimentally.

When the cells were transfected with an active (CA) Src construct, both SPIN90 and palladin rapidly re-localized to the Src-induced actin-rich membrane protrusions. SPIN90 has been previously linked directly to Src-induced cytoskeletal rearrangements (see below and Introduction) and our results suggest that also palladin plays a part in this process.

4.3. Palladin is required for Src-induced actin remodeling

Previously the knock-down of both palladin and SPIN90 by specific siRNAs has been shown to inhibit PDGF-induced membrane ruffle formation (Goicoechea et al., 2006; Kim et al., 2006). We wanted to examine whether this is true also for Src-kinase-induced actin remodeling. U251 cells were co-transfected with active Src and either with SPIN90-specific siRNA construct or with a palladin-specific siRNA oligonucleotide. The down-regulation of SPIN90 did not result in detectable cytoskeletal alterations, and the SrcCA-induced rearrangement appeared normal. This is in line with previous results which show that, although SPIN90 is a Src substrate and targets effector proteins Vav2 and p190RhoGAP to the plasma membrane, it is not needed for Src re-location and initiation of the cytoskeletal remodeling (Meng et al., 2004). However, when palladin was knocked down, the cells lost stress fibers, became more rounded, and the effects of SrcCA on the actin cytoskeleton were inhibited. Fewer membrane protrusions were formed in cells co-transfected with palladin siRNA and SrcCA, and the localization of Src, SPIN90 and cortactin remained diffuse. Activation-induced Src re-localization has been shown to require intact stress fibers and it is mediated by the SH3 domain (Fincham et al., 1996; Fincham et al., 2000). Together, these results offer a basis for a model in which Src and SPIN90 translocate after stimulation to the membrane via a palladin/stress-fiber-dependent manner. The re-localization is mediated by the interactions between the SH3 domains and palladin. Subsequently, Src phosphorylates p190RhoGAP and Vav2 via SPIN90, leading to Rho inactivation and Rac activation. This results in a loss of stress fibers and formation of lamellipodia.

4.4. Phosphorylation of palladin in Src CA-transfected cells

In order to characterize the interplay between palladin and Src in more detail, we also tested whether palladin is phosphorylated in a Src-dependent manner, as previously shown for SPIN90. Co-expression with SrcCA resulted in tyrosine phosphorylation of palladin, as verified by blotting of immunoprecipitated palladin with phospho-tyrosine antibody. Whether palladin is a direct Src substrate remains to be tested, and the phosphorylated tyrosine residues need still to be identified. However, this is the first published result showing that palladin is indeed phosphorylated on tyrosine, and that it also identifies Src as a potential effector kinase. Figure 6 and table 1 give a summary of the known palladin interactions.

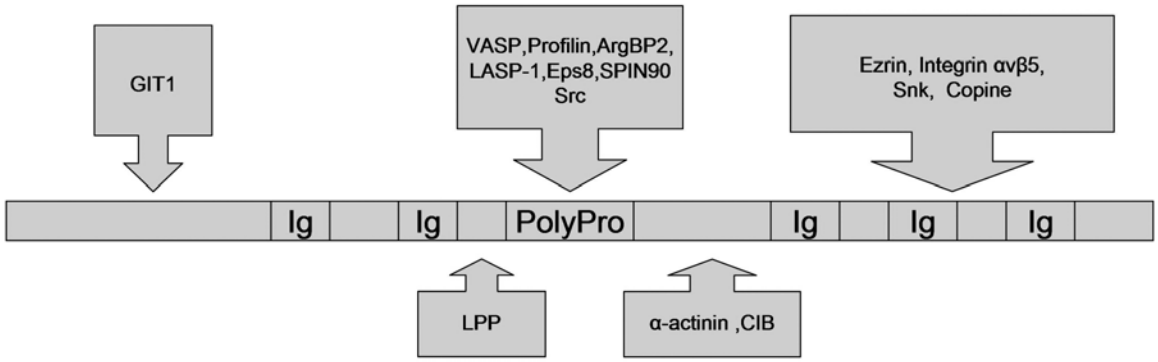

Figure 6. Palladin-interacting proteins and the rough localization of the interaction sites are summarized in the figure.All the interacting sequences identified in the shorter palladin isoforms are included in the longest 5Ig isoform.

Table 1. Palladin-interacting proteins

The published palladin-interacting proteins and some identified but unpublished binding partners and their binding sites in palladin.

Interacting protein	Interaction site in palladin	Reference
α-actinin	between amino acids 222-280, human 3Ig isoform	Parast & Otey, 2000 Rönty et al.,2004
VASP	FPXPP motifs in mouse 3Ig and 4Ig isoforms	Boukhelifa et al., 2004
Ezrin	2 carboxy-terminal IgC2 domains of human palladin	Mykkänen et al., 2001
ArgBP2	amino-terminal poly-proline sequence of human 3Ig isoform	Rönty et al., 2005
Profilin	amino-terminal poly-proline sequences of human and mouse 3Ig isoforms	Boukhelifa et al., 2006
Lipoma-preferred partner (LPP)	between amino-acids 1-81, mouse 3Ig isoform	Jin et al.,.2007
Eps8	amino-terminal poly-proline sequence of mouse 3Ig isoform	Goicoechea et al.,.2006
LASP-1	amino-terminal poly-proline sequence of mouse 4Ig isoform	Rachlin et al., 2006
Integrin αvβ5	between amino acids 298-772 of human 3Ig isoform	Lai et al., 2006
SPIN90 and Src	amino-terminal poly-proline sequences of human 3Ig isoform	Rönty et al., 2007
Arp2/3, ABP280	?	Luo et al., 2005 (unpublished)
CIB, Snk, Copine	between amino acids 222-772 of human 3Ig isoform	Rönty et al. (unpublished)
GIT1	amino terminus of human 5Ig isoform	Rönty et al. (unpublished)

5. Palladin expression is regulated in an isoform-specific manner during TGF-β1-induced myofibroblast differentiation (V)

5.1. Characterization of isoform-specific palladin antibodies

Previous studies on both humans and especially on mice have shown that palladin is expressed as multiple differentially sized isoforms (Mykkanen et al., 2001; Parast and Otey, 2000; Rachlin and Otey, 2006). The two major isoforms are called 3Ig (90kDa) and 4Ig (140kDa), since they contain 3 and 4 IgC2 type domains, respectively. According to previous results, both isoforms are expressed in U251 human glioma cells, and therefore U251 mRNA was used as a template to clone the 5'-sequence extension of the human 4Ig homologue by RT-PCR. In order to study the expression and localization of the various palladin isoforms, we raised two polyclonal antibodies against different palladin polypeptides. For antibody 3Ig (Ab-3Ig), a proline-rich amino-terminal segment of the Ab023209 sequence was used as antigen. The 4Ig (Ab-4Ig-Hu) antigen is the cloning product of the RT-PCR reaction described above. The reactivity/specificity of the new antibodies was verified by Western blot analysis of both native and palladin-transfected cells and by comparing the results obtained with a panel of previously described antibodies (Ab-4Ig-Mo, Ab953 and a mixture of palladin mAbs [4D10, 7C6, 9C12, and 1E6]). Both new antibodies recognized the transfected protein containing the specific antigen sequence and no cross-reactivity was detected. In U251 cells the antibodies reacted with several protein bands, one of which (140 kDa) was recognized by all antibodies used, thus verifying that the 4Ig sequence is expressed and recognized by the new antibodies. The previously described 3Ig (90 kDa) major isoform was recognized by Ab-3Ig, Ab953, and the mAb, but not by either 4Ig antibody. In conclusion, Ab-3Ig recognizes both the 3Ig and 4Ig isoforms, whereas the 4Ig antibodies react with the 4Ig but not the 3Ig isoform. In U251 cells the antibodies detected also some additional bands, which probably present additional differentially spliced transcripts. The immunostaining patterns of the 3Ig and 4Ig were similar in these cells.

5.2. Regulation of palladin expression during myofibroblastic differentiation

As many cytoskeletal proteins are up-regulated during TGF-β1-induced myofibroblast differentiation (Malmstrom et al., 2004), we set out to study whether this is the case also for palladin. First, we stained normal dermal fibroblasts with Ab-3Ig and Ab-4Ig-Hu antibodies. Ab 3Ig stained stress-fiber-dense regions and focal adhesions of fibroblasts, whereas Ab 4Ig-Hu did not show specific reactivity. When the cells were treated with TGF-β1 (5 ng/ml) for 12–96 hours, the staining pattern of the 3Ig isoform remained similar, but the staining became more intense. After the treatment, also Ab-4Ig-Hu showed a distinctive punctate stress-fiber staining, thus indicating that TGF-β1 induces neo-expression of the 4Ig isoform. As shown in numerous previous studies, SMA was also upregulated during the myofibroblastic differentiation (Desmouliere et al., 1993) and it co-

localized with palladin in stress fibers. Western blots of fibroblasts at different time points during the TGF-β1 treatment gave a similar result: upregulation of the 3Ig isoform and neo-expression of the 4Ig isoform. The 4Ig isoform was rapidly up-regulated by about 24 hours before the induction of α-SMA.

5.3. TGF-β1 -induced palladin expression is mediated by Smad3, p38, and ERK1/2

In order to investigate the role of the Smad and MAPK signaling cascades in the regulation of palladin expression, we utilized an adenoviral gene delivery method. An adenovirus construct of Smad3 (RAdSmad3) was used to achieve overexpression of Smad3. Dominant-negative Smad3 (RAdSmad3DN) and inhibitory Smad7 (RAdSmad7) were applied to inhibit Smad signaling. Fibroblasts were infected with the different Smad adenoviruses and treated with TGF-β1 (5 ng/ml) for 24 h. Over-expression of Smad3 enhanced the TGF-β1 -induced palladin expression, whereas the inhibitory Smad3DN and Smad7 had an opposite effect. The expression pattern of SMA was similar to that of palladin.

TGF-β1 has been shown to activate both ERK1/2 and p38 MAPK pathways in fibroblasts, and there is considerable cross-talk between the Smad and MAPK signaling cascades (Leivonen et al., 2002; Leivonen et al., 2005). Taking this into account, we checked whether ERK1/2 and p38 MAPK pathways also play a part in the regulation of palladin expression. We therefore used specific chemical inhibitors of MEK1 (the upstream activator of ERK1/2) and p38. Both PD98059, which inhibits MEK1, and SB203580, an inhibitor of p38, downregulated TGF-β1-induced palladin expression, implying that both members of the MAPK signaling cascades are involved in mediating the effects of TGF-β1 on palladin gene expression. Similar effects were also observed for SMA.

Finally, simultaneous effects of activation/inhibition of the Smad and MAPK pathways were studied. Fibroblasts were infected with different combinations of adenoviruses encoding Smad3, Smad4, wild-type p38α (RAdp38α) and constitutively active MKK3b (RAdMKK3bE), an upstream activator of p38. Activation of p38 by MKK3b induced the expression of palladin 4Ig, and this effect was further enhanced by Smad3. In addition, activation of ERK1/2 with co-expression of Smad3 resulted in marked induction in palladin 4Ig isoform expression, and this effect was further enhanced by co-expression of constitutively active MKK3b. Together, these results imply that the interplay between ERK1/2, p38, and Smad3 signaling plays an important role in regulating palladin gene expression.

5.4. Palladin is expressed in experimental rat dermal wounds

To verify the *in vitro* results *in vivo*, we used an experimental rat dermal wound model. Frozen sections of 3-, 6- and 9-day-old granulation tissue from rat dermal wounds were

stained with SMA, palladin 4Ig (Ab-4Ig-Mo) and palladin 3Ig antibodies. Palladin 4Ig was not expressed in the early stages of granulation tissue formation (3 days) but neo-expression by the fibroblasts was detected at day 6 post wounding. At this stage the fibroblasts were still negative for SMA and thus represent the proto-myofibroblast phenotype (see section on myofibroblasts). At day 9, the fibroblasts were already differentiated into mature myofibroblasts and expressed both SMA and palladin 4Ig. The findings are in agreement with the *in vitro* results, and verify that palladin is indeed up-regulated during myofibroblast differentiation.

5.5. Analysis of palladin expression *in vivo* in human tissue samples

The *in vivo* expression of palladin was also studied by immunohistochemical staining of human tissue specimens. For this purpose we chose samples from conditions associated with myofibroblastic differentiation, including fresh dermal scar and nodular fasciitis (Dayan et al., 2005). Subsequent sections of each entity were stained for α-SMA, desmin, and with two palladin antibodies: Ab-4Ig-Mo and Ab-3Ig. A clear correlation between α-SMA and palladin stainings could be detected, whereas desmin did not stain the myofibroblasts. In normal dermis the fibroblasts did not express palladin, desmin, or α-SMA. A myofibroblastic breast tumor was also stained with this antibody panel with similar results. Interestingly, the normal ductal myoepithelial cells included in the specimen showed a clear positivity for palladin 4Ig (Figure 7). This unpublished result further strengthens the correlation between palladin 4Ig and α-SMA expression, since the myoepithelium is also positive for α-SMA.

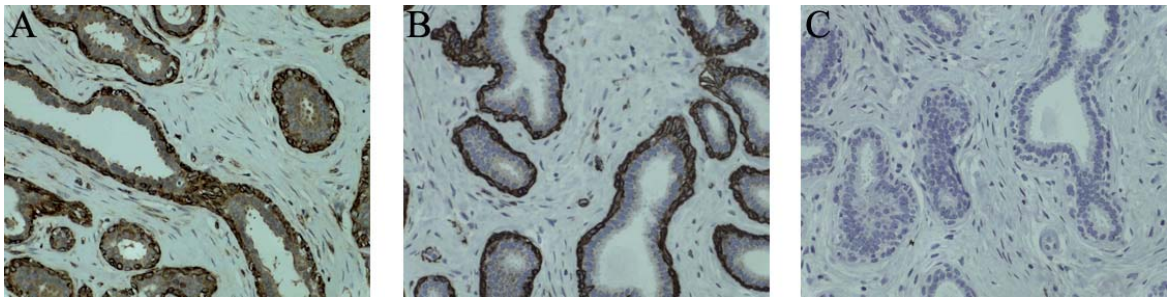

Figure 7. Breast tissue stained with A) palladin, B) SMA and C) desmin antibodies.Palladin and SMA are expressed by the myoepithelial cells lining the ductules, whereas desmin staining is negative.

Concluding remarks

This study describes the identification and initial characterization of human palladin, a microfilament-associated protein. Together with myotilin and myopalladin, palladin forms a novel cytoskeletal Ig-domain protein family. Although the family members share similarities, such as sub-cellular localization and interaction with α-actinin, there are also important differences among them. Unlike myotilin and myopalladin, palladin's expression is not limited to striated muscle, and it is ubiquitously expressed. Palladin has a very complex genetic structure giving rise to multiple differentially spliced transcripts with differing structure, tissue distribution, binding partners, and most probably function. Knock-down experiments and the generation of palladin knock-out mouse have shown that palladin has an important role in the organization and stabilization of actin stress fibers and possibly other actin-based structures, such as podosomes. It seems to play an important role in cell migration as well. Expression of palladin can be regulated by multiple mechanisms, and it seems that palladin is usually up-regulated in situations requiring a more organized actin cytoskeleton, for example, during activation of astrocytes in glial scar formation and during myofibroblast differentiation.

In this study we identified novel interactions between palladin and ezrin, ArgBp2, SPIN90 and Src. Furthermore, the previously identified interaction with α-actinin was characterized in more detail. The interaction with α-actinin probably serves to enhance the stability of the stress fibers and other actin-based structures where these two proteins co-exist. The interaction also seems to have a two-way targeting function, meaning that both proteins can target each other to the right location.

Both palladin and α-actinin probably act as molecular scaffolds that link proteins with different functional modalities to the cytoskeleton. The function of the other identified interactions is still mostly unknown. Ezrin, ArgBP2 and SPIN90 are all involved in actin cytoskeleton regulation and interact also with effectors such as kinases. Ezrin links plasma membrane to the cytoskeleton, and the interaction with palladin may enhance this linkage. Contrary to what was suggested in the intial study, ezrin and palladin co-localize and probably interact mostly in the lamellipodia of migrating cells and not in the stress fibers, since ezrin only rarely localizes to these structures. SPIN90 and ArgBP2 are scaffolding proteins like palladin, and the interactions may serve to target these proteins to specific locations such as Z-disc in the case of ArgBP2. The interaction between Src and palladin is interesting and warrants further characterization. Already in the initial report by Parast and Otey, palladin was considered to be a phosphoprotein, but no experimental data to verify this has been presented. We showed that palladin is indeed phosphorylated on tyrosine in cells expressing active Src, but here again the function of this phenomenon remains to be seen.

The finding that has probably the most direct clinical implications is the up-regulation of palladin expression in myofibroblast differentiation. Palladin expression correlates closely with SMA, which is a known mediator of myofibroblast contraction and thus implicated in tissue remodeling in fibrotic lesions. Palladin is probably also involved in myofibroblast contraction via its role in stress fiber formation and stabilization. We

showed that palladin expression is mediated both by SMAD and MAPK pathways; this is also true for SMA. The inhibition of these signaling cascades is a potential therapeutic target of anti-fibrotic treatment. In this sense the elucidation of palladin's role in conditions such as idiopathic pulmonary fibrosis is particularly interesting.

So far palladin mutations/SNPs have been detected in two disease conditions. This list will probably grow in the future, and also the function of the already identified mutations will be investigated. Since the complete genetic knock-out of palladin results in the death of the developing embryos, the generation of a conditional knock-out mouse model is surely going to be informative.

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