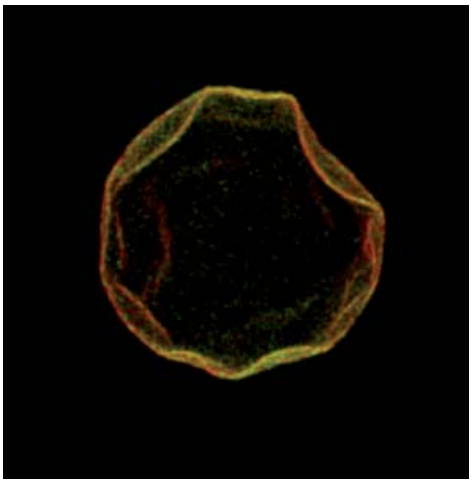
DISSERTATIONES BIOCENTRI VIIKKI UNIVERSITATIS HELSINGIENSIS

25/2011

ANETTE PYKÄLÄINEN

Generation of Flat and Curved Membranes by Inverse-BAR Domain Proteins



INSTITUTE OF BIOTECHNOLOGY AND
DEPARTMENT OF BIOSCIENCES
DIVISION OF GENETICS
FACULTY OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES AND
VIIKKI DOCTORAL PROGRAMME IN MOLECULAR BIOSCIENCES
UNIVERSITY OF HELSINKI

Generation of Flat and Curved Membranes by Inverse-BAR Domain Proteins

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ACADEMIC DISSERTATION

To be presented with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki for public examination in the auditorium 1041 of Biocenter 2, Viikinkaari 5, Helsinki on November 25th, 2011, at 12 o'clock noon

HELSINKI 2011

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Cover figure: A giant unilamellar vesicle (GUV) with ~ 30 μm radius. The flat indentations on the surface of this GUV are produced by the BAR domain of Pinkbar. In this merged capture BAR-mCherry is shown in red and NBD-labeled phosphatidylcholine in green.

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ISBN 978-952-10-7344-1 (Paperback)
ISBN 978-952-10-7345-8 (PDF)
ISSN: 1799-7372
Press: Unigrafia, Helsinki 2011


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TABLE OF CONTENTS

ABBREVIATIONS

ABBA	actin binding protein with BAIAP homology/MTSS1l
ANTH	AP180 N-Terminal Homology
APPL	adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing BAR domain protein
Arp	actin related protein
ATP	adenosine triphosphate
BAR	BIN/Amphiphysin/Rvs
CALM	clathrin assembly lymphoid myeloid leukemia
Cdc42	cell division control protein 42 homolog
CIP4	Cdc42 interacting protein 4
COP	coat protein
CRIB	Cdc42 and Rac1 interactive binding
DNA	deoxyribonucleic acid
DPH	1,6-diphenyl1,3,5-hexatriene
EHD	Eps15 homology domain
EHEC	enterohemorrhagic *Escherichia coli*
EM	electron microscopy
ENTH	Epsin N-terminal homology domain
ER	endoplasmic reticulum
ET	electron tomography
F-BAR	FHC BAR
FBP17	formin binding protein 17
FCCS	fluorescence cross-correlation spectroscopy
FCH	Fps/Fes/Fer/CIP4 homology domain
Fer	a tyrosine kinase
Fes/Fps	feline sarcoma/fujinami poultry sarcoma tyrosine kinase
FLJ22582	BAI1-associated protein 2-like 2 mouse gene name
GAP	GTPase activating protein
GDP	guanosine diphosphate
GEF	GDP/GTP exchange factor
GFP	green fluorescent protein
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
GUV	giant unilamellar vesicle
IC	intermediate compartment
IF-BAR	inverse F-BAR
I-BAR	inverse BAR domain
IRSp53	insulin receptor tyrosine kinase substrate protein of 53
IRTKS	insulin receptor tyrosine kinase substrate
kDa	kilodalton
Ld	liquid-disordered
Lo	liquid-ordered
MIM	missing-in-metastasis
mRNA	messenger RNA

NBD	7-chloro-4-nitrobenz-2-oxa-1,3-diazole
NMR	nuclear magnetic resonance
PC	phosphatidylcholine
PDGF	platelet-derived growth factor
PDZ	postsynaptic density-95 (PSD 95)/discs large/zona occludens-1
PH	pleckstrin homology
PI	phosphatidylinositol
$PI(4,5)P_2$	phosphatidylinositol 4,5-biphosphate
$PI(3,4,5)P_3$	phosphatidylinositol 3,4,5-triphosphate
PICK-1	protein interacting with C kinase 1
PE	phosphatidylethanolamine
PS	phosphatidylserine
PSD	postsynaptic density
PTPδ	protein tyrosine phosphatase δ
PX	phox homology domain
Rab	ras-related protein (originally cloned from rat brain)
Rac	ras-related C3 botulinum toxin substrate
REEP	receptor accessory protein
RHD	reticulon homology domain
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH3	src-homology 3
SM	sphingomyelin
SNX9	sortin nexin 9
Src	sarcoma
TEM	transmission electron microscopy
WAVE2	WASP family verprolin homologous
WASP	Wiscott-Aldrich syndrome protein
WH2	WASP family homology domain 2
Å	ångström

LIST OF ORIGINAL PUBLICATIONS

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

I Mattila PK, Pykäläinen A*, Saarikangas J*, Paavilainen VO, Vihinen H, Jokitalo E, Lappalainen P. 2007. Missing-in-metastasis and IRSp53 deform PI(4,5)P2-rich membranes by an inverse BAR domain-like mechanism. *J Cell Biol* **176**, 953-64.

II Saarikangas J*, Zhao H*, Pykäläinen A, Laurinmäki P, Mattila PK, Kinnunen PK, Butcher SJ, Lappalainen P. 2009. Molecular mechanisms of membrane deformation by I-BAR domain proteins. *Curr Biol* **19**, 95-107.

III Pykäläinen A, Boczkowska M, Zhao H, Saarikangas J, Rebowski G, Jansen M, Hakanen J, Koskela EV, Peränen J, Vihinen H, Jokitalo E, Salminen M, Ikonen E, Dominguez R, Lappalainen P. 2011. Pinkbar is an epithelial-specific BAR domain protein that generates planar membrane structures. *Nat Struct Mol Biol* **18**, 902-7.

* Equal contribution

Some unpublished data are also presented.

SUMMARY

Biological membranes are tightly linked to the evolution of life, because they provide a way to concentrate molecules into partially closed compartments. The dynamic shaping of cellular membranes is essential for many physiological processes, including cell morphogenesis, motility, cytokinesis, endocytosis, and secretion. It is therefore essential to understand the structure of the membrane and recognize the players that directly sculpt the membrane and enable it to adopt different shapes. The actin cytoskeleton provides the force to push eukaryotic plasma membrane in order to form different protrusions or/and invaginations. It has now became evident that actin directly co-operates with many membrane sculptors, including BAR domain proteins, in these important events. However, the molecular mechanisms behind BAR domain function and the differences between the members of this large protein family remain largely unresolved.

In this thesis, the structure and functions of the I-BAR domain family members IRSp53 and MIM were thoroughly analyzed. By using several methods such as electron microscopy and systematic mutagenesis, we showed that these I-BAR domain proteins bind to $PI(4,5)P_2$-rich membranes, generate negative membrane curvature and are involved in the formation of plasma membrane protrusions in cells e.g. filopodia. Importantly, we characterized a novel member of the BAR domain superfamily which we named Pinkbar. We revealed that Pinkbar is specifically expressed in kidney and epithelial cells, and it localizes to Rab13-positive vesicles in intestinal epithelial cells. Remarkably, we learned that the I-BAR domain of Pinkbar does not generate membrane curvature but instead stabilizes planar membranes. Based on structural, mutagenesis and biochemical work we present a model for the mechanism of the novel membrane deforming activity of Pinkbar.

Collectively, this work describes the mechanism by which I-BAR domain proteins deform membranes and provides new information about the biological roles of these proteins. Intriguingly, this work also gives evidence that significant functional plasticity exists within the I-BAR domain family. I-BAR proteins can either generate negative membrane curvature or stabilize planar membrane sheets, depending on the specific structural properties of their I-BAR domains. The results presented in this thesis expand our knowledge on membrane sculpting mechanisms and show for the first time how flat membranes can be generated in cells.

1 INTRODUCTION

1.1 Biological membranes

Biological membranes act as semi-permeable barriers that divide the cell into many compartments and, most importantly, isolate the cell from its surroundings. This enables the cell to control its biochemical functions and block the entry of any unwanted intruders. Certainly, it is of uttermost importance that these different compartments can change information at very fast pace. Therefore, a well-controlled transport system has developed to exchange material between these compartments. It is also interesting to note that structurally all biological membranes have at least three things in common; 1) Integral proteins are always associated with them, 2) Through lipid asymmetry, biological membrane compartments appear to have generally positive curvatures, and 3) Soluble cytoplasmic proteins help membranes to adopt different shapes, such as protrusions and invaginations, through non-covalent interactions.

1.1.1 Lipid composition of biological membranes

Lipids are the main components of biological membranes, and constitute to ~50 % of the mass of these structures. The remarkable diversity of lipids is the key feature of biological membranes. Different biological membranes of a eukaryotic cell are mainly composed of glycerophospholipids, sphingolipids, and sterols. The main sterol in mammalian cell membranes is cholesterol (Alberts et al., 2008).

The main class of membrane lipids is glycerophospholipids. They are generally composed of two hydrophobic fatty-acyl chains covalently attached to a glycerol backbone. The third carbon molecule is usually connected to a phosphate group joined to a hydrophilic and polar head group **(Figure 1)**. By combining several different fatty acids and head groups, cells are able to synthesize a vast number of different glycerophospholipids. The most abundant ones in mammalian cell membranes are phosphatidylcholines (PC), phosphatidylethanolamines (PE), and phosphatidylserines (PS). Also the hydrocarbon chains of fatty acids differ in length and degree of saturation, and vary between different glycerophospholipids. This backbone renders all lipid molecules in the biological membranes amphiphilic **(Figure 1)** (Kinnunen, 1991).

Shingolipids are defined by their long amino-alcohol sphingosine backbones. The amino group of sphingosine forms an amide bond with a fatty acyl group. These molecules are known as ceramides. The backbones are synthesized in the ER and further modified to give rise to the large family of sphingolipids. Additionally, more than 500 different carbohydrate structures can be added to these ceramide backbones to create the group of glycosphingolipids. Altogether, sphingolipids play significant roles in membrane biology and provide large amounts of metabolites that regulate cell function (Futerman and Hannun, 2004; Gault et al., 2010). Cholesterol has a stiff four-ring structure with 17 carbon atoms, to which an iso-octyl side chain and two methyl groups are attached (Blom et al., 2011). In mammals cholesterol is highly enriched at the plasma membrane depending on the cell type. It provides additional support and regulates membrane fluidity through its rigid structure. Furthermore, cholesterol participates in several membrane trafficking and trans-membrane signaling processes (Ikonen, 2008).

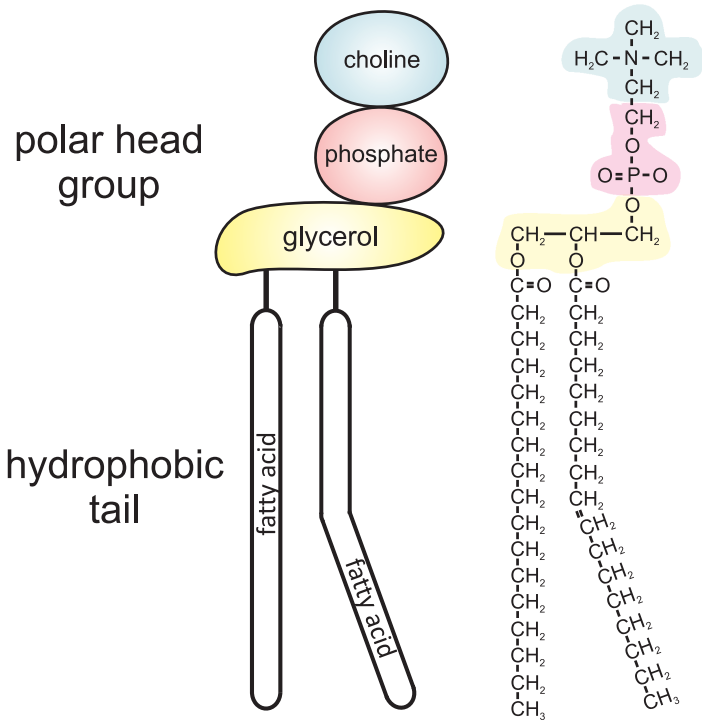

Figure 1. A schematic representation of a glycerophospholipid (left) and the molecular structure of phosphatidylcholine (right). The polar head group is hydrophilic, while the fatty acid chains are hydrophobic, rendering the molecule amphiphilic. Double bonds within the fatty acid chains induce kinks to the chain, which has an influence on the membrane fluidity.

The main force that shapes a bilayer from a mixture of amphipathic lipids is the hydrophobic force. Lipids form bilayers and micelles spontaneously, and to minimize their contact with water they face their hydrophilic head-groups towards it. This principle also applies to the insertion of membrane proteins into the bilayer (Vance and Vance, 2008). Lipids try to organize at an energy minimum by balancing the hydrophobic effect and the repulsive force of adjacent head-group association. The ability of different lipids to pack into many unique structural compositions is referred as lipid polymorphism (Frolov et al., 2011).

1.1.1.1 The lipidome and its research

Despite our increasing knowledge of the various roles that lipids play in the membranes, we are still puzzled over the extent of this diversity. Certainly, there must be certain basic principles behind lipid polymorphism and a reason why a cell would spend vast amounts of energy to sustain it? The amount of lipid species seems to be directly correlated to the complexity of the cell; prokaryotic cells contain only around 100 different lipids while different membranes of a eukaryotic cell can occupy over 1000 different lipid species (Kinnunen, 1991). The simplicity of their cellular compartments and the paucity of lipid traffic might provide the easiest explanation to this

observation. The other obvious reason is system redundancy. If there would be perturbation in a pathway leading to the synthesis of a certain type of lipid, another, similar lipid could substitute its position and function at least temporally until the problem is fixed. It might also be that the lipid complexity has more of a structural role in preventing leakage from protein-lipid interfaces (Contreras et al., 2011). Different lipids can also interact with different membrane proteins and enzymes to fine-tune their functions (Dowhan, 1997).

Any single eukaryotic membrane may contain more than 100 individual lipid species. Lipid composition in eukaryotic plasma membrane may vary significantly depending on the cellular functions, stage of differentiation, and the cell type in question. New techniques, such as shotgun mass spectrometry, have recently been used to identify an unprecedented number of different lipid species isolated (more than 300) from a single sample of 10^5 cells with one experiment (Sampaio et al., 2011). These data are becoming to be more available in different lipid databases such as The Lipid Composition Atlas (http://opm.phar.umich.edu/atlas.php).

Many methods applied to the study of proteins or genes do not convert as such to study lipids and the membranes they form. For example, a genetic mutation does not directly result in a mutated phospholipid. Instead the mutation has to be made in the biosynthetic pathway leading to a particular phospholipid, and by mutating a member of a pathway one might accidentally change many cellular processes (Dowhan, 1997). For these and other reasons the field of lipid research has been overrun by genomics and proteomics. However, in recent years membrane research has gained more interest. An increasing number of previously unsolvable membrane protein structures are being solved, the field of lipid research is rapidly expanding, and the principles of membrane organization are being revisited (Simons and Sampaio, 2011).

1.1.1.2 Lipid functions in cells

Lipids actively participate in a number of specialized functions, which also dictates their location to a certain place at a certain time. Lipids and fats are very abundant energy storage molecules. Some lipids are important for morphological reasons. They stabilize particular regions of the bilayer such as those of high curvature, or bilayers involved in the formation of a tight junction with another membrane. Certain lipids accumulate to the sites of the enzyme activities that they control. Lipids also modulate the localization and function of constitutively membrane associated proteins. Additionally, they are thought to recruit soluble proteins to membranes by serving as temporally and spatially regulated markers for lipid binding cytosolic proteins (Blom et al., 2011). Especially phosphatidylinositol (PI) and its phosphorylated analogs are involved in many regulatory functions in the cell. The sites of active membrane remodelling, such as phagocytotic sites and membrane ruffles, have been reported to have high local concentrations of PI(4,5) P_2, (Saarikangas et al., 2010).

1.1.2 Membrane bilayer structure – from the 19th century to current perspectives

Although membranes have been studied for more than a hundred years, little is still known about the complex organization and structure of membranes of living cells. However, current super-resolution techniques allow us for the first time to visualize membrane structures and their spatio-temporal dynamics in living cells. To be able to understand biological

membranes one should know at least a part of their history (Edidin, 2003a).

The interest towards studying oils and fats in nature started as early as 1880´s. During the late 19th century, cells were known to be surrounded by an outer layer of protoplasm of unknown composition and properties (Overton's Concept of a Cell Membrane 1899, Arnost, 2008). Around twenty years later Gorter and Grendel used the Langmuir´s device, by which one can control the spreading of oil on a water surface, to discover that the surface of a red blood cell is in fact a layer of fatty substances two molecules thick (Gorter and Grendel, 1925). It took many years before the first electron micrographs from cells were taken with high enough resolution to visualize the ~75-Å-thick cellular bilayer. After seeing that this similar dark double-line also surrounded other compartments of the cell, J. David Robertson was convinced that all cellular organelles share this common membrane structure (Robertson, 1959).

At that time, rapidly developing methods such as nuclear magnetic resonance (NMR) and electron spin resonance spectroscopy were able to provide much more information concerning the movement of lipids within membranes. Scepticism about the bilayer-model lasted for more than a decade before it was finally accepted as the model for the basic structure of cell membranes. During this time several laboratories concluded that membrane lipids behave as a fluid within the membrane and that, besides the lipids, it also contains many integral proteins that are free to float around in the sea of lipids (Chapman, 1975). This model, known as the fluid mosaic model, was made famous by Singer and Nicolson in 1972 and it was widely accepted by the field (Singer and Nicolson, 1972).

Soon, it was observed that lipids may not be randomly distributed in the bilayer, and the fluid mosaic model became insufficient in explaining this result. Also certain local order, visualized in model membranes, could not be explained with the Singer & Nicolson model which stated that all molecules are able to move freely within the bilayer (Jain and White, 1977). The 1990s was the golden era of the "lipid raft" model. The model proposes that specific lipids which are about to be sorted into certain membrane traffic routes, segregate into rafts which are rich in sphingolipids and cholesterol. These rafts can be created because membrane lipids can exist in multiple possible phase states depending on their structure. Solid-like phases are composed of long, saturated hydrocarbon chains like those in SM. The glycerophospholipids, on the other hand, have a higher content of unsaturated hydrocarbon chains and tend to be enriched in liquid phases. Interestingly, the abundant plasma membrane lipids PC and cholesterol can adopt two coexisting fluid phases: liquid-ordered (Lo) and liquid-disordered (Ld). Thus, plasma membrane has high order with reduced lateral mobility but also the high translational mobility of a liquid phase (Recktenwald and McConnell, 1981). The rafts are considered as platforms for the intracellular traffic of lipids and lipid-anchored proteins involved in many cellular functions, the most important of which may be signalling (Brown and Rose, 1992; Simons and Ikonen, 1997). Despite extensive work, the basis for raft formation in cell membranes as well as their size and stability are still uncertain.

The lipid raft model fails to explain the considerable differences seen between model membrane systems and *in vivo* data, as well as to give an answer to certain equally important questions: What are the dynamics of the lipids at a certain site, phase or time? What controls

the membrane trafficking and prevents it from disrupting the raft order? How is the cytoskeleton linked to the membrane? What kind of role does the lipid asymmetry between the two leaflets have? And finally, what is causing or stabilizing the membrane curvature needed for a multitude of cellular processes (Edidin, 2003b)?

Fortunately, new state-of-the-art methods such as super-resolution microscopy, single-particle tracking, and fluorescence cross-correlation spectroscopy (FCCS) have shed some light onto these questions and provided a new model for plasma membrane structure. This new model suggests that most plasma membrane-associated proteins are clustered into islands attached to the cytoskeleton. These protein islands are separated by low cholesterol and protein-free membrane. Besides the different structural characterization, the model also implies a new role for plasma membrane as a spatio-temporal regulator of signal transduction (Lillemeier et al., 2006; Lillemeier et al., 2010).

To conclude, our concept of biological membranes has dramatically changed from the fluid mosaic model to the latest model where all membrane-bound proteins are gathered together to form functional protein-lipid islands, which are connected to the underlying cytoskeleton.

1.1.3 Biological membranes are dynamic and interconnected through vesicular and tubular trafficking

Lipids are not randomly distributed within the cellular organelles. Both lipids and proteins undergo membrane-associated trafficking to allow eukaryotic cells to sustain membrane homeostasis between different cellular compartments (see the following chapter) during intracellular transport (van Meer et al., 2008). For example, the amount of sphingolipids in membranes is increased by 30% along the secretory route from the the endoplasmic reticulum (ER) to the plasma membrane. The target membrane of sphingolipids in this case is the plasma membrane and sphingolipids are depleted from retrograde carriers which are recycled back to ER (van Meer and Lisman, 2002; Klemm et al., 2009). However, it is still poorly understood, how these compositional differences between cell organelles are maintained.

Lipid sorting differs from protein sorting. While many cargo proteins are delivered as bound to their receptors, lipids use other means for their sorting. The best studied mechanism of lipid sorting is the formation of lipid rafts through lateral segregation. The rafts are thought to contain sorting proteins, and through protein sorting also the lipids – mainly sphingolipids and cholesterol – would get transported (Simons and van Meer, 1988; Lingwood and Simons, 2010). Another way to sort lipids is by coupling lipid composition to the energy used for membrane curvature. This mechanism suggests that the local membrane curvature could produce a non-homogenous lipid distribution to minimize the energy used by the cell for bending the membrane (van Meer and Lisman, 2002; Maxfield and McGraw, 2004). Recent findings point out that sorting indeed occurs during the formation of highly curved transport intermediates; vesicles and tubules (Klemm et al., 2009; Maxfield and McGraw, 2004).

1.1.4 The shapes of membranous compartments in eukaryotic cells

Membranous compartments in cells are called organelles. The lipid bilayer, which forms a semi-permeable barrier between the lumen of the organelle and the cell cytoplasm, separates an

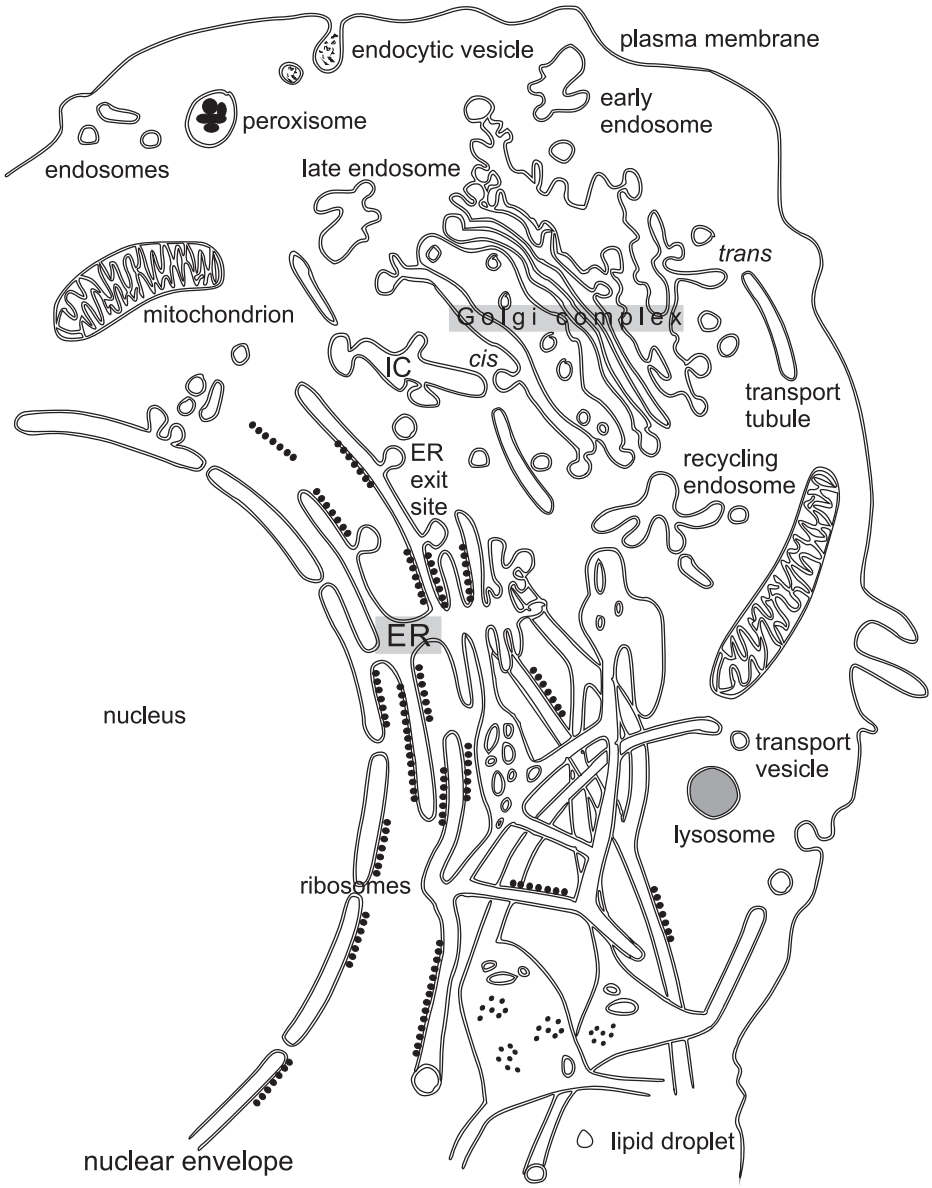

Figure 2. A cartoon showing the membranous organelles of an animal cell. See text for details.

organelle from its surroundings. Lipid droplets are the only known organelles surrounded with a lipid monolayer. These microenvironments make it easier for the cell to compartmentalize its biochemical functions. pH, ionic conditions, lipid species, and the shape of the organelles can be efficiently regulated within these compartments. Depending on motility, cell type or the phase of the cell cycle, all eukaryotic organelles can modify the shape of their membrane bilayer. For example, a planar membrane can be bent, or a tubule may be drawn out of a flat membrane. Also membrane fusion and fission can rapidly change the membrane shape (Shibata et al., 2009).

In textbooks, membranes are usually described as planar structures. This oversimplifies the actual image of biological membranes visualized in living cells. Cell membranes can form complex structures, as seen in the inner membrane of a mitochondrion or in the plastic ER. The shapes of cellular organelles vary from flat and fenestrated sheets to tubules, spheres, and branch-points **(Figure 2)**. However, when viewed from a close distance, the majority of biological membranes tend to be planar and lamellar. The reasons behind this are not well understood (Simons and Sampaio, 2011). One of the biggest challenges in modern membrane biochemistry is to understand how curvature can be generated from a planar membrane and how the stable planar membranes are maintained.

An average eukaryotic cell is thought to have at least 15 unique organelle types. The size and amount of each organelle type varies depending on the cell type. These organelles can be artificially subdivided into groups according to their shape. However, this categorization is a generalization because the majority of the organelles simultaneously display many shapes. Lysosomes (Luzio et al., 2007), multivesicular bodies (which contain many small vesicles)(Fader and Colombo, 2009), and lipid droplets (Reue, 2011) are the only spherule-shaped organelles found in a typical eukaryotic cell. Peroxisomes and transport vesicles, which are also usually placed in this category, are more tubule-shaped *in vivo* (Maxfield and McGraw, 2004; Klemm et al., 2009; Schrader et al., 2000; Baker et al., 2010). Mitochondria are composed of two distinctly different membranes; a surrounding outer membrane and an inner membrane, which forms tubular invaginations called *cristae* (Mannella, 2008). The ER is composed of a sheet-like nuclear envelope, as well as planar sheets of varying morphology and of branched tubular (three-way) networks (Puhka et al., 2007; Palade, 1956; Voeltz et al., 2006). A stack of perforated sheets is a proper description for the Golgi complex (Egea et al., 2006). The other major recycling hub in cells in addition to the Golgi complex is the recycling endosome. These hubs have the ability to bud off small vesicles and tubules and to fuse with them. The irregular-shaped and tubular intermediate compartment (IC) and the similarly shaped early and late endosomal compartments sort the incoming or leaving material prior or after the major hubs (Williams and Urbe, 2007; Saraste et al., 2009) **(Figure 2)**. All the above mentioned compartments communicate with one another throughout the cell by vesicular and tubular trafficking (see the previous chapter for more details).

Membrane shaping functions are inseparable from membrane curvature generation (McMahon and Gallop, 2005; Zimmerberg and Kozlov, 2006). In the context of cellular organelles high membrane curvature can be found in cross-sections of narrow tubules or in small transport vesicles. It is also present at the edges of closely spaced membrane sheets and at the nuclear pores. ER, nuclear envelope and the Golgi complex are mostly planar with low membrane curvature. It is still poorly understood how the characteristic shape of an organelle is achieved (Shibata et al., 2009).

1.2 Membrane curvature

The physiology of the cell requires that the dynamics and structure of different membrane shapes are properly maintained throughout the entire lifespan of the cell. Therefore, it is crucial to understand the molecular mechanisms

behind the generation of variable membrane curvatures and characteristic organelle shapes (Kozlov, 2010). When reading the following chapters it is good to keep in mind that membrane shaping is always tightly linked to the generation of membrane curvature (McMahon and Gallop, 2005; Zimmerberg and Kozlov, 2006). In addition to generation of membrane protrusions and invaginations, membrane shaping processes include membrane fusion, fission, and pore formation. These latter membrane remodelling mechanisms are not within the scope of this thesis and will not be further discussed here (for a recent review see Kozlov et al., 2010).

Membrane bilayers on their own are relatively thin (4-8 nm) structures (Hristova et al., 2001; Nagle, 2000). The thickness varies depending on the lipids that are present. For example liquid-ordered membranes are thicker than liquid-disordered membranes, and therefore also stiffer (Rawicz et al., 2008; Roux et al., 2005). A majority of the cellular membranes are relatively planar, most likely because generation of curvature requires energy. The reason why membranes resist bending is quite simple; changes in membrane topology create tension in the lipid bilayer and/or requires lateral re-organization of components that generates this topology (Zimmerberg and Kozlov, 2006). Logically, stiffer membranes are harder to bend and therefore cells must use more energy to do so (Callan-Jones et al., 2011). A patch of unsaturated lipids would be therefore relatively easy to pinch off from the surrounding saturated and stiffer lipids (Yu et al., 2010). The energy that the cells use to bend a patch of a membrane bilayer can be calculated from the elastic model of lipid membranes (Helfrich, 1973).

Most of the mechanisms introduced in the following chapters are applicable for curvature generation during vesicle budding. With the few exceptions stated, the same rules can be applied in organelle morphogenesis. Firstly, cellular organelles maintain their morphology over long time periods while vesicles are not permanent structures. Secondly, all curvature-generating proteins are soluble proteins that sculpt the lipid bilayer when needed, whereas some organelle curvature-generating proteins are integral proteins. Finally, vesicle formation occurs through local membrane curvature, while organelle membrane remodelling requires substantially larger modifications (Shibata et al., 2009).

1.2.1 Mechanisms behind shaping cellular membranes

High membrane curvature in cellular organelles is generated by two major mechanisms: scaffolding and hydrophobic insertion. These methods are also used to generate small intracellular vesicles, which possess the highest degree of curvature. Besides direct protein contact, cells use lipid-lipid connections and cytoskeleton-based force to shape organelles (McMahon and Gallop, 2005; Zimmerberg and Kozlov, 2006; Callan-Jones et al., 2011; Shibata et al., 2009). In cells, curvature of the membrane is conventionally defined as positive if the membrane is bulging towards the cytoplasm, and negative if the bulge is pointing towards either the non-cytoplasmic side of vesicle, tubule, or sheet, or the exterior of the cell (Campelo et al., 2010).

1.2.1.1 Lipid shape and asymmetry in generation of membrane curvature

The mechanisms behind local membrane curvature generation are tightly connected to lipid sorting. Here, I will briefly go through why lipid shape alone is not enough to drive membrane curvature to

the point of carrier vesicle formation. As stated in the previous chapters, lipids come in many shapes and sizes. They can be readily separated into different groups according to the intrinsic shape of the molecule **(Figure 3)**. Lipids can have cylindrical, conical, or inverted-conical shapes. Based on the steric effects, one would expect that the outer leaflet of small vesicles would be enriched in inverted-conical lipids, whereas the inner leaflet would be enriched in conical lipids. However, asymmetric lipid distribution is not able to explain the high-curvature present in small unilamellar vesicles (Zimmerberg and Kozlov, 2006). Instead, measurements of the membrane curvature preference of phospholipids revealed only weak coupling between lipid shape and leaflet curvature (Kamal et al., 2009). Moreover, membrane curvature experiments conducted using giant unilamellar vesicles (GUVs) suggest that the lipid shape alone is not enough to drive measurable lipid sorting; lipid-lipid interactions or lipid-protein interactions are necessary to amplify the curvature-based sorting (Sorre et al., 2009; Tian et al., 2009). Lipid-protein effects on membrane curvature are described in **chapters 1.2.1.3 – 1.2.1.5**.

Lipids are also differentially distributed in different organelles with respect to the orientation of the lipid to the cytosol. Whereas all lipids are symmetrically distributed between the two leaflets of the ER membrane bilayer, the Golgi complex, plasma- and endosomal membranes display an asymmetric lipid distribution. In these organelles sphingomyelin (SM) and glycosphingolipids are concentrated in the extracytoplasmic leaflet, while PS and PE are enriched in the cytoplasmic leaflet (Devaux and Morris, 2004; Daleke, 2007). The asymmetric distribution of lipids has many important functional consequences. For example, when exposed on the cell surface, PS functions as a susceptibility signal for apoptosis. The factors behind this asymmetry include the biophysical properties of lipids which dictate the ability of a lipid to cross the bilayer spontaneously, and the presence

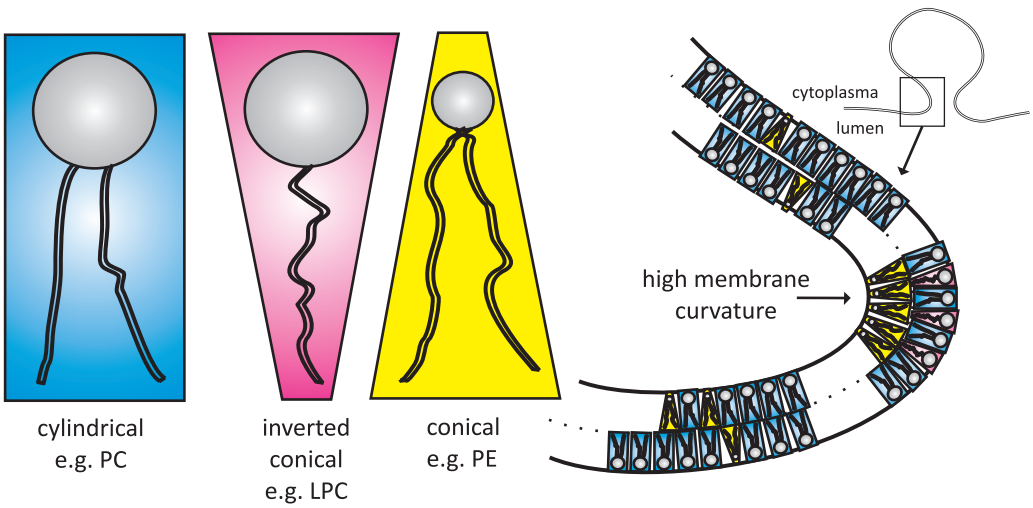

Figure 3. Lipid shape and asymmetry in generation of membrane curvature. The size of the head group and the number, shape and saturation level of the acyl chains determine the intrinsic shape of a lipid molecule (left). The degree of membrane curvature dictates the packing of lipid species between the leaflets, especially in the regions of high curvature (right).

of "flippases" that actively translocate PS and PE to the cytoplasmic surface. "Floppases", on the other hand, function in reverse direction. Certain mechanisms can also trap lipids in one leaflet of the bilayer (Pomorski and Menon, 2006). However, the very basis for this transmembrane lipid asymmetry lies in the fact that glycerolipids are primarily synthesized on the cytoplasmic and sphingolipids in the extracytoplasmic leaflet of cellular membranes. While having a higher affinity for sphingolipids than for glycerolipids, the localization of cholesterol has also an impact on the asymmetry (van Meer, 2011).

The simplest way to induce *in vivo* membrane asymmetry would be to introduce more lipid molecules into one monolayer than in the other, or by introducing more inverted cone-shaped molecules in the outer monolayer **(Figure 3)**. In both cases, the bilayer would bulge out to create membrane curvature (Devaux and Morris, 2004). However, lipid asymmetry alone is not enough to establish membrane curvature. Both lipids and proteins require physical constraints on the membrane, like frames or scaffolds, to be able to enforce this membrane curvature. To achieve this, lipids again use their polymorphism; by grouping together lipids with similar qualities, lipids create patches with different phase states. Briefly, this patch formation creates line tension which gives enough support for the patches to bulge out to generate membrane curvature (Lipowsky, 1992). A recent study presented an excellent model system to study phase-dependent curvature generation in GUVs (Yu et al., 2010). To conclude, lipids are capable of forming mild membrane curvature by themselves, but the current understanding is that high membrane curvature of cellular membranes is generated by proteins. Lipids facilitate this process by acting as inducers and by modifying the curvature-generating proteins (Devaux et al., 2008).

1.2.1.2 Mechanical force in membrane deformation

Cells can readily change their morphology simply by the assembly and disassembly of the actin cytoskeleton (Pollard and Borisy, 2003; Welch and Mullins, 2002). Actin monomers can rapidly polymerize into polar filaments with rapidly growing plus ends facing the plasma membrane and shrinking minus ends pointing towards the cytoplasm. This fast polymerization, which is used to drive the membrane forward, is tightly regulated by a large number of actin binding proteins (Pollard et al., 2000).

The cell harnesses the energy of this polymerization to create cellular protrusions, such as filopodia and lamellipodia, and cellular invaginations e.g. during the formation of an endocytic vesicle. In lamellipodium, which is located at the leading edge of a migrating cell, actin filaments form a branched or dendritic network. These branches are formed by Arp2/3 complex and provide the structural and mechanical platform to push the membrane forward (Mullins et al., 1998; Svitkina and Borisy, 1999). Filopodia are thin and relatively short-lived cellular protrusions composed mainly of actin filament bundles. Cells use filopodia for example to scout the environment for guidance signals and places to attach (Mattila and Lappalainen, 2008). Additionally, actin polymerization has many roles in the formation of endocytic vesicles. Cells use endocytosis for example for nutrient uptake from the extracellular environment and for receptor down-regulation and recycling (Doherty and McMahon, 2009). Very recently, high resolution EM images have revealed that the primary role of actin cytoskeleton

in clathrin-mediated endocytosis is to constrict and elongate the neck of the forming bud and to push the endocytosed vesicles away from the plasma membrane (Collins et al., 2011) **(Figure 4)**.

Many organelle membranes are also extremely dynamic and can change their shapes rapidly (Lee and Chen, 1988). For example, thin membrane tubules can be drawn out from planar or low-curvature membranes by molecular motors as they move along microtubules or actin filaments. It has been estimated that around 10 molecular motors are required to pull out tubules of 60 nm in diameter (Zimmerberg and Kozlov, 2006; Shibata et al., 2009). Another way of pulling out membrane tubules is by using the force exerted by polymerizing microtubules (Waterman-Storer and Salmon, 1998; Terasaki et al., 1986) **(Figure 4)**.

In mammalian cells, ER uses microtubule cytoskeleton for movement and transport. The proteins that mediate the interaction between membrane tubules and motors or microtubules have not yet been identified. This process is most likely driven by the cytoplasmic microtubule-based motor proteins dynein and kinesin-1 (Wozniak et al., 2009). Interestingly, instead of using microtubules, plant and yeast cells generate ER tubules along actin filaments. Plant-specific myosin VI has recently been shown to be the main molecular motor energizing plant ER tubule movement (Yokota et al., 2011; Ueda et al., 2010). Plant myosin mutants have been shown to display several gross morphological phenotypes, the most severe being dwarfism and reduced fecundity. Additionally, these mutations reduce organelle movement (Sparkes, 2011). Although the role of actin filaments has not been yet demonstrated in shaping ER in mammalian cells this does not mean that it is not involved in the process.

ER tubules move along the microtubules in two mechanistically distinct ways. The first mechanism uses tip-to-tip contacts and is highly dynamic (Waterman-Storer and Salmon, 1998). The second mechanism of ER tubule dynamics is referred to as sliding. Here, the tip of the ER tubule first binds to the shaft of an existing microtubule and slides along the

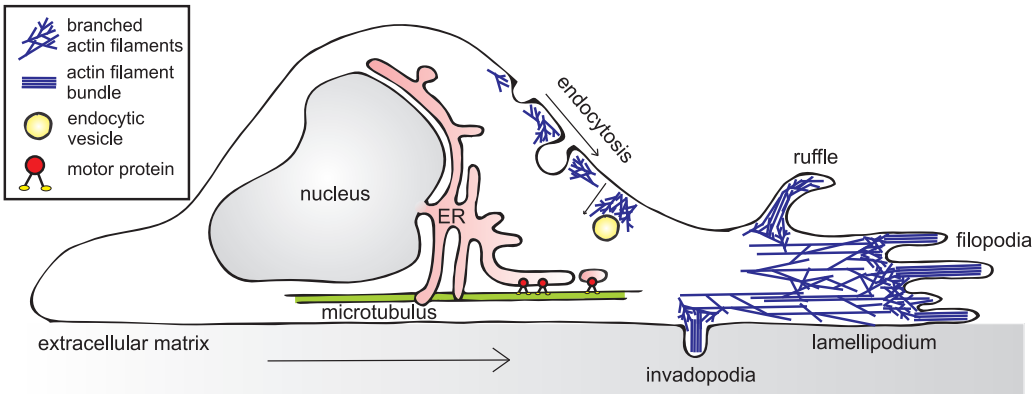

Figure 4. A schematic representation of a migrating mammalian cell. The force generated by polymerization of actin filaments and microtubules is used in many cellular events related to membrane deformation. The actin cytoskeleton is involved in e.g. the formation of filopodia, lamellipodia, membrane ruffles, invadopodia, and in endocytosis. In mammalian cells, microtubules and associated motor proteins contribute to ER membrane tubule and vesicle trafficking. See text for further details.

microtubule as the ER tubule grows (Lee and Chen, 1988). ER tubule sliding occurs more frequently and is faster than tip-to-tip dynamics. It can also progress in both directions (Waterman-Storer and Salmon, 1998; Grigoriev et al., 2008) **(Figure 4)**.

1.2.1.3 Scaffolding

The first mechanism by which membrane binding proteins directly bend membranes is scaffolding **(Figure 5 a)**. A typical scaffolding protein contains a structurally curved membrane binding domain. Usually, the curved surface has high affinity for the polar head groups of lipids, especially for those of phosphoinositides and phosphatidylserine. In order to match the protein shape, the membrane adopts a similar shape underneath the protein–lipid interface. This requires that the rigidity of the protein scaffold is higher than the membrane bending rigidity and that the energy of the membrane-protein attachment exceeds the membrane bending energy (Shibata et al., 2009). It is still not known how much energy scaffolding proteins use for membrane binding or how the elasticity of the membrane can be quantitatively characterized (Campelo et al., 2010). The membrane binding interface of a single scaffolding protein is generally not enough to generate the desired curvature. This is why many scaffolding proteins self-assemble or oligomerize into larger complexes (Frost et al., 2008; Henne et al., 2010). However, it is currently not known if the self-assembly is a requirement for a proper function of all scaffolding proteins. Many membrane bending protein families use the scaffolding mechanism to sculpt membranes. The best studied protein groups are the dynamin family of proteins, the BAR domain containing proteins, EHD proteins, the clathrin complexes, the proteins of the reticulon and REEP families, and the COPI and COPII complexes (Campelo et al., 2010). For example, certain members of the BAR superfamily (e.g. arfaptin and endophilin A1) bind to the membrane with their convex surface and generate high membrane curvature. These proteins are able to bend membranes into tubes of ~15-17 nm in diameter (Qualmann et al., 2011). These protein families and the mechanisms behind their membrane deformation are discussed in detail in **chapter 1.3**.

Besides generating curvature, some scaffold proteins act to stabilize planar membrane structures. Golgins and p180 are abundant integral membrane proteins with large cytosolic coiled-coiled domains expressed on Golgi complex and ER sheets, respectively. They have been suggested to function as linkers in stacking sheets of either Golgi complex or ER, by bridging

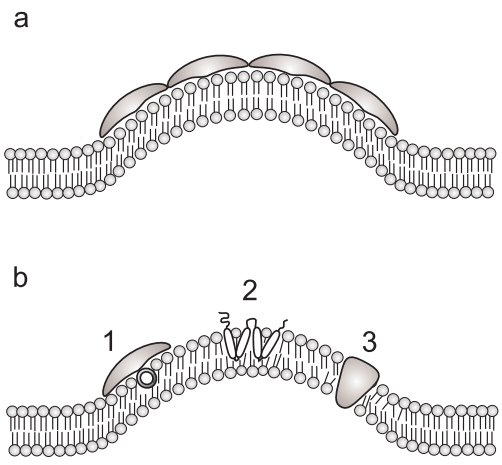

Figure 5. The mechanisms that proteins apply to generate membrane curvature. a) Generating membrane curvature through scaffolding. Scaffolding proteins additionally use oligomerization to stabilize regions of high membrane curvature. **b)** Generating membrane curvature by hydrophobic insertion can occur at least by three different mechanisms. These are insertion of **(1)** an amphiphatic helix or **(2)** amphiphatic hairpin loops and **(3)** wedging through an integral membrane protein. This figure was made according to Zimmerberg and Kozlov, 2006.

the cytosolic surfaces together. However, the details of this mechanism are not known (Shibata et al., 2010; Short et al., 2005).

1.2.1.4 Hydrophobic insertion & wedging

The other major way for proteins to bend membranes is through hydrophobic insertion or wedging **(Figure 5 b)**. A generalized view of the mechanism is that proteins generate membrane asymmetry by inserting their hydrophobic domains into the lipid bilayer matrix on only one side of a membrane, which then bulges towards the disturbed monolayer (McMahon and Gallop, 2005). Additionally, many integral wedge-shaped membrane proteins can cause asymmetry between leaflets (Kim et al., 2000). However, the local concentration of wedge-shaped integral proteins should be very high to induce noticeable curvature (Shibata et al., 2009). A theoretical analysis by Campelo and colleagues provides evidence that the highest membrane curvatures are generated by shallow insertions that penetrate the external membrane monolayer only to the depth of about 40% of a monolayer thickness (approx. the interface between the polar head groups and the hydrocarbon chains) (Campelo et al., 2010; Campelo et al., 2008; Gallop et al., 2006). To induce curvature using the hydrophobic insertion mechanism, membrane sculpting proteins generally insert either an amphipathic α-helix(es) or a short hydrophobic hairpin loop(s) into the membrane leaflet (Campelo et al., 2010; Campelo et al., 2008). Also caveolins insert a hairpin domain into only one leaflet of plasma membrane during endocytosis suggesting a universal hairpin insertion mechanism (Zurek et al., 2011).
The group of proteins which bend the membranes by inserting hydrophobic domains includes e.g. Epsins, which bind the polar head groups of $PI(4,5)P_2$, the proteins of the reticulon and REEP families, and N-BAR domains (Campelo et al., 2010). More details of these protein families and the mechanisms behind their membrane deformation activities are discussed in **chapter 1.3**. Like in the case of scaffold proteins, some hydrophobic insertion-proteins are also involved in stabilization and generation of planar membrane structures. The results from a recent study by Shibata and colleagues suggest that the reticulons and REEP proteins are mainly responsible for determining the ER morphology. These proteins can help to stabilize ER sheets by inserting hairpin loops into the high membrane curvature areas at sheet edges (Shibata et al., 2010).

1.2.1.5 Scaffolding & hydrophobic insertion

Most membrane curvature generating proteins use a combination of hydrophobic insertion and scaffolding. For example, N-BAR domains insert their N-terminal amphipathic helix into the lipid bilayer and use the concave lipid binding site to scaffold the curved membrane. Similarly, some of the loops of Dynamin-1 PH domain get embedded into the bilayer and form a spiral scaffold around the neck of the budding vesicle (Shibata et al., 2009; Campelo et al., 2010). Which of the two mechanisms is more important for a given membrane bending protein and what is the possible interplay between them are questions to be addressed (Campelo et al., 2010). In the case of N-BAR domain proteins, recent theoretical studies indicate that the amphipathic helices are both necessary and sufficient to generate membrane curvature (Campelo et al., 2008; Khelashvili et al., 2009). Curvature sensing should not be confused with curvature generation; these processes are not mutually exclusive and can thus occur simultaneously. Curvature sensing proteins tend to interact with membrane

topology that already corresponds to the shape of the protein. It is important to remember that insertions do not sense the curvature *per se*, instead they sense the stress within the bilayer. Scaffolding domains, however, sense only the membrane curvature. Interestingly, N-BAR domain of Endophilin, the C2 domain of Synaptotagmin, and Dynamin-1 with the aid of GTP hydrolysis, have all been demonstrated to be able to perform both membrane curvature generation and either membrane fusion or fission (Campelo et al., 2010).

1.3 Membrane curvature generating proteins

This section concentrates on elucidating the cellular and functional roles of some of the major membrane curvature generating or/and sensing protein families. Those proteins that promote membrane fission, fusion or pore-formation are not discussed here. The biggest challenge today in membrane modelling is to find out the common mechanistic principles of action of the various proteins. This task is difficult, because the proteins discovered thus far are so diverse in terms of their biochemical and structural characteristics. It is also plausible that one protein or protein complex can drive several types of membrane shaping and remodeling (Campelo et al., 2010).

1.3.1 ENTH & ANTH domain proteins

The Epsin N-terminal homology domain (ENTH) was first described in the Epsin protein family as a 140 aa module that binds to $PI(4,5)P_2$. The ENTH domain is responsible for recruiting the full-length epsin to the plasma membrane. By modifying membrane curvature, epsins facilitate the formation of clathrin-coated invaginations (Legendre-Guillemin et al., 2004; Stahelin et al., 2003). The ANTH (AP180 N-Terminal Homology) domain is functionally and structurally similar with the ENTH domain.

Both the ANTH and the ENTH domains interact directly with $PI(4,5)P_2$ in the cytosolic leaflet of the plasma membrane and use a conserved cluster of basic residues to coordinate the binding of $PI(4,5)P_2$ headgroup. However, the molecular locations of the lipid binding sites are distinct (Stahelin et al., 2003). The ANTH domain binds $PI(4,5)P_2$ via strong electrostatic bridging interactions controlled through basic residues in the domain surface. The ENTH domain binds $PI(4,5)P_2$ within a cleft with both basic and hydrophobic residues contributing to membrane penetration. The N-terminal helix 0 of the ENTH domain plays a critical role in the $PI(4,5)P_2$ dependent membrane penetration. The helix 0 inserts into the outer leaflet of the bilayer pushing the head groups apart, thus bending the membrane. This is a classic example of using the hydrophobic insertion mechanism to generate membrane curvature. In contrast, mammalian ANTH domains do not insert a hydrophobic helix into the membranes. They appear to generate membrane curvature through electrostatic interactions and scaffolding mechanism (Stahelin et al., 2003; Ford et al., 2002).

After the ENTH domain induces membrane curvature, the C-terminal region of full-length Epsin is necessary for recruiting clathrin coat components. It is possible that Epsin may act as initial anchor protein onto which the clathrin cage can be assembled (Horvath et al., 2007). Similarly to ENTH domain proteins, ANTH domain proteins are involved in membrane trafficking, in particular clathrin-mediated endocytosis (Nonet et al., 1999; Pryor et al., 2008; Mishra et al., 2001). In mammals, the ANTH domain

family consists of two members, clathrin assembly lymphoid myeloid leukemia (CALM) and AP180. AP180 is exclusively expressed in neurons (Morgan et al., 1999) while CALM is a ubiquitous protein found in both neuronal cells and in non-neuronal tissues (Yao et al., 2003). ANTH domain proteins have been implicated in the development of a wide range of pathologic processes, including Alzheimer's disease, leukemia and other human cancers (Rao et al., 2002). In addition, a novel group of plant ANTH domains has recently been identified with unique ENTH domain-like structure and function (Silkov et al., 2011), underlining the fact that these protein domains might be more versatile in their modes of action than previously thought.

1.3.2 Reticulons and REEPs

Two major protein families, Reticulons (a.k.a. Nogo) and REEPs (receptor expression enhancing protein), have an extensive role in maintaining and generating the tubular ER network (van de Velde et al., 1994; Wieczorek and Hughes, 1991). Although the REEP proteins (also known as DP1/Yop1p proteins) do not share detectable primary sequence homology with the reticulons, they share a highly conserved C-terminal domain, which has been named the reticulon homology domain (RHD). This abundant domain is around 200 residues long and contains two short (approximately 30 residues) hydrophobic hairpins (Oertle and Schwab, 2003). These hydrophobic hairpins insert into membrane but are short enough to span the membrane only partially without entering to the luminal side of the bilayer **(Figure 5)**. The hairpin insertion regulates the ER tubule diameter by generating regions of high membrane curvature (Zurek et al., 2011).

In vitro, both Reticulons and REEPs transform liposomes into narrow tubes of ~15 nm in diameter, which are half the size of those observed *in vivo* perhaps owing to a higher concentration of the tubule-inducing proteins used. From these results Hu and colleagues estimated that only ~10 % of the total tubular ER surface in yeast is occupied by these proteins. They explain this through the ability of Reticulons and REEPs to homo- or hetero-oligomerize into arcs or rings that would be sufficient to drive high membrane curvature (Hu et al., 2008). This implies that the oligomerization of Reticulons and REEPs is important for both their correct localization into the tubular ER and for their ability to form the tubules. Because the morphology of ER is continuously changing, the oligomers need also to be rapidly formed and disassembled, which requires energy in the form of ATP (Shibata et al., 2008).

To emphasise the significance of Reticulons in the stabilization of positively curved membranes, it is important to note that these proteins are conserved from yeast and plants to humans. The Reticulon proteins are widely dispersed and expressed in many tissues. The most abundant transcripts are highly enriched in the nervous system (Oertle and Schwab, 2003). Depletion of Reticulons and REEPs either from yeast or mammals alters the ER morphology by converting the tubules into sheets and expanding the nuclear envelope (Voeltz et al., 2006; Anderson and Hetzer, 2008).

1.3.3 EHD proteins

Dynamin-related proteins, which share a C-terminal Eps15 homology domain (EHD), have been shown to be important in endosomal receptor-recycling and membrane trafficking (Naslavsky and Caplan, 2011; Grant and Caplan, 2008). Although this domain has also been linked to membrane fission events, it will be discussed here because of its unique

mechanism to generate membrane curvature (Campelo et al., 2010).

The EH-domain is composed of 90-100 residues and it is well conserved throughout eukaryotic species. EHD proteins localize into long and tubular endosomal membrane structures. EH-domain binds phosphoinositides and self-associates into dimers and oligomers *in vitro*. The membrane binding site created by the dimerization has highly concave geometry. These observations have resulted in a model of EHD-mediated regulation of endosome morphology (Daumke et al., 2007). According to this model, EHD proteins bind ATP and dimerize. The dimers are further assembled into ring-like structures around membrane tubules. Subsequently, the hydrolysis of ATP leads to the dissociation of vesicles from the tubules. Interestingly, ATP is not required for the lipid-binding, while the oligomerization is ATP-dependent (Daumke et al., 2007). The hydrolysis of ATP has been shown to regulate the topology of the EHD-dimer lipid binding interface, and to be connected to the disassembly of membrane tubules (Campelo et al., 2010; Daumke et al., 2007). The EH-domain function is dependent on the oligomerization, which creates the scaffolding surface to generate membrane curvature.

Additionally, it was recently stated that F-BAR domain proteins Syndapin1&2 and the *C. elegans* BAR domain protein AMPH-1/Amphiphysin/Bin1 interacts with EHD proteins (Pant et al., 2009; Braun et al., 2005). Thus, it is possible that the membrane curvature generation mechanism of EHD proteins resembles the scaffolding mechanism used by BAR domain proteins (see next chapters), and that these protein families would control the cellular endocytic trafficking together (Naslavsky and Caplan, 2011; Grant and Caplan, 2008).

1.3.4 BAR domain protein superfamily

The easiest way to describe the mechanism of membrane curvature generation is through the structurally conserved BIN/Amphiphysin/Rvs (BAR) domain superfamily. BAR domain proteins use both a scaffolding mechanism and hydrophobic insertion to shape membranes. New members are added to this group of membrane-sculptors monthly, which makes it the largest group of proteins that generate membrane curvature. There are several alternative ways to divide these proteins into sub-families, and in this thesis I will use the following one: canonical BAR, N-BAR, F-BAR, and IF-BAR domains (McMahon and Gallop, 2005; Frost et al., 2009; Suetsugu et al., 2010). These sub-families will be introduced in more detail in the following chapters, with the emphasis on curvature generation.

All BAR domain proteins share common features that link them together, even though the sequence similarity might be relatively low. The crystal structure of the first BAR domain, Arfaptin, revealed that the core structure of the domain is a homo-dimer. Both monomers are solely helical, composed of three long α-helices, which are linked together with a stable overall surface area (Tarricone et al., 2001) **(Figure 6)**. However, the link between membrane curvature generation was not realized until later (Peter et al., 2004; Masuda and Mochizuki, 2010). Most of the BAR domains bind membranes and have been shown to tubulate round liposomes *in vitro* (Takei et al., 1999). Peter and colleagues were the first ones to suggest that the BAR domains bind membranes through a curved interface and that the membrane-bending feature of the BAR domains could be more widespread (Peter et al., 2004). From there on, it was thought that the intrinsic curved structure of the

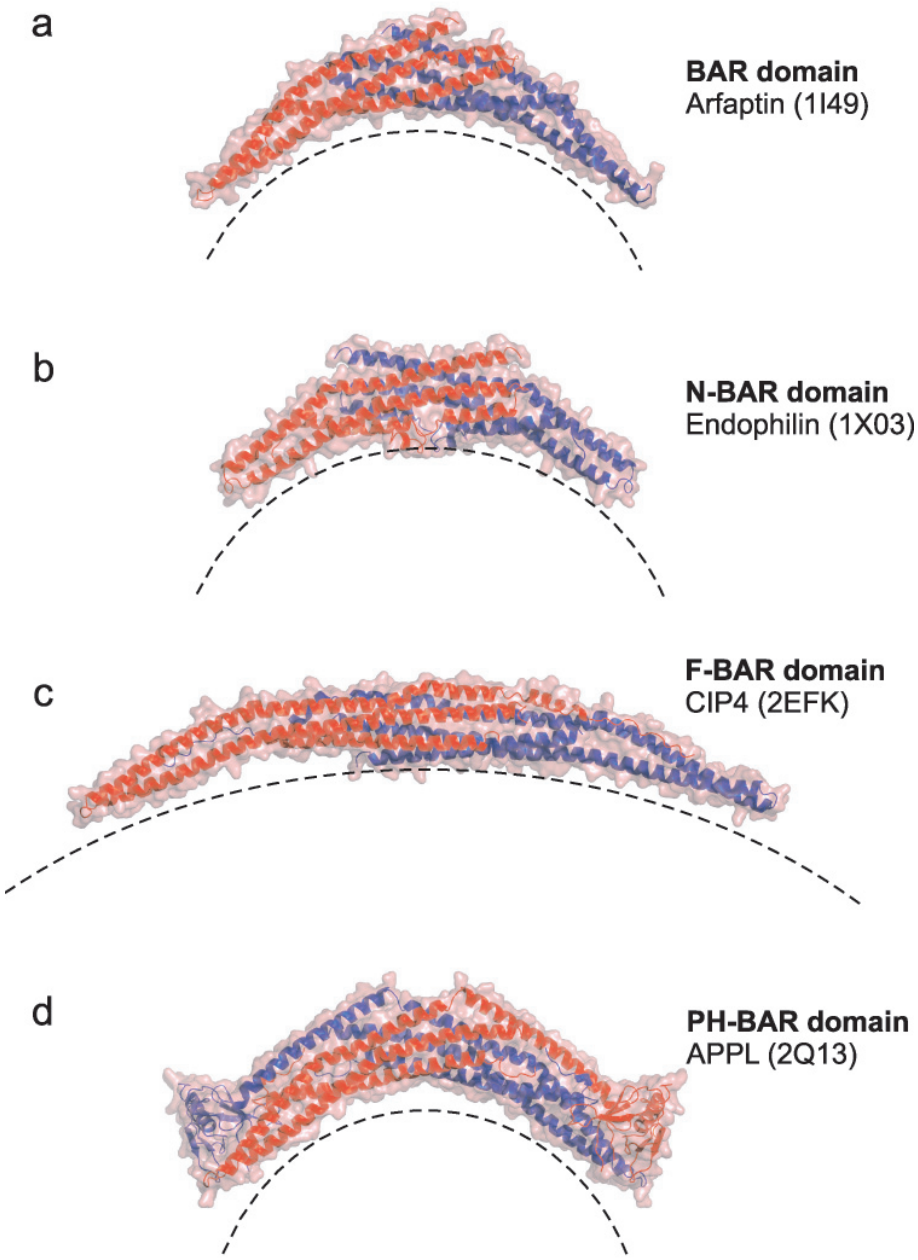

Figure 6. Structures of different BAR superfamily domains display many similar features. The core structure of each domain is a homodimer which is composed of an α-helical bundle. One monomer of each dimer is shown in red and the other one in blue. Surfaces are shown in transparent pink. Membrane bilayer is depicted by the dotted line close to the concave membrane binding surface of the BAR domain. **a)** A classical BAR domain. **b)** N-BAR domain can insert an amphipathic helix into the bilayer. **c)** F-BAR domains are longer and more gently curved as compared to canonical BAR domains. **d)** PH-BAR domain has an additional PH domain sticking out from the BAR domain core structure. The figure was created with program PyMOL.

BAR domain itself is enough to induce membrane curvature generation and/ or to prefer certain types of membrane curvature (curvature sensing). However, it has been recently shown that this "BAR domain hypothesis" is not sufficient to explain why some shallowly curved BAR domain proteins are implicated to function at sites of high membrane curvature (Qualmann et al., 2011; Taylor et al., 2011).

1.3.4.1 BAR & N-BAR domain proteins

A previously uncharacterized protein Bin1 was shown to have high N-terminal sequence similarity with human Amphiphysin and yeast Rvs proteins (David et al., 1994). Accordingly, the N-terminal region was named the BAR domain (Bin1/amphiphysin/Rvs167) (Sakamuro et al., 1996). Shortly after this, the BAR domain of Amphiphysin-1 was implicated in *in vitro* liposome tubulation. It was also shown to bind to Dynamin with its C-terminus and act together with it in endocytosis of clathrin coated vesicles (Takei et al., 1999). Takei and colleagues also suggested that Amphiphysin-1 could have an important role in sensing and supporting the curved membrane area during vesicle budding.

The BAR domain is conserved from yeast to human and is present in > 10 human proteins (Rao and Haucke, 2011). The BAR domain is an α-helical, banana-shaped homodimer with a capacity to bind negatively charged membranes with its positively charged residues at the concave surface of the dimer. The canonical BAR and N-BAR domain containing proteins usually share also SH3, PH, PX, GAP or GEF domains. The BAR/N-BAR domain is generally located in the N-terminus, but in few cases in the C-terminus (Sorting nexins and PICK-1) or in the central region (Tuba) of the full-length protein (Suetsugu et al., 2010; Suetsugu, 2010). The N-BAR domains of Endophilin and Amphiphysin have an amphipathic helix in their N-terminus (hence the name N-BAR), which can insert into the hydrophobic region of the lipid bilayer **(Figure 6)**. The inserted helix can act as a wedge promoting further membrane curvature, which can be stabilized by the N-BAR domain (Gallop et al., 2006; Masuda et al., 2006).

BAR and N-BAR domain proteins are the key regulators of membrane remodelling. They bind and sculpt the membrane by their banana shaped BAR domains and bind actin or actin-interacting proteins with their other domains. The intrinsic shape of the BAR dimer partially defines the degree of the membrane curvature it can bind to (Rao and Haucke, 2011; Suetsugu, 2010). At the subcellular level, BAR and N-BAR domain proteins generally localize to vesicles, endosomes, membrane compartments, and to plasma membrane at different stages of clathrin-coated pit formation during endocytosis. When the expression patterns of most BAR and N-BAR proteins are pooled together an interesting observation emerges. Proteins carrying this domain are typically most strongly expressed in three cell types or organs: brain, highly specialized epithelial or endocrine cell types and in hematopoietic cells (Human Protein Atlas and BioGPS). The common feature shared by these cells is the increased ability to either secrete or take in material from the extracellular environment, and these processes are tightly linked to membrane remodelling. However, some highly specialized BAR domain proteins have found their niche elsewhere. For example Amphiphysin-2 and Sorting nexin 5 are expressed in skeletal muscle where they have special roles in T-tubule biogenesis and in myotendinous junctions, respectively (Lee et al., 2002; Otsuki et al., 1999; Towler et al., 2004).

1.3.4.2 F-BAR & IF-BAR domain proteins

The Fes/CIP4 (FCH) domain was discovered as a conserved protein motif between Fer and Fes/Fps proteins and in CIP4 proteins (Aspenström, 1997). Subsequently, an extended version of the FCH domain was identified as a BAR domain-like membrane deforming protein module and named F-BAR domain (Peter et al., 2004; Aspenström, 1997; Itoh et al., 2005; Tsujita et al., 2006). The structural similarity to BAR domains was demonstrated when the first crystal structures of F-BAR domains were determined (Shimada et al., 2007; Henne et al., 2007). Interestingly, the overall structure, cellular localization, and even some functions in clathrin-mediated endocytosis appeared to be highly similar between BAR and F-BAR domains. However, despite displaying a similar α-helical banana-shaped structure to the BAR domains, the dimers of F-BAR domains display longer and shallower curvature. This indicates that F-BAR domain proteins can generate tubules with larger diameter than those generated by canonical BAR domains **(Figure 6)** (Shimada et al., 2007; Henne et al., 2007). Another feature that groups the F-BAR domain proteins together is the SH3 domain. A majority of the family members contain an SH3 domain linking the F-BAR domain proteins directly to the actin cytoskeleton and to endocytic and signaling proteins. Some members have additional protein domains, such as RhoGAP domains (srGAP1-3), Cdc42 binding sites (Toca-1, CIP4, and FBP17), and tyrosine kinase domains (Fes and Fer). Besides having many functions in different steps during endocytosis, F-BAR domain proteins have been demonstrated to have roles in other cellular events as well (Ahmed et al., 2010; Fricke et al., 2010). One F-BAR domain protein, Syndapin, have been shown to insert so called wedge-loops into the membrane bilayer. Other F-BAR domain proteins mostly use a scaffolding mechanism to bind to the membrane (Plomann et al., 2010).

No PX or PH domains, which create kinks protruding from the core helical structure in BAR domains, have been found in F-BAR domain proteins **(Figure 6)**. It is interesting to note that at least two F-BAR domain proteins, APPL and SNX9, have similar kinks in their core structure (Qualmann et al., 2011). These kinks can partially explain the differences seen in the tubule sizes created by these mildly-curved F-BAR domains. According to the BAR domain hypothesis, the increasing curvature generated during the formation of an endocytic vesicle should constantly recruit more and more highly curved BAR and F-BAR proteins. Taylor and colleagues analyzed the recruitment pattern of many endocytic proteins including 10 proteins of the BAR domain superfamily to verify this theory. However, their findings indicate that there must be also other ways than the intrinsic curvature of the domain alone to generate variable membrane curvature (Taylor et al., 2011). There is a large body of evidence of F-BAR domain self-assembly and according to the current view these oligomeric assemblies could be the functional units in BAR domain protein-mediated curvature generation, at least for some F-BAR domain proteins (Frost et al., 2008; Qualmann et al., 2011). Importantly, to make things more exciting, a recent study by Guerrier and colleagues pointed out that the F-BAR domain of srGAP2 protein generates negative membrane curvature, in contrast to positive curvature generated by other so far characterized BAR and F-BAR domains. For this reason, this domain was named as an Inverse F-BAR (IF-BAR) domain. In cells, srGAP induces filopodia-like membrane protrusions, which were reported to be important for the proper development of the central nervous system

(Guerrier et al., 2009). Thus, F-BAR domain proteins appear to have evolved to participate in many different cellular functions, although the majority of the known proteins function in endocytic events and in membrane trafficking.

1.4 I-BAR domain protein family

I-BAR domain proteins were first grouped together when a GenBank database search revealed homology between the N-terminal domains of the following five proteins: IRSp53, MIM, ABBA, IRTKS and FLJ22582 (Yamagishi et al., 2004). IRSp53 and MIM were described already during years 1999 and 2002 (Abbott et al., 1999; Lee et al., 2002), respectively, but the paper by Yamagishi and colleagues first introduced a novel N-terminal IRSp53/MIM homology Domain (IMD), which is currently known as I-BAR domain, as the common feature for these proteins. The vertebrate IRSp53/MIM homology domain family is divided into two major groups: the MIM/ABBA subfamily and the IRSp53/IRTKS subfamily (Yamagishi et al., 2004).

The secondary structure of I-BAR domain is almost completely α-helical and the domains of IRSp53 and MIM are structurally very similar, although they share only ~19.3 % sequence identity at the amino acid level (Lee et al., 2007). The I-BAR domain is an American football shaped dimer consisting of four α-helices in IRSp53 and three in MIM (Lee et al., 2007; Millard et al., 2005). The MIM/ABBA subfamily members and most IRSp53/IRTKS sub-family members have a C-terminal WASP homology 2 domain (WH2). The MIM/ABBA subfamily members also have a central poly-proline region, while IRSp53/IRTKS subfamily members share a SH3 domain instead of the poly-proline region (Yamagishi et al., 2004). When expressed in mammalian cells, the I-BAR domain alone is capable of inducing formation of filopodia and membrane ruffles (Yamagishi et al., 2004; Bompard et al., 2005) **(Figure 7)**.

1.4.1 MIM

1.4.1.1 Domain structure

In 2002, Lee and colleagues reported that a specific mRNA was not expressed in metastatic bladder carcinoma cell line, but was strongly expressed in non-metastatic bladder carcinoma cell lines. For this reason the corresponding protein was named Missing-in-Metastasis (MIM) (Lee et al., 2002). MIM gene is located in chromosome 8 in humans and chromosome 15 in mice. The gene is highly conserved in vertebrates and some similarity is found in genes of fruit fly (*Drosophila melanogaster*) and roundworm (*Caernorhabditis elegans*) (Scita et al., 2008). At least four protein coding transcripts can be spliced from the same mouse gene, and in the case of humans, the transcript number is even higher. The most common isoform is 759 amino acids long and builds up a moderate sized (82,4 kDa) protein (UniProt).

MIM has an N-terminal I-BAR domain (Yamagishi et al., 2004) and a C-terminal WH2 domain which binds ATP actin monomers with high affinity (Mattila et al., 2003; Woodings et al., 2003) **(Figure 7)**. Through its central region, MIM binds to the cytoplasmic domain of PTPδ (protein tyrosine phosphatase δ) receptor, thus forming a connection between tyrosine kinase signalling and the actin cytoskeleton. This binding was also shown to re-localize PTPδ receptor to the plasma membrane (Woodings et al., 2003; Gonzalez-Quevedo et al., 2005). A proline-rich region known to bind to the SH3-domain of Cortactin is located close to the same area. The crystal structure of

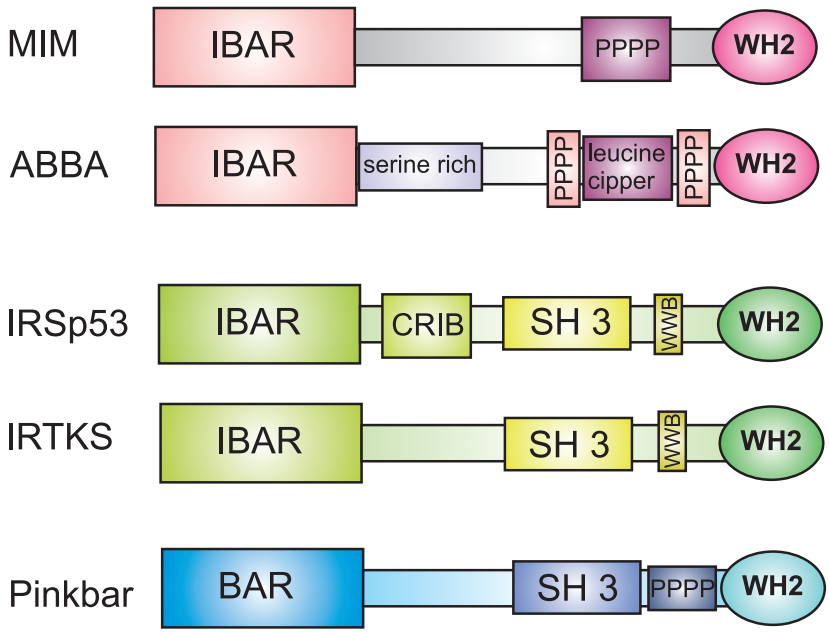

Figure 7. The domain compositions of I-BAR domain protein family members. See text for details.

MIM I-BAR domain was solved, revealing a homodimer consisting of three α-helixes in a tight bundle (Lee et al., 2007; Millard et al., 2005). The role of I-BAR domain will be further discussed in the **Results and Discussion** part of this thesis.

1.4.1.2 Cellular localization and expression pattern

MIM appears to be widely expressed in both human and mouse tissues. It is especially strongly expressed in the developing heart, myoblasts and sub-populations of neurons. In adult mice, MIM is expressed in high levels in the liver, kidney, and Purkinje cells of the cerebellum (Mattila et al., 2003; Woodings et al., 2003). At the protein level, MIM has been detected in the brain, bladder and spleen of adult mouse tissue, as well as at lower levels in the lung and liver (Bompard et al., 2005). In highly polarized epithelial cells MIM localizes to adherens junctions (Saarikangas et al., 2011). Platelet-derived Growth Factor (PDGF) stimulated tyrosine phosphorylation of MIM by Src-kinase at these sites has been shown to lead to MIM re-localization to dorsal ruffles in the plasma membrane (Wang et al., 2007b). In cultured mammalian mesenchymal cells, MIM localizes to lamellipodia, cell-cell contacts and polarized protrusions. Over-expression of MIM results in the disappearance of stress fibres and formation of microspikes, filopodia and membrane ruffles in cultured mammalian cells (Yamagishi et al., 2004; Bompard et al., 2005; Mattila et al., 2003; Woodings et al., 2003; Lin et al., 2005).

1.4.1.3 Cellular function

MIM knockout mice are viable and fertile and display no gross defects. However, in adult mice MIM gene inactivation leads to a progressive kidney disease described by abnormal tubular morphology, severe urine concentration defects, renal electrolyte wasting and bone

abnormalities as a result of leaky cell-cell contacts in kidney epithelial cell layer. MIM appears to stabilize epithelial sheets by acting as a scaffold between plasma membrane and actin cytoskeleton at cell-cell contacts (Saarikangas et al., 2011). This may also provide an explanation for why the loss or decreased expression of MIM in certain epithelial cancers is linked to increased metastatic behaviour (see the next chapter). Weak cell-cell contacts might promote epithelial-mesenchymal transition of the cancer cells (Saarikangas et al., 2011).

1.4.1.4 MIM has many faces in cancer

The original study identifying MIM as a metastasis suppressor was based on comparison of only five long-established cancer cell lines, only one of which was associated with metastatic disease (Lee et al., 2002). Shortly after this, another group concluded in a larger scale study that down-regulation of MIM expression can occur in bladder cancer cell lines but does not seem to be associated with increased invasive behaviour *in vivo* or *in vitro* (Nixdorf et al., 2004). DNA methylation seems to regulate the expression of MIM, providing a possible explanation for the large differences in expression levels between different cell lines (Utikal et al., 2006).

More recent studies imply that MIM has two contradictory roles in cancer, either metastasis suppressor or metastasis promoter, depending either on the tissue, the phase of the cancer, or both. MIM seems to act as a metastasis suppressor in gastric, lung, breast and bladder cancers (Lee et al., 2002; Folgueira et al., 2005; Wang et al., 2007a; Parr and Jiang, 2009; Nymark et al., 2011). To support this, up-regulated MIM expression in gastric, esophageal and lung cancers results in less invasive metastatic cells and therefore promotes longer overall survival age and better prognosis (Cheung et al., 2011; Xie et al., 2011; Zhao et al., 2011). In other cancers, MIM may also act as a metastasis promoter. Increased MIM expression level was reported to make colorectal and liver cancer cells more aggressive and enhance the cancer progression (Wang et al., 2011; Ma et al., 2007). However, in diseases like colorectal cancer, the over-expression of MIM in epithelial cells would favour cancerous cells. The surface epithelial cells normally have an extremely short lifespan of 2-4 days (Radtke and Clevers, 2005). MIM might thus promote the survival of nascent cancer cells in this unstable environment by making the cell-cell contacts stronger in the early stage of the disease. In support of this theory, MIM over-expression has been shown to be at its peak in 70 % of tumour samples collected from patients with early-phase hepatocellular carcinoma (Ma et al., 2007).

MIM was also earlier proposed to be involved in the Shh signalling pathway, which could also link MIM to cancer progression (Callahan et al., 2004; Bershteyn et al., 2010). However, more recent studies demonstrated that MIM knockout mice do not display defects in Shh-dependent developmental processes and that MIM does not contribute to Shh signalling in cultured cells (Saarikangas et al., 2011).

1.4.2 ABBA

1.4.2.1 Domain structure

This protein was identified through its sequence homology to MIM and named ABBA (actin-bundling protein with BAIAP2 homology/MTSS1l) (Yamagishi et al., 2004). The name is somewhat misleading, since the I-BAR domain of ABBA was recently shown to lack the proposed actin-bundling activity

(Saarikangas et al., 2008). In addition to the I-BAR and actin monomer binding WH2 domains, ABBA contains a serine-rich region, three proline-rich patches and a leucine zipper motif **(Figure 7)**. These interactions suggest a functional role for ABBA in areas of rapid actin dynamics. Currently, no other known interaction partners have been experimentally identified for ABBA (Saarikangas et al., 2008).

1.4.2.2 Expression pattern

ABBA is strongly expressed in the brain and moderately in the testis, skeletal muscle and lung. Low expression levels were also detected in the kidney, liver and heart. Expression patterns of MIM and ABBA differ especially during development. For example, MIM is highly expressed in the Purkinje cells of the cerebellum, while ABBA1 is expressed strongly in molecular layer of the cerebellum (Saarikangas et al., 2008). In the developing central nervous system, ABBA expression is the strongest in many populations of midline radial glial cells but it is absent from neuronal cells. Radial glial cells are involved in axon guidance and organizing neural tube patterning. Only some of the developmentally important glial cells survive to adulthood as specialized mature glial cells, such as the cerebellar Bergmann cells, where ABBA is abundantly expressed (Saarikangas et al., 2008; Lemke, 2001).

1.4.2.3 Cellular localization and function in glial-like cells

The I-BAR domain of ABBA localizes the protein to the interface between the cortical actin cytoskeleton and the plasma membrane. More precisely, ABBA is localized to lamellipodial structures and membrane ruffles. Knock down of ABBA in C6-R glial-like cells results in defects in lamellipodium dynamics (Saarikangas et al., 2008). Thus, according to the current view, ABBA acts as a scaffolding protein that links plasma membrane and actin dynamics during protrusive events in radial glial cells. In the future, it will be important to search for interaction partners and regulators for ABBA to understand its expert role in the regulation of radial glial cell processes.

1.4.3 IRSp53

1.4.3.1 Domain structure

IRSp53 (for insulin receptor tyrosine kinase substrate protein of 53 / BAIAP2 (BAI-associated protein 2)) protein was originally identified in a screen for substrates of insulin and insulin-like growth factor 1. The study suggested that this 53 kDa protein might be membrane-associated and also contain an SH3 domain at the C-terminus, which could link it to the cytoskeleton (Yeh et al., 1996). In addition to the C-terminal SH3 domain, IRSp53 contains various domains for protein-protein interactions, including an N-terminal I-BAR domain, a partial Cdc42 and Rac1-interactive binding (CRIB) motif, and a C-terminal PDZ [postsynaptic density-95 (PSD 95)/Discs large/zona occludens-1 (ZO-1)]-binding motif **(Figure 7)**. The longest and most abundant isoform, expressed mainly in the brain, has also an actin monomer binding WH2 domain (Scita et al., 2008). Also, certain other IRSp53 isoforms contain a WH2 domain at their C-terminus (Lee et al., 2007).

1.4.3.2 Expression pattern, cellular localization and interaction partners

IRSp53 has at least six protein coding and alternatively spliced isoforms in humans that differ e.g. in their tissue distribution and in their domain composition (Okamura-Oho et al., 2001; Alvarez et al., 2002). In the nervous system, IRSp53

mRNA is expressed in the molecular layer of mouse cerebellum, forebrain, hippocampus, olfactory bulb and striatum suggesting that the expression is restricted to areas of high synaptic plasticity (Alvarez et al., 2002; Abbott et al., 1999; Thomas et al., 2001). In cultured hippocampal neurons, IRSp53 localizes to the synapses and is highly expressed in the postsynaptic density (PSD) membrane (Abbott et al., 1999). Besides being highly expressed in the brain, IRSp53 is also abundantly expressed in the apical surface of many specialized epithelial cells including cells covering the surface of the gastrointestinal tract, kidney tubules, lung and prostate (Human Protein and BioGPS Atlas).

The IRSp53 knockout mice (-/-) do not display gross developmental/ morphological abnormalities and surprisingly have no defects in their dendritic spine densities or morphologies, suggesting that more studies are required to reveal the role of IRSp53 in dendritic filopodia formation (Sawallisch et al., 2009; Kim et al., 2009). However, instead of following Mendelian ratios (25%), fewer homozygous cubs survived the birth (6,6 – 12%). The knockout mice displayed also some mild cognitive defects in their memory, learning and fear reflexes (Sawallisch et al., 2009; Kim et al., 2009).

IRSp53 is implicated in the actin cytoskeleton remodelling through its interactions with the small GTPase Cdc42 and actin bundling/capping protein Eps8. Cdc42 has been proposed to activate the auto-inhibited IRSp53 (Krugmann et al., 2001) thus rendering the protein active and free to bind to other interaction partners involved in actin dynamics **(Table 1)** (Scita et al., 2008).

Over-expression of IRSp53 promotes filopodia formation (Krugmann et al., 2001). In neuroblastoma cells, IRSp53 was shown to induce strong neurite outgrowth (Govind et al., 2001). Furthermore, IRSp53 has been indicated to play a role in membrane ruffling by binding Rac through its I-BAR domain and to WAVE2 through its SH3 domain (Miki and Takenawa, 2002; Miki et al., 2000; Takenawa and Miki, 2001; Abou-Kheir et al., 2008). This interaction is further enhanced through Rac guanine nucleotide exchange factor Tiam1. Tiam1 has been shown to promote IRSp53 localization to Rac-induced lamellipodia rather than Cdc42-induced filopodia (Connolly et al., 2005). IRSp53 also co-localizes with WAVE2 at the tips of protruding lamellipodia and filopodia (Nakagawa et al., 2003). IRSp53 is regulated by phosphorylation of two threonine residues that promote interactions with 14-3-3 protein, which leads to the consequent inactivation of IRSp53 (Robens et al., 2010).

Collectively, these data show that IRSp53 controls actin polymerization locally close to the plasma membrane and that its activity and localization are regulated by many other proteins **(Table 1)**. The function of IRSp53 is linked to the formation of filopodia, membrane ruffles and dynamic lamellipodia.

1.4.4 IRTKS

1.4.4.1 Domain structure

Insulin receptor tyrosine kinase substrate (IRTKS/BAIAP2L1) belongs to the IRSp53/IRTKS sub-family of I-BAR domain proteins. IRTKS is a 57 kDa protein, which has a similar domain structure to IRSp53 although it lacks the central CRIB motif and 14-3-3 binding region **(Figure 7)** (Yamagishi et al., 2004; Millard et al., 2007). The first study describing IRTKS reported that its I-BAR domain functions as an actin bundling domain with a capability to bind the small GTPase Rac. Additionally, the C-terminal WH2 domain of IRTKS was shown to

Table 1. The known interaction partners of IRSp53.

Binding domain	Interaction partner	Function (in the context of IRSp53)	Reference
WH2	G-actin	binds ATP-actin monomers	Lee 2007; Scita 2008
PDZ	PSD-95	scaffolding protein, receptor clustering	Soltau 2004; Choi 2005
PDZ	MALS/Lin7	tight junction binding	Hori 2003
SH3	Atropin-1	CAG-repeats lead to DRPLA	Naito 1982; Thomas 2001
SH3	mena/VASP	formation of adherens junctions in epithelial cells	Vasioukhin 2000
SH3	mDia1/2	binds and activates IRSp53 and recruits Mena	Fujiwara 2000
SH3	Eps8	binding activates Rac	Funato 2004; Millard 2005; Disanza 2006
SH3	Espin	strong actin filament bundler protein	Sekerkova 2003; Bartles 1998; Bartles 1996
SH3	WIRE	filopodia formation via Cdc42	Misra 2010
SH3	WAVE2	promotes the binding of Rac to the I-BAR	Miki 2000; Takenawa 2001; Miki 2002; Abou-Kheir 2008; Nakagawa 2003
SH3	WISH/SPIN90	induces Rac dependent ruffling	Teodorof 2009
SH3	Shank-1	scaffolds which link receptors to actin cytoskeleton	Soltau 2002; Bockmann 2002; Redecker 2007
SH3	N-WASP	couples membrane protrusions to actin cytoskeleton	Lim 2008
SH3	BAI1	enhance localization to membranes	Oda 1999; Das 2011
SH3	EspF(U)	activates nucleation promoting factors	Weiss 2009; Vingadassalom 2009
?	synaptopodin	inhibitor of Mena:IRSp53 signalling	Yanagida-Asanuma 2007
?	14_3_3	blocks other binding sites, thus controlling IRPs53 activity	Robens 2010; Kakinuma 2008; Cohen 2011
CRIB	Cdc42	binding reveals the SH3 domain and induces filopodia formation	Govind 2001; Krugmann 2001
I-BAR	Rac	localizes IRSp53 to ruffles and lamellipodia	Miki 2000; Takenawa 2001; Miki 2002; Abou-Kheir 2008
I-BAR	F-actin	binds to F-actin	Yamagishi 2004; Millard 2005; Gonzalez-Quevedo 2005; Mattila 2007
I-BAR	Kank	suppresses lamellipodia/neurite growth by Rac inhibition	Chandra Roy 2009
I-BAR	Tir	uses IRSp53 for correct localization	Weiss 2009; Vingadassalom 2009

sequester actin monomers more weakly compared to canonical WH2 domains (Millard et al., 2007).

1.4.4.2 Expression pattern, cellular localization and interaction partners

At the protein level IRTKS is strongly expressed in the bladder, liver, lungs, testes and heart. It was also detected in trace amounts in spleen, brain and skeletal muscle (Millard et al., 2007). BioGPS gene expression database shows mouse IRTKS mRNA to be expressed also in stomach, whole intestine, myoblasts, placenta, nasal cavity epithelium, brain and eye. Besides these organs the human IRTKS gene also appears to be highly expressed in lymfoblasts and leukemic cells (BioGPS).
In non-polar cells, instead of being expressed in lamellipodia and filopodia as IRSp53, IRTKS resides in membrane ruffles and in short actin filament containing regions in lamellae (Robens et al., 2010; Millard et al., 2007). By swapping SH3 domains between IRSp53 and IRTKS Robens and colleagues studied the role of different binding partners that would be responsible for the correct sub-cellular localization of the full-length protein. Accordingly, they concluded, that while the SH3 domain of IRSp53 functions to localize the protein to lamellipodia, the related IRTKS SH3 domain is not able to do so. The SH3 domains of these proteins are 62% similar at the sequence level and they were shown to bind mostly different interaction partners (Robens et al., 2010). The shared interaction partners between IRSp53 and IRTKS are generally highly expressed in the intestinal epithelial cells and in certain specialized brain cells - the same places where both IRTKS and IRSp53 are functional (The Human Protein Atlas and BioGPS).

1.4.4.3 Cellular function

The epithelial tissues and cells form a barrier layer against the external environment. This is consistent with the possible functions of IRTKS in the context of pathogen infection (Weiss et al., 2009; Vingadassalom et al., 2009; Morita-Ishihara et al., 2009). Recent studies identified IRTKS and IRSp53 as key host modulators connecting two bacterial proteins together and triggering an actin polymerization cascade. Actin polymerization and membrane remodelling are needed for the enterohemorrhagic *Escherichia coli* (EHEC) bacteria to remain tightly bound to the host membrane, and to be able to multiply before getting flushed out from the intestinal epithelia. The host IRTKS and IRSp53 proteins interact with membrane-bound bacterial Tir protein through their I-BAR domains, localizing Tir to the correct site of EHEC attachment. The SH3 domain of IRTKS binds to the poly-proline rich region of EHEC $EpsF_U$ protein which can also activate the actin nucleation promoting factors WASp and N-WASP (Vingadassalom et al., 2009). While IRTKS seems to be recruited to bacterial adhesion sites in cells, the Tir:IRTKS pathways seem not to be essential for N-WASP recruitment or EHEC lesion formation *in vivo* (Crepin et al., 2010).

1.4.5 Pinkbar

The fifth member of the I-BAR family, FLJ22582 (also known as brain-specific angiogenesis inhibitor 1-associated protein 2 like 2 a.k.a. BAIAP2L2), was first introduced by Yamagishi and colleagues (Yamagishi et al., 2004). This protein was shown to have an N-terminal I-BAR domain, a central region SH3 domain, a poly-proline rich region, and a C-terminal WH2 domain **(Figure 7)**. However, the biochemical, cell biological or functional

properties of this protein were not characterized before this thesis work. The gene encoding the proteins, which we named Pinkbar, is located in the human chromosome 22. According to UniProt two isoforms are encoded from this gene. The major encoded splice variant is 59 kDa in size (UniProt).

2 AIMS OF THE STUDY

The goal of this study was to characterize a novel group of membrane deforming proteins - the Inverse-BAR domain proteins (I-BARs). Moreover, prior to this work, practically nothing was known about the yet uncharacterized I-BAR domain family member FLJ22582. Thus, the specific aims of this study were:

1. To reveal the possible membrane binding properties of the I-BAR domain and to map the precise membrane binding site of MIM I-BAR domain
2. To elucidate the similarities and differences between the biochemical properties of different I-BAR domains
3. To characterize the biochemical and cellular functions of the novel I-BAR family member FLJ22582, which we named Pinkbar

3 MATERIALS AND METHODS

Table 2. Methods which I personally applied in this study. Roman numerals are used to refer to the publication in question.

Method	Publication
Purification of rabbit muscle actin	I
Recombinant protein expression and purification	I, III
Reverse transcription polymerase chain reaction	III
SDS-PAGE	I, III
Actin filament and liposome co-sedimentation assays	I, III
Preparation of synthetic lipid vesicles	I, III
Western blotting	I, III
Transmission electron microscopy (TEM)	I, II, III
Mammalian cell culture and transfection	III
Fluoresence light microscopy	III
Laser scanning confocal microscopy	III
Time-lapse imaging of cells or vesicles	III
Northern blot-analysis	III
Amino acid sequence alignments	III
Phylogenetic tree generation	III
Radioactive in situ-hybridization	III
Histological staining(s) and microscopic analysis of tissue sections	III
Standard RNA and DNA techniques	I, II, III
Sucrose gradient-centrifugation	III
GUV preparation	III
Site-directed mutagenesis and plasmid construction	I, II, III
Image analysis programs (e.g Photoshop, Imaris Bitplane, TILL VisION)	III

4 RESULTS AND DISCUSSION

4.1 Identification of the I-BAR domain as a membrane binding domain with affinity to PI(4,5)P_2 (I)

I-BAR domain proteins were first described to function as actin filament bundling proteins that promote the formation of cellular protrusions or filopodia (Yamagishi et al., 2004; Millard et al., 2005; Gonzalez-Quevedo et al., 2005). The I-BAR domain proteins can by divided into two sub-groups, one consisting of MIM and ABBA and the other of IRSp53 and IRTKS. The "out-cast" family member, Pinkbar, forms its own branch and will be introduced in publication **III**. Relatively soon after the initial discovery of the I-BAR family, the structures of MIM and IRSp53 I-BAR domains were determined (Lee et al., 2007; Millard et al., 2005). Surprisingly, the structure of the α-helical dimer core of the I-BAR domain resembled the membrane sculpting BAR domains (Tarricone et al., 2001; Peter et al., 2004).

Our studies revealed that, unlike reported earlier (Yamagishi et al., 2004; Millard et al., 2005; Gonzalez-Quevedo et al., 2005), MIM and IRSp53 I-BAR domains do not bundle actin filaments under physiological conditions. Thus, we tested whether the MIM and IRSp53 I-BAR domains would bind membranes like the canonical BAR domains. First, we tested the lipid specificity by a native polyacrylamide gel electrophoresis assay, where the interaction of MIM I-BAR and different lipid species could be monitored. This experiment revealed that MIM I-BAR domain indeed binds lipids, and interacts strongest with PI(4,5)P_2. This was also confirmed by co-sedimentation assays, where both MIM and IRSp53 I-BAR bound PI(4,5)P_2-rich synthetic lipid vesicles (liposomes). Binding was also observed with PI(3,4,5)P_3, however, the interactions appeared to be higher with PI(4,5)P_2 (I, Fig. 1 A and B). This membrane binding activity directly linked I-BAR domain proteins functionally closer to the canonical BAR domain proteins, which are involved in events that induce membrane curvature in cells.

I next mapped the membrane binding site of the MIM I-BAR domain. Canonical BAR domains are known to bind the membrane through the concave surface of the banana shaped dimer. Point mutations in positively charged amino acid residues close to the distal ends of the dimer have been previously shown to diminish the membrane binding affinity of the Arfaptin BAR domain (Peter et al., 2004). Thus, twenty well-conserved charged or hydrophobic amino acids on the surface of the MIM I-BAR domain were chosen for site-specific alanine scanning mutagenesis and the binding of purified mutant proteins on PI(4,5)P_2-rich vesicles was studied by co-sedimentation assays. Interestingly, the membrane binding surface of MIM I-BAR mapped on the convex surface of the domain, forming relatively large positively charged patches at the distal ends of the dimer (I, Fig. 2). Taken together, these experiments revealed that the I-BAR domain is a membrane binding protein motif, which interacts with negatively charged PI(4,5)P_2-rich membranes mainly through electrostatic interactions.

4.2 The I-BAR domain proteins generate negative membrane curvature and are involved in PI(4,5)P_2-dependent filopodia formation (I)

When mixed together with liposomes, the BAR and N-BAR domains can generate positive membrane curvature by tubulating the small round liposomes into outwards-protruding long membrane tubules (Peter et al., 2004). This encouraged us to test whether I-BAR domains are also capable of shaping membranes similarly to BAR domains. We used transmission electron microscopy (TEM) to study the protein-liposome interactions and found that the I-BAR domain of MIM did indeed tubulate liposomes. However, there was one clear difference as compared to BAR and N-BAR domains. Electron tomography (ET) analysis of thick sample sections revealed that the tubules in the BAR domain containing samples invaginated towards the interior of the liposome (I, Fig. 1 C, D and E). This indicates that I-BAR domains are able to generate negative membrane curvature, in contrast to positive membrane curvature generated by BAR and F-BAR domains. To understand the mechanism, we had a closer look into the membrane-binding surface mapped by mutagenesis analysis. Importantly, although the membrane binding surfaces of the I-BAR and BAR domains point to the same direction, the intrinsic curvatures of the membrane binding sites are different (I, Fig. 7 A). BAR domains bind membranes through their concave surface, while Inverse-BAR domains (I-BAR) bind membranes through a convex surface. The ability of I-BAR domains to bind membranes with their convex surface was further supported by the fact that the diameter of the tubules generated by MIM I-BAR domain (78 nm with a SD of 7 nm) were comparable to those when an imaginary circle was drawn by using the convex surface of the MIM I-BAR domain (95 nm) (I, Fig.1 and Fig. 7 A, dotted line).

Previous studies revealed that the MIM I-BAR domain localizes to plasma membrane and into filopodia, where it was thought to bundle actin filaments (Yamagishi et al., 2004; Millard et al., 2005; Gonzalez-Quevedo et al., 2005). Our results, however, showed that the MIM I-BAR domain does not co-localize with actin bundles in filopodia, but instead localizes to the interface between plasma membrane and F-actin bundles (I, Fig. 4). Cell biological studies with specific mutants revealed that PI(4,5)P_2-binding by the MIM I-BAR domain is required for filopodia formation (I, Fig. 3). We demonstrated this by transfecting human osteosarcoma cells (U2OS) with either wild-type GFP-tagged MIM I-BAR domain, or with mutant domains defective in actin filament and/or PI(4,5)P_2 binding. Mutant “12+15” K138A / K139A / K146A / K149A / K150A with severe defects in both actin filament and PI(4,5)P_2-binding sites was no longer capable of inducing filopodia formation when over-expressed in cells. Mutant “14+17” L145A / L147A / L170A with moderate defects in PI(4,5)P_2-binding, but normal affinity towards actin filaments, displayed reduced filopodia formation compared to the wild-type.

4.3 I-BAR domains are not equal in their mechanisms of membrane curvature generation (II)

Our hypothesis that I-BAR domains bind to the inner leaflet and promote the formation of membrane protrusions warranted further experiments. This was tackled by using two different methods: live cell imaging of giant unilamellar vesicles (GUVs) and cryo-EM. GUVs resemble small liposomes but are generally around 100 times larger in diameter. They have the approximate size of an average cell or organelle (5-80 μm) (Sens et al., 2008), and can be used as a model to study changes in membrane morphology and dynamics under fluorescent light microscope. We established a system where fluorescently labeled GUVs containing 10% $PI(4,5)P_2$ and 90 % PC were placed under the microscope in sucrose buffer and continuously imaged while gently adding a low concentration of purified recombinant proteins. Almost immediately (within tens of seconds) after addition of the N-BAR domain of Amphiphysin, a rapid formation of membrane tubules protruding outwards from the GUV surface was observed. This *in vitro* system resembles an *in vivo* situation where tubules grow towards the interior of the cell during endocytosis. Addition of I-BAR domains triggered an opposite reaction, where the tubules were oriented towards the interior of the GUV, thus supporting our hypothesis which implies that I-BAR domains bind to the inner leaflet of membrane bilayer (II, Fig. 1 A and B). Again, in cells this would be equivalent to formation of filopodia. However, to be certain that the I-BAR domains are indeed located in the inner leaflet of membrane tubules, we applied cryo-EM on tubules and small liposomes. Although we were not able to visualize the individual protein domains bound to the inner leaflet, we detected an increase in the electron density difference between the inner and outer leaflets resulting from the bound protein (II, Fig. 1 C-F). These results supported our hypothesis that the I-BAR domains generate membrane curvature to the opposite direction compared to canonical BAR domains.

While imaging GUVs containing fluorescent labeled $PI(4,5)P_2$ and PC, we noticed that $PI(4,5)P_2$ seemed to form clusters upon addition of either N-BAR or I-BAR domains. We quantified the clustering ability by using a fluorometric $PI(4,5)P_2$-assay based on self-quenching of bodipy-TMR-label attached on $PI(4,5)P_2$, and found that all tested I-BAR domains were able to cluster $PI(4,5)P_2$ efficiently. Furthermore, by using a MIM I-BAR mutant "12+15" defective in $PI(4,5)P_2$ binding, we revealed that the mechanism behind the $PI(4,5)P_2$ clustering depends on electrostatic interactions between the protein and the membrane (II, Fig. 2). In this mutant five positively charged hydrophilic lysines were replaced with neutral hydrophobic alanines (see chapter **4.2**).

Interestingly, the tubules created by different I-BAR domains vary in size *in vitro*. MIM and ABBA I-BAR domains induced approximately 40 % wider tubules as compared to those generated by IRSp53 and IRTKS (II, Fig. 3). This finding was further supported by analyzing the sub-cellular localization pattern of I-BAR domains in double-transfected cells. When over-expressed together in a same cell the MIM and ABBA I-BAR domains shared a similar localization pattern in as did IRSp53 and IRTKS (II, Fig. 3 D and S3). Both *in vitro* and *in vivo* results indicated that the I-BAR sub-groups use partially different mechanisms to generate membrane curvature. To find out what these mechanisms would be, we carried out many parallel assays.

First, by increasing the salt concentration from physiological values up to 400 mM, we learned, that besides electrostatic interactions, the MIM and ABBA I-BAR domains use some other mechanism for membrane interactions (II, Fig. 4 A). The structure of MIM I-BAR domain (Lee et al., 2007) and the results from two N-BAR domain studies (Gallop et al., 2006; Masuda et al., 2006) encouraged us to test whether I-BAR domains would also insert a part of their structure into the membrane bilayer. Diphenylhexatriene (DPH) anisotropy measurements indicated that MIM and ABBA I-BAR domains most likely insert part of the domain into the membrane bilayer, and truncation of their N-terminal amphipathic α-helix by 11 residues abolished this insertion. We also measured the depth of the membrane insertion by placing brominated tags into different positions at the acyl-chain. These experiments suggested that the tryptophans at positions 30 and 4 in the N-terminal region of the MIM I-BAR domains insert at depths of ~7,3 Å and ~10,8 Å from the center of the bilayer, respectively (II, Fig. 4 B-F). The N-terminal deletion mutant was still able to tubulate membranes, but the width of the tubules now resembled those generated by IRSp53 and IRTKS (II, Fig. 5). This suggests that the MIM and ABBA sub-group of I-BAR domains uses both scaffolding and hydrophobic insertion mechanisms to generate the membrane curvature (see **chapter 1.2.1.5** and II, Fig. 7).

4.4 Pinkbar is a BAR domain protein with a novel mechanism to deform membranes - functional plasticity revealed within the family (III)

Next, we set forth to identify the potential membrane modeling capacity of a previously uncharacterized protein closely related to the other I-BAR domain proteins (see **chapter 1.4.5**). We named the protein Pinkbar (for planar intestinal- and kidney-specific BAR domain protein, see below). Because of the novel functions of the Pinkbar I-BAR domain compared to other I-BAR domains, the domain will be referred as a BAR domain in the following chapters.

4.4.1 The structure of the BAR domain of Pinkbar

The crystal structure of the BAR domain of Pinkbar was determined by our collaborator Roberto Dominguez´s laboratory in University of Pennsylvania, USA (III, Table 1). At first look, the structure of the BAR domain of Pinkbar resembles those of MIM and IRSp53 I-BAR domains. The domain is composed of two identical monomers, and each monomer consists of three α-helices. However, certain special details emerge upon closer examination. First, the BAR domain of Pinkbar is shorter than the other I-BAR domains (Pinkbar 164 Å vs. MIM 185 Å). Second, the domain contains blubs at the distal ends of the dimer, which are not present in other I-BAR domains. Third, the overall structure of the domain is relatively flat when compared to the MIM and IRSp52 I-BAR domains (III, Fig. 3 A and B).

4.4.2 The BAR domain of Pinkbar stabilizes planar membranes rich in $PI(4,5)P_2$

To dissect the membrane binding potential of the Pinkbar BAR domain, we applied similar methods to those used and introduced in publications **I** and **II**. With the structure in hand, we were also able to perform a systematic mutagenesis analysis to map the lipid binding surfaces of the molecule (III, Fig. S8 B). In fluorometric

assays and vesicle co-flotation assay the BAR domain of Pinkbar was able to bind to and cluster PI(4,5)P_2-rich membranes and decrease the membrane fluidity similarly to other I-BAR domains. Mutagenesis data revealed that the membrane binding surface of the Pinkbar BAR domain was oriented towards the same direction as the membrane binding surfaces of other I-BAR domains (III, Fig. 4 A and B and Fig. S3 A). Additionally, an increase in the salt concentration weakened this interaction, suggesting that the binding is electrostatic (III, Fig. S8 A).

To test if this BAR domain is capable of inserting into the membrane bilayer, four additional mutants were generated. First, we tried to neutralize the amphipathic nature of the potential N-terminal helix by changing 19I and 20M to 19E and 20E. However, this mutant turned out to be insoluble and unstable (data not shown). We then created two N-terminal deletion mutants, one lacking the first 9 residues and the other lacking 16 residues. Of these mutant proteins, only the former was soluble. DPH anisotropy analysis using the wild-type and the N-terminal 9 aa deletion domain suggested that the N-terminal α-helix inserts into the lipid bilayer (III, Fig. S4 B and Fig. S3 B) and that the insertion is not specific for any certain phosphoinositide (III, Fig. S4 A). To evaluate the depth of this insertion we generated a mutant where two tryptophans of the domain were replaced by leucines and a new tryptophan was introduced in the N-terminal α-helix at position 9 (Y9W / W141L / W216L). With this mutant, we detected a change in the tryptophan fluorescence in acyl chains brominated at positions 11 and 12 corresponding to a distance of ~ 6,3 Å from the center of the bilayer (III, Fig. S4 B).

Although these experiments suggested that Pinkbar shows similar properties with other I-BAR domain proteins, its membrane deformation activity is clearly different. While other I-BAR domains tubulated liposomes in an EM analysis, the BAR domain of Pinkbar showed no signs of membrane tubulation. Instead, it clustered liposomes together and generated membrane sheets (III, Fig. S5 A and B). Electron tomography (ET) analysis revealed that the observed liposome clusters are composed of stacks of membrane bilayers. The more electron dense protein signal originating from BAR domain clusters appeared to be missing from the regions with high membrane curvature (III, red arrow heads in Fig. 2 A) indicating that the BAR domain of Pinkbar prefers to bind less curved or planar membranes. Additionally, the mutant R145A / K146A / R147A domain with defects in PI(4,5)P_2 binding had a severe defect in the ability to cluster PI(4,5)P_2-rich liposomes *in vitro* (III, Fig. S7 D). A liposome content mixing assay revealed that the Pinkbar BAR domain does not induce liposome fusion within these clusters **(Figure 8)**.

Next, we carried out live imaging assays of NBD-PC labeled GUVs in the presence of Cherry-tagged BAR domain of Pinkbar. Similarly to the smaller liposomes examined by EM, no tubules were detected protruding towards the interior of GUVs. The binding of the protein onto the GUV was rapid and cooperative, since not all the GUVs accumulated protein on their surface (III, Fig. 2 C-D). Importantly, we observed planar or gently curved regions on Pinkbar containing GUV surfaces that were not present in the control samples (III, Fig. 2 C-D and S5 C). To conclude, the BAR domain of Pinkbar does not tubulate membranes like other I-BAR domains. Instead, it induces the formation of planar or gently curved membrane structures.

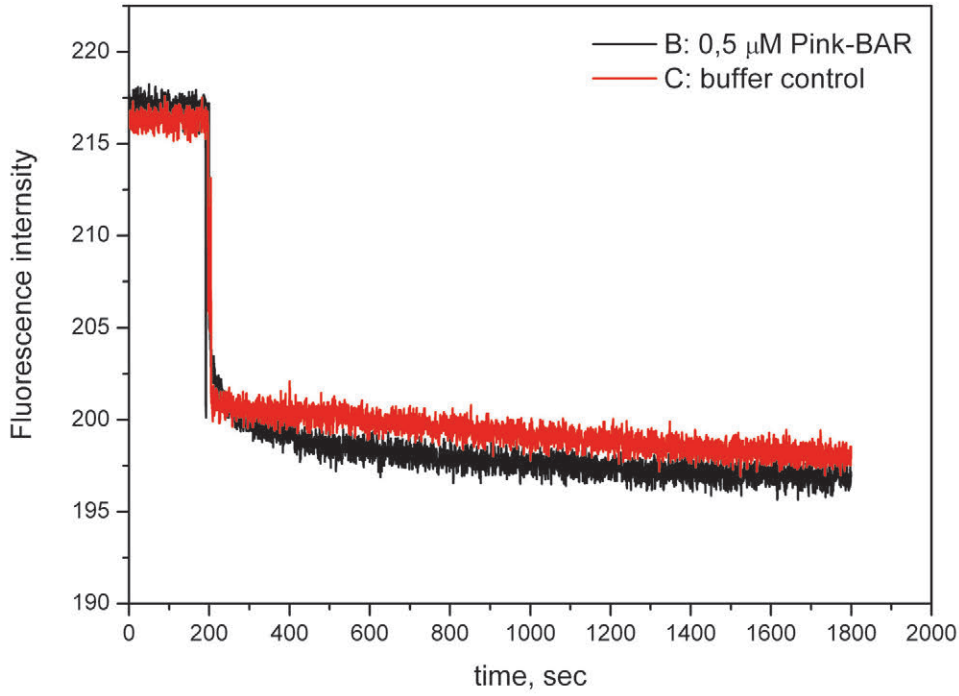

Figure 8. Liposome content mixing assay. The BAR domain of Pinkbar does not induce significant membrane fusion as detected by an ANTS/DPX content mixing assay (Ellens et al., 1984). 0.5 μM protein or buffer (control) were added to a 100 μM 9:1 mixture of vesicles containing DPX or ANTS. The large unilamellar vesicles consisted of a POPC/POPE/POPS/PIP2 (50:20:20:10) mixture.

4.4.3 The mechanism of Pinkbar-induced membrane sculpting

The BAR domain of Pinkbar, as a recombinant protein, was more difficult to purify as compared to the other I-BAR domains. The main reason behind this was proteins aggregation or oligomerization which occurred after a critical concentration was exceeded (III, Fig. S7 A). Increasing the salt concentration or addition of glycerol reduced this effect slightly, but this self-assembly was clearly stronger than previously reported for other I-BAR domains. These oligomeric structures were also visible in both EM and GUV samples [III, Fig. S5 B (sheets) and C (red protein clusters)]. The molecular mechanism behind the self-assembly was not clear until the crystal structure of the domain was determined. In the crystal lattice, the BAR domain of Pinkbar is arranged to sheet-like oligomers (III, Fig. 3 C). Interestingly, similar oligomeric coats were previously reported in the cryoEM analysis of F-BAR domain induced membrane tubules (Frost et al., 2008).

A surface exposed tryptophan (W141) appears to be important for stabilizing the interdomain interactions in the crystal lattice. Importantly, replacement of W141 by serine (S) decreased domain oligomerization also in solution as detected by dynamic light scattering (III, Fig. S7 B). The W141S mutant also displayed displayed severe defect in membrane deformation assay (III, Fig. S7 C), although the membrane binding affinity was not altered (III, Fig. 4 A). It

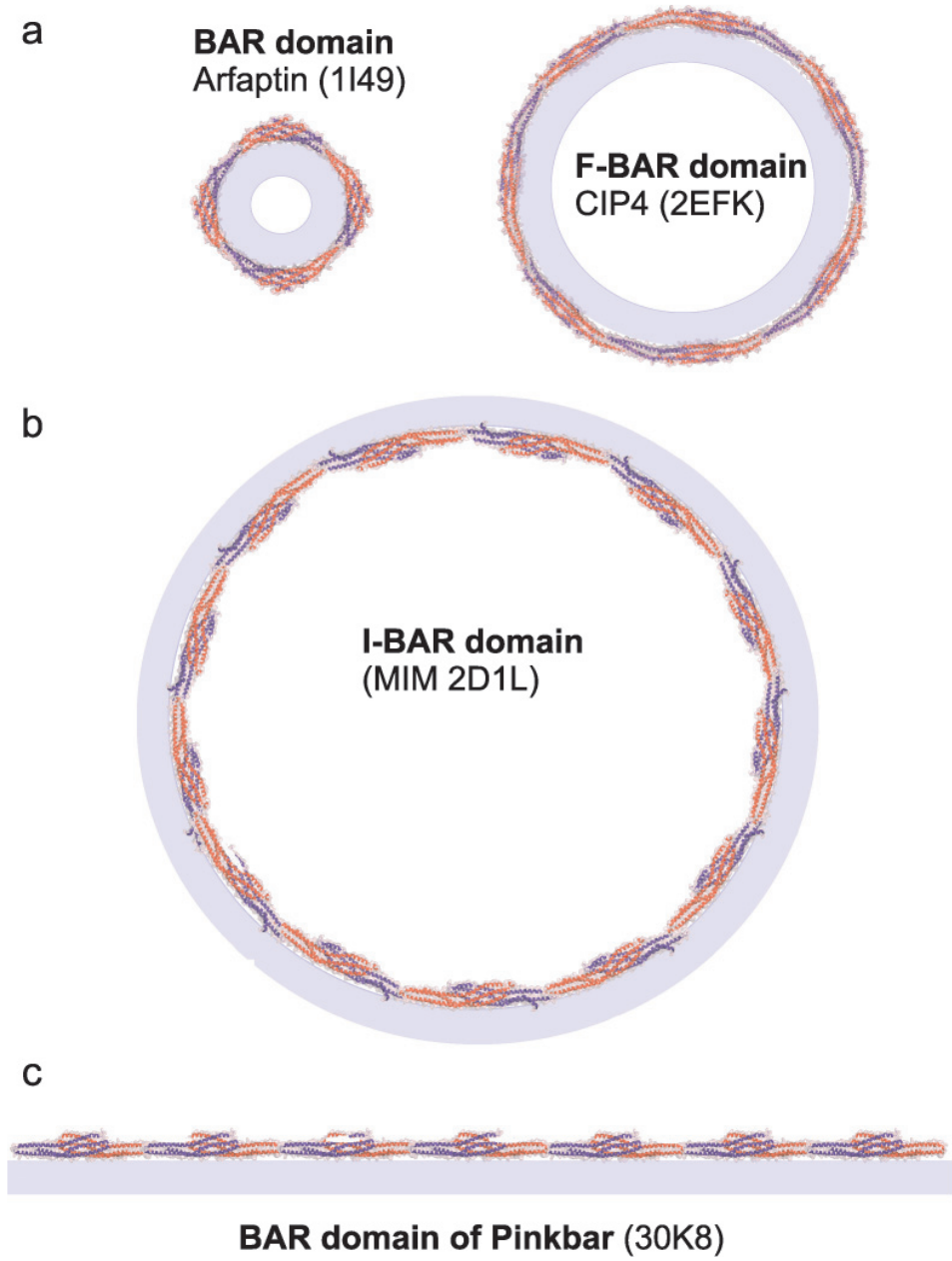

Figure 9. Comparison of membrane curvature/stabilization mechanisms by different domains of the BAR domain superfamily. The light violet area depicts a 4 nm thick membrane bilayer. The size of the BAR domains is in proportion with the membrane. **a)** BAR and F-BAR domains form a coat around the outer leaflet. The widths of the tubes generated by Arfaptin (~ 28 nm) and CIP4 (~ 58 nm) domains correspond well with the literature (Peter et al., 2004; Frost et al., 2008; Qualmann et al., 2011; Shimada et al., 2007). **b)** The Inverse-BAR domains (I-BAR) bind to the inner leaflet and generate wider tubules in diameter. **c)** The BAR domain of Pinkbar stabilizes planar membranes with its relatively flat membrane binding surface.

is important to note that this tryptophan residue is unique to Pinkbar and is not conserved among the other I-BAR domain proteins (III, Fig. 6 C). Surface exposed tryptophans are rare and often linked to structural roles such as formation of protein-protein interactions and W141 position fits perfectly to support this hypothesis (III, Fig. 3 D). Together, these results suggest that oligomerization of the Pinkbar of BAR domain is not critical to vesicle clustering, but is important for the formation of planar membrane sheets by this domain (III, Fig. 3 and Fig. S7).

Individual BAR domains are aligned in the planar oligomer observed in the crystal structure in a way that leaves their membrane binding surfaces accessible (III, Fig. 3 C). In other words, the BAR domain is able to bind the membrane in its planar oligomeric form (III, Fig. 4 C). Thus, the nearly planar membrane binding surface of the BAR domain combined to the oligomerization and membrane insertion seems to promote the formation of flat membrane structures **(Figure 9)**. These data also suggest that the Pinkbar BAR domain uses three different membrane sculpting mechanisms simultaneously to achieve its function: 1) specific geometry of the lipid binding interface at the domain, 2) amphipathic insertion and 3) domain oligomerization.

4.4.4 The expression pattern and sub-cellular localization of Pinkbar

Next, we examined where and how Pinkbar utilizes its novel membrane stabilizing activity in cells. *In situ* hybridization analysis carried on mouse tissues revealed that expression of Pinkbar mRNA was confined to the epithelial layer of the

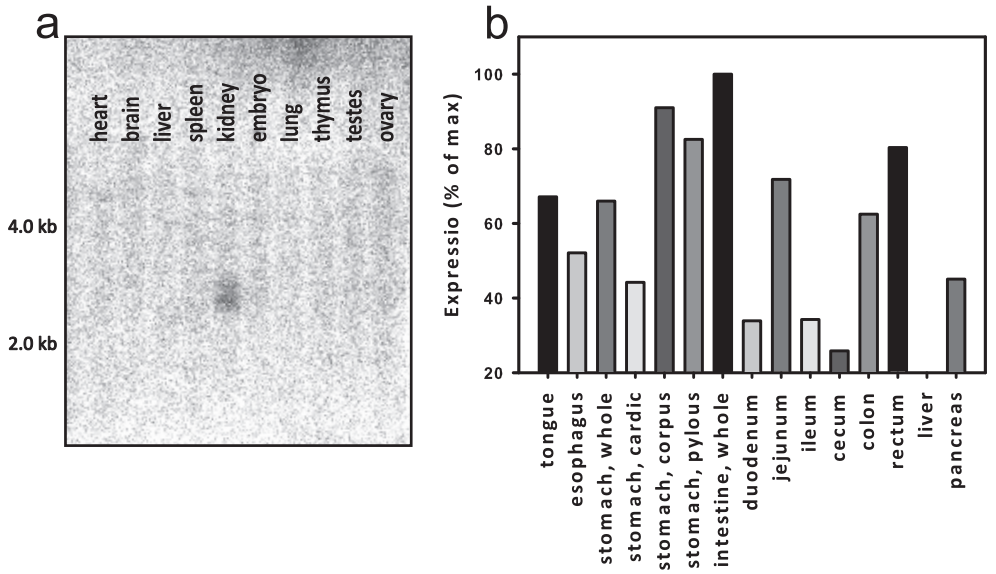

Figure 10. Northern blot analysis revealed that Pinkbar mRNA is specifically expressed in kidney and in the gastrointestinal tract. a) The Mouse blot contains 2 µg of poly(A) RNA per lane (Ambion). The hybridization of a radioactive DNA-probe could be detected only in the kidney lane. **b)** A more thorough Mouse blot from different digestive tissues contains 20 µg of total RNA per lane (Zyagen). The data was quantified with Aida software. Pinkbar mRNA is expressed at highest levels in the stomach, intestine, and rectum.

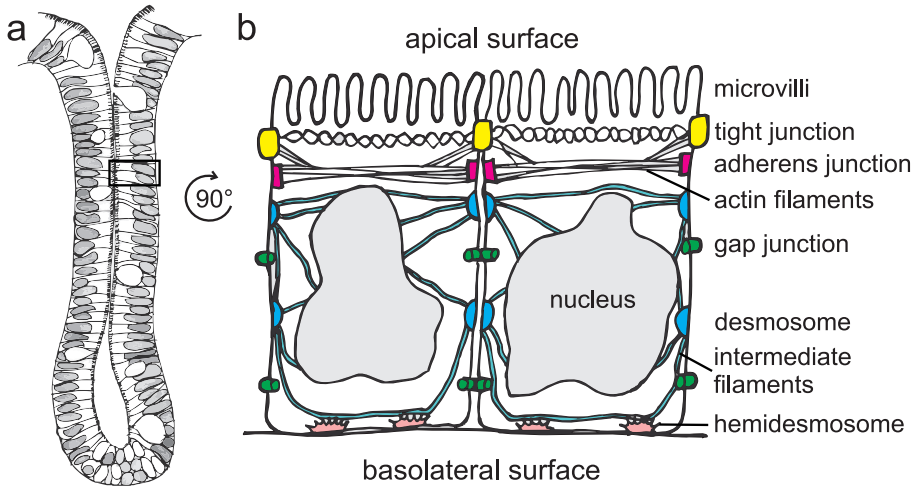

Figure 11. Tissue organization of intestinal enterocytes. a) A schematic representation of the intestinal epithelial cell layer consisting of epithelial cells a.k.a. enterocytes and of mucus secreting goblet cells (white cavities). This figure was made according to www.bartleby.com/107/pages/page1173.html: Anatomy of The Human Body. **b)** A close-up of two enterocytes. These cells are highly polarized with an apical surface dense with microvilli and a basolateral surface. These cells are specialized in nutrient absorption from the intestinal lumen. They also function as a barrier against the outer environment. The cell-cell contacts, known as tight junctions, prevent any unwanted leakage through the epithelial layer. This figure was made according to Tsukita et al., 2001.

intestine and to specific regions of the kidney (III, Fig S1 B and C and BioGPS). Northern blot analysis of different mouse organs also detected Pinkbar expression in the kidney **(Figure 10 a)**. A more thorough Northern blot analysis using tissues containing different parts of the mouse gastrointestinal (GI) tract, demonstrated that Pinkbar is expressed in varying amounts throughout the entire GI tract. The expression was the highest in the lower parts of stomach, the middle part of small bowel (jejunum), colon, and rectum **(Figure 10 b** and Human Protein Atlas**)**. We were also able to detect the Pinkbar protein in human colon epithelial layer by immunohistochemistry using a specific antibody (III, Fig S1 D).

Human epithelial colorectal adenocarcinoma cells (Caco-2) were used as *in vivo* cell model since the protein was endogenously expressed only in this cell line from the ones we tested. Caco-2 cells are highly polar when mature, displaying a specialized apical surface with microvilli, tight cell-cell contacts, and a basolateral surface which attaches the cell layer onto the substratum **(Figure 11)**. Antibody staining showed that Pinkbar is localized close to cell-cell contacts both in Caco-2 cells and in a human colon sample. It displayed a punctuate localization pattern and was occasionally localized to tight junctions (III, Fig S2 A and B).

4.4.5 Hypothetical function of Pinkbar in intestinal epithelia cells

The cytoplasmic punctuate localization pattern of Pinkbar did not fit with the planar membrane supporter role suggested by the structure and the biochemical analyses. Thus, we aimed to identify these punctuate structures by performing multiple co-stainings with a full-length Pinkbar-GFP

construct and some well-known organelle markers e.g. dextran for endosomes, transferrin for recycling compartments, and bodipy fatty-acid for lipid droplets. None of the tested lipid markers co-localized with Pinkbar although some mild overlap was seen with the cholesterol stain Filipin (data not shown). However, systematic analysis of Pinkbar localization compared to small Rab GTPases tagged with a fluorescent probe revealed colocalization of Pinkbar and Rab13 at tubular and punctuate cytoplasmic structures in nonpolarized and polarized Caco-2 cells, respectively (III, Fig. S2 C). The same was demonstrated with endogeneous proteins in Caco-2 cells (III, Fig. 1 E). To conclude, Pinkbar localizes to Rab13-positive vesicles and to the plasma membrane at intercellular contacts in intestinal epithelial cells (enterocytes). Interestingly, the BAR domain of Pinkbar also has a high affinity towards cholesterol (data not shown), which is enriched in the enterocyte plasma membrane.

The Rab small GTPases have many functions in regulating various intracellular vesicle trafficking pathways. Rab proteins are known to recruit effectors to the sites of vesicle formation and membrane fusion. They are also tightly linked to phagosome formation and to early endocytic pathways. New studies have also identified Rab proteins as key factors in membrane fusion events together with the SNARE-complex (Ohya et al., 2009; Hutagalung and Novick, 2011). Rab8, a close relative of Rab13, has an important role in exocytic membrane traffic from Golgi complex to the plasma membrane (Chen and Wandinger-Ness, 2001). Rab8 is found on tubular structures that participate in recycling of membrane to the plasma membrane. These structures showed a significant overlap with Rab13 (III, Fig. S2 C)(Hattula et al., 2006). Rab13 is highly expressed in enterocytes and localizes mainly to cell-cell contacts. It was reported to function in transport from the *trans*-Golgi network and recycling endosomes to tight junctions in enterocytes (Zahraoui et al., 1994). Consequently, it plays an important role in regulating both the structure and function of tight junctions (Marzesco et al., 2002).

Enterocytes have two important roles; they form the inner protective layer against pathogens and absorb nutrients from the digested food. This means that the cell-cell contacts must be tightly sealed to prevent major leakage. However, tight junctions are not fully sealed; they are selectively permeable and able to discriminate between solutes on the basis of size and charge (Shen et al., 2011). Two major structural proteins, Claudin and Occludin, connect the neighboring cells together within tight junctions. Rab13 regulates the transport of these structural building blocks to and from tight junctions (Yamamura et al., 2008). Also other proteins introduced in this thesis earlier (e.g. Reticulons, BAR domain proteins, and EHD proteins) have been linked to various Rab proteins in the intracellular recycling routes (Audhya et al., 2007; Miaczynska et al., 2004; Naslavsky et al., 2006). Thus, it is possible that also Pinkbar is transported to/from tight junctions in Rab13 positive vesicles. The importance of Pinkbar in cell-cell junctions is also supported by recent studies demonstrating that the related I-BAR domain proteins MIM and IRSp53 are involved in the maintenance of intercellular junctions in other types of epithelial cells (Saarikangas et al., 2011; Massari et al., 2009).

Based on our data, I propose the following hypothetical model for the function of Pinkbar in enterocytes: The enterocyte lifecycle is relatively short, 2-4 days, which means that new cells are continuously needed to replace the old ones from the

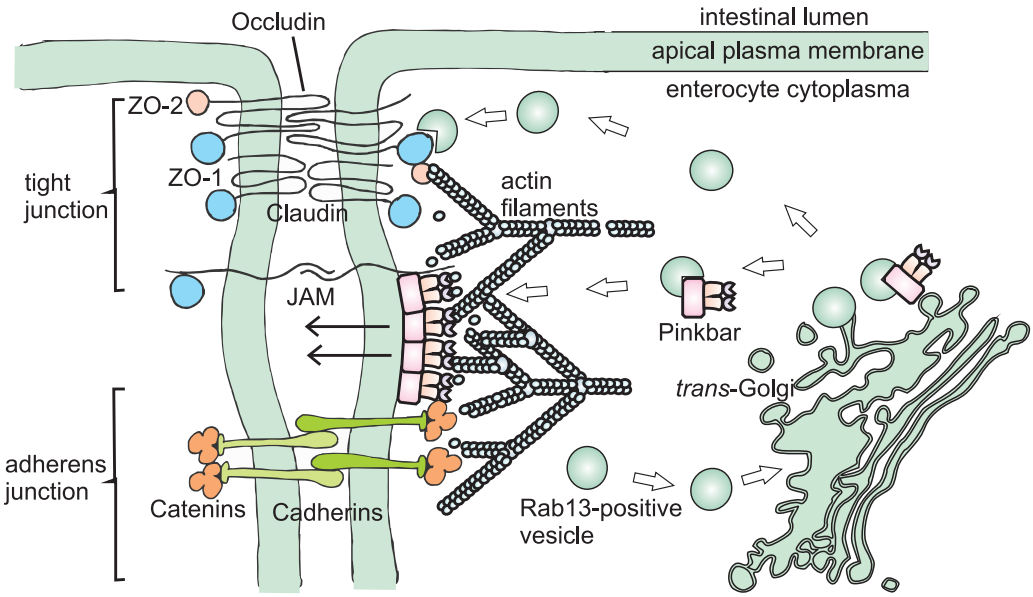

Figure 12. Hypothetical functional role for Pinkbar in intestinal epithelial cells. Pinkbar is transported from *trans*-Golgi network close to cellular junctions in Rab13 positive vesicles. At cell-cell contacts Pinkbar self-assembles into planar platforms, which may stabilize these contact sites. Additional support may be provided by the growing actin filaments attached to the Pinkbar platform. JAM: Juctional Adhesion Molecule and ZO-1 & 2: Zona Occludens proteins 1 & 2.

apical surface (Radtke and Clevers, 2005). Pinkbar expression is the highest when these cells are mature and localized to the surface facing the luminal environment (III, Fig. S1 E). The Rab13-positive vesicles localize the Pinkbar protein either in the vicinity of cellular junctions or as a part of a complex supporting them. At these sites the plasma membrane is relatively planar and may need additional support for the other junctional proteins to perform their tasks properly. I propose that inactive Pinkbar is transported from *trans*-Golgi network close to cellular junctions in Rab13-vesicles, where it becomes activated and released from the vesicle **(Figure 12)**. At cell-cell contacts Pinkbar self-assembles into planar platforms, which together with Pinkbar´s interaction partners stabilize these contact sites. Pinkbar may directly also interact with the growing actin filament ends through its actin monomer binding WH2 domain. Elongating actin filaments might provide additional structural support by pushing against the Pinkbar platform. In this way, Pinkbar may be involved in regulating the intestinal permeability or nutrient absorption. However, the exact function of Pinkbar at these sites remains unresolved. Revealing the precise role of Pinkbar for the stability of intercellular contacts in intestinal epithelial cells would require generation and analysis of Pinkbar-deficient mice. Moreover, no interacting proteins for Pinkbar have yet been identified.

5 CONCLUSIONS

By using *in vitro* model biomembranes (liposomes and GUVs), we have shown that a majority of the I-BAR domains bind to membranes and deform them into tubular structures through a convex lipid binding surface composed of positively charged amino acids (**I**). Thus, the membrane-binding surface of I-BAR domains displays an opposite geometry to that of the BAR, N-BAR and F-BAR domains. These structural differences partially explain why I-BAR domains induce plasma membrane protrusions, whereas BAR, N-BAR and most F-BAR domains induce plasma membrane invaginations.

We also discovered that a subset of I-BAR domains can insert an α-helical motif into the membrane bilayer, which has important consequences for their membrane binding, bending, and/or stabilizing functions (**II**). By inserting a helix into one leaflet of the membrane bilayer, I-BAR domain proteins MIM and ABBA induce wider tubules than those generated by IRSp53 and IRTKS.

In mammals, there are five I-BAR domain containing proteins. Of these five, MIM, IRSp53, IRTKS, and ABBA induce filopodia formation when expressed in cells, and deform $PI(4,5)P_2$-rich membranes to tubular structures *in vitro*. Remarkably, the BAR domain of the fifth mammalian family member Pinkbar binds membranes through similar electrostatic interactions as other I-BARs, but does not deform membranes to tubules. Instead it stabilizes them into planar sheet-like structures (**III**). Structural and mutagenesis analysis revealed that the BAR domain of Pinkbar has a relatively flat lipid-binding interface and that it assembles into sheet-like oligomers in crystals and in solution, which probably explains its unique membrane deforming activity. Pinkbar is expressed predominantly in intestinal and kidney epithelial cells, where it localizes to Rab13-positive vesicles and to the plasma membrane at intercellular junctions. My results suggest that Pinkbar could be involved in the formation of specific membrane structures at the intercellular junctions of intestine epithelial cells that may e.g. regulate the intestinal permeability or nutrient absorption.

In conclusion, our work describes the mechanisms by which I-BAR domain proteins deform membranes and provides new information about the biological roles of these proteins. Intriguingly, this work also provides evidence that significant functional plasticity exists within the I-BAR domain family. I-BAR domain proteins can either generate negative membrane curvature or stabilize planar membrane sheets, depending on the specific structural properties of their I-BAR domains. To our knowledge, this work also introduces the first example of a protein domain involved in the stabilization/generation of planar membrane structures. In the future, it will be especially important to reveal the cellular and physiological roles of the five mammalian I-BAR domain proteins, and to elucidate how the membrane sculpting activities of these proteins are linked to actin dynamics and intracellular signaling pathways.

6 ACKNOWLEDGEMENTS

This study was carried out at the Institute of Biotechnology (BI), University of Helsinki, with the financial support from the Viikki Doctoral Programme in Molecular Biosciences (VGSB) and the Biomedicum Helsinki Foundation. I wish to thank directors Mart Saarma and Tomi Mäkelä for the great atmosphere and the excellent research facilities at the BI. I also want to thank the computational (Iikka), maintenance (Repe and Petri), administrative, and Media Kitchen staff (Riitta) for their assistance and invaluable help.

Professor Pekka Lappalainen, you have been the best supervisor a PhD student could hope for. You have been able to create a stimulating environment and attract all the coolest researchers, my dear colleagues, to work in your lab. Your enthusiasm towards science is admirable – actually, nearly everything I have learned from being an academic researcher comes from you. I can remember our countless corridor conversations and your excitement after seeing some of the preliminary results. Although sometimes I sure hoped that I would own a recorder. When I struggled with my research, you made me feel better by saying that I don´t need to know everything about everything and that it is OK if I only take 10 images from one cell instead of hundred. With respect, Ansku.

I had the privilege to study in the best division that University of Helsinki can offer – the Division of Genetics. Professors Tapio Palva, Jim Schröder and Juha Partanen together with Docent Pekka Heino made it easy for me to get excited about genomics and its applications. However, part of the reason behind my good success in courses was the excellent VGSB grad school and its amazing coordinators Sandra Falck and Nina Bergelin and the coolest principal, Professor Dennis Bamford. I also want to thank Anita Tienhaara for the always updated information and for the cover layout for this thesis. Thank you all for organizing such a great events and courses throughout the years. Timo Päivärinta alias Tinde is thanked for the superb layout.

My sincere thanks go to the excellent pre-examiners Docent Varpu Marjomäki and Docent Vesa Olkkonen for reviewing this thesis with a fast schedule. Your excellent (mielettömät) comments and suggestions improved this work a great deal. I am grateful for my follow-up group members Docent Maria Vartiainen and Professor Elina Ikonen for the guidance throughout these years. Maria, I am happy that you came back and for all the scientific and not so scientific conversations we have shared.

I want to thank the best possible colleagues and co-authors one can hope for: Pieta, Juha, Hongxia, Ville and Essi. Working with you made me realize why I love this work and why I just can´t see myself doing anything else. Additionally I want to give my biggest gratitude for all the collaborators and co-authors: Malgorzata Boczkowska, Grzegorz Rebowski, Maurice Jansen, Janne Hakanen, Johan Peränen, Helena Vihinen, Eija Jokitalo, Marjo Salminen, Elina Ikonen, Roberto Dominguez, Pasi Laurinmäki, Paavo Kinnunen and Sarah Butcher. The valuable help and numerous constructs from Docent Johan Peränen have made my life much easier, thank you. The colleagues and personnel at the EM and LM units have taught me pretty much everything I know about imaging, thank you for that!

Pieta, you were my first supervisor in the practical lab work. The way to pipet, to make the perfect agarose gel and all the small things that matter, I learned from you. You are my friend and a mentor whom I respect tremendously. Thank you for choosing me. Miia, I know that at first you were a bit upset that I was hired instead of some cute guy. But can you now imagine a life where we would not be ´sydämen ystäviä´ – I can´t. I love you dearly, thank you for all the help in matters related to lab work, school and especially, matters of heart. You are my solid rock and a mirror against which I can reflect my own feelings and see a familiar reflection.

Minna, you are the best! Thank you dear friend for choosing Pekka´s lab for your postdoctoral studies. When you enter the room the sun starts to shine. I sometimes wonder if you know how huge impact you leave in the hearts of people you encounter during your life. I was blessed to be one of the lucky ones. I also want to thank you for the critical reading and for the constructive suggestions you have given me during my thesis and grant application writing. You are my good police. Janne, you are my bad police. Without the strict deadlines and constant reminder emails I would have failed. I am in debt to you, Minna and muru (the best police and the kielipoliisi). You all improved the quality of my thesis and I am ever so grateful for it. Hongxia, you have taught me everything I know about lipids and membranes. You are the big sister I never had and belong to my extended family. Hugs, Ansku.

I also had the privilege to be a part of and work together with two Lappalainen sub-groups. I don´t know any other scientific group that would have such a good spirit than ours. I might be biased, but still I think we rule! The first people I got to know were (in random order): Pieta (the machine), Pirta (the lovely PI), Miia (my Miia), Enni (energy bunny), Ville (the coolest teacher), Aneta (warm at heart), Elisa (Vuh) and Perttu (ghost). Gradually, when people graduated we got some new blood in to our group and soon the whole group was changed: Maarit (loyal friend), Hongxia (my big sister), Martina (the wild-one), Sari (the silent gun), Geri (the scientist), Minna (the soul and heart of the group), Elena (the precise mom to be), Markku (superman), Essi, (the best student) and former students Kirsi, Juho, and Nitai. Anna-Liisaa haluan kiittää kaikesta avusta näiden kuuden vuoden aikana.

Haluan kiittää kaikkia ystäviäni kaikesta siitä tuesta ja avusta mitä teiltä olen saanut tämän prosessin aikana. Ilman teitä ja kaikkia ihania tapahtumia en olisi jaksanut painaa pitkiä päiviä ja viikonloppuja. Kiitos myös siitä, että olitte ymmärtäväisiä silloin kun minun piti lähteä ruokkimaan soluja kesken kaiken tai poistua juhlista aikaisemmin työhöni vedoten. Kiitos Manski, Pale, Touko, Laura, Timo, Ella, Paula, Aino, Esa, Sohvi, Minna P, Phillip, Mikko A, Sari, Minna T, Kirsi T, Kirsi M, Kirsi P, Tuukka, Kari, Tuukka A, Make, Jukka, Jenni, Tuomas, Jenni, Johanna, Antti V, Antti I, Kaisa, K-P, Guillaume, Joseph, Lotta, Jussi, Maiju (kiitti erityisesti työtilasta), Ontrei, Hude, Martina, Satu, Essi, Heini, Mari, Meri, Joona, Vilppu, M&M, Vilma, Mikko M, Paavo, Heidi ja Miriam. Unohdin varmaan jonkun, mutta annathan sen minulle anteeksi. Kiitän sinua silti.

Ennen kaikkea haluaisin kiittää perhettäni; iskää, äitiä ja Eekulaa. Kiitos siitä, että jaksoitte aina uskoa osaamiseeni ja taitoihini silloinkin kun en itse kyennyt. Äiti, sinun vankka uskosi minun osaamiseeni on potkinut minua eteenpäin ja aina tähän pisteeseen saakka. Iskä, ilman sinun namnam-hyrinäruokaa ja huomaamatonta huolehtimista minulta olisi loppunut energia jo ajat sitten. Pikkusiskoani haluan kiittää vankasta tuesta huonoina ja hyvinä hetkinä. On ihana tietää, että sinä olet aina olemassa minulle sellaisena kun olet. Olet voimakas ja sydämellinen ihminen, josta ammennan voimaa omaankin jaksamiseeni. Haluan myös kiittää Jannea siitä, että otat meidät aina ilomielin vastaan kotiisi. Sinun öttiäistesi tarjoama rapsuterapia on myös ladannut akkujani enemmän kuin arvaatkaan. Kiitos Anu ja Kari kun olette aina lähellä. Teidän vankkumaton tukenne ja monenmoiset illalliset ovat tehneet minusta vahvemman ihmisen.

Viimeiseksi ja tärkeimmäksi haluaisin kiittää sydämeni valittua, omaa Anttiani. Sinun uskomaton positiivisuutesi ja epäitsekkäät tekosi ovat pitäneet minua pystyssä silloinkin kun olin jo valmis luovuttamaan. Kiitos, että jaksoit tukea minua läpi tämän koko prosessin ja että pidit minut lämpöisenä ja kylläisenä. Odotan innolla meidän uutta elämäämme Kalifornian lämmössä; siitä tulee niin mageeta! Maailmassa ei ole mitään mitä en voisi kokea yhdessä sinun kanssasi. Rakastan sinua.

Helsinki, November 2011

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