Meeting report: Second International Conference on F-BAR Proteins

October 1-3, 2009 at Rånäs Slott, Sweden

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Running title: F-BAR conference
Keywords: membrane tabulation, actin polymerization, F-BAR domain, Rho GTPases, phosphoinositides

Architectural beauty attracts many travellers to Europe. The participants of this conference were also attracted by architectural beauty, however the focus in our case was on the architectural beauty of the living cell. The living cell is not only an architectural masterpiece, but possesses fluidity and dynamism even the most gifted artisans would have found impossible to represent in stone. The architectural beauty of the living cell is created by a complex interplay between lipid bilayer membranes and the proteins that lie underneath them at the cortex. The architecture of the living cell is extraordinarily responsive to changes that occur within cells (intrinsic events) and in the extracellular environment (extrinsic events). Complex and interwoven signalling pathways link the intrinsic and extrinsic events to changes in cell shape and behaviour and those that have been identified and studied probably represent only a minority. What has become apparent, however, is the central and unique role of a group of phospholipid-binding and signalling proteins in these various pathways: the BAR, F-BAR, and I-BAR domain proteins.
The founding member of these families was the BAR domain. The BAR domain is a class of phospholipid binding domain that possesses the ability to sense membrane curvature. In conjunction with other phospholipid binding domains BAR domains can also generate membrane curvature, e.g. by drawing the membranes of large unilamellar vesicles out into fine membranous tubules. The name BAR is derived from the first letter of the three original members of the BAR domain protein family, mammalian tumour suppressor Bin1, mammalian neuronal protein amphiphysin, and the yeast protein Rvs167p. The 3D structures of several BAR domains have been solved and in each case the monomer consists of a bundle comprising three $\alpha$-helices in a coiled-coil arrangement. Kinks in two of the $\alpha$-helices ensure that the monomer possesses some curvature. The curved monomers associate at an angle with respect to each other thus generating increased curvature in the dimer such that it has a shape reminiscent of a banana. BAR domain dimers associate with membranes via their concave surfaces and induce curvature $^{1,2}$.

A second group of proteins with predicted $\alpha$-helical structure were originally called the Pombe Cdc15 Homology (PCH) proteins based on amino acid sequence similarity to the fission yeast ($Schizosaccharomyces pombe$) cell cycle regulatory protein Cdc15. What only became apparent when the 3D structures of the first PCH proteins were elucidated was that the predicted $\alpha$-helical region of each PCH protein forms a bundle of three $\alpha$-helices and these assemble to form banana-shaped dimers reminiscent of the 3D structure of the BAR domain. F-BAR domains interact with membranes via their concave surface to generate curvature. The PCH proteins include a group of vertebrate non-receptor protein tyrosine kinases (FES/FER) and the mammalian actin cytoskeleton and signalling protein CIP4. The $\alpha$-helical regions of these proteins had been given the name FCH (FES/CIP4 Homology) domain. The new domain shared by the PCH proteins is now referred to as the F-BAR domain. The F-BAR domain differs from the BAR domain in that the individual monomers and the dimer are both more elongated in the F-BAR structure. Secondly, the monomers are set at a wider angle in the dimer giving the dimer a more gentle curvature $^{3,4,5}$. 
Similarly, when the 3D structure of members of a third group of proteins with predicted α-helical structure, including the signalling protein IRSp53, were elucidated it became clear that these proteins also contain a related domain. However, in this third group the dimer is without an obvious bend (more like a zeppelin than a banana). It can interact with membranes via its convex surfaces and induce the opposite curvature to that induced by BAR and F-BAR domains. For this reason, the domain represented by these proteins is now known as an inverse BAR (or I-BAR) domain\textsuperscript{6,7}.

The 1st International Conference on PCH/F-BAR Proteins was organised by Werner Müller-Esterl and Ann Siehoff-Icking and was held at Schloß Waldthausen, Germany from October 10-13, 2007. The first conference focussed on PCH proteins, which feature an F-BAR domain. However, for this second conference we made a decision to widened the scope and include the related proteins with BAR and I-BAR domains.

\textit{Session 1: F-BAR proteins coordinate cytoskeletal regulation and membrane dynamics}

The meeting commenced with a wonderful presentation by Britta Qualmann (Friedrich-Schiller University, Jena, Germany) on the role of the dynamin I- and N-WASP-interacting F-BAR protein syndapin I (PACSIN1) in neuromorphogenesis and neuronal network formation. Neuromorphogenesis and neuronal network formation are critical for the normal development of the nervous system in the vertebrate embryo. To form neuronal networks neurons must create two distinct types of polarised cellular processes: long, unbranched axons and shorter and often highly branched neurites. The axons and neurites must then be connected in the correct combinations to form functional synapses.

Normal development of neurons requires the assembly of new actin filaments. The rate-limiting step in actin filament assembly from free actin monomers is the formation of the initial short
filaments comprising only three monomers, a process known as nucleation. In contrast, growth of short filaments by addition of further monomers at each end is relatively rapid. The Arp2/3 complex, a highly conserved seven-subunit protein complex containing two actin-related proteins (Arp2 and Arp3), promotes the nucleation of actin filament assembly. The Arp2/3 complex is inactive unless activated by one of a number of Arp2/3 activators. There are a number of known Arp2/3 activators and the reason for the existence of multiple Arp2/3 activators is elusive.

One of the first Arp2/3 activators to be discovered is the neuronal Wiskott-Aldrich Syndrome Protein (N-WASP). N-WASP exists in an auto-inhibited state and requires activation to drive Arp2/3-dependent actin filament assembly. The regulation of N-WASP intracellular targeting and activation during neuromorphogenesis is not yet well understood. Qualmann showed that N-WASP and syndapin I function together to regulate the decision by neurons whether to promote the formation of neurites or axons. In hippocampal neurons over-expression of N-WASP to increase Arp2/3-mediated actin filament nucleation results in an increased number of neurites being formed and an increased branching of each neurite at the expense of axons (i.e. neuronal arborisation). Conversely, reduction of cortical Arp2/3-mediated actin filament nucleation in hippocampal neurons by knockdown of either N-WASP or Arp3 or expression of a dominant-negative truncated form on N-WASP (N-WASP-CA) that binds and inhibits the Arp2/3 complex all resulted in longer axons. The axons also showed aberrant branch formation (axonal arborisation).

Qualmann then showed that in hippocampal neurons syndapin I is the key factor promoting Arp2/3-mediated actin filament nucleation at the cortex. A gain-of-function mutation of syndapin I induced neuronal arborisation in hippocampal neurons similar to N-WASP over-expression. The effect of the syndapin I gain-of-function mutant was dependent on N-WASP expression as knockdown of N-WASP prevented neuronal arborisation induced by mutant syndapin I. Conversely, knockdown of syndapin I resulted in axonal arborisation with aberrant axonal branch formation, similar to what
was observed for knockdown of N-WASP or Arp3 or Arp2/3 dominant-negative inhibition. This suggests that syndapin 1 may activate N-WASP and thereby promote Arp2/3-dependent actin filament assembly to drive neurite formation.

The ability of the syndapin I gain-of-function mutant protein to confer neuronal arborisation in hippocampal neurons required not only its Src Homology 3 (SH3) domain (which mediates syndapin I binding to N-WASP) but also its N-terminal F-BAR domain. The isolated syndapin I SH3 domain was shown using a pyrene-actin assembly assay to be sufficient to activate N-WASP for Arp2/3-dependent actin filament assembly in vitro. The N-terminal F-BAR domain of syndapin I was shown to be sufficient for direct binding to liposomes in vitro and to have a preference for liposomes that contain phosphatidylserine (PS). In an elegant experiment in which the F-BAR domain was substituted for sequences that confer constitutive membrane association by directing protein palmitoylation the role of the F-BAR domain in neuronal arborisation was shown to be membrane recruitment.

The role of syndapin I and N-WASP in neuromorphogenesis is specific. Abp1 is another protein that, like syndapin I, can bind and regulate N-WASP activity. Knockdown of Abp1 in hippocampal neurons also resulted in longer axons. Intriguingly, however, knockdown of Abp1 differed from knockdown of either syndapin I or N-WASP in that it did not result in aberrant axonal branch formation. This suggests that not all Arp2/3 activators play the same role in neuromorphogenesis and suggests a possible reason for the existence (even in a single cell type like a neuron) of multiple types of Arp2/3 activator.

F-BAR domain proteins not only function in cortical actin filament assembly, but also in membrane traffic, e.g. in uptake of extracellular material and cell surface receptors by endocytosis. Most work on F-BAR proteins in endocytosis has focused on their role in uptake through clathrin-coated pits.
However, Shiro Suetsugu (Institute of Molecular and Cellular Biosciences, University of Tokyo, Japan) has identified an F-BAR protein that localises to caveolae. This work is unpublished so the identity of the F-BAR domain protein found to function in caveolae cannot be revealed. In this meeting report we will refer to this protein only as F-BAR(x).

Suetsugu showed that F-BAR(x) localises to caveolae in HeLa cells (as detected by immuno-labelling for endogenous caveolin-1) and exhibits much less colocalisation with clathrin-coated pits (as detected by immuno-labelling for clathrin). Suetsugu presented the 3D structure and the results of a thorough mutational structure-function analysis of the F-BAR domain of F-BAR(x). A notable feature of the F-BAR(x) F-BAR domain is its greater curvature compared to other F-BAR domains. Two assays for membrane deforming activity were employed - one *in vivo* and one *in vitro*. The *in vivo* assay involved transient over-expression of the wild type or mutated F-BAR(x) F-BAR domain in HeLa cells and examining their ability to induce the appearance of cell surface protrusions known as microspikes. Wild type F-BAR(x) was shown to localise to the cell surface proximal neck of each microspike. Over-expression of wild type F-BAR(x) also induced the appearance of networks of membrane tubules in the cytoplasm. Sulphorhodamine labelling revealed that these membrane tubules retain a connection to the plasma membrane. Caveolin-1 was evenly distributed throughout the length of membrane tubules, whereas F-BAR(x) gave a punctate distribution that colocalised with caveolin-1 only at the tubule tips. Using TIRF microscopy the tubule diameter was estimated as 50-100 nm. The *in vitro* assay tested the ability of the wild type or mutated F-BAR domain to associate with and tubulate liposomes. The tubules formed by F-BAR(x) *in vitro* had a diameter of 40-170 nm. This is consistent with the prediction from the 3D structure of the F-BAR(x) F-BAR domain that it would form membrane tubules with a diameter of ~40 nm.

Consistent with what has been shown previously for BAR domains, basic charged residues exposed on the concave surface of the F-BAR(x) F-BAR domain were found to play a critical role in both
microspike and cytoplasmic tubule induction \textit{in vivo} and liposome tubulation \textit{in vitro}. Substitution of these basic residues on the concave surface generally had only modest effects on the ability of the F-BAR domain to bind membranes. As expected, charged basic residues located on the convex surface of the F-BAR(x) F-BAR domain were found to be less important for induction of membrane curvature \textit{in vivo} and \textit{in vitro}. Substitution of acidic residues with basic residues on the concave surface resulted in mutant proteins with enhanced ability to generate membrane curvature \textit{in vivo} and \textit{in vitro}. Interestingly, the mutations in the F-BAR domain that abolished microspike and cytoplasmic tubule induction \textit{in vivo} and liposome tubulation \textit{in vitro} also abolished subcellular localisation of F-BAR(x) with caveolae \textit{in vivo}.

In the presence of liposomes of suitable diameter, F-BAR proteins have been shown to promote Arp2/3-dependent actin filament assembly. In the presence of liposomes F-BAR(x) binding to N-WASP via its C-terminal SH3 domain was shown using \textit{in vitro} pyrene-actin polymerisation assays to result in significant stimulation of actin filament assembly. The activation observed with F-BAR(x) is much weaker, however, than that observed in the presence of liposomes with another F-BAR protein known to bind and activate N-WASP, Toca-1 (FBP17). This may be explained on the basis that Toca-1 is known to function in endocytosis through clathrin-coated pits, whereas F-BAR(x) functions in uptake through caveolae. Clathrin-dependent endocytosis is associated with extensive actin filament assembly, whereas uptake through caveolae may require only modest levels of actin assembly.

I-BAR domain proteins and their role in actin filament assembly and membrane curvature was the focus of the presentation by Pekka Lappalainen (University of Helsinki, Finland). I-BAR domains bind phospholipids and like BAR domains show a preference for phosphatidylinositol (4,5)P2 (PIP2). When I-BAR domains associate with liposomes they do so only on the inside (lumenal) side of tubules and they drive tubule growth inward (i.e. into the liposome lumen). This is in contrast to
BAR and F-BAR domains that bind to the outer side of tubules and drive tubule growth outward (i.e. away from the liposome surface). Consequently, the charged basic residues of the I-domain that mediate membrane binding are on the opposite surface compared to BAR and F-BAR domains. Bodipy-TMR-PIP2 was used as a fluorescent label for giant unilamellar vesicles (GUVs) and it was demonstrated that coincident with tubule formation the I-BAR domain induced PIP2 clustering (as monitored by fluorescence self-quenching).

The focus of the presentation then turned to phylogenetic relationships between the various known I-BAR domains. Several subfamilies of I-BAR domains could be defined based on amino acid sequence homology. Members of distinct subfamilies of I-BAR domain were shown to differ in their biochemical properties in vitro. For example, the salt-sensitivity of I-BAR domain association with liposomes differed between different subfamilies of I-BAR domains. This suggests that some I-BAR domains may rely on hydrophobic interactions as well as hydrophilic interactions mediated by charged basic residues for their membrane association. Moreover, I-BAR domains from different subfamilies induced formation of membrane tubules of different diameters in vitro and in vivo. The subcellular localisation of members of different subfamilies of I-BAR domain protein also varied. Although all I-BAR domain proteins localise to filopodia in vivo, different I-BAR domain proteins showed distinct localisations within the filopodia.

The biochemical and functional differences between I-BAR domain proteins of different subfamilies are attributable to the presence or absence of an N-terminal amphipathic α-helix adjacent to the I-BAR domain. The insertion of this α-helix into the membrane provides salt-resistant membrane association and at the same time alters the diameter of the membrane tubules the I-BAR domain protein forms. A model was presented in which I-BAR domain proteins are initially recruited to the plasma membrane by electrostatic interactions. They then cause clustering of PIP2 within the membrane, which generates membrane curvature by virtue of the convex
geometry of the lipid-binding surface of the I-BAR domain. Finally, some I-BAR domains can insert their N-terminal amphipathic α-helix into the membrane and this lowers the degree of membrane curvature.

The expression patterns of I-BAR domains in different adult tissues and during embryonic development suggest *in vivo* roles for I-BAR domain proteins in the developing nervous system as well as adult liver and kidney. Knockout of the I-BAR domain protein Missing-in-Metastasis (MIM) in mice results in no strong phenotypes. However, a more detailed analysis of the knockout mice revealed an altered renal histology including disruption of cell-cell contacts. The mice also exhibit a severe urinary concentration defect and excessive urination (polyuria) - a defect that often culminates in end-stage renal failure. It was proposed that MIM has a role in maintenance of the integrity of kidney epithelia. Experiments using Madine-Derby Canine Kidney (MDCK) cells showed that MIM displays dynamic localisation to adherens junctions (a type of cell-cell contact) and recruits or assembles actin filaments to these sites in a mechanism dependent on its membrane-binding I-BAR domain and actin-monomer-binding WASP Homology 2 (WH2) domain.

Sohail Ahmed (Institute of Medical Biology, Singapore) presented on the I-BAR domain protein IRSp53, which acts in formation of cell surface protrusions known as filopodia. The mechanisms by which IRSp53 promotes formation of filopodia are not yet clear and the presentation described recent progress in identifying these. IRSp53 physically interacts with Cdc42 and is a Cdc42 effector. Although Cdc42 has many effectors, IRSp53 is unique in that it is the only Cdc42 effector that can induce filopodia.

It is important to have a widely accepted definition of filopodia as there may be multiple types of cell surface protrusion. Ahmed shared his definition of filopodia, which is based on imaging of filopodia labelled with GFP-actin and induced by expression of constitutively active (GTP-locked)
mutant Cdc42(G12V) or over-expression of IRSp53 or endogenously generated. To be classified as filopodia, cell protrusions should be dynamic with a lifetime of <2 min. They should also have a length of 8-15 µm and a uniform width of 0.6-1.2 µm.

Over-expression of full-length IRSp53 in N1E115 cells induces filopodia of a uniform length and shape. The I-BAR domain alone is sufficient for induction of cell protrusions, however, expression of the isolated I-BAR induces filopodia that although sometimes dynamic are variable in morphology and in most cases lack filamentous actin (F-actin). The cell protrusions induced by the isolated I-BAR domain often have a half-life of >10min and tend to have reduced diameter and length compared to those induced by full-length IRSp53. Various known IRSp53 SH3 domain interactors (WAVE1, WAVE2, Mena, Eps8, mDia1, mDia2) were tested for interaction with IRSp53 specifically within the filopodia of living cells using Fluorescence Resonance Energy Transfer (FRET). Of these, only WAVE2, Mena, Eps8, and mDia1 showed FRET signals that localised specifically within filopodia. The IRSp53 Cdc42/Rac Interactive Binding (CRIB) domain that mediates binding to Cdc42 is essential for its ability to induce filopodia. This suggests that Cdc42 interaction with IRSp53 plays an essential role in formation of filopodia. IRSp53 was proposed to act downstream of Cdc42 to couple actin filament assembly by its SH3 domain interactors WAVE2, Mena, Eps8, and mDia to membrane protrusions formed by its I-BAR domain to generate filopodia at the cell surface.

The F-BAR domain protein Toca-1 is another Cdc42 effector. Expression of Toca-1 alone does not induce cytoplasmic tubules or cell protrusions in vivo. However, co-expression of Toca-1 with N-WASP results in massive induction of cytoplasmic tubules and vesicles. Toca-1 and N-WASP colocalise on both cytoplasmic tubules and on vesicles. The vesicles may arise from cleavage of tubules in a process dependent on the large GTPase dynamin. In support of this, co-expression of
Toca-1 and N-WASP in dynamin-deficient cells results in accumulation of cytoplasmic tubules but not vesicles.

Both Toca-1 and N-WASP associate with F-actin and F-actin can be seen at the tips of cytoplasmic tubules and on vesicles induced by Toca-1 and N-WASP co-expression. The CRIB domain of Toca-1 is dispensable for cytoplasmic tubule induction, however the N-WASP CRIB domain is essential for both interaction with Toca-1 and for membrane tubulation in vivo. The N-WASP VCA (or WA) domain is not necessary for interaction with Toca-1, but is essential for cytoplasmic tubule induction. This implicates the Arp2/3 complex in cytoplasmic tubule formation as the N-WASP VCA domain is critical for Arp2/3 binding and activation.

Activated GTP-locked Cdc42(G12V) mutant protein co-expression with Toca-1 and N-WASP does not affect the induction of cytoplasmic tubules, but the tubules are not dynamic. In contrast, expression of the dominant-negative Cdc42(T17N) mutant protein with Toca-1 and N-WASP blocks the formation of cytoplasmic tubules. This suggests Toca-1 and N-WASP function downstream of Cdc42 in membrane tubulation.

Giorgio Scita (FIRC Institute for Molecular Oncology and University of Milan, Milan, Italy) presented a functional analysis of the orthologs of F-BAR proteins Toca-1/FBP17 and Toca-2 found in the nematode worm Caenorhabditis elegans (CeToca-1 and CeToca-2). The amino acid sequence of the C. elegans orthologs suggested the presence of an N-terminal F-BAR domain as found in mammalian Toca-1 and Toca-2. Consistent with the presence of an F-BAR domain, co-expression of CeToca-1 and CeToca-2 in cultured mammalian cells resulted in cytoplasmic tubule formation. CeToca-1 and CeToca-2, like mammalian Toca-1 and Toca-2, possess C-terminal SH3 domains. In the case of CeToca-1 the SH3 physically associates with both mammalian and C. elegans N-WASP (CeWSP-1). The Toca-2 SH3 does not associate with N-WASP or CeWSP-1, but instead with both
mammalian and *C. elegans* WAVE (CeWVE-1). This suggests CeToca-1 and CeToca-2 may regulate actin filament assembly driven by N-WASP/CeWSP-1 and CeWVE-1, respectively. Immuno-staining with CeToca-1 and CeToca-2 antibodies revealed expression of both proteins in embryos and in the adult germ line where they localised to intracellular vesicular structures and on the plasma membrane.

CeToca-1, CeToca-2, and CeToca-1 CeToca-2-deficient mutant lines were created. Both single and double mutants exhibited phenotypes consistent with a defect in clathrin-mediated endocytosis. In particular, oocytes were defective in receptor-mediated endocytosis of the yolk protein vitellogenin (YP170). YP170 binds to its cell surface receptor RME-2 and the receptor-ligand complex is internalised by endocytosis. As a consequence of the endocytic defect, vitellogenin/YP170 accumulated in the body cavity of the adult worm and there was a reduction in both the number of vitellogenin/YP170 positive oocytes and in the total number of eggs laid. Worms that arose from these defective eggs exhibited an arrest in embryonic development.

In mammals, N-WASP and WAVE1 are often viewed as being somewhat specialised for endocytosis and formation of cell protrusions, respectively. However, in *C. elegans* CeWSP-1 (N-WASP ortholog) and CeWVE-1 (WAVE1 ortholog) are both important for receptor-mediated endocytosis of vitellogenin/YP170 by oocytes. Other members of the CeWVE-1 protein complex (CeABI-1, CeGEX-2, and CeGEX-3) are also important for vitellogenin/YP170 uptake. Consistent with the idea that CeToca-2 regulates CeWVE-1, RNAi-mediated knockdown of either CeWVE-1 or other members of the CeWVE-1 protein complex like CeABI-1, CeGEX-2, or CeGEX-3 (but not CeWSP-1) showed genetic interactions with the CeToca-2 mutation.

Loss of CeWVE-1, CeABI-1, CeGEX-2, or CeGEX-3 function due to mutation results in a failure of ventral enclosure during embryonic development. As a consequence, these mutants display a Gut
on the Exterior (Gex) phenotype. The CeToca-1 CeToca-2 double mutant also displays a Gex phenotype. Re-expression of CeToca-2 in these mutant worms is sufficient to rescue this defect. Further analysis revealed altered intestinal morphology during embryonic development in CeToca-1 CeToca-2 deficient worms. In particular, defects in the adherens junctions were observed, including delocalisation and increased expression of the adherens junction markers AJM-1 (ortholog of mammalian JAM1) and CeDLG-1, a disrupted F-actin distribution, and loss of normal cell-cell contacts.

The embryonic lethal phenotype of CeToca-1 CeToca-2 deficient worms is enhanced by RNAi-mediated knockdown of CeWVE-1, but not CeWSP-1. This suggests the major contributing factor in the embryonic lethality of CeToca-1 CeToca-2 deficient worms is reduced CeWVE-1 function. Interestingly, however, CeToca-1 may also function (at least in part) through CeWVE-1. Although only CeToca-2 binds CeWVE-1 directly, CeToca-1 binds CeABI-1, which is a component of the CeWVE-1 protein complex.

Cross-talk between the N-WASP- and WAVE-dependent actin filament assembly pathways may also exist in mammals where Toca-1 is known to associate indirectly with WAVE2 because of its direct interaction with ABI-1 (a component of the WAVE2 protein complex). Moreover, in mammalian cells Toca family members were shown, in preliminary experiments, to localise to cell-cell junctions and to regulate the formation and/or maintenance of tightly sealed cell monolayers, presumably by controlling the localization of ABI-1 (and presumably the rest of the WAVE2 protein complex) at junctions. This is consistent with the Gex phenotype observed for CeToca-1 CeToca-2 mutants in C. elegans. It was proposed that in both C. elegans and mammals Toca-1 and Toca-2 promote tissue morphogenesis by coordinating clathrin-mediated endocytosis with N-WASP- and WAVE-dependent actin filament assembly.
Jaeda Coutinho-Budd (Franck Polleux group, University of North Carolina, Chapel Hill, NC, USA) is investigating the role of slit-robo GTPase Activating Protein 2 (srGAP2), a member of the F-BAR family of proteins, in membrane remodelling during embryonic development.

The development of the cerebral cortex of the brain is a good process in which to explore the role of membrane remodelling and F-BAR proteins in tissue formation. Radial glial cells/neuronal stem cells form within the cortical ventricular zone of the brain during day 11 in mouse embryonic development. A subset of radial glial cells then polarise, sending out a leading process which grows outwards toward the outer cortical plate of the brain. In this way the radial glial cells form a structural scaffold used by neurons during their radial migration to the cortical plate. Neurons destined for the cortical plate form transient cell-cell contacts with the polarised radial glial cells and migrate along the radial glial cell until they reach the cortical plate. Upon reaching the cortical plate this cell-cell contact is broken and the neurons form synapses with other neurons. Successive waves of migration of neurons from the cortical ventricular zone to the cortical plate occur. In each successive wave the new neurons migrate past (invade) the layer formed by the previous wave of migrating neurons such that older neurons are deep within the cortex and younger neurons are more peripheral. These successive cell migrations form the six layers of the cerebral neocortex. The migration properties of the cortical neurons are under the general control of the transcription factor neurogenin-2. A downstream target, srGAP2, plays an important but more specific role in membrane remodelling during these waves of directed neuronal cell migration.

There exist three subtypes of srGAP (srGAP1-3). Each subtype has a slightly different expression pattern in the brain. srGAP1 binds slit/robo and plays a role in neuronal guidance. srGAP3 binds WAVE and functions in regulation of actin filament assembly. Less is known about the role or interactions of srGAP2. srGAP2 has an N-terminal F-BAR domain, a central GAP domain (Rac1-specific), an SH3 domain, and a coiled-coil domain at the C-terminus. srGAP2 is expressed
throughout the period of neuronal migrations during mouse brain cortex development consistent with it having a physiological role in this process. Ectopic expression of srGAP2 in COS-7 cells induces the formation of spike-shaped (filopodia-like) cell protrusions of 80nm diameter whose entire length stains for F-actin. Some of these protrusions are highly dynamic while others are not. Interference with actin filament assembly does not prevent induction of these cell protrusions, however it does affect their dynamics. The F-BAR domain is both necessary and sufficient for induction of these spike-shaped cell surface protrusions. The F-BAR domain is also sufficient for subcellular localisation to these protrusions, although the isolated F-BAR domain is not found exclusively in protrusions and some shows an even distribution over the cell surface. Transient over-expression of srGAP2 in neurons also induces spike-shaped cell surface protrusions and srGAP2 distributes throughout the length of these protrusions.

Liposome binding experiments showed that the srGAP2 F-BAR domain acts more like an I-BAR domain and induces the formation of scalloped morphology on the surface of liposomes and inward membrane tubule growth (like IRSp53). Only when the permeability barrier of the liposomes is broken and the srGAP2 F-BAR domain can gain access to the lumen can it induce the growth of tubules outward from the liposome.

A novel ex vivo cortical electroporation and slice culture methodology was developed to track neuronal cell production and migration in the developing brain. In this technique, DNA encoding green fluorescent protein (GFP) is electroporated into the neuronal progenitors prior to neuronal migration. The migration of the GFP-tagged neurons through to the cortex could then be tracked using GFP fluorescence. Knockdown of srGAP2 increases radial migration of neurons and decreases the frequency of neurite branch formation. Conversely, srGAP2 over-expression reduces radial migration of neurons. At the level of individual neurons, srGAP2 regulates neuronal migration by regulating leading process branching. Excessive formation of protrusions and
branches at the leading process, caused by srGAP2 over-expression, delays cell migration. Reduced formation of protrusions and branches at the leading process caused by srGAP2 knockdown increases the rate of cell migration.

Mutation of the srGAP2 GAP domain such that it still binds Rac but no longer inactivates Rac GTPase activity had only an intermediate effect on the ability of over-expressed srGAP2 to inhibit radial neuronal migration. In contrast, a point mutation in the SH3 domain that prevents binding to partner proteins abolished inhibition of radial neuronal migration by srGAP2 over-expression. In contrast, complete deletion of the SH3 domain and flanking C-terminal sequences restored the ability of srGAP2 over-expression to inhibit radial neuronal migration. This suggests that the binding of a partner protein to the srGAP2 SH3 domain may break an srGAP2 auto-inhibitory intramolecular interaction involving the SH3 domain and be important for srGAP2 activity. This model is currently being tested experimentally.

Session 2: F-BAR proteins and vesicle trafficking

Following his elucidation of the 3D structure of the Drosophila amphiphysin BAR domain (Peter et al., 2004) and human FCHo2 F-BAR domain (Henne et al., 2007), Harvey McMahon (MRC LMB, University of Cambridge, Cambridge, UK) has created a website called the "Bar Superfamily" (http://www.bar-superfamily.org/). At this website are image files with the 3D structures and links to the original PDB database for all BAR-, F-BAR-, and I-BAR-domain proteins whose 3D structure has been solved. The "Bar Superfamily" website should be considered a review article and properly cited whenever data contained in it are referred to in other research articles. The remainder of McMahon's presentation consisted of unpublished data on the function of FCHo2 that cannot be described here but will be published elsewhere in due course.
The BAR domain proteins amphiphysin and endophilin play an important role in synaptic vesicle recycling in the nervous system. Both proteins have a C-terminal SH3 domain that binds the high molecular weight GTPases dynamin. Dynamin is expressed by three genes in mammals. Dynamin 1 and 3 are neuronal proteins, while dynamin 2 is the ubiquitous isoform. The lab of Pietro De Camilli (Howard Hughes Medical Institute/Yale University, New Haven, CT, USA) has generated conditional KO mice for all three dynamin isoforms. In the talk, De Camilli presented an analysis of fibroblastic cells that lack dynamin. To obtain such cells, their first generated dynamin 2 KO cells from dynamin 2 conditional KO mice. Surprisingly, they found that such cells also expressed dynamin 1. Thus, they generated dynamin 1 and 2 double conditional knockout (KO) mice. Then, fibroblasts from these mice were cultured and dynamin double KO cells were obtained via a tamoxifen-inducible activation of Cre recombinase in the cultures. This strategy allowed for the acute generation of cells lacking dynamin.

Double KO cells lived in vitro for several weeks in spite of the absence of dynamin. However, endocytosis through clathrin-coated pits was arrested, as shown by a block of clathrin coated pit dynamics and of transferrin receptor internalization. Electron microscopy and electron tomography revealed a dramatic accumulation of aberrant pits with very long (up to 1 micron) tubulated necks.

These changes were accompanied by a striking accumulation along the tubules of the BAR domain containing protein endophilin, as well as of other BAR proteins, such as amphiphysin, Snx9, and Tuba. These findings indicate that endophilin and other BAR proteins function upstream of dynamin in clathrin-mediated endocytosis and provide genetic evidence for the role of this class of proteins in the dynamics of the necks of endocytic clathrin coated pits. The tubules were also positive for the Arp2/3 complex and its activators (e.g. N-WASP). Knock down of clathrin and of the Arp2/3 complex prevented tubule formation and acute disruption of the actin cytoskeleton with latrunculin induced their collapse. Thus, BAR domain dependent tubulation in cell that lack
dynamin is triggered by clathrin coated pits and requires actin. Overall, this study highlighted the role of BAR proteins during clathrin-mediated endocytosis and revealed a critical function for the actin cytoskeleton in coordination with BAR proteins at the neck of clathrin-coated pits. These findings raise interesting mechanistic questions concerning the coupling between actin polymerisation and BAR protein mediated membrane tubulation. Further results were presented on the development of an in vitro system to further probe such questions.

A novel endocytosis pathway that is clathrin-independent has recently been characterised. Internalisation via this pathway involves an endocytic structure known as a CLIC (clathrin-independent carrier) or GEEC (GPI-enriched early compartment). These endocytic structures label with cholera toxin indicating that GPI-linked receptors including the cholera toxin receptor use this endocytic pathway for their internalisation. The CLIC pathway is also the major pathway by which fluid-phase markers (e.g. dextran) are taken up by cells. Several BAR domain proteins are required for endocytosis through the CLIC pathway, including the related BAR domain proteins GRAF1-3 (GTPase regulator associated with focal adhesion kinase/FAK) and oligophrenin/OPHN1. Richard Lundmark (Umeå University, Umeå, Sweden) described his work exploring the role of GRAF1 in the CLIC pathway.

GRAF1 possesses a predicted BAR domain, GAP domain, and plextrin homology (PH) domain. The 3D structure of the GRAF1 BAR domain is not yet determined, however, consistent with the presence of a BAR domain GRAF1 binds liposomes that contain PIP2 in vitro. Compared to other BAR domain proteins GRAF1 has a preference for liposomes of smaller diameter. When transiently expressed at high level in vivo GRAF1 induces the formation of cytoplasmic tubules. The GRAF1 GAP domain is active on both Cdc42- and Rho-GTPases. The GAP activity of GRAF1 is subject to auto-inhibition by interaction with the BAR domain. Hence, intact GRAF1 stimulates GTPase activity only modestly in comparison to its isolated GAP domain, a truncated GRAF1 protein
containing only the GAP and neighbouring PH domain, or full-length GRAF1 protein after partial trypsin digestion. Endogenous GRAF1 localises to tubular endosomes that are highly dynamic, of variable length, and positive for cholera toxin (CTxB). GRAF1 does not colocalise with caveolin, transferrin (Tf), Arf6, or class I MHC. This is consistent with subcellular localisation of GRAF1 to the CLIC/GEEC compartment.

GRAF1 is important for the function of the CLIC pathway as knockdown of GRAF1 results in reduced uptake of fluid-phase endocytic markers that use the CLIC pathway (e.g. dextran) but does not affect the uptake of markers that use the clathrin-coated pit pathway (e.g. Tf). One might predict that the GRAF1 lipid-binding BAR or PH domains would specify subcellular localisation. However, this is only partially the case as a truncated form of GRAF1 comprising only the BAR and PH domains does not colocalise as efficiently with internalised GPI anchor proteins/CTxB as full length GRAF1. Dynamin may play a role in the fission of the GRAF1-positive tubular compartment as dynamin also localises to these compartments. Dynamin labels the ends of the tubules (presumably sites of fission). In contrast, GRAF1 is evenly distributed along the entire length of the tubule. GRAF1 also physically associates with dynamin via its C-terminal SH3 domain. GRAF1 also binds and colocalises with GIT1 (a GAP specific for ARF1 and ARF6). The remainder of the Lundmark presentation consisted of unpublished data on the function of GRAF1 that cannot be described here, but will be published elsewhere in due course.

CIP4 was one of the founding members of the F-BAR protein family and its role in development of the wing in *Drosophila* was the subject of a presentation by Sven Bogdan (University of Münster, Münster, Germany). Key activators of the Arp2/3 actin nucleation complex are members of the WASP- and WAVE-families of proteins. In mammals, activation of Arp2/3 by WASP-family proteins is more important for endocytosis and phagocytosis, whereas activation by WAVE-family proteins plays the major role in formation of lamellipodia. WASP- and WAVE-family proteins
form distinct protein complexes \textit{in vivo}. These two complexes are conserved between mammals and flies (e.g. \textit{Drosophila}). WAVE-family proteins are present in \~440kDa complexes while WASP-family proteins are present in \~250kDa complexes.

\textit{Drosophila} possesses a single gene encoding the F-BAR protein DmCIP4, the only member of the CIP4/Toca-1/FPB17 subfamily in \textit{Drosophila}. DmCIP4 shares 35\% amino acid sequence identity with mammalian Toca-1, FBP17 and CIP4. In pyrene-actin polymerisation assays DmCIP4 was found to promote DmWASP-dependent actin filament assembly. Liposome-binding experiments showed that the DmCIP4 F-BAR domain binds and tubulates membranes \textit{in vitro}. Over-expression of DmCIP4 induces membrane tubulation \textit{in vivo}. DmCIP4 also localises to these tubules. A pool of DmCIP4 is also found on vesicles and when these undergo actin-dependent movement through the cytoplasm an actin comet tail is formed. DmCIP4 binds DmWASP directly and binding requires the C-terminal SH3 domain of DmCIP4. DmCIP4 also binds DmABI and this results in physical association of DmCIP4 with the DmWAVE complex. DmCIP4 acts to recruit DmWASP and DmWAVE to actin comet tails (recruit to vesicles to induce actin comet tails) on vesicles. Consistent with this, the formation of actin comet tails and actin-dependent movement of CIP4-GFP labelled vesicles are both dependent on an intact DmCIP4 SH3 domain. Consistent with these results, knockdown of DmCIP4 affects E-cadherin endocytosis thus demonstrating that DmCIP4 plays an important role in endocytosis. It is not yet clear, however, if DmCIP4 regulates endocytosis via interactions with the DmWAVE complex.

To test DmCIP4 function \textit{in vivo} a transposon- (P-element) mediated deletion of the DmCIP4 gene was isolated. Flies deficient in DmCIP4 are viable, but exhibit defects in the wing epithelium. The \textit{Drosophila} wing is composed of hexagonal epithelial cells, each of which has a single hair associated with it. In the DmCIP4-deficient flies, instead of one wing hair each epithelial cell had 2-3 wing hairs. In contrast to these findings with DmCIP4, mutation or RNAi-mediated knockdown
of DmWASP resulted in no change to wing hair number or appearance. This suggests that DmCIP4-dependent regulation of wing hair number may require DmWAVE, but not DmWASP. Further support for this came from the finding that knockdown of DmWAVE in the DmCIP4-deficient flies enhanced the DmCIP4 mutant phenotype, but knockdown of DmWASP in DmCIP4-deficient flies did not. Over-expression of either DmWAVE or DmWASP in DmCIP4-deficient flies rescued the defects. Thus although DmCIP4 normally works through DmWAVE, high-level expression of DmWASP can compensate for reduced DmWAVE function. In summary, DmCIP4 appears to act through DmWAVE to regulate wing epithelium development in Drosophila.

Mammalian CIP4 was discovered on the basis of its physical association with the Rho-family GTPase Cdc42. Consistent with possible physical association of DmCIP4 with DmCdc42 in Drosophila, expression of a constitutively active (GTP-locked) mutant form of Cdc42 resulted in efficient recruitment of DmCIP4 and the associated DmWAVE complex in vitro (not shown), but loss of Cdc42 (MARCM mutant clones) function resulted in loss of DmCIP4 apical localisation. This resulted in the development of multiple wing hairs as observed in DmCIP4-deficient flies. A block in endocytosis imposed by a mutation in dynamin also resulted in the formation of multiple wing hairs. The C-terminal SH3 domain of DmCIP4 is important for DmCIP4 function as expression in wing epithelium of a mutant form of DmCIP4 lacking an SH3 domain DmCIP4(ΔSH3) resulted the development of multiple wing hairs. Expression of DmCIP4(ΔSH3) may induce this developmental phenotype by blocking endocytosis because when DmCIP4(ΔSH3) was tagged with GFP it could be seen to localise to long tubular invaginations of the plasma membrane.

Lennart Brodin (Karolinska Institutet, Stockholm, Sweden) uses the giant reticulospinal axon synapse of the lamprey to investigate synaptic vesicle recycling. This experimental model is being used to investigate the relative contribution of transient and partial fusion of synaptic vesicles with
the plasmalemma (i.e. "kiss and run") vs irreversible and complete synaptic vesicle fusion followed by recycling in neurotransmitter release at synapses. The giant reticulospinal axon was also used to first show that the BAR domain protein amphiphysin plays a key role in synaptic vesicle recycling. Currently, Brodin is using this experimental model to study the function of epsin. Epsin has an N-terminal epsin N-terminal homology (ENTH) domain followed by a UIM ubiquitin-binding motif, a clathrin- and AP2-binding (CLAP) domain, and finally an NPF tripeptide motif (potentially capable of binding an EH domain). A specific antibody was raised against the ENTH domain of epsin. By immuno-EM epsin was found to localise to synaptic vesicles. Injection of anti-epsin antibody into the giant reticulospinal axon inhibited coat formation at clathrin-coated pits resulting in a reduction in the length of the membrane that displayed a coat. An antibody was also raised to the CLAP domain of epsin. Injection of this antibody also affected clathrin coat formation, however in this case it enhanced clathrin coat formation resulting in an increase in the length of the membrane that displayed a coat, i.e. regions of the synapse that are not normally coated became coated.

Next, attention was turned to the role of the F-BAR protein syndapin/PACSIN in synaptic vesicle recycling. Lamprey syndapin has the same domain structure as mammalian syndapin with an N-terminal F-BAR domain, centrally located NPF tripeptide motif (potentially capable of binding an EH domain), and a C-terminal SH3 domain. At the lamprey giant reticulospinal synapse syndapin can be detected at vesicle release sites on the plasmalemma.

An antibody was raised to lamprey syndapin. Following stimulation of the lamprey reticulospinal axon with K⁺, immuno-EM reveals syndapin localisation at the peri-active zone. Injection of anti-syndapin IgG into the lamprey reticulospinal axon resulted in a reduction in the F-actin accumulation at the peri-active zone that is otherwise observed upon axonal stimulation. This suggests a possible inhibition of synaptic vesicle endocytosis at the active zone. Injection of anti-syndapin IgG did not significantly affect synaptic vesicle recycling in the first round when the axon
was subjected to mild electrical stimulation (0.2 Hz). However, if electrical stimulation was increased (5 Hz) there was a depletion of free synaptic vesicles and an accumulation of membrane cisternae that still exhibited continuity with the plasma membrane. Following stimulation at 5 Hz a selective accumulation of coated pits on these cisternae was observed. These observations indicate that the injected anti-syndapin IgG induced a severe defect in the recycling of synaptic vesicles such that the plasmalemma expanded (resulting in membrane cisternae) while free synaptic vesicles were depleted.

An antibody was raised to the SH3 domain of lamprey syndapin. Injection of anti-syndapin SH3 Fab fragments into the lamprey reticulospinal synapse had similar effects on synaptic vesicle recycling as injection of the anti-syndapin IgG. Only following a massive electrical stimulation of the axon (20 Hz) did the injected anti-syndapin SH3 Fab fragments significantly reduce the number of free synaptic vesicles formed at the plasmalemma and result in the accumulation of membrane cisternae.

What is the role of syndapin in synaptic vesicle recycling? Does syndapin function in the fast synaptic-vesicle recycling pathway that is dependent on clathrin-mediated endocytosis? The experiments performed using the lamprey reticulospinal axon provide little support for such a proposed role. If syndapin were to function in the fast synaptic-vesicle recycling pathway with clathrin and dynamin the injection of anti-syndapin IgG should have resulted in significant synaptic vesicle depletion and the accumulation of invaginated coated pits in the first round following even mild electrical stimulation. The data are more consistent with a specific role for syndapin in bulk membrane endocytosis. Bulk membrane endocytosis is the slow synaptic-vesicle recycling pathway that recovers membrane material from the cisternae that form whenever the rapid synaptic-vesicle recycling pathway is over-loaded (e.g. following multiple rounds or particularly strong electrical stimulation of the axon). However, an alternative role for syndapin in stabilising the plasmalemma
to prevent membrane cisternae formation cannot formally be excluded. The remainder of Borodin’s presentation consisted of unpublished data on a syndapin 1 interacting protein he identified that cannot be described here, but will be published elsewhere in due course.

Session 3: F-BAR proteins in health and disease I

Scott Soderling (Duke University, Durham, NC, USA) is using the mouse model to investigate the role of the srGAP subfamily of F-BAR proteins in the development and function of the CNS. His presentation consisted of unpublished data that cannot be described here, but will be published elsewhere in due course.

Seth Corey (Northwestern University, Chicago, IL, USA) presented the latest results from his ongoing phenotypic analysis of mouse lines that harbour a knockout of the gene encoding the F-BAR domain protein CIP4. The presentation consisted of unpublished data that cannot be described here, but will be published elsewhere in due course.

One important subfamily of F-BAR domain proteins is defined by the presence of a tyrosine kinase domain, and includes FES & FER. The FES/FER tyrosine kinases were the topic of a presentation by Andrew Craig (Queen's University, Kingston, ON, Canada). The presentation started with a brief description of what is known about the most widely expressed member of this subfamily of F-BAR proteins, the FER tyrosine kinase. FER plays an important role in the formation of lamellipodia and regulates cell migration\textsuperscript{9,10}. The domain structure includes an N-terminal F-BAR domain, a phosphotyrosine-binding Src Homology 2 (SH2) domain, and a C-terminal tyrosine kinase domain. Insights into modes of regulation of FES/FER localization via their F-BAR domains, and kinase activation via their SH2 domains have emerged recently. Interestingly, the SH2 domain plays a regulatory role in FES activation: binding of the SH2 domain to tyrosine phosphorylated ligands results in activation of FER tyrosine kinase activity. The 3D structure of FES has been elucidated
and suggests a mechanism by which ligand binding by the SH2 domain stabilizes the active site of the kinase domain\textsuperscript{11}. Thus, FES/FER binding to "primed" SH2 ligands could promote their kinase activities and lead to further FER/FES-induced phosphorylation of additional tyrosine residues on the SH2 ligand protein or other substrates.

While no 3D structure has been reported yet for the F-BAR domains of FER/FES, homology modelling suggests FES residues 1-300 fold into an F-BAR domain. Consistent with the N-terminal region of FES containing an F-BAR domain, a FES N-terminal fragment binds phosphoinositides \textit{in vitro} \textsuperscript{12}. FER has been shown by Toshiki Itoh to have an FX domain flanking the F-BAR domain. Similarly, FES also possesses an FX domain adjacent to the F-BAR domain. The presence of the FX domain alters the lipid-binding specificity of FES. The F-BAR domain is required for FES activation downstream of the IgE receptor and for membrane targeting of FES in mast cells.

Mast cells are an excellent cell type in which to study the role of F-BAR proteins as dramatic alterations in the actin cytoskeleton, microtubule cytoskeleton, and membrane morphology accompanies the activation of mast cells. These alterations are required to promote both endocytosis and exocytosis. F-BAR proteins may link changes in the actin and microtubule cytoskeletons to changes in membrane morphology. FES functions in signalling downstream of the c-Kit tyrosine kinase in mast cells. Activation of FES kinase appears to be highly regulated and involves interaction with membranes, F-actin, and microtubules. FES is recruited to activated c-Kit at the plasma membrane via binding of the FES SH2 domain to phospho-Tyr in activated c-Kit. Therefore, the FES F-BAR and SH2 domains both contribute to the recruitment of FES to membranes in mast cells. Consistent with a role for FES in c-Kit signalling, FES regulates SCF-induced spreading, polarisation and chemotaxis of mast cells\textsuperscript{13}. The focus of the remainder of the presentation turned to the F-BAR adaptor protein Toca-1, and its role in regulating actin structures.
and epidermal growth factor (EGF)-induced cell migration. The presentation consisted of unpublished data that cannot be described here, but will be published elsewhere in due course.

A feature common to those F-BAR proteins that lack GAP or kinase domains is the presence of a C-terminal SH3 domain. A C-terminal SH3 domain is present in the F-BAR protein PSTPIP1, although not in the related protein PSTPIP2 (also known as MAYP)\textsuperscript{14,15,16}. PSTPIP2 is predominantly expressed in macrophages, osteoclasts, and mast cells\textsuperscript{17,18,19} and Richard Stanley (Albert Einstein College of Medicine, Bronx, NY, USA) presented his work with Violeta Chitu and collaborators on the role of PSTPIP2 in the regulation of the differentiation and function of macrophages and osteoclasts. PSTPIP2 is predominantly expressed in macrophages, osteoclasts, and mast cells. The cytokine colony stimulating factor-1 (CSF-1) plays a critical role in survival and differentiation of haematopoietic cells. By synergizing with other haematopoietic cytokines, CSF-1 stimulates haematopoietic stem cells (HSCs) to become determined to the myeloid lineage and differentiate initially into the committed macrophage precursor, colony forming unit-macrophage (CFU-M). Under the influence of CSF-1 alone, CFU-M differentiate into circulating monocytes, then into tissue macrophages. Exposure of monocytes to the cytokines RANKL and CSF-1 in combination induces their differentiation into osteoclasts that resorb bone. CSF-1 also has roles apart from macrophage and osteoclast differentiation, e.g. in the female reproductive tract CSF-1 produced in the oviduct and uterine wall (together with ovarian estradiol [E2] and progesterone [P]) regulates both foetally derived (trophoblast) and maternally derived (decidual) cells, the former regulation being important for innate immunity at the maternal-foetal interface.

CSF-1 is a ligand for the CSF-1-receptor (CSF-1R), encoded by the protooncogene c-fms. Knockout of the CSF-1R in mice, when homozygous (Csf1r -/-), results in a similar overall phenotype to that of mice homozygous for a knockout of CSF-1, but the CSF-1R-deficient mice have more severe osteoporosis. The extracellular portion of CSF-1R has an N-terminal CSF-1 binding domain
followed by a dimerization domain. CSF-1R has a single transmembrane domain. On the intracellular domain has a tyrosine kinase domain interrupted by a kinase insert and contains 7 known tyrosine phosphorylation sites. Autophosphorylation of Tyr residues within the cytoplasmic domain creates sites that are recognized by various adaptor proteins with phospho-Tyr-binding SH2 domains, e.g. Src, Grb2 and Mona.

When extracts were prepared from macrophages before or after CSF-1 stimulation and phospho-Tyr-containing proteins were then isolated by anti-phospho-Tyr affinity purification followed by denaturing S6-size exclusion chromatography, 181 unique proteins with CSF-1-stimulated phospho-Tyr were identified. Of these, 95 were cytosolic, 49 were membrane-associated and 37 were found in both fractions. Pull-down of F-actin using the F-actin-specific toxin phalloidin showed that PSTPIP2 is the major phospho-Tyr-containing protein in the cytosolic fraction that associates with F-actin. The alternative name for PSTPIP2, MAYP, is an acronym for macrophage actin-associated and tyrosine phosphorylated protein.

Increased PSTPIP2 expression in macrophages is associated with changes in the cellular response to CSF-1. These changes include increased chemotaxis, increased filopodia formation, and decreased membrane ruffling/lamellipodia formation in response to CSF-1. The macrophages lose ruffling and instead spread and become more polarised. Immuno-EM reveals that PSTPIP2 exhibits subcellular colocalisation with cortical and filopodial F-actin. Transmission EM with rotary shadowing of the actin cytoskeleton shows that PSTPIP2 bundles F-actin in vivo. However, macrophages isolated from PSTPIP2-deficient mice had no defect in CSF-1R endocytosis (Chitu et al., unpublished). This suggests that PSTPIP2, unlike several other F-BAR domain proteins, may not function in endocytosis.
Mutations in PSTPIP2 cause auto-inflammatory disease (e.g. the *Lupo* mutation, which is associated with auto-inflammatory disease, results in the amino acid substitution I282N). The *Lupo* mutant cells express only 30% of wild type levels of PSTPIP2 protein\(^\text{18}\). Another PSTPIP2 mutation (resulting in amino acid substitution L98P) is associated with chronic multifocal osteomyelitis in mice (known as the *cmo* mouse). The *cmo* mutation causes complete loss of PSTPIP2 protein expression in macrophages, osteoclasts, and mast cells\(^\text{19}\). The *cmo* mice have kinked tails and inflamed paws in which some tissue is necrotic. There is a massive macrophage infiltration into the inflamed tissue. The disease can be transferred to a healthy mouse by bone marrow transplant, suggesting the major defect resides in bone-marrow-derived cells, e.g. macrophages. The data are consistent with the view that the major function of PSTPIP2 is anti-inflammatory. What is the molecular mechanism that promotes inflammation in *cmo* mice? In *cmo* mice prior to disease onset there is already an increased number of multipotent colony forming cells (HPP-CFC). There are also increased numbers of determined macrophage progenitors (CFU-M) and primitive myeloid cells, which hyperproliferate. CSF-1 stimulates ERK1/2 kinase and STAT1 activation in macrophages. PSTPIP2 attenuates CSF-1-stimulated ERK1/2 activation and cell proliferation. These data suggest that PSTPIP2 negatively regulates monopoiesis by counteracting ERK1/2 function. PSTPIP2 was also shown to negatively regulate STAT1 expression\(^\text{19}\).

Stanley presented their model for the pathology observed in PSTPIP2-deficient *cmo* mice. In PSTPIP2-deficient mice there is an increased proliferation of HPP-CFC in the bone marrow. This results in an increased number of HPP-CFC and CFU-M cells in the spleen such that the spleen becomes "primed". Upon tissue damage, which acts as a local trigger, tissue macrophages and become activated and produce excessive amounts of IL-6 and MIP-1\(\alpha\). MIP-1\(\alpha\) promotes further recruitment of monocytes while IL-6 promotes the expansion of HPP-CFC\(^\text{19}\).
The kinked tail phenotype of cmo mice also suggested that inflammatory bone resorption may occur and hence the bone structure of cmo mice and the role of PSTPIP2 in osteoclast differentiation and function has also been intensively investigated and was also discussed.

Session 4: F-BAR proteins in health and disease II

The Keynote Lecture was by Tadaomi Takenawa (Kobe University, Kobe, Japan). The Takenawa group is interested in the tyrosine kinases that contain an F-BAR domain. In particular, his group has been investigating how signalling lipids activate FER kinase. The Keynote Lecture began with a brief historical perspective of the signalling lipids field. Takenawa described the original discovery by Michael Berridge and Yasutomi Nishizuka that hydrolysis of PIP2 by phospholipase C (PLC) generates the second messengers inositol (1,4,5)-trisphosphate (IP3) and diacylglycerol (DAG). He then described the discovery of the lipid kinase phosphatidylinositol 3-kinase (PI3K) by Lewis Cantley in 1988. PI3K generates a range of phosphoinositides, including PtdIns(3)P, PtdIns(3,4)P2, PtdIns(3,5)P2, and PtdIns(3,4,5)P3. Unlike PIP2, these phosphoinositides are not substrates of PLC. However, these 3-phosphorylated phosphoinositides were predicted (and later shown) to be a new class of signalling lipids. Takenawa then described the discovery by Ingrid Lassing and Uno Lindberg in 1985 that PIP2 can bind to the actin sequestering protein profilin in addition to the plasma membrane and thereby regulate actin filament assembly. A number of actin cytoskeleton and focal adhesion proteins were found to specifically bind PIP2, including α-actinin and vinculin. Subsequently, most actin regulatory proteins have been shown to bind PIP2.

Takenawa then described the discovery, starting in the 1990s, of various protein domains that bind phosphoinositides, including the PH, PX, FERM, C2, PTB, FYVE, and PDZ domains. The 3D structures of many of these domains have now been elucidated. Phosphoinositide-binding domains have several known functions. Some play a role in targeting of signalling lipids to the plasma membrane. Some phosphoinositide-binding domains, by binding and sequestering signalling lipids,
alter the activity of the protein effectors of these lipids. Other phosphoinositide-binding domains, such as the BAR, F-BAR, and I-BAR domains, either deform membranes or sense membrane curvature.

The focus then changed to the ENTH domain, which is a PIP2-binding domain involved in endocytosis. The ENTH domain is distinct from any of the various BAR or BAR-like domains, yet like these domains it has the ability to deform membranes into tubules \textit{in vitro}. The 3D structure of the ENTH domain has been elucidated using NMR. Upon binding of the ENTH domain to PIP2, some chemical shifts in the ENTH domain NMR spectrum can be seen to change. The residues whose chemical shifts were affected by PIP2-binding were mapped. The ENTH domain contains three sites involved in PIP2 binding and these are known as site 1, 2, and 3. The perturbed residues were found to coincide with basic and hydrophobic residues located in sites 1 and 3. Substitutions of these residues prevented PIP2 binding (e.g. R63A and W71A in site 3). This shows that basic and hydrophobic residues in sites 1 and 3 of the ENTH domain are important for PIP2 binding.

Why is there such a diversity of phosphoinositide-binding domains and what distinguishes one from another? For example, how is lipid binding by the ENTH domain different from lipid binding by the PH domain? Why can the ENTH domain deform membranes into tubules but the PH domain cannot? In this case the difference has been determined. The PH domain does not have an amphipathic $\alpha$-helix to insert into membranes and only binds PIP2. In contrast, the ENTH domain has an amphipathic $\alpha$-helix, which it inserts into the membrane to stabilise membrane curvature.

Another class of phosphoinositide-binding domains are the BAR domains that bind PIP2 and are found in proteins such as amphiphysin 1 and 2, endophilin 1-3, nadrin/RICH1, SH3BP1, oligophrenin-1, and GRAF2. BAR domains have the ability to bind liposomes that contain PIP2 and convert them into membrane tubules. Takenawa has an interest in the signalling protein IRSp53. IRSp53 has an I-BAR (also known as RCB) domain. Shiro Suetsugu in Takenawa’s group
elucidated the 3D structure of the IRSp53 I-BAR domain and found it resembles that of the BAR domain.

Another area of interest of Takenawa’s is the Extended FCH (EFC) domain (FCH domain plus flanking coiled-coil regions). The EFC domain is now known as the F-BAR domain. Kazuya Tsujita in Takenawa’s group screened for novel phosphoinositide-binding domains and identified the EFC/F-BAR domain. Like the BAR and I-BAR domains, the EFC/F-BAR domain binds PIP2 and when incubated with liposomes containing PIP2 it forms tube-like membrane structures. F-BAR domains are found in Toca-1, FBP17, CIP4, syndapin/PACSIN1-3, Nwk1 and 2, FBP2, ARHGAP4, WRP, FPS/FES, and FER. Most F-BAR domains, including those of Toca-1/FBP17 and CIP4, have lipid binding and membrane deforming ability. Takenawa’s group elucidated the 3D crystal structure of the F-BAR domains of FBP17 and CIP4 with the aim of determining the mechanism by which these domains tubulate membranes. They found that, like BAR domains, F-BAR domains fold into curved bundles of α-helices that then assemble into banana-shaped dimers. Compared to BAR domain dimers, the F-BAR domain dimers possess a more gentle curvature. Moreover, F-BAR domain dimers can further associate end-to-end connected by hydrogen bonds to form filaments. Cryo-TEM was used to show that F-BAR domains form striated structures on the membrane tubules they generate from liposomes in vitro. The striations have a regular spacing of 42 nm. These striations represent tight F-BAR spirals that cover the tubular membranes.

In Takenawa’s group, Toshiki Itoh has been exploring the functions of the F-BAR domain protein FER in regulation of cell morphology. Over-expression of some F-BAR domain proteins (e.g. srGAP1 and srGAP2) induces the formation of filopodia-like cell protrusions. In contrast, over-expression of FER in COS-7 cells induces lamellipodia. FER over-expression also induces tyrosine phosphorylation of cortactin specifically at lamellipodia in COS-7 cells. For many F-BAR domain proteins that have been studied, the isolated F-BAR domain alone is sufficient for induction of
striking alterations in cell morphology, e.g. formation of filopodia by srGAP1 and srGAP2. It was therefore somewhat unexpected to discover that over-expression of the isolated FER F-BAR domain did not induce any remarkable change in cell shape. Consistent with this, the tyrosine kinase activity of FER was shown to be required for induction of lamellipodia. Over-expression of the kinase-dead D742R mutant FER did not induce lamellipodia in COS-7 cells. However, induction of lamellipodia also requires the FER F-BAR domain. Induction of lamellipodia by FER is dependent on the GTPase Rac. Expression of a dominant-negative mutant form of Rac prevented induction of lamellipodia by FER. Moreover, the expression of a mutant form of Rac that is GTP-locked and therefore constitutively active induced lamellipodia in COS-7 cells without over-expression of FER.

Toshiki Itoh also examined the ability of the FER F-BAR domain to bind liposomes in vitro. The F-BAR domain of FER is not sufficient for liposome binding in vitro. A flanking region, which has been given the name of FX domain, is required for liposome binding. The FX domain alone is sufficient to bind liposomes, however efficient liposome binding requires both FX and F-BAR domains. The FX domain binds phosphatidic acid (PA) more strongly than phosphoinositides. The FX domain is essential for lamellipodia formation by FER. Full-length FER and a FER fragment that contains only the F-BAR and FX domains both preferentially bind to liposomes that contain PA in vitro and this lipid binding specificity is due to the presence of the FX domain. Why is the F-BAR domain essential for formation of lamellipodia? The F-BAR domain may play a role in supporting the binding of the FX domain to PA or it may only be important for dimerisation of FER.

PA induces the tyrosine kinase activity of FER. In vitro, addition of liposomes that contain PA stimulates tyrosine phosphorylation of the FER kinase substrate cortactin. This is dependent on the kinase activity of FER. When a kinase-dead (AAA) mutant form of FER was tested in this assay
liposomes that contain PA showed no ability to stimulate phosphorylation of cortactin. PA plays an important role in lamellipodia induction by FER. Induction of FER kinase activity and the ability of FER over-expression to induce lamellipodia were both attenuated by treatment of cells with t-butanol to inhibit phospholipase D (PLD)-mediated PA production. This supports the idea that induction of FER kinase activity by PA plays an important role in lamellipodia induction.

The importance of FER interaction with PA for its in vivo function was tested. RNAi was first used to knockdown endogenous FER. A mutant form of FER that harbours a mutation in the FX domain that renders it unable to bind PA was then expressed in the FER-knockdown cells. These experiments showed that FER interaction with PA is essential for cell migration. The cell migration defect could be efficiently rescued by expression of wild type FER, but not by expression of the kinase-dead AAA mutant FER. FER was proposed to function in lamellipodia formation and cell migration downstream of integrins, PLD1 and/or PLD2, and PA and upstream of two pathways, one the Rac signalling pathway downstream of Vav2 and the other an F-actin assembly pathway downstream of cortactin. This pathway that FER is proposed to function in is distinct from the PLD1 and/or PLD2 and PA signalling pathway involving the kinase mTor that regulates protein synthesis and cell growth. In conclusion, it is proposed that the mechanism by which the PLD-PA signalling pathway promotes cell migration involves FER-kinase-dependent stimulation of actin filament assembly.

F-BAR domain proteins are not unique to metazoans. They are also found in simple unicellular eukaryotes, such as yeast. Indeed, the former name of the F-BAR domain protein family was the Pombe Cdc15 Homology (PCH) family because its founding member was the fission yeast (Schizosaccharomyces pombe) cell division protein Cdc15. Yeast are not motile and cannot form either filopodia or lamellipodia. However, yeast cells do have an actin cytoskeleton and they take up extracellular fluid and cell surface receptors by endocytosis. Moreover, membrane remodelling
plays a critical role in the final stage of the cell division cycle when one cell body divides into two - a process known as cytokinesis. *S. pombe* Cdc15 functions in cytokinesis, as does its mammalian ortholog PSTPIP1.

There were no presentations on *S. pombe* Cdc15 or mammalian PSTPIP1, however Alan Munn (Griffith University, Gold Coast, QLD, Australia) presented work from his group and that of Barbara Winsor (University of Strasbourg, Strasbourg, France) on the Cdc15 ortholog in budding yeast (*Saccharomyces cerevisiae*). This F-BAR domain protein is known as homolog of (cdc) fifteen 1 protein, Hof1p (formerly cytokinesis-defective 2 protein, Cyk2p). Hof1p is one of at least three F-BAR domain proteins in *S. cerevisiae* (the others are Bzz1p and Syp1p) (for reviews see refs 21 and 22). Budding yeast Hof1p has previously been shown to be expressed specifically in dividing cells and to localise to a ring at the bud neck (equivalent to the cleavage furrow in mammalian cells). Hof1p plays an important, although non-essential, role in cytokinesis. Deletion of the gene that encodes Hof1p results in viable, but slow growing cells. Hof1p-deficient cells are enlarged, misshapen, and form clusters and chains due to inefficient cytokinesis. In mammalian cells, PSTPIP1 also localises to the cleavage furrow and interacts with proteins essential for cytokinesis.

The focus of the presentation was on the C-terminal SH3 domain of Hof1p. The Hof1p SH3 domain interacts with proline-rich sequences in verprolin/Vrp1p (the yeast ortholog of human WASP-Interacting Protein, WIP). Mutation of the verprolin proline-rich sequences or loss of verprolin results in defects in both Hof1p localisation and cytokinesis. However, the Hof1p SH3 domain is non-essential for cytokinesis. This suggests that the role of the Hof1p-verprolin interaction cannot simply be to bring the two proteins together into a multi-protein complex. A model was proposed in which loss of interaction with verprolin either 1) induces a persistent change in the 3D structure of the Hof1p SH3 domain or 2) a change in its pattern of interactions with other proteins and this
interferes with cytokinesis. In this model, interference in cytokinesis would result despite the Hof1p SH3 domain having no essential role in cytokinesis. This model is supported by the efficient suppression of the cytokinesis defect that is observed when the Hof1p SH3 domain is deleted. This suggests that inhibition of cytokinesis is responsible for the cytokinesis defect and furthermore that this inhibition is dependent on the Hof1p SH3 domain. The remainder of the presentation consisted of unpublished data that cannot be described here, but will be published elsewhere in due course.

The F-BAR-domain containing protein kinases FES and FER, and in particular their role in oncogenesis, were the topic of a presentation by Peter Greer (Queen's University, Kingston, ON, Canada). FES and FER are the products of two different genes, but both have a similar domain structure with an N-terminal F-BAR domain, central FX and Src Homology 2 (SH2) domains, and a C-terminal protein tyrosine kinase domain. The fer gene also encodes a related protein tyrosine kinase known as FERT that possesses only the SH2 and protein tyrosine kinase domains. Finally, there exist powerful oncogenic retroviral protein known as GAG/FES or that are the products of in-frame fusions of the cellular fes and retroviral gag genes (reviewed in 23). Mice that are homozygous for a knockout of either fes or fer are healthy, have a normal life span, and are fertile.

A major question that remains unanswered is whether the gene that encodes FES is an oncogene or a tumour suppressor gene. On the one hand, the cellular fes gene was originally identified as an oncogene from avian and feline retroviruses. Expression of the retroviral GAG-FES fusion protein in transgenic mice induced tumours. Finally, transgenic mice that express a kinase-activated mutant form of FES display a hypervascularity phenotype. These all suggest a possible role as an oncogene or factor that contributes to angiogenesis. On the other hand, however, fes knockout mice show a hyper-inflammatory phenotype. In the MMTV-PymT murine breast cancer model24, knockout of FES results in an earlier onset of tumours compared to wild type mice. Kinase-inactivating mutations of FES have been reported in human colon cancer and there is evidence that FES
expression may be downregulated in human colon cancer in a mechanism dependent on promoter methylation. These data all suggest a role for FES as a tumour suppressor.

One major interest in Greer's group is the stromal role of FES in tumorigenesis. To investigate this, his group has been using the orthotopic breast carcinoma cell mouse engraftment model and AC2M2 breast carcinoma cells. AC2M2 cells are a basal-like breast carcinoma tumour model with a human growth factor (HGF)/Met autocrine loop. In the engraftment model, AC2M2 cells are grown in culture and then 7,500 cells are injected into the fat pad of wild type (\(fes^{+/+}\)) or FES knockout (\(fes^{-/-}\)) nude mice (\(nu/nu\)). Then the growth of the grafted tumour is monitored over time by measuring tumour volume. In the FES knockout mice the tumour volume increases more slowly than in wild type mice. This is despite the fact that the genotype of the tumour cells is the same in each case. The only difference is in the genotype of the stromal cells (cells that surround the tumour cells). Not only is tumour growth affected, but the frequency of lung metastases is also reduced in the FES knockout mice.

Next, the tumour cell intrinsic role of FES in AC2M2 tumours was investigated. AC2M2 breast carcinoma cell lines were generated that express either wild type FES or hyperactive or kinaseinactive mutant forms of FES. These tumour cells were then injected into the fat pad of nude mice and the growth of the tumour was monitored over time. The results showed that the FES genotype of the tumour had no significant effect on tumour growth. When the frequency of metastasis was investigated, there was only a marginal effect of FES genotype.

The presentation then changed focus to FER and its role in tumorigenesis. This analysis used the MMTV-Neu transgenic mouse model of breast cancer\(^{25}\). Transgenic mice carrying the \(MMTV\text{-}Neu\) transgene on a homozygous FER mutant (\(fer^{DR/DR}\)) background were constructed. The results
showed that *MMTV-Neu* induced breast tumours took longer to appear in the FER-deficient mice compared to wild type mice. Moreover, when tumours did arise in the FER-deficient mice they grew more slowly (~30-40% wild type growth rate). Hence, loss of FER attenuates tumorigenesis in a Her2/Neu over-expressing breast tumour model.

Does attenuation of tumorigenesis in FER-deficient mice reflect a tumour cell intrinsic or stromal role for FER in tumorigenesis? FER has been implicated in epidermal growth factor signalling, so this provides a possible mechanistic basis for a tumour cell intrinsic role for FER in tumorigenesis. However, there may be a stromal role for FER in breast tumorigenesis. FER-deficiency in the stroma may reduce tumour growth in a Her2/Neu model of breast cancer. For example, the F-BAR domain protein syndapin 1/PACSIN1 is phosphorylated by FER when co-expressed in Cos-1 cells. Moreover, in FER-deficient (fer<sup>DR/DR</sup>) murine embryonic fibroblasts (MEFs) syndapin2/PACSIN2 phosphorylation is defective. Changes in the phosphorylation of syndapin2/PACSIN2, which is known to play an important role in endocytosis, may alter the response of stromal cells to tumours.

Interestingly, in FES-null macrophages LPS-stimulation resulted in prolonged surface expression of Toll-like receptor 4 (TLR4/MD2). This suggests a possible defect in TLR4/MD2 internalisation by endocytosis in FES-deficient stromal cells. Hence, one important in vivo role for FES (and perhaps FER) in stromal cells is regulation of the endocytic function of syndapin2/PACSIN2. The data presented suggest that both FES and FER have excellent potential as therapeutic targets in breast cancer.

Finally, Igor Kovacevic (Goethe University, Frankfurt am Main, Germany) presented his work on a zebrafish model to investigate the role of the Nostrin subfamily of F-BAR proteins. His presentation consisted of unpublished data that cannot be described here, but will be published elsewhere in due course.
ACKNOWLEDGEMENTS

Work in the authors’ laboratories is made possible by research grant funding from the Australian Centre for HIV and Hepatitis Virology Research (to AM) and from the Swedish Research Council and the Cancer Foundation (to PA).

REFERENCES


Aspenström P. (Ed.) The pombe Cdc15 homology proteins. Landes Bioscience; Austin, Texas, USA; 2009.


actin and BAR proteins upstream of dynamin at endocytic clathrin coated pits. Dev Cell 2010 in press


Munn AL, Winsor BAT The budding yeast PCH/F-BAR proteins, In: The pombe Cdc15 homology proteins (P Aspenström, editor), Landes Bioscience; Austin, Texas, USA; 2009; 21-38.


