The inverse BAR-domain protein IBARa drives membrane remodelling to control osmoregulation, phagocytosis and cytokinesis

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Abstract
Here, we analyzed the single I-BAR family member IBARa from D. discoideum. The X-ray structure of the N-terminal I-BAR domain solved at 2.2 Å resolution revealed an all-α helical structure that self-associates into a 165 Å zeppelin-shaped antiparallel dimer. The structural data are consistent with its shape in solution obtained by small-angle X-ray-scattering. Cosedimentation, fluorescence-anisotropy as well as fluorescence and electron microscopy revealed the I-BAR domain to bind preferentially to phosphoinositide-containing vesicles and drive the formation of negatively curved tubules. Immunofluorescence labelling further showed accumulation of endogenous IBARa at the tips of filopodia, the rim of constricting phagocytic cups, in foci connecting dividing cells during the final stage of cytokinesis, and most prominently at the osmoregulatory contractile vacuole (CV). Consistently, IBARa-null mutants displayed defects in CV formation and discharge, growth, phagocytosis and mitotic cell division, whereas filopodia formation was not compromised. Of note, IBARa-null mutants were also strongly impaired in cell spreading. Together, these data suggest IBARa to constitute an important regulator of numerous cellular processes intimately linked with the dynamic rearrangement of cellular membranes.

Introduction
Numerous cellular processes including endocytosis, cell migration or division require the dynamic remodelling of the plasma membrane and the underlying actin cytoskeleton (Keren, 2011). The superfamily of Bin/Amphipysin/Rvs (BAR)-domain proteins is characterized by their ability to sculpt membranes after recruitment and clustering by acidic phospholipids (Saarikangas et al., 2010; Qualmann et al., 2011). BAR domains are grouped into three subfamilies: BAR and F-BAR-domain proteins in combination with actin polymerization were shown to be key players in initiating and stabilizing endosomal vesicles by generating positive membrane curvature bending the plasma membrane inwards (Peter et al., 2004; Frost et al., 2008), whereas the subfamily of the inverse BAR-domain (I-BAR)-proteins induce negative membrane curvature to promote cell protrusions (Millard et al., 2005; Suetsugu et al., 2006a; Mattila et al., 2007; Saarikangas et al., 2009).
The mammalian I-BAR domain protein family is comprised of the five members IRSp53, MIM, ABBA, IRTKS and Pinkbar, which additionally harbour protein-protein interaction modules such as CRIB, SH3 or actin monomer-binding WH2-domains that link them to signalling pathways or directly to actin-based processes (Scita et al., 2008; Pykäläinen et al., 2011). Previous studies suggested I-BAR proteins to regulate the formation of actin protrusions through interactions with membranes, actin and other cytoskeletal regulators, for instance as described for IRSp53 with VASP (Vaggi et al., 2011) or the Diaphanous-related formin mDia1 and the Scar/WAVE-complex subunit WAVE2 (Goh et al., 2011). In addition, pathogens such as enterohaemorrhagic *E. coli* (EHEC) use these interactions to recruit the actin assembly machinery for formation of actin pedestals (de Groot et al., 2011). Although the precise functions of IRSp53 and MIM have not yet been fully resolved, both proteins were proposed to link Arp2/3-complex mediated actin assembly with formation of cell protrusions. Notably, IRSp53 interacts with the small GTPases Cdc42 and Rac through its N-terminal region and with WAVE2 through its central SH3 domain (Krugmann et al., 2001). Consequently, IRSp53 was suggested to regulate Arp2/3-complex activity through interaction with WAVE complex and be involved in lamellipodia and filopodia formation (Nakagawa et al., 2003; Biyasheva et al., 2004; Suetsugu et al., 2006b). In mice, IRSp53 deficiency leads to defects in the development of the eye, central nervous system and the formation of filopodia (Kim et al., 2009; Sawallisch et al., 2009; Chauhan et al., 2009; Disanza et al., 2013). MIM was originally identified as a putative tumour suppressor since it is expressed in nonmetastatic, but absent from metastatic cancer cells (Lee et al., 2002). Knockout studies revealed that MIM functions at the interface of the plasma membrane and the actin cytoskeleton to promote the maintenance of intercellular junctions in epithelial tissues and contributes to B-cell lymphogenesis (Saarikangas et al., 2011; Yu et al., 2012). Thus, I-BAR domain proteins may directly link membrane deformation with actin polymerization in migration and cell morphogenesis.

To date, our knowledge of I-BAR domain protein function comes largely from studies of the five mammalian orthologues, which are likely to have redundant functions. In
contrast, the genome of the model organism *Dictyostelium discoideum* encodes one putative 357 residue I-BAR domain protein termed IBARa (Eichinger et al., 2005). A conserved domain search predicted an N-terminal I-BAR domain and a C-terminal SH3 domain, but no WH2-domain, thus conforming to the domain architecture of an IRSp53-like protein (Clarke et al., 2010). Since phylogenetic analysis revealed that IBARa from *D. discoideum* is rooted just before the branch point where IRSp53 and MIM-like subgroups of I-BAR domain proteins have evolved (Veltman et al. 2011), this ancestral protein is likely to display all basic functions of this protein family. For this reason, here we explored the functions of IBARa at the structural, biochemical and physiological level.

**Results**

**Structure and shape determination**

To learn more about the structure and shape of the predicted I-BAR domain we crystallized the N-terminal fragment of *D. discoideum* IBARa (aa 1-259, termed here IBARa-N) and determined the 2.2 Å crystal structure by a single-wavelength anomalous dispersion experiment (SAD) on selenomethionine substituted protein. Experimental phasing and density modification lead to well-interpretable electron density for all four polypeptide chains (two dimers) of IBARa-N, 323 water molecules and 6 non-solvent molecules in the asymmetric unit. The structure could be refined to an R value of 0.194 (Rfree= 0.230) with good stereochemistry (see Table 1).

IBARa-N is an almost entirely α-helical protein consisting of three antiparallel helices α1-α3 comprising residues 8-63, 68-142 and 147-221, respectively, followed by a shorter C-terminal helix α4 (aa 227-239, Fig. 1A). It auto-assembles into a slightly bent homodimer with chains oriented opposite to another. The main helices α1-α3 of each monomer form a twisted ellipsoid with an interacting surface of approximately 3700 Å². The interface of the two interacting monomers is mostly hydrophobic (Fig. 1C and Supplementary Fig. S3), and the dimeric assembly has maximum dimensions of ~165 Å x 25 Å x 25 Å (Fig. 1A). Density for the loop region connecting α2 and α3 (residues ~140-155) was very weak indicating that these loops are flexible and thus disordered in the
crystal. Also the N-terminal 8 residues of IBARα-N (+GPLGS overhang resulting from the GST-fusion) and the C-terminal 20 residues were not traceable. Each of the 4 copies of IBARα-N in the unit cell has slightly different starts and ends of the weak density areas (see Supplementary Fig. 1 for details) - the best traceable electron density could be found for chain D and contains the residues 8-239. Fig. 1B shows the solvent accessible electrostatic surface of IBARα-N in the same orientation as in Fig. 1A. IBARα-N is a highly charged protein (Supplementary Fig. S2) and the protein shows patches of positively charged areas on the convex side which is necessary for membrane binding, but also a highly acidic patch in the middle of the concave side (mainly residues Glu-195, Glu-197 and Glu-199). Thus IBARα-N is showing a dipole structure as also observed in other BAR-domains. The obvious differences in positive charges on the convex side of the protein (Fig. 1B, bottom) are resulting from the fact that each of the chains in the asymmetric unit shows slight differences in side chain orientation and loop completeness. A symmetric dimer model created from chain D only by replacing chain C with a copy of chain D shows a symmetric distribution of charges on the convex backbone area (Supplementary Fig. S4). The structural comparison of IBARα-N with IRSp53 (de Groot et al., 2011) shows that both proteins are highly similar (RMSD 1.67 Å, Fig. 1D) and comparison of IBARα-N with MIM (Millard et al., 2005; Lee et al., 2007) also underlines the structural similarity (Supplementary Fig. S5).

To analyze the structure of IBARα-N in solution, we performed small-angle X-ray experiments with the same construct as used in the crystallization setups. The shape of the scattering curve of IBARα-N already suggests an elongated shape of IBARα-N (e.g. Volkov and Svergun, 2003). The radius of gyration calculated from guinier analysis (s*Rg <1.3) of Rg=4.04 nm (Supplementary Fig. S6) is in good agreement with the theoretical Rg calculated from the crystal structure (Rg = 3.96 nm) which lacks 33 residues present in the soluble construct due to weak electron density (loop regions, N- and C-terminus). The theoretical scattering curve calculated with crysol of the IBARα-N dimer has the same shape as the measured data confirming that the overall shape of the crystal structure is also present in solution (Fig. 1F). Moreover, we can rule out a monomeric state of the protein in solution (Supplementary Fig. S7). We then calculated
independent sets of *ab initio* models from the SAXS-data. All models were highly similar and we were able to dock the crystallographic dimer into the final averaged and filtered *ab initio* shape with a good fit (Fig. 1E). The *ab initio* models of IBARa-N show a slightly more pronounced curvature being in good agreement with our data showing IBARa-N to evaginate and bend membranes. Since the structure of IBARa-N shows the typical twisted ellipsoid $\alpha$-helical arrangement, previously found in inverse BAR-domains (Saarikangas et al., 2010), we next analyzed the lipid binding and bending properties of IBARa-N.

**IBARa binds and evaginates PIP$_2$ containing membranes**

Previous studies established I-BAR domains to interact with phospholipid-rich membranes through positively charged patches located at the distal ends of the I-BAR domain (Suetsugu et al., 2006a; Mattila et al., 2007; Saarikangas et al., 2008; Pykäläinen et al., 2011). The specificity of IBARa binding to a variety of phospholipids was assessed by cosedimentation assays using liposomes of different lipid compositions. IBARa-N bound to a variety of phospholipids, with a preference to phosphoinositides. Based on a cosedimentation assay carried out with different phosphoinositides, I-BARa-N appeared to display highest affinity towards PI(4,5)P$_2$ (Figs. 2A,B). To analyze and quantify the PI(4,5)P$_2$-clustering activity of IBARa-N, the self-quenching of Bodipy-TMR-PI(4,5)P$_2$ resulting from clustering upon addition of protein was monitored by measuring fluorescence intensity. Addition of IBARa-N resulted in self quenching of the fluorescent probe molecules (Fig. 2C). The signals were plotted to obtain values for PI(4,5)P$_2$ clustering at different concentrations. These data suggest that the positively charged ‘l lipid-binding interface’ in the very N-terminus in the I-BAR domain of IBARa is capable to efficiently cluster PI(4,5)P$_2$. The rotational diffusion of the fatty acyl chains was evaluated by fluorescence anisotropy, with DPH as a probe. This is a rod-like, highly hydrophobic molecule that locates into the hydrophobic core of a lipid bilayer without affecting the physical properties of the membranes and can thus be used for monitoring changes in the trans-gauche isomerization of phospholipid acyl chains in the membrane interior (Zaritsky et al., 1985). Anisotropy changes were interpreted here in terms of fluidity or variation in microviscosity. Binding of the MIM I-BAR domain as a control to
PI(4,5)P₂-containing membranes induced a significant increase in DPH anisotropy, because the N-terminal amphipathic α-helix inserts into the acyl chain region of the bilayer and therefore modulates its microviscosity (Saarikangas et al., 2009). Notably, IBARa-N had no significant effect on DPH anisotropy, strongly suggesting that this I-BAR domain does not insert into the acyl chain region of the bilayer (Fig. 2D). This is consistent with the X-ray structure which does not show any N-terminal amphipathic α-helices which could insert into membranes (see Fig. 1).

Since the “zeppelin-like” shape and structure of IBARa groups the protein into the subfamily of inverse BAR-domain proteins, in the following we analyzed its lipid bending mode using large unilamellar vesicles (LUV). The addition of 5 µM IBARa-N to LUV solution with a total lipid concentration of 200 µM was sufficient to cause marked fragmentation of the LUVs (Figs. 2E). From this experiment it became obvious that IBARa-N was also able to initiate and elongate membrane tubules (Figs. 2E, white arrows). To assess whether IBARa-N drives the formation of either positive or negative membrane curvature the protein was incubated together with preformed fluorescently labelled giant unilamellar vesicles (GUV), and analyzed by fluorescence microscopy. After addition of 1 µM IBARa-N to the GUVs invaginations of the membrane were observed, demonstrating that IBARa generates negative membrane curvature (Fig. 2F). Of note, in the absence of PIP₂ no tubulation of the membrane was observed (data not shown).

**Localization of endogenous IBARa**

To analyze the subcellular localization and dynamics of IBARa, we generated N- and C-terminal fusions with GFP. However, *Dictyostelium* cells transfected with these construct displayed only diffuse signals, suggesting that fusions with GFP are not functional. To corroborate this notion, we raised specific polyclonal antibodies against a C-terminal IBARa fragment (aa 225-357). After affinity purification, the antibody was used to analyze the localization of endogenous protein by indirect immunofluorescence microscopy. Interestingly, IBARa prominently localized at large vacuolar structures interconnected by a tubular network (Fig. 3A; Supplementary Movie S1 and Fig. S8),
presumably the contractile vacuole (CV) which is an osmoregulatory organelle (Gabriel et al., 1999). To confirm this, fixed cells were additionally stained for endosomes as well as the CV marker csA-Rh50 after ectopic expression of this construct in WT cells (Mercanti et al., 2005). As shown in Fig. 3A, IBARa-localization was clearly distinct from p80-stained endosomes, but perfectly colocalized with csA-Rh50 corroborating its presence on the CV system. Additionally, IBARa was seen at the distal tips of filopodia (Fig. 3B). In summary, this showed that IBARa is associated with dynamic regions in membranes and suggested the protein to be implicated in osmoregulation and filopodia formation.

**Generation and analysis of IBARa-null mutants**

To assess the contribution of IBARa to these processes, the ibrA-gene was disrupted by homologous recombination. The inactivation of the ibrA-gene was validated by PCR using specific primers (Fig. 3C), and the absence of IBARa protein was additionally confirmed by Western Blotting (Fig. 3D). Due to its localization in filopodia tips and the implication of the I-BAR protein IRSp53 in filopodia formation (Millard et al., 2005, Vaggi et al., 2011), we quantified the total number of filopodia in randomly migrating IBARa-null and WT cells using phase contrast microscopy. However, and consistent with a recent report (Veltman et al., 2011), we could not find a significant difference as both cell lines formed about 6 filopodia on average (Fig. 3E). Additionally, we monitored the protrusion and retraction of filopodia in WT and mutant cells expressing the filopodial tip marker protein GFP-myosin VII (Tuxworth et al., 2001). Again, no differences between WT and mutant cells were observed, as in both cell lines filopodia protruded with a speed of ~ 0.4 µm/s (Fig. 3F). Together, these findings suggest IBARa not to be critically involved in filopodia formation.

**The null mutant displays substantial defects in osmoregulation**

Since endogenous IBARa most prominently decorates the CV (Fig. 3A), next we quantified the number of this organelle in WT and mutant cells using the live cell dye FM2-10 (Health and Insall, 2008). Analysis by confocal microscopy revealed that in the absence of IBARa the cells displayed a significantly increased number of CVs as
compared to control (Figs. 4A,B), indicating a functional defect of this osmoregulatory organelle, either in filling, discharge or fusion with the plasma membrane. To monitor dynamics and discharge of the CVs in WT and mutant cells, ectopically expressed GFP-Dajumin was used as a CV marker suitable for live cell imaging, as this probe was previously reported to label the entire CVs, whereas the endosomal compartment was not stained (Gabriel et al., 1999). First, we tested whether the CVs of the null-mutant cells were still able to expel liquid. Therefore, the cells were incubated in low osmolarity phosphate buffer to stimulate CV activity supplemented with the soluble fluorescent dye tetramethylrhodamine (TAMRA). Confocal time-lapse imaging revealed that the mutant cells were still able to fill and discharge their CVs, albeit it appeared to take longer in the IBARa-mutant (Fig. 4C, Supplementary Movie S2). Notably, with the beginning of the CV discharge, we noticed an influx of exterior liquid, as visualized by diffusion-driven incorporation of TAMRA into the compartment, demonstrating the formation of unspecific channels or pores when the CV fuses with the plasma membrane (Fig. 4C, white arrows). Of note, we noticed a broader cellular distribution of the GFP-Dajumin probe in these experiments as compared to the CV structures labelled by IBARa antibodies. To correlate the localization of both probes, the GFP-Dajumin expressing WT cells were immunostained with anti-IBARa antibodies (Fig. 4D). Confocal microscopy showed that IBARa and GFP-Dajumin colocalize at CVs, however, GFP-Dajumin was also seen on other structures, for instance around the nucleus, presumably comprising the ER.

Next, we compared the survival rate of IBARa-null and WT cells by incubation under hypotonic conditions for different time periods. This assay revealed that after 5 hours in water more than 40% of the IBARa-null cells have died, whereas the number of viable WT cells remained almost constant, indicating that absence of IBARa causes a strong defect in osmoregulation (Fig. 4E). To quantify this defect in more detail in the least invasive way, we imaged WT and mutant cells moderately flattened by an overlaying thin agar sheet by high-speed phase-contrast imaging. Interestingly, about 10% of all IBARa-null cells failed to form or maintain a CV after transfer into hypotonic buffer and subsequently lysed (Fig. 4F upper panel, Supplementary Movie S3). These cells were
morphologically distinct from the others as evidenced by their grainy cytoplasm as well as a swollen and round appearance. Unexpectedly, upon contact, they also became detectable as potential prey for neighbouring IBARa-null cells containing CVs (Fig 4F, lower panel, Supplementary Movie S4), suggesting markedly altered cell surface properties in the diseasing cells. The majority of IBARa-null still formed functional CVs, however, these were larger in size and displayed a diminished discharge rate by 23% as compared to the CVs of WT cells (Fig. 4G). Together these findings establish IBARa as a critical factor in CV function.

**IBARa effects growth, adhesion and phagocytosis**

Since defects in the CV system of *Dictyostelium* cells are frequently associated with defects in growth or cytokinesis (Kwak et al., 1999; Gerald et al., 2002; Damer et al., 2005, 2007), we compared growth in bacterial lawns on agar plates as well as in shaken suspension (Figs. 5A,B). In both conditions, growth of the null-mutant was markedly slowed down when compared to WT cells. In shaken suspension the null mutant reached only a density of ~8 x 10^6 cells/ml, while the WT became stationary above 1.3 x 10^7 cells/ml. Together these findings suggest defects in cytokinesis, endocytosis or adhesion either alone or in combination.

Next, we therefore compared the efficiency of uptake of the fluid phase marker TRITC-Dextran in WT and IBARa-null cells. However, no defects in macropinocytosis between WT and mutant cells were measurable in our assays (Fig. 5C). Then we compared the efficiency of phagocytosis in WT and IBARa-null cells. Phagocytic activity was measured by the uptake of fluorescently labelled yeast particles (FLY), as well as nocodazole treated, fluorescently labelled, dumbbell shaped yeast particles (NTY). We observed an impaired uptake of both types of particles by the null mutant (Fig. 5D), and as expected, ingestion of NTY was less efficient when compared to the spherical FLY particles. In line with these findings, IBARa was detected at the rim of constricting phagocytic cups (Fig. 5E; Supplementary Movie S5). Thus in endocytosis, IBARa contributes predominantly to phagocytosis.
As numerous cellular processes require an adaptation of the cell morphology, which for instance during cytokinesis is also linked to substantial changes in volume, we analyzed the dynamic of cell shape changes by time-dependent measuring of the adhesion area by reflection interference contrast microscopy (RICM). WT cells showed highly dynamic contracting and spreading behaviour resulting in a highly fluctuating cell adhesion area (Figs. 5F,G upper panel). By contrast, IBARa-null cells remained relatively static (Figs. 5F,G lower panel). Interestingly, the median size of the adhesion area in WT and null-mutant did not differ significantly (Fig. 5F), while time resolved distribution of the measured areas was markedly broader in the WT as opposed to the mutant (Fig. 5F). These data do not favour a general defect in adhesion, but rather suggest a defect in adaption of the cellular volume in response to morphological changes.

**IBARa is required for normal cytokinesis**

Previous work revealed that constriction of the cleavage furrow is accompanied by a drastic efflux of water as evidenced by highly active contractile vacuole formation during cytokinesis (Fukui and Inoué, 1991). Therefore, we analyzed dividing WT and mutants cells by phase contrast time-lapse microscopy. We found that IBARa-null cells were frequently unable to finish separation into daughter cells (Fig. 6A). Contrary to the WT which divides by symmetric constriction, the mutant frequently began to separate into four or more daughter cells at the beginning of anaphase. Some of them, however, failed to separate completely and reunited into the same cell body, resulting in fewer daughter cells due to this asymmetric cell division (Supplementary Movies S6,S7). To substantiate this defect in cytokinesis, we quantified the number of nuclei after DAPI staining in cells grown on plates or in shaken suspension (Fig. 6B,C). In both conditions, IBARa-null cells became highly multinucleated and as expected, the cytokinesis defect was more pronounced in cells grown in shaken suspension, during which the cells lack adhesion to the substratum and are exposed to strong shear forces. In the latter case 38% of IBARa-null cells contained 3 or more nuclei, while this value was less than 3% in the WT (Fig. 3C). Finally, we explored the subcellular localization of IBARa in dividing myosin II-null cells, since in adherent culture these cells divide by regular cytokinesis comparable to the WT (Neujahr et al 1997). However, due to their increased adhesion to the substrate,
a much larger fraction of cells can be captured in various stages of cytokinesis (Weber et al. 1999). During mitosis the CV system was much more dispersed into smaller vesicles when compared to its morphology in interphase cells as assessed by immunolabeling with IBARa antibodies. We only observed an occasional enrichment of IBARa-containing vesicles in the vicinity of the cleavage furrow region stained by the actin-bundling protein cortexillin during telophase (Weber et al., 1999). Most strikingly, however, during final stages of cytokinesis, IBARa became highly enriched in foci connecting the separating daughters cells (Fig. 6D, Supplementary Movies S8-S10). These findings strongly suggest IBARa to actively contribute to membrane remodelling during mitotic cell division.

Discussion

The x-ray structure of *D. discoideum* IBARa-N shows high similarity to existing structures of I-BAR-domains like IRSp53 (de Groot et al., 2011) and MIM (Lee et al., 2007). Two IBARa-N monomers auto assemble into an antiparallel dimer forming the typical I-BAR architecture having only a small angle of approximately 10° between the monomers in comparison to the more bent structures of F- or N-Bar domains (Frost et al., 2009). Small-angle X-ray scattering data support that the conformation of IBARa-N observed in the crystal structure is also present in solution, and at the same time rule out the existence of significant amounts of monomers. The dipole structure orients IBARa-N at the membrane by electrostatic interactions of the positive side with the phospholipids and repulsive effects of the positive far side. As we could not identify any regions that are potentially inserted into the membrane, for instance as seen with the N-terminal amphipathic helix in MIM (Saarikangas et al., 2009), IBARa-N presumably binds to membranes exclusively by electrostatic interaction with negatively charged PIP₂, thereby inducing membrane deformation similar to IRSp53 (Mattila et al., 2007). This mode of membrane interaction is also supported by the fluorescence anisotropy experiments using DPH-labelled lipids, showing no effect on anisotropy similarly to IRSp53 (Saarikangas et al., 2009), but in stark contrast to MIM which causes increased anisotropy due to insertion into the membrane (Saarikangas et al., 2009). As a consequence, the far side of IBARa-N is oriented towards the cytosol with the negatively charged patch in the middle.
of the IBARa-N concave side being accessible for potential protein-protein interactions. As previously reported for other proteins (Mercanti et al., 2005), the acidic patch might also serve as a localization signal to the CV.

**Localization of IBARa**

The great advantage of analyzing GFP-fusion proteins is the capability of following the dynamics of the proteins by live cell imaging to understand their cellular function. Recent work using this approach reported on the function of IBARa in phagocytosis and clathrin-coated pinocytosis (Clarke et al., 2010; Veltmann et al., 2011). However, with the exception of a moderate enrichment of IBARa in constricting phagocytic cups (Clarke et al., 2010), and endocytic vesicles (Veltmann et al., 2011), a distinct cellular localization of IBARa was not seen. In our hands, both N-terminal and C-terminal GFP fusions with IBARa were more or less uniformly distributed in the cytoplasm, even when the proteins were expressed at low levels, suggesting that in this case fusions with GFP are not functional. This notion was further confirmed by immunolabeling of the endogenous IBARa protein in fixed cells, which in turn was found to prominently accumulate in the membrane of the CV, in filopodia and in constricting phagosomes. Thus, the only localization of IBARa seen with both, antibody labelling and expression of the GFP-fusion protein were constricting phagosomes enclosing dumbbell-shaped NTY yeast particles. What could be the reason for that? We hypothesize that due to the atypical form of the NTYs, the *Dictyostelium* cells vehemently attempt to close the phagocytic cup after engulfing the first halve of the NTY. However, since this fails due to presence of the neck region of the NTY, the cells appear to counter this by localizing excess IBARa protein to this region. This notion is also consistent with massive accumulation of coronin in constricting phagosomes of *Dictyostelium* cells engulfing NTYs as assessed by immunolabeling or expression of GFP-coronin (data not shown). Therefore, even a poorly functional GFP-IBARa fusion construct might yield a detectable signal in this case.
**IBARa is dispensable for filopodium formation**

Due to the localization of IBARa in the distal tips of filopodia, its domain architecture and our finding showing that IBARa is able to generate negative curvature to evaginate membranes, in *Dictyostelium* cells the protein therefore appears to carry out an IRSp53-like function in filopodium formation (Krugmann et al., 2001; Millard et al., 2005; Mattila et al., 2009). However, and consistent with recent work (Veltmann et al., 2011), inactivation of IBARa did not abolish the formation of filopodia. Thus, this IMD-domain protein is either not essential and carries out only modulatory functions in this process, or other membrane sculpting proteins execute overlapping functions with IBARa in the formation of filopodia. Of note, although F-BAR-containing proteins are expected to participate in the induction of invaginations, in mammalian cells two F-BAR proteins, srGAP/MEGAP and PACSIN/syndapin, have been reported to induce filopodia-like membrane protrusions (Wasiak et al., 2001; Guerrier et al., 2009; Carlson et al., 2011). The *Dictyostelium* genome encodes six F-BAR proteins including four MEGAPs and one syndapin-like protein, from which only MEGAP1 and MEGAP2 have been characterized and shown to be implicated in cell motility and tubulation of CV system (Eichinger et al., 2005; Health and Insall, 2008). Thus, it remains to be seen whether some of the remaining F-BAR proteins contribute to filopodium formation.

**IBARa function in cytokinesis**

Why is IBARa, residing most prominently on the CV (see below), involved in other processes such as cytokinesis? A microscopic analysis of cytokinesis in *Dictyostelium* cells previously revealed that constriction of the cleavage furrow is accompanied by an efflux of water, as evidenced by highly active contractile vacuole formation during cytokinesis (Fukui and Inoué, 1981). This implies a critical function for the CV system in *Dictyostelium* cytokinesis and may explain the phenotypic defects reported for genetic knockout mutants lacking the CV constituents CpnA and LvsA (Kwak et al., 1999; Gerald et al., 2002; Damer et al., 2007). This function is also consistent with the substantial defect of IBARa-null cells in mitotic cell division, since these cells display impaired removal of water, as evidenced by their sensitivity towards incubation in hypotonic conditions. However, alike cell spreading and cell migration (Traynor and
Kay, 2007), cytokinesis is also intimately linked to considerable remodelling of the plasma membrane (Neto et al., 2011), that is most prominently seen in later stages, during which fusion and fission of the curved lipid bilayer is required to constrict the mother cell in two. Since IBARα interacts preferentially with PI(4,5)P₂, which in turn has been shown to be enriched in the cleavage furrow and required for cytokinesis (at least in *Drosophila* and mammalian cells) (Field et al., 2005; Wong et al., 2005), we monitored the distribution of IBARα during cytokinesis by immunofluorescence microscopy. Consistent with previous work, the CV system was found to be fragmented in mitotic cells (Zhu et al., 1993; Gabriel et al., 1999). However, only in some cases we found dispersed IBARα-containing vesicles in the vicinity of the cleavage furrow. Notwithstanding this, during the final stage of cytokinesis, IBARα became highly concentrated in small foci at the cell periphery connecting the separating daughter cells. This enrichment appears to be transient, as it was not always observed. These findings therefore strongly suggest that IBARα primarily facilitates membrane fission and/or fusion of membrane sheets during mitotic cell division.

**IBARα function in CV discharge**

IBARα localizes most prominently at the CV membrane. The CV system is an osmoregulatory organelle composed of cisternae and interconnecting ducts (Gabriel et al., 1999). In regulating the osmolarity of the cytosol, the CV periodically fuses with the plasma membrane, forming pores to expel excess water from the cell, thus allowing cells to survive hypotonic stress (Heuser et al., 1993; Du et al., 2008). IBARα-null cells contain a substantially elevated numbers of CVs and show defects in osmoregulation. Despite the fact that numerous CV components including vacuolar proton pumps (Heuser et al., 1993), actin and unconventional myosins (Zhu and Clarke, 1992; Heuser, 2006; Jung et al., 2009) as well as proteins implicated in membrane trafficking (Gerisch et al., 2002; Essid et al., 2012) have been identified, the molecular mechanism of CV discharge remained elusive. How could IBARα operate in this process? Of note, the F-BAR proteins MEGAP1 and MEGAP2 have been previously also shown to localize at the CV membrane (Health and Insall, 2008). Interestingly, MEGAP1/2- mutants empty their CVs less efficiently and have three times more CVs as compared to wild type. As
assessed by time-lapse imaging, these proteins localize transiently to the CV membrane, and subsequently fragment into smaller vesicles as the vacuole empties (Health and Insall, 2008). Provided that IBARa as well as MEGAP1 and MEGAP2 (and possibly other F-BAR proteins) act on the same side of the CV membrane, we surmise that I-BAR and F-BAR proteins might synergize in CV discharge because appearance of negative curvature is accompanied by positive curvature and vice versa. In our proposed model, we hypothesize that IBARa, residing most - if not all of the time - on the CV membrane, could serve to constitutively generate invaginations in the CV membrane resulting in a highly folded surface. This, in turn could facilitate the pinching of vesicles from the positively curved sites of the folded CV membrane by transiently binding F-BAR proteins, such as MEGAP1 and MEGAP2 (Fig. 7). Thus, IBARa may drive F-BAR-mediated vesicle formation to dismantle the CV membrane during acto-myosin driven contraction. Future work will aim to address whether different types of F-BAR proteins, presumably containing different curvatures of their respective F-BAR domains, alternate in their entry on the CV to promote its discharge.

Materials and Methods

Cell culture and transformation

Cultivation and transformation of D. discoideum AX2 wild-type (WT) cells and derived mutants strain was as described previously (Linkner et al., 2012).

Plasmids

The ibrA-cDNA (DictyBase ID DDB0266618) was amplified from a λgt11 cDNA library and inserted into the pJet1.2 vector (Fermentas) using the primers IBARa-H3U 5´-GCGAAGCTTATGTCAAACGCTAAAAAACAAC-3´ and IBARa-BD 5´-GCGGGATCCTACGTATTCAATATAAGTTGAAGG-3. For construction of the ibrA gene targeting vector, a 5´ BamHI/PstI fragment and a 3´ HindIII/SalI fragment were amplified from genomic AX2 WT DNA by PCR. The primers used for the 5´ fragment were 5´-GCCGGCATCCATGTCAAAAACGCTAAAAAAACAACAAAAATC-3´ and 5´-GCGCTGCAGAAAAGTTAGAGGGAGGGAGGAGAGTG-3´ and the primers for
the 3’ fragment were 5’-GCGAAGCTTGATAGAGATCAAATGAGACAAGATATT-3’ and 5’-GCGGTCGACTTATGCATATCAATACGATATGCATC-3’. Both fragments were gel purified after cleavage with BamHI/PstI and HindIII/Sall, and cloned into the corresponding sites of pLPBLP containing a Blasticidin S resistance cassette (Faix et al., 2004). The resulting vector was cleaved with BamHI and SalI and used to disrupt the ibrA gene in WT cells. Null mutants were screened by PCR as described previously (Linkner et al., 2012).

For expression constructs, the coding sequence of IBARa (aa 1-259) was amplified by PCR using the primers IBARa-1BU 5’-GCGGGATCCATGTCAAACGCTAAAAAACAAC-3’ and IBARa-259*SD 5’-GCGGTCGACTTATGCATATGAAATACGATATGCATC-3’. The coding sequence of the C-terminal fragment of IBARa (aa225-357) was amplified by PCR using the primers IBARa-225BU 5’-GCGGGATCCCTTACCACTTGAAATGGAATCAATGATT-3’ and IBARa-357*SD 5’-GCGGTCGACTTATACGTATTCAATATAAGTTGAAGG-3’, both fragments carried BamHI and SalI sites to facilitate cloning. The digested PCR fragments were inserted into the corresponding sites of pGEX-6P1 (GE-Healthcare). For the expression of N-terminal fusions to GFP the coding sequence of IBARa was amplified by PCR using primers IBARa-BU+2 5’-GCGGGATCCGCATGTCAAACGCTAAAAAACAAC-3’ and IBARa-357*SD. After digestion with BamHI/SalI the PCR fragments were inserted into the corresponding sites of pDGFP-MCS-Neo (Faix et al., 2001). For C-terminal GFP-fusion constructs the coding sequence of IBARa was amplified by PCR using primers IBARa-H3U 5’-GCGAAGCTTAAAAATGTCAAACGCTAAAAAACAAC-3’ and IBARa-BD 5’-GCGGGATCCCTACGTATTCAATATAAGTTGAAGG-3’. After digestion with HindIII/BamHI the PCR fragments were inserted into the corresponding sites of pB15GFPXSac1A (Gräf et al., 2000). All constructs were verified by sequencing. The plasmids for expression of GFP-Dajumin, GFP myosin VII and csA-Rho50 were described previously (Gabriel et al., 1999; Tuxworth et al., 2001; Mercanti et al., 2005).

**Protein purification**
IBARa (aa 1-259, also referred herein as IBARa-N) and IBARa (aa 225-357) fragments were purified from *E. coli* host BL21 DE3 as an N-terminally GST-tagged fusion protein. Briefly, the fusion protein was first purified on glutathione sepharose (GE-Healthcare), eluted with buffer A containing 20 mM Tris/HCl pH 8.0, 300 mM NaCl, 1 mM EDTA, 1 mM DTT supplemented with 30 mM reduced glutathione. The GST-tag from IBARa-N was subsequently cleaved with PreScission protease (GE-Healthcare). The resulting IBARa-N protein carries the extra amino acids GPLGS at its N-terminus. After cleavage, the protein fragments were separated by size-exclusion chromatography on a Superdex-S200 26/60 column (GE-Healthcare) equilibrated with buffer A. The protein was then dialyzed three times against buffer B containing 20 mM Hepes pH 7.3, 300 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 0.01% NaN₃ for crystallization. Selenomethionine substituted protein for crystallization was expressed in the Met auxotroph *E. coli* B831 as described in Hendrickson et al. (1990) and purified as described above with the addition of 5 mM β-mercaptoethanol to all buffers used in purification and 2 mM DTT to the final storage buffer.

**Generation of IBARa antibodies**

Polyclonal antibodies were obtained by immunizing female white New Zealand rabbits with recombinant GST-tagged IBARa (aa 225-357) together with complete Freund's adjuvant (Sigma). Specificity of the antibodies for IBARa was assessed by Western blotting.

**Preparation of fluorescently labeled yeast particles**

*Saccharomyces cerevisiae* were grown in shaking suspension in YPDA at 30°C. For the generation of dumbbell shaped yeast, 10 µg/ml nocodazole was added during the logarithmic growth phase, and the cells were incubated for another 90 min to arrest them in telophase. Nocodazole treated yeast (NTY) and untreated yeast cultures, were pelleted and resuspended in distilled water, incubated at 100°C for 20 min and labeled with tetramethylrhodamine isothiocyanate TRITC followed by vigorously washing to remove free dye. Cell density was adjusted to 5 x 10⁸ cells/ml and aliquots were stored at -20°C.
**Determination of growth rates, phagocytosis and pinocytosis**

Quantitative fluid-phase uptake and determination of growth rates on bacterial lawn and in shaken suspension were performed as described by Dumontier et al. (2000). Phagocytosis assays were carried out according to Maniak et al. (1995).

**Osmotic shock treatment**

Axenically grown cells were harvested at a density of not more than 5 x 10^6 cells/ml, washed in 17 mM Soerensen phosphate buffer; pH 6.0 (PB) and transferred into bidistilled water (ddH2O). The cell density was adjusted to 3 x 10^6 cells/ml and shaken in 25 ml of ddH2O at 150 rpm at 23°C for 24 h. After various time points of incubation, the cells were diluted into PB and 100 cells/plate were plated on SM-agar together with a solution of *K. aerogenes*. After incubation for 48 h at 23°C colonies were counted.

**Microscopy**

For imaging, axenically grown cells were seeded onto glass bottom dishes (MatTek) and incubated in 17 mM phosphate-buffer pH 6.1, for 1 h to reduce autofluorescence and to stimulate CV activity. The CVs of live cells were visualized with the lipophilic dye FM2-10 (Molecular Probes) as described (Health and Insall, 2008), and imaged using an Olympus Fluoview FV1000 confocal microscope (Olympus) at a rate of 1 frame/5 sec.

The topography of the ventral cell surface, including filopodia and cell-to-substratum contacts, was imaged by RICM using a LSM 510 confocal microscope (Zeiss) and the 633 nm line of the He-Ne laser. Immunolabeling of fixed cells with polyclonal anti-IBARa antibodies or monoclonal anti-cortexillin mAb 241-71-3 antibody was essentially performed as described (Faix et al., 2001). Endosomes were labelled with monoclonal antibody H161 (Ravanel et al., 2001) and CV marker csA-Rho50 with anti-csA monoclonal antibody 41-71-21 (Bertholdt et al., 1985). GFP was enhanced with Atto488-conjugated nanobodies (Chromotek). F-actin was visualized by fluorescent phalloidin (Molecular Probes) and DNA stained with Dapi or TO-PRO-3 (Molecular Probes).

Retracting and protruding filopodia as well as discharging CVs were filmed with an Olympus IX-81 fluorescence microscope equipped with phase contrast optics (Olympus) and a CoolSnap EZ camera (Photometrics) at a rate of 1 frame/sec. For the latter
experiment, the cells were allowed to adhere to 3 cm diameter glass-bottom dishes (Ibidi), subsequently washed with PB, and modestly compressed between a thin sheet of 1.5% agarose in PB and the glass surface essentially as described (Fukui et al. 1987). The CV discharge rate was quantified as follows: randomly picked CVs of WT and mutant cells were selected, and their diameters determined for each frame. Corresponding volumes were calculated and plotted over time. The centroid of each single curve was translated into the origin and thereby pooled into a single data set for each strain. Finally, the respective CV discharge rates were determined by linear regression of the data.

**Vesicle cosedimentation assays**
Large unilamellar vesicles were prepared according to Saarikangas et al. (2009), and vesicle cosedimentation assays were performed as described (Zhao et al, 2010). The final concentrations of IBARa-N and liposomes were 3 μM and 1 mM, respectively, in 20 mM Hepes, pH 7.5, 100 mM NaCl buffer.

**Negative stain electron microscopy**
Samples for transmission electron microscopy were prepared by mixing 5 μM proteins with 200 μM unilamellar vesicles (Ø=400 nm) in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl at room temperature for 15 min. This mixture was applied to the glow-discharged collodion- and carbon-coated copper grids and stained with 3% uranyl acetate. At each step, excess solution was removed by filter paper. The membrane morphologies were examined with a Tecnai 20 FEG electron microscope (FEI Corp.) operating at 80 kV. Images were recorded with a 4k x 4k Ultrascan 4000 CCD camera (Gatan Corp.) with a magnification of 13,000x.

**Tubulation of giant unilamellar liposomes**
Giant unilamellar vesicles (GUV) were prepared as described (Saarikangas et al., 2009). The lipid composition of the GUVs was POPC:POPE:POPS:PIP2:TopFluor-PIP2 (50:20:20:9:1). GUVs were mixed with the same osmolarity buffer (approx. 200 mM glucose in 5 mM Hepes buffer, pH 7.5) in 1:3 ratio prior to imaging. The total volume of
the samples during imaging was 100 μl and the final concentration of protein added to the GUVs was 1 μM.

**Bodipy quenching experiment**

The fluorescence measurements were performed in quartz cuvettes with 3 mm path length. Fluorescence spectra were measured with a Perkin-Elmer LS 55 spectrometer (Perkin-Elmer). Bodipy-FL-PI(4,5)P₂ fluorescence was excited at 495 nm and the emission spectra were recorded from 503 to 530 nm in the presence of different concentrations of protein. Emission and excitation band passes were set at 4 nm. All experiments were carried out in 20 mM Hepes, 100 mM NaCl, pH 7.5 at room temperature. The percentage of quenching was calculated as: % quenching=(1-F/F₀)×100, where F is the fluorescence intensity of Bodipy-FL-PI(4,5)P₂ in the presence of protein, and F₀ is the fluorescence intensity of Bodipy-FL-PI(4,5)P₂ in the absence of protein. The lipid composition was POPC:POPE:POPS:PI(4,5)P₂:Bodipy-FL-PI(4,5)P₂=50:20:20:9,5:0,5 and the lipid concentration was 30 μM. 1-Palmitoyl-2-oleoyl-sn-glycerol-3-phosphatidylcholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphatidylethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphatidylserine (POPS), and L-a-phosphatidylinositol-4,5-bisphosphate, PI(4,5)P₂ were purchased from Avanti Polar Lipids. Bodipy-FL-PI(4,5)P₂ was purchased from Echelon Biosciences.

**Fluorescence anisotropy of DPH**

The lipid composition was POPC:POPE:POPS:PI(4,5)P₂:DPH=50:20:20:10:0.2 and the lipid concentration used was 40 μM. 1,6-diphenyl-1,3,5-hexatriene (DPH) was obtained from Invitrogen. Fluorescence anisotropy for DPH was measured with a Perkin-Elmer LS 55 spectrometer. The excitation was set at 360 nm and emission at 450 nm, using 10 nm bandwidths. The experiments were carried out in 20 mM Hepes, 100 mM NaCl, pH 7.5 at room temperature.

**Crystallization and structure determination**
Crystals of selenomethionine substituted protein were obtained in hanging drop geometry using 20% PEG3350, 0.25 M CH₃COONH₄ and 15% (v/v) glycerol as reservoir solution. 1.5 µl of reservoir was added to an equal volume of IBARa-N (10 mg/ml) in storage buffer supplemented with 2 mM TCEP and incubated at 20°C. Crystals were backsoaked in mother liquor containing 20% (v/v) ethylene glycol for cryo-protection. Data collection at the synchrotron was carried out at 100 K at selenium peak wavelength determined by a fluorescence scan. Diffraction data were indexed, integrated and scaled using the XDS-package (Kabsch, 1993). Phases were obtained by autoSHARP (Vonrhein et al., 2007). Density modification was done using PIRATE and PARROT (Zhang et al., 1997). The high-resolution data allowed automatic model building using buccaneer (Cowtan, 2006). The preliminary model was refined in cyclic rounds of manual model building in COOT (Emsley and Cowtan, 2004) and refinement using PHENIX (Adams et al., 2010). The final model showed no outliers in the Ramachandran plot and reasonable R-factors and geometries. Atomic coordinates and structure factors were deposited in the Protein Data Bank with accession number 4NQI. Data collection and refinement statistics are listed in Table 1. Figures were prepared with PyMOL (DeLano, 2008).

**Small-angle X-ray scattering (SAS)**

SAS data were collected at EMBL/DESY P12 and X33 (Hamburg, Germany) and ESRF ID14-3 (Grenoble, France). Protein samples were measured at different protein concentrations between 1 and 9 mg/ml. Scattering data of the running buffer of the size exclusion chromatography were used for buffer correction. The scattering data were analyzed using the ATSAS package (Petoukhov et al., 2007) as described (Putnam et al., 2007). No particle interaction or aggregation could be detected in the observed concentration range (c.f. Supplementary Fig. S7). Radius of gyration (Rg) and the extrapolated scattering intensity at zero angle for molecular weight determination (Io) were obtained from Guinier-plot analysis (log(I(s) vs. s²) with s*Rg < 1.3 (Putnam et al., 2007). Calculation of the theoretical scattering curve from the IBARa-N crystal structure was done using CRYSOL (Svergun et al., 1995) (ZITAT). Sets of 16 independent *ab initio* models were each calculated using GASBOR (Svergun and Petoukhov, 2001) and DAMMIF (Franke and Svergun, 2009), filtered and averaged using DAMAVER (Volkov
and Svergun, 2003). Figures were prepared using SITUS (Wriggers and Chacón, 2001) and UCSF Chimera (Pettersen et al., 2004).

Acknowledgments
We thank the staff of X06SA at the Swiss Light Source (Villigen, Switzerland), ESRF ID14-3 (Grenoble, France) and DESY/EMBL X33/P12 (Hamburg, Germany) for help and support during data collection, M. Winterhoff and J. Greipel for stimulating discussions and help during image processing. We further thank A. Müller-Taubenberger for plasmid pDGFP-Dajumin, M. Titus for pTX-GFP-Ddmyosin VII plasmid and R. Gräf for plasmid pB15GFPXSac1A. We also thank the Dicty stock centre (http://dictybase.org/StockCenter/StockCenter.html) for csA-Rho50 expression construct pFL674 and its depositor F. Letourneur. We are grateful to G. Gerisch for providing anti-csA antibody. This work was supported by grants to J.F. (330/5-1) and G.W. (GRK1721, 3717/2-1) from the Deutsche Forschungsgemeinschaft, and to P.L. and H.Z by the Academy of Finland.

Author Contribution
JL, GW, HX, BJ PW and JF performed experiments. JL, GW, PL and JF designed experiments and wrote the manuscript.

References


Figure legends:

Figure 1: Structure of IBARa.
(A) Overall structure of the IBARa-N dimer shown as ribbon representation in two orientations. The helices $\alpha_1$-$\alpha_4$ are labeled in the respective colors. (B) The solvent accessible electrostatic surface (red -5 KT/e – blue +5kT/e) calculated with APBS (Baker et al., 2001) shows a positive charged surface on the convex “backbone” of the dimer and a negatively charged patch on the concave side. (C) Dimerization of IBARa-N. The figure shows the contact surface area of one monomer (hydrophobic residues colored in dark grey, charged residues in orange) and the second monomer shown as cartoon model (red). The dimerization is mainly driven by hydrophobic interactions between the two monomers. In comparison, the outside of the dimer shows a more balanced distribution of hydrophobic and charged residues. (D) Superposition of one chain of IBARa-N and one chain of IRSp53 (pdb code 2YKT) shows a high structural similarity between the two proteins (RMSD 1.67 Å). (E) The final volume of IBARa-N in solution obtained by ab initio modeling from SAXS data with docked crystallographic IBARa-N dimer show a good fit. Please note that the curvature of the proteins seems to be slightly more pronounced in solution. (F) The theoretical scattering curve of IBARa-N calculated with CRYSOL and the measured data support that the shape of IBARa-N is highly similar in crystal and solution.

Figure 2: The Dictyostelium I-BAR domain binds and tubulates phosphoinositide-containing vesicles. (A) Proteins cosedimented with vesicles were present in the pellet fraction (P), and unbound free proteins were detected in the supernatant (S) by SDS-PAGE and Coomassie Blue staining. (B) Quantification of membrane bound IBARa-N in the presence of vesicles with different lipid compositions. The final concentrations of the protein and lipids were 3 $\mu$M and 1 mM, respectively. Lipid compositions were POPC:POPE=8:2, POPC:POPE:POPS=6:2:2, and POPC:POPE:POPS:PIP$_x$=5:2:2:1. (C) Quenching of bodipy-FL-PI(4,5)P$_2$ demonstrated that IBARa-N sequesters PI(4,5)P$_2$ at the membrane. (D) The Dictyostelium I-BAR domain does not affect steady state DPH anisotropy. This suggests that the lipid-binding of this protein does not influence membrane fluidity. (E) The Dictyostelium I-BAR domain causes tubulation of PIP$_2$.
containing membranes (right). Transmission electron microscopy analysis of large unilamellar vesicles containing 10% PI(4,5)P2 incubated with IBARa-N. Control vesicles in the absence of protein (left). The membrane morphology of vesicles was visualized by negative staining. The lipid composition was POPC:POPE:POPS:PIP2=50:20:20:10 and the final concentrations of protein and lipids were 5 µM and 200 µM, respectively. Bar, 5 µm. (F) Control giant unilamellar vesicle in the absence of protein (left). 1 µM of I- BARa-N induced membrane invaginations of giant unilamellar vesicles (right). The lipid composition was POPC:POPE:POPS:PIP2:TopFluor-PIP2 (50:20:20:9:1).

**Figure 3: Endogenous IBARa localizes to contractile vacuoles and the tips of filopodia** (A) Confocal images of cells stained for IBARa and F-actin with fluorescent phalloidin (top panel), IBARa and endosomes (middle panel), or IBARa and the CV marker csA-Rho50 (lower panel). Bars represent 10 µm. (B) Endogenous IBARa also localizes to the distal tips of filopodia (white arrow heads). Magnified inset in the upper left corner shows IBARa (green) at the tips of filopodial F-actin bundles (red), and the inset in the right upper corner shows IBARa at filopodial tips without the phalloidin staining to better illustrate accumulation of the protein in the tips. Bar, 10 µm (C) Generation of IBARa-null mutant by gene disruption. Genetic knock-out (KO) of the ibrA-gene was confirmed by two PCRs to screen for disruption (left panel) or the presence of the WT allele (right panel) using specific primer pairs as indicated by arrows in D. (D) Absence of IBARa in the mutant was confirmed by Western-blotting using anti-IBARa antibodies. (E) The number of filopodia per cell is not altered in IBARa-null mutant. The average number of filopodia for WT and IBARa-null cells was determined from phase contrast time-lapse movies, which allowed discrimination between retraction fibres and filopodia. There was no statistically significant difference (p = 0.218). The box boundaries represent the upper and lower quartile, the whiskers show the upper and lower percentile, the median is shown as line. (F) WT and IBARa-null cells were transfected with GFP-myosin VII as a marker for canonical filopodia and subjected to time-lapse imaging using confocal microscopy to score protruding and retracting filopodia. Tip localization of GFP-myosin VII in a representative IBARa-null cell is shown left (white
arrows). Bars represent 10 μm in the left panel and 1 μm in the gallery of the time-lapse series. Time is shown in seconds.

**Figure 4: Impaired CV function in IBARa-null mutants.** (A) Morphology of CVs in WT and IBARa-null cells visualized by the lipophilic dye FM2-10. (B) The null-mutant showed a significantly increased number of vacuoles. The box boundaries represent the upper and lower quartile, the whiskers show the upper and lower percentile, the median is shown as dashed line. The difference is statistically significant (p < 0.0001). (C) The CVs of IBARa-null mutant cells were still able to expel their content. Loaded CVs in WT and IBARa-null cells attached to the membrane prior to their discharge (white asterisk), and subsequently an entry of TAMRA into the CV was observed (white arrow heads). Confocal sections of the vacuolar network visualized by GFP-Dajumin are shown. Time is shown in seconds. (D) GFP-Dajumin labels the CV (white arrow heads) and other membranous compartments such as the ER (orange arrow heads). GFP-Dajumin expressing cells were fixed and labelled with anti-IBARa polyclonal antibodies (red), GFP-nanobodies (green), and TO-PRO-3 to visualize the nucleus (blue). (E) IBARa-null mutants are more sensitive to hypotonic stress. Considerably less mutant cells survived incubation in water after 5 or 24h. The relative mean values of four independent experiments for each cell line are shown. Error bars indicate the s.e.m. (F) Phase-contrast imaging of IBARa-null cells in low osmolarity PB buffer overlaid with a thin sheet of agar (top panel). White arrow heads point toward a cell lacking a CV, which lysed under these conditions. (lower panel) Autophagy of IBARa-null cells devoid of CVs by CV-containing cells upon establishing contact. Time is shown in min and sec. (G) (left) CV discharge rate of WT and IBARa-null cells in PB buffer. (right) Maximal volume distribution of tracked vacuoles prior to discharge in picoliters by box plot representation. Bars in A, C, D, and F represent 10 μm.

**Figure 5: IBARa-null cells show defects in growth, adhesion and phagocytosis.** (A, B) Reduced growth rates of IBARa-null cells. (A) IBARa-null cells formed considerably smaller colonies in bacterial lawns when compared to WT cells, indicating a substantial growth defect. Mean values of four independent experiments for each cell line are shown.
Error bars indicate the s.e.m. (B) Reduced growth was also observed in shaken suspension culture. (C) Pinocytosis was not affected in the absence of IBARa. Fluid phase uptake was measured by the addition of fluorescent dextran (10µg/ml) to nutrient medium. (D) Phagocytosis was instead strongly impaired in IBARa-null mutants. Uptake of FLY particles by WT (closed symbols) and mutant (open symbols) cells was assayed in shaken suspension in growth medium. The uptake of either untreated (circles) or nocodazole treated dumbbell shaped yeast cells (NTY, triangles) was strongly reduced in the null mutant, when compared to control. (E) IBARa was found at the rim of constricting phagocytic cups. 3D reconstructions from confocal sections are shown. Bar, 10 µm. (E, F) Reduced dynamics of cell spreading and contraction in IBARa-null mutants. (E) WT and mutant cells display a similar median adhesion area. The box plot representation of time resolved measured values showed a broadly spread distribution of the contact areas for the WT, whereas values for mutant cells accumulated in a narrow range. The size of the contact area for each cell was determined every 8 seconds for an 8 minute period. The box boundaries represents the 25th and 75th percentile, the whiskers the 10th and 90th percentile, and dots represent max and min values. (F) WT cells behaved highly dynamic by continuously contracting and spreading their cell bodies on a glass surface as compared to IBARa-null cells which were considerably less dynamic. Time-lapse recording of the contact areas were obtained by reflection interference contrast microscopy (RICM). Note substantial changes in the adhesion area of WT cells, while IBARa-null mutants appear more static. Time is shown in seconds. Bar, 10 µm.

Figure 6: IBARa-null cells display a marked defect in cytokinesis. (A) Contrary to WT cells, which regularly divide into two daughter cells (upper gallery), multinucleated mutant cells frequently broke up into multiple fragments, some of which reunited again into a single cell (middle and lower gallery). Time series using phase-contrast microscopy of mitotic cells beginning at the anaphase are shown. Time is given in seconds. Bar, 10 µm. (B) In WT and mutant cells cultivated with nutrient medium either on petri dishes or in shaken suspension, the nuclei were stained with DAPI to illustrate the relation of mono- to multinucleate cells. Bar, 10 µm. (C) Histogram showing the distribution of the number of nuclei in WT cells and IBARa-null cells. Over 700 nuclei
were counted for each strain. (D) IBARa localization in diving myosin II-null cells. (top panel) During early telophase IBARa-containing vesicles (green) accumulated occasionally in the cleavage furrow region of dividing myosin II-null cells (white arrows). The cleavage furrow marker protein cortexillin (red) was employed to score mitotic cells (Weber et al., 1999), and the nuclei were stained with TO-PRO-3. (middle panel) In the final stages of cytokinesis, IBARa became transiently enriched in small areas connecting the separating daughter cells. (lower panel) In about 50% of the cells, IBARa was seen only in one of the daughter cells. Bar, 10 µm.

**Figure 7: Hypothetical model of IBARa function in CV discharge.** After filling, the CV approaches the plasma membrane (PM) for discharge of the content through a porous channel. Provided that IBARs and MEGAPs (and possibly other F-BAR proteins) act both on the cytosolic side of the CV membrane, these proteins drive the formation of different curvatures (highlighted in the insets), and could cooperate in CV discharge by the coordinated removal of CV membrane during its release. The size of the CV is scaled up and its tubular network has been omitted for reasons of clarity.
Figure 2
Figure 3
Figure 4
Figure 7
Table 1: Data collection and refinement statistics

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| **Refinement**             |                   |
| **Resolution**             | 48.4-2.21         |
| **No. of reflections**     | 99167             |
| **Rwork/Rfree**            | 19.4/23.0         |
| **No. of atoms**           | 7559              |
| Protein                    | 7254              |
| Ligands                    | 29                |
| Water                      | 276               |
| **B-factors**              |                   |
| Protein                    | 53.8              |
| Ligands                    | 68.2              |
| Water                      | 49.0              |
| **R.m.s. deviations**      |                   |
| Bond lengths (Å)           | 0.004             |
| Bond Angles (deg)          | 0.766             |
| **Ramachandran (%)**       |                   |
| favourite                  | 97.9              |
| allowed                    | 2.1               |
| outliers                   | 0                 |
| **PDB Accession Code**     | 4NQI              |

*Values in parentheses are for highest resolution shell*