BANFF: bending of bilayer membranes by amphiphilic α-helices is necessary for form and function of organelles

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<th>Journal:</th>
<th>Biochemistry and Cell Biology</th>
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<td>Manuscript ID:</td>
<td>bcb-2018-0150.R2</td>
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<tr>
<td>Manuscript Type:</td>
<td>Mini Review</td>
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<tr>
<td>Date Submitted by the Author:</td>
<td>05-Sep-2018</td>
</tr>
<tr>
<td>Complete List of Authors:</td>
<td>Könnel, Anne; Universitat des Saarlandes, Molecular Plant Biology Bugaeva, Wassilina; Universitat des Saarlandes, Molecular Plant Biology Gügel, Irene Luise; Ludwig-Maximilians-Universitat Munchen Fakultat fur Biologie, Department Biology I; Stiftung caesar, Computational Neuroethology Phillippar, Katrin; Universitat des Saarlandes, Molecular Plant Biology</td>
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<tr>
<td>Keyword:</td>
<td>membrane curvature, mitochondrion, chloroplast, fatty acid/lipid transport, amphiphilic α-helix</td>
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<td>Is the invited manuscript for consideration in a Special Issue?:</td>
<td>CSMB Special Issue</td>
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**BANFF:** bending of bilayer membranes by amphiphilic α-helices is necessary for form and function of organelles

Anne Könnel, Wassilina Bugaeva, Irene L. Gügel, and Katrin Philippar

**Abbreviations:**

ALPS, ArfGAP1 lipid packing sensor; CURT, curvature in thylakoids; DGDG, digalactosyldiacylglycerol; ENTH, epsin N-terminal homology domain; ER, endoplasmatic reticulum; IE, inner envelope membrane chloroplast; FA, fatty acid; FAX, fatty acid export protein; IM, inner membrane mitochondrium; LBS, lipid binding site; MICOS, mitochondrial contact site and cristae organizing system; MTL, mitochondrial transmembrane lipoprotein complex; N-BAR, N-terminal Bin/Amphiphysin/Rvs domain of endophilin; OM, outer membrane mitochondrium; OE, outer envelope membrane chloroplast; PE, phosphatidyl-ethanolamine; TAG, triacylglycerol; TEM, transmission electron microscopy

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Abstract:

By binding to and inserting into the lipid bilayer, amphiphilic α-helices of proteins are involved in curvature of biological membranes in all organisms. In particular, they are involved in establishing the complex membrane architecture of intracellular organelles like endoplasmatic reticulum, Golgi apparatus, mitochondria and chloroplasts. Thus, amphiphilic α-helices are essential for maintenance of cellular metabolism and fitness of organisms. Here we focus on the structure and function of membrane-intrinsic proteins, which are involved in membrane curvature by amphiphilic α-helices, in mitochondria and chloroplasts of the eukaryotic model organisms yeast and Arabidopsis thaliana. Further, we propose a model for transport of fatty acids and lipid compounds across the envelope of chloroplasts, in which amphiphilic α-helices might play a role.

Keywords: Amphiphilic α-helix, membrane curvature, mitochondrion, chloroplast, fatty acid/lipid transport
Résumé:

En se liant et s’insérant dans la bicouche lipidique, les hélices α-amphiphiles des protéines sont impliquées dans la liaison et la courbures des membranes biologiques de tous les organismes. Elles sont en particulier impliquées dans la mise en place de l’architecture complexe des membranes des organelles intracellulaires comme le réticulum endoplasmique, l’appareil de Golgi, les mitochondries ou les chloroplastes. De ce fait, les hélices α sont essentielles au maintien du métabolisme cellulaire et au bon fonctionnement des organismes. Ici nous nous concentrerons sur la structure et la fonction des protéines membranaires, qui via des hélices α sont impliquées dans la courbure de la membrane des mitochondries et des chloroplastes, et ce chez deux organismes modèles eucaryotes, la levure et Arabidopsis thaliana. Dans un second temps nous présenterons un modèle pour le transport des acides gras et des composants lipidiques à travers l’enveloppe des chloroplastes qui pourrait être dépendant des hélices α-amphiphiles.

Mots-clés: hélice α-amphiphile, courbure de la membrane, mitochondrie, chloroplaste, transport de acides gras/lipides
Compartmentalization of the cell as well as subdivision inside organelles are vital for the various cellular functions and fitness of all eukaryotic organisms. This partitioning of cellular space is achieved by complex intracellular membrane architectures such as tubules of the endoplasmatic reticulum (ER), cisternae and stacks of the Golgi network, as well as inner membrane invaginations in mitochondria and thylakoid membrane systems in chloroplasts, the latter two perfectly adapted for energy production. Biological lipid bilayer membranes, which delimit cells and organelles, however, are not only envelopes or boundaries but also provide the environment for membrane-intrinsic proteins. These proteins mediate transport and exchange of polypeptides, metabolites and ions and accomplish signaling, contact, and communication within the cell. To fulfill these tasks, lipid bilayer membranes show specific composition and complex shapes, thereby reflecting the complexity of intracellular processes (Bigay and Antonny 2012; Block et al. 1983; Kozlov et al. 2014; Marechal et al. 1997; van Meer et al. 2008). Further, membrane dynamics in a cell like membrane budding, tubulation, fission and fusion are necessary and associated with changes in membrane curvature (Boucrot et al. 2012). Thus, localization, activity and signaling properties of membrane-intrinsic and -associated proteins are regulated directly through shape and composition of membranes. *Vice versa*, proteins, which are able to sense and create membrane curvature, may change the properties of membranes. Here three different ways are described how proteins can accomplish bending of membranes (Kozlov et al. 2014): (i) by molecular motors and cytoskeletal filaments, e.g. for the strong membrane curvature of tubular components of Golgi- and ER networks; (ii) by hydrophilic so-called scaffold protein domains, which adhere to the surface of one leaflet of the bilayer and thereby create asymmetry and thus membrane curvature; (iii) by embedding hydrophobic and/or amphiphilic protein domains into the membrane matrix that again asymmetrically perturbs membrane bilayer structure and thereby induces membrane bending. In particular, the insertion of amphiphilic (or amphipathic) α-helices
into the hydrophobic phase of one leaflet of a lipid bilayer is proposed to be a general biophysical mechanism for curvature generation (Peter et al. 2004).

**Membrane curvature by amphiphilic α-helices**

Amphiphilic membrane insertion motifs in general contain a hydrophilic part that opposes a hydrophobic part and thereby insert asymmetrically into one leaflet of the bilayer membrane, which in turn causes membrane bending and curvature (Madsen and Herlo 2017). In protein polypeptide chains these amphiphilic motifs are represented by α-helices, which distribute their different amino acid residues in a way that centers polar residues on one side and hydrophobic side chains on the opposite side. Being part of many proteins and peptides, the amphiphilic α-helix is known for its membrane-binding capacity. It provides the association of peripheral proteins to membranes, serves as a membrane-destabilizing agent or as a promoter of membrane curvature (Drin et al. 2007). The two opposite faces created by the arrangement of hydrophobic and polar residues along the axis of an amphiphilic α-helix are similar to the chemistry and organization of the lipid bilayer membrane, i.e. the presence of a hydrophobic core of acyl chains and exterior polar head groups. Hence, the orientation of a "classical" amphiphilic α-helix (type II, Fig. 1) will be parallel to the lipid bilayer membrane. Its central axis positions at the level of the phospholipid glycerol group, because hydrophobic residues are embedded within fatty acyl chains and polar residues meet lipid polar heads groups. Thus, the adsorption to the membrane is driven by the hydrophobic effect and electrostatic interactions of negatively charged lipid head groups and positively charged amino acids in the amphiphilic α-helix (Drin et al. 2007; Madsen and Herlo 2017). The strength of interaction also determines if α-helices can induce membrane curvature or just sense already curved membranes. Amphiphilic helices that do not fit the perfect curvature-inducing design and for example show less positively charged residues are thus rather
sensing membrane curvature than bending the bilayer. Depending on the grade and distribution of hydrophobic residues along the axis four different types of α-helices in membranes are distinguished (Gkeka and Sarkisov 2010; Fig. 1): Type I helices have two parallel strands of polar and hydrophobic residues along the helical axis with the hydrophobic face being dominant (Fig. 1A). These amphiphilic α-helices are known to insert vertically into the lipid bilayer and in oligomeric arrangements their polar faces can form a water-filled channel pore (Fig. 1B). For example, such a structure is predicted for the protein translocon Tic110 in the inner envelope membrane of chloroplasts (Balsera et al. 2009; Kovacs-Bogdan et al. 2010). For type II helices, the hydrophobic face is either of the same size or smaller than the hydrophilic face (Fig. 1A). These helices insert parallel to the membrane surface (Fig. 1B) and align with the hydrophobic hydrocarbon tails and polar lipid head groups of membrane lipids from one leaflet (Madsen and Herlo 2017). This embedding, also called "shallow insertion", causes a strong local asymmetry and in consequence a powerful membrane curvature. Further, the bending is dependent on the concentration of helices inserted: the more shallow insertions are embedded per unit area of membrane surface, the stronger is the overall membrane curvature (Kozlov et al. 2014). In intracellular organelles, however, very often strong membrane curvature by shallow insertions of type II amphiphilic α-helices has to be stabilized by strongly curved protein scaffolds (Kozlov et al. 2014). Examples for these type II amphiphilic α-helices are the N-terminal helices of N-BAR endophilins and ENTH-epsin proteins as well as Mic60 from mitochondria inner membranes (IM) and CURT from thylakoid membrane systems in chloroplasts (see below). For type III helices the distribution of hydrophobic side chains along the axis is not uniform and cylindrical from top to the bottom but conical, i.e. pronounced at one helix end (Fig. 1A). These helices are transmembrane, however, they span the bilayer not vertically but in a tilted fashion (Fig. 1B). One example for a tilted helix in the membrane would be Mic10 in the IM of mitochondria (see
Type IV α-helices are hydrophobic throughout but might have polar residues at the top and bottom (Fig. 1A). These helices insert vertically into the lipid bilayer and represent true transmembrane helices (Fig. 1B), which strongly anchor proteins inside the membrane.

**The role of amphiphilic α-helices in vesicle formation**

Amphiphilic α-helices that sense membrane curvature can be found in small GTPase-activating proteins (GAP). For instance the Golgi-associated protein ArfGAP1 (shows a membrane-adsorbing amphiphilic α-helix with a weakly charged polar face containing mainly serine and threonine residues – the so-called ArfGAP1 lipid packing sensor (ALPS). This architecture gives the specificity of ArfGAP1 for curved versus flat lipid membranes (Drin et al. 2007). ArfGAP1 is a GTPase-activating protein for Arf1, a small G protein, which in its GTP-bound conformation binds strongly to lipid membranes and promotes the assembly of the COPI coat. COPI coats are shell-like protein scaffold structures which in turn deform membranes into transport vesicles (McMahon and Mills 2004). By catalyzing GFP hydrolysis on Arf1, ArfGAP1 can promote disassembly of the COPI coat. Although they show a low conservation level in sequence, the weak amphiphilic character and the accumulation of Ser/Thr residues of the ArfGAP1 α-helix is evolutionary conserved. Further, this motif can be found in several other proteins with various functions at Golgi membranes, in sterol binding or in nucleoporins (Drin et al. 2007). In general, ALPS motifs form a family of membrane-associated amphiphilic helices that are defined by the abundance of serine, glycine, and threonine residues in their polar face. Binding of ALPS motifs to lipid membranes is directed by characteristic membrane features like the degree of curvature or lipid composition (Bigay and Antonny 2012; Bigay et al. 2005; Drin et al. 2007). A recent analysis on evolution of eukaryotic endomembrane trafficking shows that ArfGAP proteins are conserved in the plant lineage as well (Barlow and Dacks 2018).
In contrast to the curvature-sensing of Arf1GAP, N-BAR-domain (N-Bin/Amphiphysin/Rvs) proteins like endophilin induce membrane bending, which is supported by fixed membrane scaffolds that impose their bent shape on the membrane. In general, endophilins have a function in forming transport vesicles in different trafficking pathways (Kjaerulff et al. 2011). The cytoplasmic, human N-BAR-domain containing protein endophilin A1 is enriched at synapses and is implicated in membrane curvature during synaptic vesicle endocytosis. Human endophilin A1 possesses an N-terminal amphiphilic α-helix directly followed by the BAR domain, which is coupled by a variable linker to the C-terminal SH3 domain (Gallop et al. 2006; Kjaerulff et al. 2011; Masuda et al. 2006). *In vitro*, the N-BAR domain of endophilin can tubulate liposomes. Biophysical as well as crystallographic analysis showed that the N-terminal amphiphilic α-helix inserts into the membrane bilayer and can generate curvature together with the BAR domain, which acts as scaffold in stabilizing the curvature (Gallop et al. 2006; Masuda et al. 2006). In general, a dimeric or oligomeric curved N-BAR-domain framework and a C-terminal SH3 domain, which in addition binds to the GTPase dynamin and to the phosphatase synaptojanin, are necessary to curve membranes. Stabilization of the dimer and fixation of the curvature is accomplished by an additional BAR-domain-internal amphiphilic helix. Thus, there are two synergistic ways endophilin induces membrane curvature: asymmetry between inner and outer leaflets of the bilayer, which causes a positive curvature, is created by shallow insertion of the type II, N-terminal amphiphilic α-helix, whereas the concave face of the scaffold BAR-domain fixes membrane curvature (Gallop et al. 2006; Kjaerulff et al. 2011; Masuda et al. 2006).

Members of the epsin family, which play an important role as accessory proteins in clathrin-mediated endocytosis in mammalian cells, are also able to induce membrane curvature via a type II amphiphilic α-helix (Horvath et al. 2007). Evolutionary well conserved epsin orthologs are present in all eukaryotes including the plant lineage. In general, epsins are distributed in the
cytosol, but show a punctuated accumulation at the plasma-membrane, which might represent endocytically incompetent coated pits. Epsin proteins fulfill several functions like curving membranes, acting as adaptor proteins, coupling of various components of the clathrin-assisted uptake, selecting and recognizing cargo and blocking vesicle formation during mitosis. In plant cells epsin-like proteins that localize to growing cell plates during cytokinesis as well as to the plasma membrane and endosomes in non-dividing cells bind to the membrane lipid phosphatidic acid in dependence on the membrane curvature (Putta et al. 2016; Song et al. 2012). The conserved ENTH-domain (epsin N-terminal homology) at the N-terminus of epsin proteins shows an all α-helical structure out of seven α-helices. The first step in bending membranes during endocytosis in mammals is binding of the phospholipid phosphatidylinositol-4,5-bisphosphate by the ENTH domain. Subsequently, so far unstructured residues at the N-terminus of epsin form an amphiphilic α-helix (Horvath et al. 2007). This helix inserts in the inner leaflet of the plasma membrane and thereby induces membrane curvature. In contrast, the C-terminal region of epsin is essential for recruiting clathrin coat components, driving the clathrin-assembly and sorting of cargo (Horvath et al. 2007). Thus, epsin not only induces curvature formation through its ENTH domain, but also stimulates clathrin-assembly and may act as initial anchor protein onto which the clathrin cage can be assembled.

Not only for membrane vesicle formation and traffic, but also to maintain functionality of organelles membrane bending and curvature is a prerequisite. In particular, this applies to the complex membrane architecture of mitochondria and chloroplasts, which harbor the electron transport chains for respiration and photosynthesis that are directly coupled to ATP-synthesis. In both organelles in the last years substantial progress has been made in identification of membrane-bending proteins. In the remainder of this review we will thus focus on specific
examples of the membrane-bending capacity of amphiphilic α-helices, which are present in membrane-intrinsic proteins of mitochondria and chloroplasts. For a comprehensive overview we mainly focus on the model organisms *Arabidopsis thaliana* (thale cress plant) and *Saccharomyces cerevisiae* (bakers' yeast). Further, we develop a hypothesis for the transport of fatty acid/lipid compounds via membrane curvature and contact that might be mediated by α-helical membrane-intrinsic proteins in mitochondria and chloroplasts.

**Membrane-bending proteins in mitochondria**

Mitochondria harbor two membranes of different architecture and function. While the relatively smooth outer membrane (OM) surrounds the organelle and is crucial for exchange of molecules and communication with the rest of the cell, the inner membrane (IM) has a largely increased surface and harbours the protein complexes of the respiratory chain and the F$_1$F$_0$-ATP synthase. For import of nuclear-encoded proteins, both OM and IM are equipped with multisubunit protein-translocon complexes (Backes and Herrmann 2017; Murcha et al. 2014; Neupert and Herrmann 2007). Metabolites and ions are transported via channel pores in the OM and several transporters in the IM (Becker and Wagner 2018; Checchetto and Szabo 2018; Noskov et al. 2016; Palmieri and Monne 2016; Palmieri and Pierri 2010). The structural design of the protein-rich IM can be classified by two domains: (i) the inner boundary membrane, directly adjacent to the OM and (ii) the cristae membranes that fold into numerous invaginations of different size and shape (Frey et al. 2002; Schwarzländer and Fuchs 2017; Vogel et al. 2006). Contact, cooperation and dynamic exchange between the OM and IM is essential to maintain functions like import of nuclear-encoded proteins as well as lipid transfer from the endoplasmic reticulum (ER; Lahiri et al. 2015; Michaud et al. 2017; Tatsuta et al. 2014). Subunits of the protein translocon complex are therefore enriched in the OM-adjacent and straight inner boundary membrane (Chacinska et al.
Cristae of the mitochondrial IM in contrast have a complex architecture with tubular invaginations and small openings, virtually forming three-dimensional little membrane bags. Within these "bags", the respiratory chain complexes and the F$_1$F$_0$-ATP synthase are sitting in the IM, thereby operating in a micro-compartment, which is optimized for chemi-osmotic coupling during oxidative phosphorylation (Mannella, 2006; Schwarzländer and Fuchs 2017; Zick et al. 2009). The cristae junctions are narrow, neck-like structures connecting cristae and inner boundary membrane. The protein-built "necklace", which restricts and forms these cristae membrane bags, is called MICOS for mitochondrial contact site and cristae organizing system (Pfanner et al. 2014; Schorr and van der Laan 2018; van der Laan et al. 2016). In the model yeast mitochondria, MICOS is a large multi-subunit protein complex that can be subdivided into two subcomplexes of distinct functions. Together with a peculiar membrane lipid composition, e.g. the mitochondria specific cardiolipin (Rampelt et al. 2018), MICOS is essential for form and function of cristae and thereby mitochondrial performance in cellular metabolism (Nunnari and Suomalainen 2012; Rampelt et al. 2017b; Zick et al. 2009). The two MICOS subcomplexes are named by the core proteins involved in membrane bending and shaping, i.e. Mic10 and Mic60 (Rampelt et al. 2017b).

**Mic10 in yeast mitochondria**

Yeast Mic10 spans the mitochondrial IM by two hydrophobic transmembrane domains connected by a short soluble loop with three positively charged amino acid residues (Barbot et al. 2015; Bohnert et al. 2015). Thereby Mic10 proteins arrange in a hairpin topology within the IM (Fig. 2A) and thus might represent the topology of type III amphiphilic $\alpha$-helices. Further, glycine-rich motifs in both membrane-spanning helices allow Mic10 proteins to form large oligomers. Oligomerization of Mic10 proteins then forms the scaffold of the virtual necklace, thereby stabilizing cristae membrane bags and inducing a strong negative membrane curvature at the
cristae junctions (Fig. 2B). Depletion or mutation of Mic10 causes a massive loss of cristae junctions and abnormal cristae structure. This phenotype could be rescued by expressing wildtype Mic10 in mic10 knockout cells (Barbot et al. 2015). In Mic10 overexpressing yeast strains in contrast, mitochondria showed considerably elongated and deformed cristae (Bohnert et al. 2015). Compared to N-BAR endophilin or ENTH epsin (see above), Mic10 per se does not need a superimposed scaffold to stabilize the curvature. However, Mic10 oligomers are associated with other proteins of the MICOS complex and these are coupled via Mic60 (see below) to the OM as a membrane organizing and stabilizing structure (Barbot and Meinecke 2016; Schorr and van der Laan 2018). Further, Mic10 together with Mic26/27 is described to bind to the F1F0-ATP synthase complex in the cristae membranes and thereby determine cristae architecture (Eydt et al. 2017; Rampelt et al. 2017a; Rampelt and van der Laan 2017; Rampelt et al. 2018).

**The function of Mic60 in yeast and plant mitochondria**

Mic60, which is inserted in the cristae junction membrane by one N-terminal α-helical membrane-spanning domain (Fig. 2C), is able to bind and remodel membranes and thus plays a role in cristae junction formation and IM cristae shaping. Furthermore, Mic60 in yeast mitochondria is able to build contact sites between IM and OM by interaction with the OM protein translocases TOM and SAM (Hessenberger et al. 2017; Rampelt et al. 2017b). Mic60 in fungi acts as dimer and together with its partner Mic19 was shown to regulate membrane remodeling and form cristae junctions (Hessenberger et al. 2017). Crucial for the membrane binding and shaping of Mic60 is a type II amphiphilic α-helix called LBS1 (lipid binding site 1) located between the C-terminal coiled-coil and mitofilin domains (Fig. 2C). The soluble part of Mic60 (coiled-coil, LBS1, LBS2, and mitofilin domains) forms stable dimers and is able to tubulate liposomes in vitro (Hessenberger et al. 2017). Subsequently, Hessenberger and coworkers could show that a conserved motif with a positively charged amino acid (arginine or
lysine) followed by a hydrophobic side chain (phenylalanine or tryptophane; see Fig. 2C) is responsible for the amphiphilic character of the LBS1 helix and for tubulation of liposomes. Via ultrastructure analysis Mic60-mutant yeast strains were examined to test MICOS functionality. Wild-type mitochondria showed clearly defined cristae with extensive contact to the inner boundary membrane via cristae junctions. Deletion of the entire Mic60 gene or removal of only the Mic60 mitofilin-domain led to a grossly aberrant mitochondrial ultrastructure with increased IM surface and detached, lamellar cristae membranes (Hessenberger et al. 2017). A similar phenotype was observed when the LBS1 region in Mic60 was deleted. Here the IM showed an increased surface region and virtually no cristae junctions. Even a mutant with an inactivated "positive-hydrophobic" motif in LBS1 showed the same phenotype (Hessenberger et al. 2017). Mic60 overexpressing cells in contrast displayed a mitochondrial ultrastructure with an increased branching of cristae (Bohnert et al. 2015; Rabl et al. 2009). In conclusion, the following model was developed (Fig. 2B): Compared to the epsin ENTH-domain (see above) the Mic60 coiled-coil domain as intramolecular scaffold helps LBS1 in curving the mitochondrial membrane. The C-terminal mitofilin domain of Mic60 shows a similar function as the SH3-domain of endophilin in controlling membrane tubulation by intramolecular interactions or binding to other MICOS proteins, i.e. the Mic19 CHCH-domain (Hessenberger et al. 2017). Further, the mitofilin domain of yeast Mic60 connects to the OM by interacting with TOM and SAM protein translocases (see Fig. 2B).

Thus, yeast Mic60 is crucial for connecting mitochondrial IM and OM and for formation of cristae junctions, while Mic10 forms the structural basis of cristae junctions. In general, deletion of any MICOS protein subunit except for Mic26 leads to a phenotype in cristae architecture, like detachment of most cristae membranes from the inner boundary membrane and formation of internal membrane stacks (Pfanner et al. 2014; van der Laan et al. 2016). Deletion of the core
subunits Mic10 and Mic60, however, causes the most pronounced phenotypes and no characteristic changes in morphology are obtained in cells where MICOS subunits other than Mic10 and Mic60 are overexpressed (Bohnert et al. 2015).

Interestingly, in plant mitochondria Mic60 was described to be involved in lipid transfer from chloroplasts during phosphate starvation (Michaud et al. 2016). In general, major components of mitochondrial membranes are made essentially of lipids synthesized by other organelles. Thus, to build their membranes, mitochondria have to import most lipids from other compartments. Unlike in yeast, where extensive lipid exchange of mitochondria with the ER and the vacuole has been demonstrated (Kawano et al. 2018; Lahiri et al. 2015; Tatsuta et al. 2014), knowledge about this inter-organellar lipid traffic in plant cells is still scarce (Michaud et al. 2017). Under phosphate limitation, however, it is well known that the plastid-made galactolipid digalactosyl-diacylglycerol (DGDG) compensates for the loss of phospholipids in cellular membranes including mitochondria (Jouhet et al. 2004; Nakamura 2013). Here, the number of plastid-mitochondria contacts increases and phosphatidyl-ethanolamine (PE) is exported from mitochondria for phosphate recycling in the cytosol whereas DGDG from plastids is imported (Michaud et al. 2017). An extensive membrane biochemical approach identified the Arabidopsis Mic60 at the IM of plant mitochondria to play an important role during this lipid traffic (Michaud et al. 2016). In this study, At-Mic60 was identified as central component of a protein complex that retains $^{14}$C-labeled DGDG after in vitro lipid uptake at phosphate starvation. As in yeast, At-Mic60 is an essential part of the MICOS complex and together with the OM β-barrel protein TOM40, which forms the protein import pore, connects the OM and IM in mitochondria. Michaud and co-workers in addition described At-Mic60 to be part of a large lipid-binding complex (mitochondrial transmembrane lipoprotein, MTL) and to mediate DGDG flow from
plastids into mitochondria as well as PE export during phosphate starvation (Michaud et al. 2016; Michaud et al. 2017). In MTL complexes from mitochondria lacking At-Mic60, the content of DGDG and PE was significantly reduced. Further, *in vitro* lipid-binding as well as liposome extraction and leakage assays demonstrated that the soluble part of At-Mic60 (i.e. the mature protein, lacking the transmembrane domain) is able to bind to cardiolipin lipids and to destabilize lipid bilayer membranes. The respective soluble part of Mic60 from the thermophilic fungus *Chaetomium thermophilum* was used by Hessenberger and co-workers to demonstrate membrane tubulation *in vitro* (see above and Hessenberger et al. 2017; Michaud et al. 2016).

In summary, the membrane binding activity and the ability to connect to the OM allows Mic60 proteins to regulate shaping of cristae junctions as well as lipid traffic across plant mitochondrial membranes. Whether yeast Mic60 is involved in lipid transfer as well still remains an open question.

**Organization of membranes and bending of thylakoids in chloroplasts**

The plant cell is highly compartmentalized, containing at least seven different types of organelles, which are involved in metabolism and cellular maintenance. In many cases metabolic pathways and other cellular processes are not confined to one organelle but require the cooperation of several of them, e.g. for mitochondria, chloroplasts and peroxisomes during photorespiration or the delivery of plastid galactolipids to other organelle membranes during phosphate limitation. Metabolic networking between compartments thus requires directed and coordinated exchange of biochemical pathway intermediates across organelle membranes (Philippar and Soll 2007). Due to their endosymbiotic origin chloroplasts and mitochondria are unique since like their prokaryotic ancestors they are surrounded by two membranes. The inner membrane of both organelles is derived from the bacterial plasma membrane. Surprisingly the outer membrane
largely originated from and still resembles the outer membrane of the Gram-negative like bacterial endosymbiont (Block et al. 2007). In the inner chloroplast envelope membrane, numerous metabolite transport proteins were identified and characterized to large detail with respect to their physiological impact and molecular mechanisms (Marchand et al. 2018; Weber and Linka 2011). These transporters are hydrophobic, mainly α-helical membrane proteins facilitating the exchange of metabolic precursors, intermediates, and final products between plastids and the cytoplasm. In contrast, the characteristic channels of the outer membranes in bacteria, chloroplasts as well as in mitochondria, span the membrane in the form of β-strands that are organized to form a barrel-like pore structure (Zeth and Thein 2010). Proteins sharing this 3-D arrangement were implied in a variety of functions, including protein import (Bredemeier et al. 2007). In addition, solute pores like the voltage dependent anion channel (VDAC) in mitochondria (Checchetto and Szabo 2018; Colombini 2012) or the outer envelope proteins OEP21, OEP24, OEP37 and OEP40 ( Bölter et al. 1999; Goetze et al. 2006; Harsman et al. 2016; Pohlmeyer et al. 1998) in chloroplasts are essential parts of the outer membrane permeom of these organelles (Breuers et al. 2011; Duy et al. 2007).

Outer (OE) and inner envelope (IE) membranes in chloroplasts are close to each other (about 10-20nm) and in contrast to mitochondria both membranes have a smooth and flat appearance that delimits the organelle. As for mitochondria, during evolution, the chloroplast has lost most of its genetic information to the nucleus. In consequence more than 90% of the chloroplast intrinsic proteins have to be imported from the cytosol. Here OE and IE directly cooperate and harbor the protein translocon machineries TOC and TIC ( Bölter and Soll 2016; Nakai 2015; Schwenkert et al. 2018). The protein complexes for light harvesting, the electron transport chain of photosynthesis as well as the F_{1}F_{0}-ATP synthase are placed in the third chloroplast membrane system, the thylakoids. Similar to the IM of mitochondria, thylakoids show a complex membrane
architecture, consisting of a continuous membrane network that encloses a single luminal space (Shimoni et al. 2005). Thylakoids are divided in grana, cylindrical stacks of thylakoid membrane discs, and stroma lamellae, which connect the grana stacks (Mustardy et al. 2008; Pribil et al. 2014). To capture this architecture, thylakoid membranes are heavily bent at the margins of each membrane in the grana stacks. Thereby, a tremendously increased membrane surface as well as a microenvironment in the thylakoid lumen, best suited for the light reactions of photosynthesis and ATP production, is created. Membrane curvature of thylakoids in chloroplasts as well as the level of grana stacking is dependent on the protein CURT1 (curvature in thylakoids; Pribil et al. 2014).

In *Arabidopsis* four CURT1 (CURT1 A, B, C and D) proteins are known, which homo-oligomerize and highly enrich at grana margins (Fig. 2D, E; Armbruster et al. 2013). Grana architecture correlates with the protein level of CURT1 and plants without CURT1 proteins show flat, lobe-like thylakoids with fewer grana, while CURT1 overexpressors show an increased number of membrane stacks. Thus, at the grana margins CURT1 is necessary for membrane bending and thereby determines the architecture of the thylakoid network (Pribil et al. 2014). In addition, for CURT1A and B interaction with other thylakoid proteins could be shown (Armbruster et al. 2013). CURT1 is evolutionary conserved and CURT1 from *Arabidopsis* can partially replace an ortholog in the model cyanobacterium *Synechocystis* sp PCC 6803 (Armbruster et al. 2013). Interestingly, cyanobacteria that today represent the evolutionary precursors of chloroplasts and whose thylakoids do not differentiate into grana regions also contain a single CURT1 protein named CurT (Heinz et al. 2016). The cyanobacterial CurT protein is essential for shaping thylakoid membranes and promotes efficient assembly of photosystem II at the cell periphery. Furthermore, Heinz and co-workers show that CurT has a critical role in response to osmotic stress. CURT proteins in *Arabidopsis* and *Synechocystis* both
have a conserved N-terminal amphiphilic α-helix and two transmembrane domains (Fig. 2D). While the membrane-spanning domains anchor the protein in the thylakoid membrane, the amphiphilic α-helix is supposed to be involved in membrane-bending and induction of membrane curvature (Fig. 2E). The type II amphiphilic character (compare Fig. 1) of this helix is clearly shown by spatial separation of polar and charged from hydrophobic amino acid residues in helical wheel analysis (Armbruster et al. 2013; Heinz et al. 2016). Investigation of the effect of curt1 mutants on architecture of the thylakoid system was done by transmission electron microscopy (TEM) in Arabidopsis. Grana stacks in wild-type chloroplasts show a smaller diameter, but are higher than in curt1abcd mutants. In the latter thylakoids were disorganized with extended stretches of unstacked membranes and broader stacks built by fewer layers than in wild type. Combinations in knockouts of two-three CURT1 isoforms show occasionally curved thylakoids instead of forming horizontal layers and also contain vesicular structures. CURT1A overexpressing plants show slimmer, but higher grana stacks than wild type (Armbruster et al. 2013). In Synechocystis the CurT-mutants exhibit a massive disorganization of the thylakoid membrane system (Heinz et al. 2016). In all mutant-strains cells, the thylakoid system appeared as disordered sheets that traversed the cytoplasm and completely lacked the typical convergence zones with the corresponding biogenesis centers at the periphery. Thus, CurT is necessary to establish normal thylakoid membrane architechure in Synechocystis. Pribil and co-workers speculate that in addition to CURT oligomers at grana margins, proteins in the granum core as well as still unknown margin-related factors might affect interaction of thylakoid membranes, grana architecture and stacking (Pribil et al. 2014). In comparison to the membrane-bending mechanisms described above these additional proteinaceous factors would represent scaffolds that stabilize stacking and membrane curvature at thylakoid grana margins.
Whereas above we summarized specific examples of proteins involved in membrane bending in mitochondria and chloroplasts, in the following we will develop a hypothesis for the function of amphiphilic $\alpha$-helices in transport of fatty acids across lipid bilayer membranes.

**FAX proteins: fatty acid/lipid transport by membrane-bending?**

In all organisms, fatty acids (FAs) are building blocks for acyl lipids, which in the form of phospho-, galacto- or sphingolipids are assembled into bilayer membranes or as triacylglycerol (TAG) oils represent a major form of carbon storage. In plants, stress conditions like cold temperatures or phosphate starvation require FA/lipid remodeling and turnover to protect against freezing or to replace phospholipids by plastid-derived galactolipids, respectively (Moellering and Benning 2011; Nakamura 2013). Further, FA breakdown by $\beta$-oxidation in plant peroxisomes and yeast mitochondria plays an essential role in energy metabolism. Thus, proper maintenance and regulation of FA/lipid metabolism is not only crucial for membrane function but also essential for development, growth and fitness of organisms. Lipid metabolism therefore has been subjected to intensive studies and was found to involve hundreds of enzymatic reactions, occurring in several subcellular compartments (Li-Beisson et al. 2013; Manan et al. 2017; Tatsuta et al. 2014). In turn, this requires exchange of FAs, lipids and their metabolic intermediates between organelles and membrane systems. Because lipophilic molecules cannot move freely in an aqueous cellular environment and their transport has to be directional, several modes of intracellular lipid traffic exist. These include membrane contact sites, diffusion within the same membrane system, vesicular trafficking and direct transport of molecules (Bates 2016; Block and Jouhet 2015; Du et al. 2016; Hurlock et al. 2014; Salminen et al. 2016). In plants substantial progress has been made in identifying membrane-intrinsic proteins, which either actively transport FA/lipid compounds or mediate their intracellular traffic (Li-Beisson et al. 2017; Li et
al. 2016). However, it is noteworthy, that for lipid traffic in general most likely membrane contacts as well as tight co-operation between transport and biosynthesis are required. Feeding of lipids into metabolism often drives transport, e.g. during plastid FA export where membrane-transporter mediated facilitated diffusion of FAs most likely is activated by the flow of free FAs into acyl-CoA and subsequent lipid metabolism.

**Fatty acid transport from plastids to the ER**

Since in the plant cell all long-chain FAs are made in plastids and assembled into lipids either in plastids or in the ER, and ER-derived lipids are components of plastid intrinsic membranes, extensive FA/lipid transport between these two organelles is required. *De novo* FA synthesis occurs in the plastid stroma where acyl chains grow while attached to the acyl carrier protein ACP (Li-Beisson et al. 2013; Troncoso-Ponce et al. 2015). Part of these FAs is integrated into lipids inside plastids (prokaryotic pathway), leading to galactolipids such as monogalactosyl-diacylglycerol (MGDG), digalactosyl-diacylglycerol (DGDG) and the phospholipid phosphatidyl-glycerol. These lipids mainly constitute the plastid intrinsic thylakoid and IE membrane systems. The majority of plastid-made FAs, however, is exported to the ER for further elongation, acyl editing and lipid assembly via the eukaryotic pathway. Here, the phospholipids phosphatidyl-choline -ethanolamine, and -inositol as well as sphingolipids, TAG oils and precursors for complex extracellular lipophilic compounds such as wax, cutin, suberin and sporopollenin are produced (Li-Beisson et al. 2013). However, a considerable amount of these eukaryotic lipids as well is relocated into plastids for lipid assembly. In consequence, a eukaryotic-type acyl-glycerol backbone has to be imported into plastids (Wang and Benning 2012).

Once synthesized in the plastid stroma acyl residues are either integrated into lipids via plastid-resident acyltransferases or released as free FAs by hydrolysis via acyl-ACP thioesterases at the
stroma-side of the plastid IE (Li-Beisson et al. 2013; Troncoso-Ponce et al. 2015). Free FAs are toxic and therefore a quick flow into metabolism or export from the organelle is required. In general, it is agreed that free FAs are shuttled across plastid envelopes, although it was a matter of debate whether this occurs by simple diffusion or if membrane transport proteins are involved (Koo et al. 2004; Li et al. 2016). Insight into a membrane protein mediated mechanism of plastid FA-export in plants was provided by the identification of Arabidopsis FAX1 (fatty acid export 1), which inserts into the chloroplast IE by α-helical membrane-spanning domains (Li et al. 2015). FAX1 function is crucial for biomass production, male fertility and distribution of FA-derived compounds such as lipids, ketone waxes or pollen cell wall materials. ER-derived lipids decreased when FAX1 was absent, but levels of plastid-produced species increased. FAX1 overexpressing lines showed the opposite phenotypes, including a rise of TAGs in flowers and leaves. In yeast mutants, FAX1 further complements for FA transport. Thus, in IE membranes FAX1 could function in a process called vectorial acylation that is represented by a passive, carrier-like transport mechanism. This FAX1-mediated facilitated diffusion most likely is driven by a strong gradient of free FAs across the envelope membranes. Subsequently, these FAs are fed into lipid metabolism and activated via coupling to coenzyme A (CoA) at the cytosolic face of the plastid envelope (for details of potential transport mechanisms, see Li-Beisson et al. 2017; Li et al. 2016). The long-chain acyl-CoA synthetase LACS9 at the OE is unique in plastids, catalyzes about 90% of the plastid activity for acyl-CoA synthesis, and thus might drive vectorial acylation for plastid FA-export (for discussion see Jessen et al. 2015; Li-Beisson et al. 2017). Finally, ABCA9, a half-size Arabidopsis ABC transporter of subfamily A (for overview see Hwang et al. 2016; Theodoulou and Kerr 2015), was described to be directly involved in FA/acyl-CoA import into the ER (Kim et al. 2013). ABCA9 is localized to ER membranes and is expressed in seeds at storage lipid accumulation stages. Besides the action of membrane-intrinsic proteins such as
FAX1 (IE) and ABCA9 (ER) as well as LACS enzymes at both compartments, it is discussed that plastid to ER transfer of lipid compounds can occur via membrane contact sites. In principle, it is likely that both, membrane contacts and membrane transport are necessary for efficient transfer of lipid compounds (Block and Jouhet 2015).

**FAX proteins: FA transport via amphiphilic α-helices?**

In *Arabidopsis* FAX1 belongs to a family of seven proteins, which are not only predicted to localize to plastids, but also to membranes of the secretory pathway (Li et al. 2015). In general, FAX proteins group into the TMEM14 protein family with four conserved α-helical domains. When comparing the amino acid sequences of these four predicted α-helices, mature At-FAX1 is most related to At-FAX5 and At-FAX6 (see Fig. 3). The proteins At-FAX5 and At-FAX6 are highly similar in sequence (80% identical amino acids) and both are predicted to insert into membranes of the secretory pathway, most likely in the ER (preliminary results by *in vivo* GFP-targeting). Thus, we assume that both proteins are redundant in their potential FA/lipid transport function. The secondary structure predictions of At-FAX1 and At-FAX5/FAX6 nicely fit to the NMR structures of human TMEM14C and 14A proteins, respectively (Li et al. 2015). TMEM14C and TMEM14A also have four α-helices and according to NMR data helix 3 of TMEM14C and helix 1 of TMEM14A are described to be amphiphilic and align parallel to the membrane surface (Klammt et al. 2012). In vertebrates and yeast, FAX relatives of the TMEM14 family are annotated as mitochondrial membrane proteins. In zebrafish human TMEM14C was identified to co-express with the core machinery of heme biosynthesis and its knockdown caused anaemia in embryos (Nilsson et al. 2009). Human TMEM14A instead was described to stabilize the mitochondrial membrane potential and inhibit apoptosis in yeast (Woo et al. 2011). A precise biological function of both proteins, however, is so far unknown.
Current structural modeling of mature At-FAX1 and At-FAX5/FAX6 with a high confidence (99.9%, Phyre\textsuperscript{2} Protein Homology/analogY Recognition Engine V 2.0; Kelley et al. 2015) suggests that all three FAX proteins fit to both NMR structures of TMEM14C and TMEM14A with the same probability. Thus, helix 1 - as predicted for TMEM14A - and helix 3 - as predicted for TMEM14C - of FAX1 and FAX5/6 might both be amphiphilic. The *Arabidopsis* membrane protein database Aramenon (Schwacke et al. 2003), however, annotates helix 3 of At-FAX1 and At-FAX5/6 to have the highest amphiphilic character. Helix 2 and 4 of At-FAX1, At-FAX5/6 in contrast, are mostly hydrophobic and predicted to be vertical transmembrane domains of type IV (compare Fig. 1). To analyze the amphiphilic character of FAX1 and FAX5/6 helices 1 and 3 in more detail, we aligned protein sequences from 10 different plant species, including green microalgae, mosses, monocotyledonous and dicotyledonous plants (Fig. 3). Interestingly, these helices show sequence motifs, which are highly conserved throughout evolution of eukaryotic plant organisms. Strikingly, positively charged amino acid chains (lysine, arginine) are clustered at the rims of the helices. Next to helix 3 of FAX1 and FAX5/6 are highly conserved “hydrophobic-positive” motifs, similar to the one in LBS1 from Mic60 (compare Fig. 3B, D with Fig. 2C). Further, features and conserved motifs of helix 1 from At-FAX1 and At-FAX5/6 are almost identical. Helical wheel projection analysis in Fig. 3 shows that all four helices have an amphiphilic character. However, whereas the charged and polar residues of helix 1 are clustered at the N-terminal end (Fig. 3A, C), the hydrophilic residues of helix 3 are more evenly distributed along the cylinder (Fig. 3B, D). Thus, we suggest that the amphiphilic character of helix 1 of FAX1 and FAX5/6 proteins is of type III and helix 3 represents a "classical" amphiphilic helix of type II (compare Fig. 1 and Fig. 4).

In addition to the four α-helices, FAX1 proteins show an N-terminal 28 amino acid long α-helical domain (helix 0). Helical wheel projection shows that the character of helix 0 is mostly polar,
with only a very limited hydrophobic surface “wedge” (Fig. 4A). Positive and negative charges alternate and cluster to one side of the helix. Furthermore, the sequence at the very C-terminal end of FAX1 is highly conserved with a motif containing three proline residues, followed by three positive lysine residues (“PPPKKK motif”, Fig. 4B). Since proline in polypeptide chains due to its structural restraints as imino acid usually alters secondary structures, these three residues at the FAX1 C-terminus might function in positioning the two positive charges of the lysine residues for interaction with the negatively charged head groups of the IE lipid bilayer and/or unknown protein partners (Fig. 4C). This accumulation of positive charges is reminiscent of the “KRR” motif in the loop between both transmembrane helices of Mic10 (compare Fig. 2A).

In summary, our analysis leads to the following predicted membrane topology of FAX1 and FAX5/6 proteins (Fig. 4C, D). The first helix of all three proteins is most likely a type III amphiphilic helix and thus might span the lipid bilayer membrane in a tilted manner. The third helices are type II amphiphilic α-helices and display a “shallow” insertion into one leaflet of the IE and ER lipid bilayer membranes, respectively. Helix 2 and 4 are hydrophobic (type IV, see Fig. 1), membrane-spanning helices. This predicted topology of FAX1 and FAX5/6 (Fig. 4C, D) positions the positively charged residues as well as the “hydrophobic-positive” motifs at the polar headgroups of the lipid bilayer, similar to the membrane-bending proteins Mic10, Mic60 and CURT1 (compare Fig. 2). We therefore predict that FAX1 and FAX5/6 might be able to induce membrane curvature. Helix 0 and the C-terminal motif “PPPKKK” of FAX1 could assist in membrane bending or curvature stabilization and/or interact with protein partners at the OE/intermembrane space or in the stroma, similar to the mitofilin domain of Mic60 that interacts with OM proteins (compare Fig. 2B and Fig. 4C).
Experimental indications for the membrane-bending capacity of At-FAX1 are visible in transmission electron micrographs (TEM) from plastids of FAX1 over-expressing plants (Fig. 5). When analyzing plastids in stem tissue of the strong FAX1 over-expressor FAX1ox#4 (Li et al. 2015), we were surprised to find the IE membrane to be strongly tubulated and extending far into the stroma until the thylakoid membranes (Fig. 5B, D, E). In contrast, these structures were absent in wild-type plastids (Fig. 5A, C). These tubulations were restricted to the IE membranes, OE membranes showed normal smooth and flat appearance (Fig. 5D, E). Sometimes the unusual IE membrane architecture arranged in tubules (Fig 5D), sometimes in a regular honeycomb like network (Fig. 5E). In the literature, it is described that during overproduction of IE membrane proteins chloroplasts generate more IE membrane layers, however, these additional layers are smooth, flat, and adjacent to the OE and “original” IE (see Tic40 overexpression in Breuers et al. 2011). Thus, it seems likely, that this unusual tubulation of the IE in FAX1ox#4 is due to intrinsic features of the FAX1 protein. Compared to overexpression of the membrane-bending proteins Mic10, Mic60 and CURT1, which show highly tubulated mitochondrial IMs and more grana stacks in thylakoids, respectively (see above), we conclude that At-FAX1 might be implicated in membrane bending as well.

In conclusion, we think that by a predicted membrane binding capacity via their amphiphilic α-helices FAX1 and FAX5/6 proteins might be able to establish membrane contacts and thereby contribute to FA transport across lipid bilayers. The role might be similar to that of Mic60 in lipid transport in plant mitochondria during phosphate starvation. Thus, membrane curvature and contacts with proteins as well as other bilayers seem to contribute to shuttling of lipophilic compounds across organellar membrane systems.

**Outlook**
In summary, membrane intrinsic proteins that contain amphiphilic α-helices in the mitochondrial IM and plastid IE besides shaping the membrane (Mic60) seem to be implicated in fatty acid (FAX1) or lipid transport (Mic60) across the two envelope membranes of both organelles. Direct experimental evidence, however, for the membrane-bending capacity and membrane topology of FAX proteins is still lacking. In addition, also the identification of conserved amino acid residues that play a critical role in FAX function will be a task for the future. Another open question is the role of the TMEM14 FAX-relatives and Mic60 during lipid homeostasis and transport in yeast mitochondria.
Acknowledgements: We thank the BSc student Arthur Naas for collecting plant FAX protein sequences and for French translation of the abstract. A.K., W.B. and the work on FAX proteins are funded by the Deutsche Forschungsgemeinschaft (DFG) grant PH73/7-1, and in the framework of the International Research Training Group 1830 (IRTG1830) to K.P. Transmission electron microscopy by I.L.G. was funded by Munich Center for Integrated Protein Science (CIPSM), LMU Munich, Germany.
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Figure legends

Fig 1. Different types of amphiphilic α-helices.

(A) Distribution of polar (white) and hydrophobic (grey) residues along the vertical helix axes (dotted lines). (B) Insertion of the respective α-helices from (A) into a lipid bilayer membrane. Hydrophobic acyl chains are depicted in dark, polar head groups in light blue, respectively. According to Gkeka and Sarkisov (2010), amphiphilic α-helices can be subdivided into four types, depending on the amount and distribution of hydrophobic amino acid side chains. Type I has equally distributed polar and hydrophobic residues along the vertical helix axis (A, I). However, hydrophobic residues are dominant and thus oligomers of these helices can arrange in the lipid environment in a way that their polar faces form ion-conducting, water-filled pores as depicted in (B). For type II polar and hydrophobic faces are either of similar size or the polar face is larger (A, II). These are the so-called "classical" amphiphilic α-helices, which plunge into the bilayer parallel to the membrane surface (B). In type III the hydrophobic residues are distributed in a conical fashion, i.e. more on one end of the helix (A, III). This generates a membrane-spanning helix that is tilted vertically inside the membrane (B). Type IV α-helices are mostly hydrophobic (A, IV), but can have a polar top and bottom and thus integrate vertically as transmembrane helices in the lipid bilayer (B).
Fig. 2. Membrane topology and amphphilic α-helices of membrane-bending proteins in mitochondria and chloroplasts.

The polypeptide chain of each protein is depicted as grey line, α-helices as cylinders. Hydrophobic cores of bilayer membranes and surface regions of α-helices are grey, negatively charged or polar head groups of membrane lipids are colored in turquoise for mitochondrial IM and green for chloroplast thylakoid membranes, respectively. The two leaflets of the lipid bilayer are indicated by dashed lines. Type II amphphilic α-helices have a hydrophobic surface in grey and a polar surface in white. Conserved amino acid residues, which are essential for membrane binding and curvature are indicated by red squares (positively charged side chain) and yellow circles (hydrophobic residue). Please note that dimensions of domains and helices are not drawn to scale. (A) Membrane topology of yeast Mic10 in the IM of mitochondria (according to Barbot et al. 2015; Bohnert et al. 2015). The two hydrophobic α-helical transmembrane domains of Mic10 insert into the IM in a wedge-like manner, N- and C-terminus point to the cristae junction (cj). This topology is promoted by the three highly conserved positively charged Lys and Arg residues ("KRR"-motif, red squares) in the short loop between both helices at the matrix side of the membrane. Further, Mic10 proteins assemble in a highly oligomeric state, driven by Gly residues in the oligomerisation motif "GxGxGxG" in their two helices. These features enable Mic10 to induce a highly negative membrane curvature and to stabilize cristae junctions in mitochondrial IMs. (B) Bending of the IM at cristae junctions by oligomers of Mic10 and dimers of Mic60 (adapted from Wollweber et al. 2017). Proteins are described in (A) and (C). Mic60 (orange color) is able to interact with TOM and SAM translocons at the OM. CM cristae membrane; IBM: inner boundary membrane. (C) Predicted membrane topology of yeast Mic60 in the IM of mitochondria (according to Hessenberger et al. 2017). Mic60 is anchored in the mitochondrial IM via an N-terminal transmembrane helic (TM), the N-terminus points to the
matrix. The coiled-coil domain (CC) is oriented to the cristae junction (cj), whereas the mitofilin domain (MF) interacts with OM proteins and thereby can bridge the intermembrane space (ims, compare (B) and Schorr and van der Laan 2018). The amphiphilic type II α-helix LBS1 (lipid biding site 1) contains a highly conserved positively charged amino acid residue (red square), followed by a hydrophobic amino acid (yellow circle). This "positive-hydrophobic" motif is proposed to be positioned by the amphiphilic LBS1 helix in the IM membrane in a manner where the hydrophobic face dips into the hydrophobic IM core and the positive residue interacts with the polar lipid head groups. Mutation of both residues of this motif into negatively charged aspartate interferes with the lipid binding and membrane tubulation capacity of Mic60 (Hessenberger et al. 2017). (D) According to predictions by Armbruster et al. (2013) and Heinz et al. (2016), CURT proteins in thylakoid membranes (THY) of Arabidopsis and Synechocystis contain two hydrophobic transmembrane α-helices (TM). Further, an N-terminal type II amphiphilic α-helix (ampH) at the stroma side is proposed to align with the hydrophobic core and the polar head groups of the thylakoid lipid-bilayer and thereby induce membrane curvature around the thylakoid lumen. (E) Oligomers of CURT proteins enrich at grana margins and thus are involved in tylakoid membrane bending and grana stacking (compare Pribil et al. 2014).
**Fig. 3.** Amphiphilic α-helices of plant FAX1 and FAX5/6 proteins.

*(Left)* Alignment of amino acid sequences from helix 1 and helix 3 of FAX1 (A, B) and FAX5/FAX6 (C, D) proteins from land plants and green microalgae. Amino acid residues are numbered according to their position in the preprotein At-FAX1 (At3g57280) and At-FAX5 (At1g50740). Predicted α-helices (each 21 amino acids) are boxed by green lines. Helix predictions are according to the Aramemnon database "TM alpha helix prediction consensus" for At-FAX1 and At-FAX5 (AramTmCon, http://aramemnon.uni-koeln.de; Schwacke et al. 2003). Conserved amino acid residues are shaded in black (100%), dark (50%) and light grey (25%), respectively. Highly conserved motifs with positively charged (red box), negatively charged (blue triangle), polar, uncharged (green diamond), and hydrophobic (yellow circles) amino acid side chains are indicated by symbols. The "positive-hydrophobic" motifs near helix 3 are highlighted by arrow heads. Please note that α-helix 1 of At-FAX1 and At-FAX5 are almost identical in sequence and that polar/charged residues cluster at the N-terminal end. Tyr, Phe are depicted as orange, small Gly residues as pale yellow and Pro as grey cycles, respectively. At: *Arabidopsis thaliana* (thale cress); Br: *Brassica rapa* (bok choy); Gm: *Glycine max* (soybean); Mt: *Medicago truncatula* (barrel clover); Ps: *Pisum sativum* (garden pea); Sl: *Solanum lycopersicon* (tomato); Os: *Oryza sativa* (rice); Zm: *Zea mays* (corn); Pp: *Physcomitrella patens* (spreading earthmoss); Cr: *Chlamydomonas reinhardtii*.

*(Right)* Helical wheel projections of helix 1 and helix 3 of *Arabidopsis* FAX1 (A, B) and FAX5/FAX6 (C, D). Helical wheels were produced by NetWheels software (http://lbqp.unb.br/NetWheels; settings: α-helix with 3.6 amino acid residues per helix turn). Positively charged (red box), negatively charged (blue triangle), polar, uncharged (green diamond), and hydrophobic (yellow circles) amino acid residues are indicated by symbols.
Conserved Gly and Pro residues are depicted as pale yellow as grey cycles, respectively. The blue dashed line in (B, D) indicates the separation of hydrophobic and polar helix surface regions.
**Fig. 4.** Predicted membrane topology and conserved domains in FAX1 and FAX5/6 proteins.

Additional conserved domains of FAX1 (A, B) and the predicted membrane topology of At-FAX1 (C) and At-FAX5/6 (D) are depicted. (A) A 28 amino acid stretch at the N-terminus of the mature FAX1 proteins (amino acids 45-72 of the preprotein At3g57280) is predicted to form an additional α-helix (helix 0) with low hydrophobicity, but high content of charged amino acid residues (Phyre² Protein Homology/analogY Recognition Engine V 2.0; Kelley et al. 2015). Helical wheel projection analysis (same parameters as in Fig. 3) shows a weak amphiphilic character with charged residues clustering on one large face of the helix cylinder and hydrophobic residues forming a small wedge (dashed lines). Positively charged (red box), negatively charged (blue triangle), polar, uncharged (green diamond), and hydrophobic (yellow circles) amino acid side chains are indicated by symbols. (B) The C-terminus of FAX1 relatives in plants just behind helix 4 (amino acids 205-226 of the preprotein At-FAX1) shows a highly conserved motif containing three proline residues followed by three positively charged lysine residues (for plant species see Fig. 3). The highly conserved motif contains two small glycine (yellow circles), followed by a polar, uncharged arginine (green diamond), three proline (grey circles) and the three positively charged lysine residues (red box). (C) Predicted membrane topology and conserved polar motifs of the At-FAX1 protein in the inner envelope membrane of plastids (IE). According to predictions and distribution of amino acid residues (see Fig. 2 and text), helix 1 (H1) of FAX1 most likely represents a type III amphiphilic helix and spans the IE membrane in a tilted manner. Helix 3 (H3) instead shows a "classical", type II amphiphilic character and most likely is embedded perpendicular to the membrane surface with its hydrophobic face inside the hydrophobic core and the polar and charged amino acid residues interacting with the polar head groups of the membrane lipids. Helix 2 and 4 are predicted to be truly hydrophobic transmembrane helices. Interestingly between H2-H3 and inside H3 FAX1
contains two highly conserved "hydrophobic-positive" motifs as described for Mic60 (see Fig. 2C). The additional N-terminal helix of FAX1 (H0) as well as the C-terminal conserved "PPPKKK" motif might interact with still unknown protein partners in envelope membranes and/or the stroma/intermembrane space. Please note that so far the orientation of FAX1 in the membrane is still unknown. Preliminary data on proteolysis of IE vesicles, however, point to the N-terminus in the intermembrane space and the C-terminus in the stroma. (D) FAX5 and FAX6 are predicted to localize to membranes of the secretory pathway (Aramemnon database) and according to preliminary in vivo GFP-targeting, they most likely insert into the ER membrane. For both proteins the N-terminus is predicted in the cytosol (Aramemnon database). Except the additional N-terminal domain, the predicted membrane topology of FAX5 and FAX6 proteins is highly similar to that of FAX1 in (C). The polypeptide chains of FAX1 and FAX5 are depicted as grey lines, α-helices as cylinders. Hydrophobic cores of bilayer membranes and surface regions of α-helices are grey, negatively charged or polar head groups of are colored in light green and blue for IE and ER membrane lipids, respectively. Amphiphilic α-helices have a hydrophobic surface in grey and a polar surface in white. Conserved amino acid residues are indicated by red squares (positively charged side chain) and yellow circles (hydrophobic residue). Please note that dimensions of domains and helices are not drawn to scale.
**Fig. 5.** Inner envelope membranes from plastids of FAX1 over-expressors show honeycomb-like vesicular structures.

*(A-E)* Transmission electron microscopic (TEM) pictures of plastids from Col-0 wild type (A, C) and FAX1ox (B, D, E) tissue. (A, D, E) are from stem hypodermis, (B) from stem phloem/cambium, and (C) from leaf cells. Whereas outer envelope membranes (oe) of FAX1ox plastids form one regular, mostly smooth lipid bilayer, IE membranes (ie) show multiple invaginations that extend into the stroma (B, D, E). As indicated by arrowheads, these additional membranes derived as a continuum from the IE membrane and in FAX1ox plastids form vesicular/tubular (B, D) and/or honeycomb-like structures (E), which are absent in wild-type plastids (A, C). As highlighted by arrows in (C) OE and IE membranes in wild-type plastids form single, mostly straight layers. Round and electron-dense plastoglobuli (pg) as well as thylakoid membrane systems (thy) are normal in wild-type and FAX1ox plastids. cyt: cytosol; cw: cell wall; vac: vacuole; str: stroma; to: tonoplast membrane. Scale bars are 500 nm and 50 nm in (A, B) and (C, D, E), respectively. TEM pictures were recorded on stem tissue of the strong FAX1 overexpressing line ox#4 (about 200-fold increase in transcripts, highly elevated protein level) as described in Li et al. (2015).