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REVIEW

Components of the CtBP1/BARS-dependent fission machinery

Carmen Valente · Alberto Luini · Daniela Corda

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Abstract The brefeldin A ADP-ribosylated substrate, a member of the C-terminal-binding protein family that is referred to as CtBP1/BARS, is a dual-function protein that acts as a transcriptional co-repressor in the nucleus and as an inducer of membrane fission in the cytoplasm. In this review, we first discuss the mechanisms that enable CtBP1/BARS to shift between the nuclear transcriptional co-repressor and the cytosolic fission-inducing activities. Then, we focus on the role of CtBP1/BARS in membrane fission. CtBP1/BARS controls several fission events including macropinocytosis, fluid-phase endocytosis, COPI-coated vesicle formation, basolaterally directed post-Golgi carrier formation, and Golgi partitioning in mitosis. We report on recent advances in our understanding of the CtBP1/BARS membrane fission machineries that operate at the *trans*-side and at the *cis*-side of the Golgi complex. Specifically, we discuss how these machineries are assembled and regulated, and how they operate in the formation of the basolaterally directed post-Golgi carriers.

Keywords CtBP1/BARS · Membrane fission · Golgi complex · Golgi checkpoint · Mitosis · ADP ribosylation

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CtBP1/BARS: a key player in membrane fission

The C-terminal-binding protein/brefeldin A ADP-ribosylated substrate (CtBP1/BARS) is a dual-function protein that acts in the nucleus as a transcriptional co-repressor and in the cytoplasm as a regulator of membrane fission and entry into mitosis (Corda et al. 2006; Chinnadurai 2009).

As a transcriptional co-repressor, CtBP1/BARS appears to act as a metabolic sensor that is activated by NADH and is involved in a large range of functions. Among these, the best studied are cell growth and differentiation. Here, CtBP1/BARS favours tumorigenesis and tumour progression, promotes epithelial–mesenchymal transition, inhibits apoptosis, and represses several tumour-suppressor genes (Corda et al. 2006; Chinnadurai 2009).

As a fission-inducing protein, CtBP1/BARS is required for various intracellular membrane trafficking steps and for the partitioning of the Golgi complex during mitosis (Corda et al. 2006). Membrane fission is a ubiquitous process in cell physiology that leads to the division of one continuous bilayer into two separate membranes and is essential in a large number of intracellular processes (Corda et al. 2002; McNiven and Thompson 2006). A central example is intracellular membrane transport, which occurs by virtue of transport carriers that bud, protrude, and detach by fission from donor compartments and then later fuse with acceptor organelles. Fission proceeds through intermediate stages whereby the membrane changes shape and loses its continuity (Kozlovsky and Kozlov 2003). The involved perturbation of membrane integrity is an extremely thermodynamically unfavourable event, and the required forces are provided by cooperative contributions of lipids and proteins as well as by force-generating enzymes (Chernomordik and Kozlov 2003; Kozlovsky and Kozlov 2003).

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Indeed, membrane fission appears to rely on multiple mechanisms. Many fission events are driven by the mechanochemical properties of dynamin. This large GTPase can be recruited onto the lipid bilayer, where it polymerises as a collar around the base of a forming membrane bud, finally cutting the membrane by virtue of its GTP-hydrolysis-dependent change in conformation (Campelo and Malhotra 2012). Other fission events are promoted by the shallow hydrophobic insertion of amphipathic α-helices or hydrophobic protrusions of proteins into the outer membrane leaflet. Examples of this hydrophobic insertion mechanism include not only endophilin, epsin, and amphiphysin (Gallop et al. 2005; Ford et al. 2002; Peter et al. 2004), but also the two GTPases ARF1 and SAR1, which contribute to the fission reaction during the formation of COPI and COPII vesicles, respectively (Campelo and Malhotra 2012). Lipid-phase separation represents another mechanism that has been proposed to induce membrane fission. Here, the segregation of the membrane lipids into microdomains can generate fission-prone regions at the borders between different lipid phases (Lenz et al. 2009; Roux et al. 2005). Recently, actin polymerisation alone has been reported to trigger fission in a dynamin-independent mechanism via reorganisation of the membrane lipids into these fission-prone domains during clathrin-independent endocytosis (Romer et al. 2010). Fission processes driven by actin and actin-related proteins have also been documented for the Golgi complex (Miserey-Lenkei et al. 2010; Salvarezza et al. 2009).

CtBP1/BARS appears to be involved in dynamin-independent fission events. In vitro, CtBP1/BARS has been shown to activate the fission of Golgi tubular-reticular domains into fragments of variable sizes (Weigert et al. 1999). This fission event is preceded by the formation of structures where tubules show sections of extreme constriction (i.e. a 'string of beads'), and which most likely represent intermediates in the fission process (Weigert et al. 1999). Interestingly, these CtBP1/BARS effects are structurally different from the in vitro effects on isolated membranes that are induced by the GTPase dynamin (Hinshaw 2000; McNiven 1998; McNiven et al. 2000; Sever et al. 2000), which suggests that the events involving each of these two proteins are mechanistically distinct. In vivo, the analysis of the dynamics and structure of transport carriers has revealed and defined the role of CtBP1/BARS in promoting different membrane fission events. These include transport from the Golgi complex to the basolateral plasma membrane, fluid-phase endocytosis (Valente et al. 2012; Bonazzi et al. 2005), macropinocytosis (Liberali et al. 2008; Amstutz et al. 2008), and retrograde transport of the KDEL receptor to the endoplasmic reticulum (ER) via COPI-coated vesicles (Yang et al. 2005). Moreover, Golgi partitioning during mitosis requires CtBP1/BARS-dependent fission (Colanzi et al. 2007; Hidalgo Carcedo et al. 2004).

The CtBP family

The CtBP family of proteins is ubiquitously expressed (Chinnadurai 2009). Invertebrate and plant genomes only contain a single copy of the *CtBP* gene (Folkers et al. 2002; Nibu et al. 1998), which codes for the different protein isoforms as a result of its RNA processing (Mani-Telang and Arnosti 2007). In contrast, vertebrates have two CtBP-encoding genes: *CtBP1* and *CtBP2*. Early studies have demonstrated that the CtBP proteins are necessary for vertebrate development and that CtBP1 cannot compensate for the function of CtBP2. Indeed, in mice, CtBP2 deletions are embryonically lethal, and the mice die by E10.5, while CtBP1 knockout mice are viable and fertile, although they are small (Hildebrand and Soriano 2002). As a result of alternative RNA splicing, the *CtBP1* gene gives rise to two protein isoforms: CtBP1-L (long) and CtBP1-S (short) (Chinnadurai 2003; Corda et al. 2006), which differ only in their first 11 N-terminal amino acids.

CtBP1-L was initially identified as a protein that binds the C-terminal region of the adenovirus E1A oncoprotein (Boyd et al. 1993; Schaeper et al. 1995), which contains the PxDLS amino acid sequence. Subsequently, CtBP1-L was characterised as a transcriptional co-repressor in *Drosophila* (Nibu et al. 1998). CtBP1-S was identified and cloned independently as a substrate of the ADP ribosylation induced by the fungal toxin brefeldin A and hence named as brefeldin A ADP-ribosylated substrate (BARS) (De Matteis et al. 1994). Soon after, BARS was shown to be a member of the CtBP family (Spano et al. 1999) and shown to have important roles in membrane fission (Weigert et al. 1999; Bonazzi et al. 2005; Yang et al. 2005; Liberali et al. 2008; Valente et al. 2012) and in Golgi partitioning during mitosis (Hidalgo Carcedo et al. 2004; Colanzi et al. 2007; Colanzi and Corda 2007).

The *CtBP2* gene codes for three protein isoforms: CtBP2-L (Katsanis and Fisher 1998) and CtBP2-S (Verger et al. 2006), which share 79 % identity with CtBP1, and RIBEYE (Schmitz et al. 2000). CtBP2-S lacks the N-terminal nuclear localisation signal (NLS) of CtBP2-L (Verger et al. 2006). RIBEYE has a different organisation, as it contains a large N-terminal domain that is otherwise unrelated to the CtBPs. This is linked to a form of CtBP2 that is devoid of the 20 N-terminal amino acids (Schmitz et al. 2000). Therefore, CtBP2-L is the only CtBP isoform that contains a NLS. CtBP2-L has a predominantly nuclear localisation, while CtBP2-S has a cytosolic localisation (Verger et al. 2006). The CtBP1-L and CtBP1-S/BARS isoforms localise to the nucleus and to the cytosol (Verger

et al. 2006). Recently, CtBP1-L was found to be present in the synaptic ribbon and in the mammalian synapse, but its function remains to be determined (tom Dieck et al. 2005).

However, the observation that CtBP1-L behaves similarly to CtBP1-S/BARS in different intracellular transport assays (Corda et al. 2006; Liberali et al. 2008) indicates that their functions are very likely to overlap. Of note also, most of the tools used in the different assay systems cannot discriminate between the CtBP1-L and CtBP1-S/BARS isoforms (Valente et al. 2005). Moreover, as the identity between these two CtBP1 isoforms is very high (97 %) and the role of the 11 amino acids at the N-terminus of CtBP1-L has not been characterised, we refer to both proteins collectively as CtBP1/BARS.

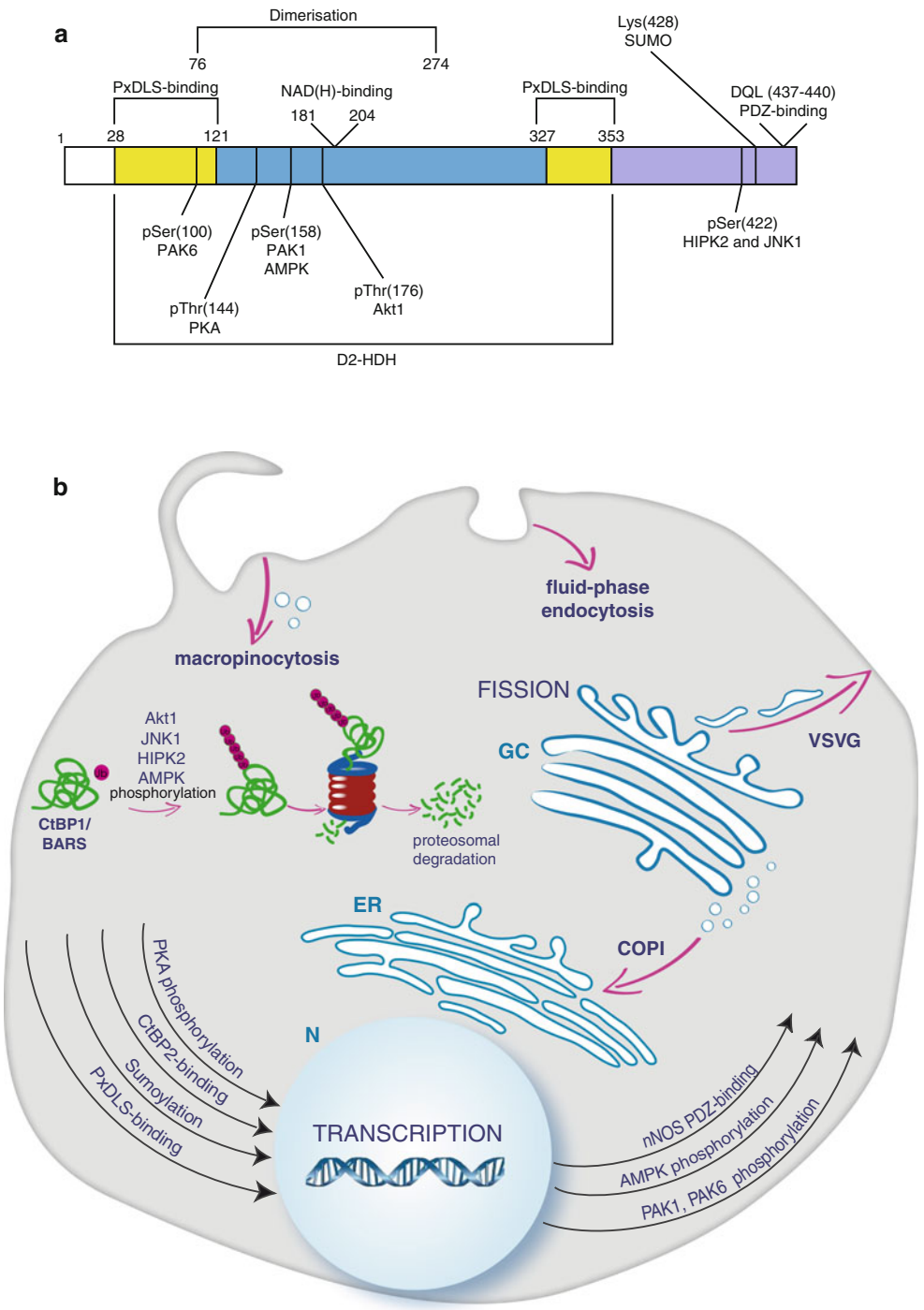
In the following, we focus on the role of CtBP1/BARS in membrane fission. We describe the structure of CtBP1/BARS and summarise the mechanisms that regulate the CtBP1/BARS-driven fission activity versus the transcriptional co-repressor activity, discussing the multiple factors that appear to regulate its location to different cell compartments. Then, we discuss more recent advances in the understanding of the CtBP1/BARS membrane fission machineries that operate at both the *trans*-side and *cis*-side of the Golgi complex, and we describe some of the molecular steps through which these complexes are assembled and operate. Through this approach, we provide new insights into the mechanisms of action of CtBP1/BARS in membrane fission.

CtBP1/BARS structure

Investigations are continuing into how the CtBPs recruit their protein partners in the nucleus or in the cytoplasm, to explain their dual function. Here, a key role in the transcriptional co-repressor activity of CtBPs has been ascribed to their ability to dimerise. Dimerisation of the CtBPs has been described through crystal structure analyses of CtBP1/BARS and the CtBP2 isoforms, all in complexes with NAD(H) (Kumar et al. 2002; Nardini et al. 2003).

Relating to CtBP1/BARS, to further delineate the structural basis of its fission activity and/or transcriptional co-repressor activity, the truncated form of the rat protein (t-CtBP1/BARS) was crystallised with NAD(H) (Nardini et al. 2003). The structure of t-CtBP1/BARS is very similar to that of the D-stereoisomer-specific 2-hydroxyacid NAD-dehydrogenases (Kumar et al. 2002), with which CtBP1/BARS shares <20 % sequence identity (Nardini et al. 2003). The t-CtBP1/BARS–NAD(H) binary complex forms an elongated homodimer in which each t-CtBP1/BARS monomer consists of two compact domains separated by a deep cleft. In accordance with the literature on enzymes with similar structures (Kumar et al. 2002), these two domains have been referred to as the nucleotide-binding domain (NBD) and the substrate-binding domain (SBD) (Nardini et al. 2003). The NBD contains all of the residues for NAD(H) binding, and it is involved in the dimerisation, while the SBD contains the binding cleft for the PXDLS peptide (Fig. 1a). The crystal structure of t-CtBP1/BARS lacks the C-terminal region, which is the third CtBP domain, and has been defined as the C-terminal domain (80 amino acids; Fig. 1a) (Nardini et al. 2006). Two NBDs build the central core of the dimer, whereas the two SBDs and the two C-terminal domains of each monomer are at opposite poles of the assembled dimer (Nardini et al. 2003, 2006). The dimer association interface is composed mainly of hydrophobic residues. In solution, CtBP1/BARS has a tetrameric conformation, and not the dimeric conformation seen in the crystal structure (Nardini et al. 2006). This difference is consistent with an interaction between the C-terminal domains, as confirmed by the observation that purified C-terminal domains of CtBP1/BARS can dimerise in solution (Nardini et al. 2006). The amino acid sequence of this region is rich in Gly and Pro residues, and 56 % of its sequence is composed of 'disorder-promoting' residues (especially Ala, Gly, Pro and Ser), as has been described for intrinsically disordered proteins (Nardini et al. 2006). ^{1}H-nuclear magnetic resonance analyses of CtBP1/BARS have indicated that the C-terminal domain shows dynamic fluctuations, and it lacks a well-defined three-dimensional structure (Nardini et al. 2006). Moreover, bioinformatic calculations have assigned properties to this domain that are consistent with a dynamic, collapsed, disordered state (Nardini et al. 2006).

Structural and biochemical analyses indicate that acyl-CoAs bind to CtBP1/BARS in the same pocket where NAD(H) is bound, with an affinity in the low μM range, and both NADH and NAD^{+} are competitors of this binding. The acyl-CoA molecule would disrupt the swapping-interaction domain that stabilises the CtBP1/BARS dimer. Indeed, in this dimeric NAD(H)-bound structure, due to the opposing dimer subunits, there is no space available for the accommodation of the acyl-CoA aliphatic tails (Nardini et al. 2003). Thus, it has been proposed that NAD(H) binding promotes a 'closed conformation/dimerisation' of CtBP1/BARS, whereas acyl-CoA binding induces an 'open conformation/monomerisation' (Nardini et al. 2003, 2009). This mechanism might represent the 'functional' molecular switch between the transcriptional activity and fission activity of CtBP1/BARS. Indeed, the activity of CtBP1/BARS in gene transcription and in membrane fission can be regulated in different ways. Oligomerisation status and intracellular localisation are the major processes that induce this functional switch. CtBP1/



BARS shuttles between the nucleus and the cytoplasm, and it is probably the regulation of this movement that controls the functions of CtBP1/BARS. These functions are controlled by post-translational modifications, co-factor binding, and protein–protein interactions (Corda et al. 2006; Fig. 1b, and see below).

The mechanisms behind the CtBP1/BARS functional switch

The switching of proteins between two roles is often connected with changes in their intracellular localisation, oligomerisation state, binding to different ligands and/or

◀ **Fig. 1** CtBP1/BARS: domain structure and mechanisms of its functional switch. **a** Domain structure of CtBP1/BARS illustrating the sequences that form the PxDLS-binding cleft at the N-terminal and C-terminal regions (*yellow*; residues 28–121 and 327–353); the dimerisation interface in the dinucleotide-binding domain (residues 76–274); the NAD(H)-binding domain (residues 181–204); the residues subjected to phosphorylation by the indicated kinases (residues 100, 144, 158, 176, 422); the SUMOylation site (residue 428); the PDZ-binding motif in the C-terminus (residues 437–440); and the dehydrogenase homology region (D2-HDH) (residues 28–353). **b** Representative scheme of the CtBP1/BARS functions mediated by a change on its oligomerisation state, intracellular localisation, and post-translational modifications. In the cytoplasm, CtBP1/BARS drives membrane fission in several dynamin-independent trafficking steps (*magenta arrows*): fluid-phase endocytosis, macropinocytosis, trans-Golgi network (TGN) to basolateral-plasma membrane-directed VSVG cargo in epithelial cells, retrograde transport of the KDEL receptor to the endoplasmic reticulum (ER) by COPI-coated vesicles, fragmentation of the Golgi complex (GC) during mitosis (not shown in the scheme). Mechanisms for cytoplasm localisation of CtBP1/BARS might depend on binding to PDZ domain of nNOS, and to AMPK, PAK1, and PAK6 phosphorylation (see *black arrows on the low right*). In the nucleus (N), CtBP1/BARS functions in the assembly of multiprotein repressor complexes involved in the modulation of gene expression (not shown in the scheme). Mechanisms for nuclear localisation of CtBP1/BARS might depend on its oligomerisation with CtBP2, binding to transcription factors containing the PxDLS motif, PKA phosphorylation, and SUMOylation (see *black arrows on the low left*). CtBP1/BARS is targeted for ubiquitylation followed by proteasome-mediated degradation upon Akt1, JNK1, HIPK2, and AMPK phosphorylation (*green protein*; see text for details)

proteins, and/or post-translational modifications (Jeffery 1999). CtBP1/BARS also shows similar behaviour, as it appears to shuttle between the nucleus and the cytoplasm, and the regulation of this movement appears to control the functions of this protein (Fig. 1b). This regulation is modulated through post-translational modifications, as well as small molecules, protein binding, and/or the formation of multiprotein complexes (see below). In addition, it has been shown that the evolution of a second function is not always without adverse effects on the ability of the protein to perform its first function. In agreement with this, it has been reported that the binding of acyl-CoAs to the NAD(H)-binding site of CtBP1/BARS favours the monomeric conformation of CtBP1/BARS and its membrane fission activity. These will thus all affect the NAD(H)-dependent co-repressor nuclear function of CtBP1/BARS (Corda et al. 2006; Yang et al. 2005; Nardini et al. 2003).

Regulation by co-factor binding: NAD(H) and acyl-CoAs

Two important NAD(H)-dependent factors can affect the co-repression activity of CtBP1/BARS: its dehydrogenase activity and its structural conformation. NAD(H)-dependent dehydrogenase activity has been detected in recombinant CtBP1/BARS (Kumar et al. 2002), although the role of this activity in transcriptional co-repression remains controversial (Kumar et al. 2002; Zhang and Arnosti 2011; Mani-Telang and Arnosti 2007). Instead, the role in transcription of the NAD(H)-dependent structure of CtBP1/BARS has been more clearly defined. Indeed, structural modelling studies have shown that NAD(H) binding to CtBP1/BARS leads to stable dimer formation that, in turn, promotes the binding of PxDLS-containing transcriptional co-repressors (Kumar et al. 2002; Nardini et al. 2003) and in parallel decreases the interactions of CtBP1/BARS with some non-PxDLS-containing proteins (Mirnezami et al. 2003). Conversely, mutation of Gly172 in the NAD(H)-binding site of CtBP1/BARS disrupts the dimer (Nardini et al. 2003, 2009) and results in the loss of the aggregation scaffold role for multimeric transcriptional co-repression complexes (Turner and Crossley 2001). These results indicate that NAD(H) can modulate protein–protein interactions through the regulation of the oligomerisation state of CtBP1/BARS.

Similarly, the absence of the NAD(H) molecule bound to CtBP2 abrogates its ability to dimerise, and consequently its co-repressor activity (Thio et al. 2004). Also, in the absence of this NAD(H) binding, *Drosophila* CtBP lacks biological activity (Mani-Telang and Arnosti 2007). However, NADH binds to CtBP1/BARS with an approximately 100-fold greater affinity compared to NAD^+, which suggests a role for CtBP1/BARS as a metabolic sensor of redox status that can link changes in NADH levels to gene regulation (Fjeld et al. 2003; Zhang et al. 2002). Moreover, NAD(H) binding to CtBP1/BARS inhibits the CtBP1/BARS membrane fission function (Yang et al. 2005). Indeed, it has been shown that the role of CtBP1/BARS in COPI-coated vesicle formation requires its interaction with ARFGAP1; in turn, this interaction is oppositely regulated by acyl-CoAs and NAD(H) binding (Yang et al. 2005).

Regulation by protein–protein interactions

The cytoplasmic and nuclear localisation of CtBP1/BARS can be regulated by its interactions with other proteins, including CtBP2, transcription factors, and neuronal nitric oxide synthase (nNOS) (Fig. 1b).

CtBP2 contains a NLS, which is not found in CtBP1/BARS, and which allows CtBP2 to shuttle between the nucleus and the cytoplasm (Verger et al. 2006). Interestingly, CtBP1/BARS and CtBP2 can homo-dimerise and hetero-dimerise (Verger et al. 2006), and indeed, they can be co-purified in the same transcriptional co-repressor complex (Shi et al. 2003). It has been shown that the redistribution of CtBP1/BARS from the cytosol to the nucleus is influenced by this hetero-dimerisation with CtBP2, by virtue of the NLS-containing motif of CtBP2. Mutation of the two positively charged amino acids Lys10

and Arg11 in the NLS of CtBP2 fails to redistribute CtBP1/BARS to the nucleus, which indicates that these two residues that are conserved also in dCtBP in *Drosophila* are critical for nuclear localisation (Verger et al. 2006). Additionally, binding to nuclear proteins such as BKLF, Net, and HIC-1 (PxDLS-motif-containing transcriptional factors) redistributes CtBP1/BARS into the nucleus (Verger et al. 2006; Criqui-Filipe et al. 1999; Deltour et al. 2002). Conversely, binding between the C-terminus DQL-containing sequences of CtBP1/BARS (Fig. 1a) and the PDZ domain of nNOS directs it to the cytosol, which makes CtBP1/BARS available for its cytosolic functions and inaccessible for transcriptional regulation (Riefler and Firestein 2001).

Finally, what effects does NAD(H) binding have on CtBP1/BARS localisation? It has been shown that mutation in the NAD(H) binding site of CtBP1/BARS (Fig. 1a) abolishes the nuclear accumulation driven by CtBP2, while it does not affect CtBP1/BARS nuclear recruitment by PxDLS-motif-containing transcription factors (Verger et al. 2006). This is in agreement with the previous structural and biochemical analyses, which have indicated that NAD(H) binding can promote the formation of a CtBP1/BARS dimer with the PxDLS consensus peptide-binding sites placed at the two distant N-terminal regions (Nardini et al. 2003; Mirnezami et al. 2003; Wang et al. 2006).

Regulation by post-translational modifications

The shuttling of CtBP1/BARS between the nucleus and the cytoplasm is also controlled by post-translational modifications, namely phosphorylation and SUMOylation (Fig. 1b).

CtBP1/BARS is phosphorylated at Ser422 upon UV irradiation by two different kinases: the homeodomain-interacting protein kinase-2 (HIPK2) and c-Jun NH_2-terminal kinase 1 (JNK1) (Fig. 1a). Both of these events result in the targeting of CtBP1/BARS for ubiquitin-mediated degradation (Fig. 1b). The subsequent decrease in the CtBP1/BARS protein levels is sufficient to trigger apoptosis in p53-null cells (Zhang et al. 2005; Wang et al. 2006). Similarly, CtBP1/BARS phosphorylation by Akt1 at Thr176 (Fig. 1a) appears to decrease its dimerisation, thereby resulting in decreased transcriptional repression, which promotes its ubiquitylation, followed by proteasome-mediated degradation (Merrill et al. 2010; Fig. 1b).

Upon metabolic stresses, AMP-activated protein kinase (AMPK) phosphorylates CtBP1/BARS on Ser158 (Fig. 1a), which leads to the nuclear export of CtBP1/BARS and to an increase in its ubiquitination and degradation (Kim et al. 2013; Fig. 1b). The same Ser158 residue (in CtBP1-L, corresponding to Ser147 in CtBP1-S) is phosphorylated by PAK1 (Fig. 1a), which results in similar nuclear export effects (Fig. 1b) to those shown by a phospho-mimetic mutant that localises mostly to the cytosol, and by a phospho-depleted mutant that localises predominantly to the nucleus (Barnes et al. 2003; Liberali et al. 2008). Moreover, this phosphorylation has two important effects: it blocks CtBP1/BARS co-repressor activity (Barnes et al. 2003), and it changes its oligomerisation status, shifting it towards the monomeric state that is active in membrane fission (Liberali et al. 2008). This PAK1-mediated phosphorylation might provide a mechanistic link between the two activities of CtBP1/BARS: PAK1 switches CtBP1/BARS on for membrane fission, and at the same time, it turns it off as a co-repressor (Barnes et al. 2003; Liberali et al. 2008). In addition to PAK1, PAK6 can also phosphorylate CtBP1/BARS at Ser100 (Fig. 1a), with similar PAK1-dependent phosphorylation effects (Dammer and Sewer 2008; Fig. 1b).

Conversely, increased nuclear relocalisation of CtBP1/BARS is mediated by the cAMP-dependent protein kinase (PK) A. Indeed, a PKA-dependent phosphorylation of CtBP1/BARS at Thr144 (Fig. 1a) results in dimerisation and accumulation of CtBP1/BARS in the nucleus (Dammer and Sewer 2008; Fig. 1b).

In addition to phosphorylation, the other known modification of CtBP1/BARS is the SUMOylation at Lys428 (Fig. 1a), which, like PKA-dependent phosphorylation, has been proposed as a mechanism for nuclear retention and to be critical for co-repressor activity of CtBP1/BARS (Kagey et al. 2003; Lin et al. 2003; Fig. 1b). Notably, the PDZ domain of nNOS inhibits the SUMOylation of CtBP1/BARS, and this correlates with the known inhibitory effects of nNOS on the nuclear accumulation of CtBP1/BARS (Lin et al. 2003; Fig. 1b).

CtBP1/BARS-dependent fission machinery at the *trans*-Golgi network

CtBP1/BARS has been shown to be involved in the fission step during the formation of large VSVG-containing post-Golgi carriers (PGCs) that leave the Golgi complex for the basolateral plasma membrane (Bonazzi et al. 2005; Valente et al. 2012). These PGCs are variably sized (from 100 to 200 nm, to several microns long), pleiomorphic, and often tubule-shaped containers that appear devoid of any visible coat and form '*en bloc*' by fission of elongated membrane precursors that extrude along microtubules from specialised *trans*-Golgi network (TGN) export domains (Polishchuk et al. 2003; De Matteis and Luini 2008; Rodriguez-Boulan and Musch 2005). While a lot is known about the molecular components and the organisation of small, coated vesicles (e.g. COPI or COPII vesicles, or clathrin-dependent vesicles; Rothman 2002; Schekman 2002),

PGCs are mechanistically incompletely understood. The molecular components so far implicated in the formation of PGCs include the following: phosphatidylinositol 4-kinase IIIβ (PI4KIIIβ), with its product of phosphatidylinositol 4-phosphate (PtdIns4*P*) (Bruns et al. 2002; Godi et al. 2004); ADP ribosylation factor (ARF), with its recruitment of PI4KIIIβ to Golgi membranes and its activation (Godi et al. 1999); neuronal calcium sensor-1 [NCS-1, which is also known as frequenin, which stimulates PI4KIIIβ (Haynes et al. 2005); the glycolipid-transfer protein FAPP2, which binds both ARF and PtdIns4*P* and has been proposed to facilitate membrane bending and tubulation (Godi et al. 2004; D'Angelo et al. 2007; Cao et al. 2009; see also Vieira et al. 2005 for a discussion on the baso-lateral vs. apical role of FAPP2); and GOLPH3, which is recruited to the TGN by PtdIns4*P* and then binds MYO18A/F-actin, which results in the molecular apparatus required for efficient tubular precursor formation, with these then pulled out of the Golgi area along microtubules by specific kinesin motors (Dippold et al. 2009; De Matteis and Luini 2008; Kreitzer et al. 2000). This set of molecules appears to be primarily involved in the initiation and protrusion of the tubular precursors (Fig. 2).

Another set of proteins is instead mostly involved in the fission of these precursors. These include the following: protein kinase D (PKD), which is recruited to the TGN by ARF (Pusapati et al. 2010), with its activators and modulators (the beta-gamma subunit of trimeric G proteins, PKCε, PLC) (Baron and Malhotra 2002; Diaz Anel 2007; Diaz Anel and Malhotra 2005; Jamora et al. 1999; Liljedahl et al. 2001; Yeaman et al. 2004); myosin II and Rab6 (Miserey-Lenkei et al. 2010); and CtBP1/BARS (Bonazzi et al. 2005; Valente et al. 2012).

These numerous components must therefore act in concert to form PGCs. We have recently investigated how these components assemble and disassemble dynamically throughout the process of carrier extrusion and carrier fission (Valente et al. 2012). As described previously, fission occurs along the carrier precursor during the protrusion process, a few seconds after the carrier precursor has emerged out of the Golgi mass (Polishchuk et al. 2003). Fission is thus spatially and temporally linked with the budding process in some specific way. To understand this coordination, we analysed the physical and functional interactions of the fission inducer CtBP1/BARS with components of the carrier budding machinery. Indeed, using CtBP1/BARS as bait (by immunoprecipitation and pull-down approaches), we isolated several components that were already known to have crucial roles in carrier formation (ARF, PI4KIIIβ, PKD, PAK1, NCS-1), as well as some components that were novel, such as the 14-3-3γ protein (Valente et al. 2012). 14-3-3γ is specifically involved in CtBP1/BARS-dependent transport from the TGN to the plasma membrane, as it acts as a dimeric scaffold in the assembly of the tripartite PI4KIIIβ–14-3-3γ(×2)–CtBP1/BARS core complex. The 14-3-3γ bridging role is in line with the demonstrations that: (1) 14-3-3 proteins have well-known roles as adaptors/scaffolds in many intracellular pathways (Aitken 1996, 2006); (2) 14-3-3-dependent associations of two proteins have been reported previously (Braselmann and McCormick 1995; Vincenz and Dixit 1996; Van Der Hoeven et al. 2000); and (3) 14-3-3 proteins interact with several components that mediate post-Golgi trafficking (e.g. PI4KIIIβ and PKD; Hausser et al. 1999, 2006; Demmel et al. 2008).

This PI4KIIIβ–14-3-3γ(2)–CtBP1/BARS complex is stabilised by reversible phosphorylation that is mediated by two of the other CtBP1/BARS complex components, PKD and PAK1, which suggests that the complex assembles and disassembles dynamically (Valente et al. 2012). In this process, PKD phosphorylates PI4KIIIβ at Ser294 in a RXXpS 14-3-3-binding motif, resulting in the creation or stabilisation of 14-3-3 binding to PI4KIIIβ (Hausser et al. 2006). This 14-3-3 binding is reported as a positive regulator of PI4KIIIβ activity, through protecting the lipid kinase from active-site dephosphorylation, keeping the kinase in an active state, and thereby ensuring a continuous supply of PtdIns4*P* to the Golgi compartments. This is supported by the demonstration that Ser294-phosphorylated PI4KIIIβ is exclusively associated with the Golgi compartment (Szivak et al. 2006). The equivalent phosphorylation in CtBP1/BARS occurs on a similar RXXpS motif at Ser147, again stabilising its binding with 14-3-3γ. Here, the kinase involved is PAK1. The following recruitment of the PAK-dependent phosphorylated form of CtBP1/BARS in a complex with 14-3-3γ dimer–PI4KIIIβ then leads to the fission of the tubular carriers (Valente et al. 2012).

Indeed, on the functional side, the formation of the PI4KIIIβ–14-3-3γ(2)–CtBP1/BARS core complex appears to be crucial for the fission of PGCs, as indicated by our findings that manipulations that impair this complex, such as 14-3-3γ knock-down, prevent CtBP1/BARS binding to 14-3-3γ, or disruption of the ability of 14-3-3γ to dimerise, all result in the formation of VSVG-containing tubules that elongate out of the Golgi complex, but cannot undergo fission. Of note, this is a phenotype that is very similar to that seen upon inhibition of CtBP1/BARS (Bonazzi et al. 2005) or PKD (Bossard et al. 2007; Yeaman et al. 2004; Liljedahl et al. 2001), in line with the concept that all of these manipulations affect the same machinery.

What is the functional role of this complex? Considering that PI4KIIIβ with its activator and effector molecules (ARF, NCS-1, PtdIns4*P*, FAPP2) has been proposed to trigger TGN-export domain formation, while CtBP1/BARS and its interactors (e.g. PLD; Haga et al. 2009) operate in

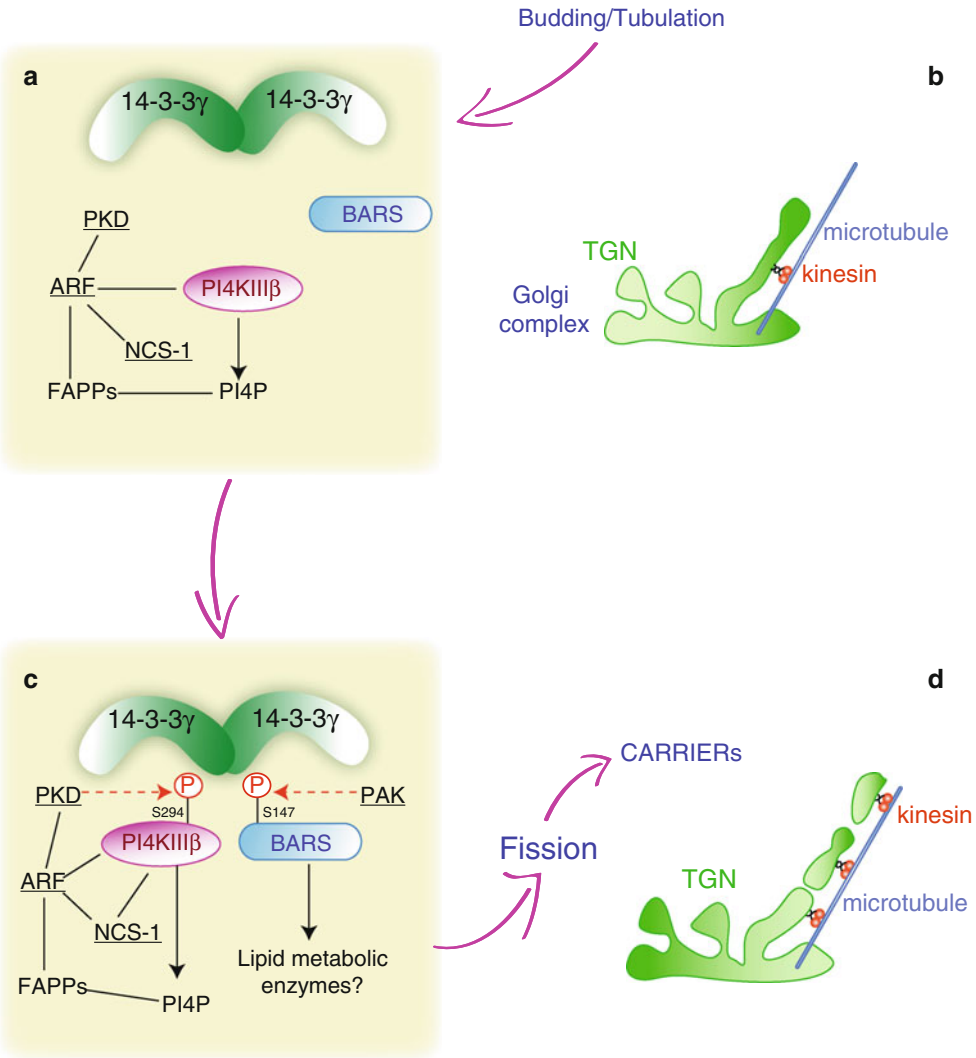


Fig. 2 Proposed model for the CtBP1/BARS-dependent, post-Golgi carrier (PGC) formation. The scheme represents a possible model based on the literature and our data, depicting the molecular steps through which the PI4KIIIβ–14-3-3γ dimer–CtBP1/BARS protein complex is assembled. **a** The activation of ARF induces recruitment of PKD (Pusapati et al. 2010) and PI4KIIIβ (Godi et al. 1999) onto Golgi membranes, and the activation of PI4KIIIβ to stimulate PtdIns4*P* synthesis (Godi et al. 1999). PI4KIIIβ is also activated by NCS-1 (a well-known ARF interactor; Haynes et al. 2005; Zhao et al. 2001). Then, both ARF and PtdIns4*P* mediate the recruitment of FAPPs, which can lead to membrane bending and budding/tubulation (Cao et al. 2009; Beck et al. 2008; Krauss et al. 2008; Lundmark et al. 2008). **b** Schematic representation of the budding/tubulation process at the Golgi complex, produced as detailed in **a**, where tubular carrier precursors, originated from specialised TGN-export domain, are extruded by kinesins along microtubules. **c** PI4KIIIβ and CtBP1/BARS, phosphorylated at Ser294 by PKD and at Ser147 by PAK1, respectively, bind the two main interactor-binding surfaces of the 14-3-3γ dimer. This creates a physical link between PI4KIIIβ and CtBP1/BARS, which is essential for fission (see text). In addition, also essential for fission is the activity of lipid-modifying enzymes, indicated in the scheme with question marks, since the identification of the enzyme directly interacting with CtBP1/BARS is under investigation. **d** Schematic representation of the fission of the tubular carrier precursors to generate free post-Golgi carriers. *Solid lines* protein interactions; *solid arrows* chemical modifications; *dashed arrows* protein phosphorylation

membrane fission (Bonazzi et al., 2005), the main effect of the CtBP1/BARS–PI4KIIIβ complex might be to anchor the fission machinery to the site of tubule budding. This would enhance the efficiency of the fission machinery, such that disrupting the complex would unlink these two processes and result in the observed tubulation phenotype. Thus, this complex links the budding, tubulation, and the fission processes and provides the first molecular scheme for the formation of large pleiomorphic PGCs.

At the molecular level, the formation of the CtBP1/BARS–PI4KIIIβ complex might correspond to the bringing together of a number of lipid metabolic enzymes to the site of carrier formation. Indeed, several such reactions are connected with this complex. For instance, ARF-activated

PI4KIIIβ can generate PtdIns4*P*, which can then recruit the glucosylceramide-transfer protein FAPP2 (Godi et al. 2004; D'Angelo et al. 2007). This results in local enrichment of both PtdIns4*P* and glucosylceramide. Both ARF and FAPP2 have been proposed to induce membrane deformation (Lundmark et al. 2008; Krauss et al. 2008; Beck et al. 2008; Cao et al. 2009) in a PtdIns4*P*-dependent manner (Krauss et al. 2008; Cao et al. 2009). In addition, PtdIns4*P* can also stimulate a flippase activity at the TGN, which should induce positive curvature in the membrane, and therefore facilitate the tubule budding process (Natarajan et al. 2009). Thus, ARF, FAPP2, and the flippase might act together, leading to budding/tubulation of transport carriers. In addition, the concentration of ARF at local sites on the budding membrane can also function as an important receptor for the fission-inducing protein PKD (Pusapati et al. 2010). Like ARF, PKD might insert its N-terminal hydrophobic stretch into the outer leaflet of the membrane bilayer, to facilitate budding/tubulation (Campelo and Malhotra 2012). Interestingly, along this line, both CtBP1/BARS and 14-3-3γ bind directly to PtdIns4*P* (Yang et al. 2008; Roth et al. 1994), which raises the possibility that PtdIns4*P* has a direct role in allowing the anchoring of the complex-bound CtBP1/BARS to a suitable site for tubule budding. Here, PAK1-mediated phosphorylation of Ser147 activates CtBP1/BARS, to drive fission of these tubular carrier precursors, and to generate free pleiomorphic carriers. Free energy calculations based on molecular dynamics simulations of non-phosphorylated and phosphorylated CtBP1/BARS have shown that the phosphorylation of CtBP1/BARS by PAK1 can be predicted to reduce stability of the dimeric conformation in favour of the monomeric fission-driven active form of CtBP1/BARS (Liberali et al. 2008; Corda et al. 2006).

In terms of the mechanisms by which CtBP1/BARS induces membrane fission, recent studies have shown that PAK1-induced phosphorylation activates CtBP1/BARS, which then binds and stimulates PLD (synergistically with ARF) (Haga et al. 2009). This activation of PLD is involved in fission, most likely through an increase in the membranes in phosphatidic acid (PA), a lipid that offers opportunities for rapid, reversible membrane deformation, that can lead (a) to fission during export from the TGN (Chen et al. 1997; Siddhanta et al. 2000), (b) to COPI-coated vesicle formation (Yang et al. 2008), and (c) to macropinocytosis (Haga et al. 2009). Thus, CtBP1/BARS might act as an adaptor to recruit PLD (and possibly other lipid metabolic enzymes) during the tubulation-fission sequence.

Of note, 14-3-3γ is a key player in the overall process, as it behaves both as an essential adaptor to assemble the fission-active complex through the bridging of active PKD and phosphorylated PI4KIIIβ kinase and, possibly, also as a positive modulator of CtBP1/BARS, through its protection of the PAK1-mediated Ser147 phosphorylation site from dephosphorylation (as for 14-3-3 binding to PI4KIIIβ; Hausser et al. 2006). This thus ensures the continuously active, phosphorylated state of CtBP1/BARS that has been proposed to be involved in fission (Liberali et al. 2008).

A further question concerns whether and how CtBP1/BARS interfaces with other fission-promoting machineries at the TGN. Indeed, in this context, it is important to note that inhibition of either CtBP1/BARS (e.g. by dominant-negative mutants; Bonazzi et al. 2005) or 14-3-3γ (e.g. by siRNAs or antibody injection; Valente et al. 2012) does not lead to a complete block of PGC exit from the Golgi complex (i.e. it is reduced by about 60 %). This suggests that additional fission factors are required for PGC formation in the CtBP1/BARS-dependent pathway. Thus, a comprehensive picture of the relationships between the different fission machineries operating at the Golgi complex appears to be far from complete. The fission events that are coordinated by CtBP1/BARS are distinct from those that require dynamin (Bonazzi et al. 2005), and our data indicate that the fission-inducing protein PKD is part of the CtBP1/BARS-dependent fission machinery.

An interesting open question for future investigations is the possibility that the actin cytoskeleton and its associated proteins can provide the additional fission factors required for CtBP1/BARS-dependent PGC formation. Among these factors, the myosins represent possible candidates (Dippold et al. 2009; Miserey-Lenkei et al. 2010). Indeed, Miserey-Lenkei et al. (2010) demonstrated that the actin-based motor myosin II regulates the fission of VSVG-containing PGCs, even if this myosin II inhibition does not lead to a complete block of PGC exit from the Golgi (i.e. it is reduced by about 50 %).

It is interesting to note that during EGF-receptor-activated macropinocytosis, PAK1 phosphorylates CtBP1/BARS and myosin VI, promoting their recruitment to membranes (Liberali et al. 2008; Buss et al. 1998). PAK1 phosphorylates myosin VI within the actin-binding region in the motor domain, and phosphorylation in the corresponding position in *Acanthamoeba* myosin Ia and Ib regulates their actin-activated ATP activities (Brzeska et al. 1997). In addition, PAK1 is involved in EGF-dependent myosin II phosphorylation (Even-Faitelson et al. 2005). In common with what occurs during macropinocytosis, there might also be physiological coupling at the Golgi complex between PAK1, myosin II/myosin VI, and possibly the CtBP1/BARS-dependent fission machinery. PAK1, myosin II, and myosin VI localise at the Golgi complex (Paglini et al. 2001; Sahlender et al. 2005; Valente et al. 2012) and are all involved in the formation of VSVG-containing PGCs (Valente et al. 2010, 2012). Here, PAK1 might coordinate two important

aspects at the TGN during the fission of PGCs: (1) local fine regulation of actin dynamics through phosphorylation of myosin II/myosin VI and (2) activation of the CtBP1/BARS-dependent fission machinery.

The role of CtBP1/BARS during formation of COPI-coated vesicles

At the *cis*-side of the Golgi complex, CtBP1/BARS controls the fission of COPI-coated vesicles (Yang et al. 2005). COPI is considered one of the best-characterised coat complexes, which acts in both transport from the Golgi to the ER, and in intra-Golgi transport (Yang et al. 2011).

Initial studies revealed that activation of ARF1 by the binding of GTP recruited coatomer into Golgi membranes, to initiate the formation of COPI-coated vesicles. This indicates that ARF1 and coatomer are the core components of the COPI coat (Orci et al. 1993). Subsequently, using an in vitro reconstitution system with purified components and Golgi membranes, additional key factors were identified. CtBP1/BARS, PLD2, and lysophosphatidic acid acyltransferase (LPAAT) activities have been shown to be required for COPI vesicle formation, specifically at the fission stage (Yang et al. 2005, 2006, 2008, 2011). However, recent evidence has indicated that ARF1 is also involved in COPI vesicle fission (Beck et al. 2011). Moreover, using CtBP1/BARS as bait, it has been shown that all of these proteins are components of the CtBP1/BARS-driven fission machinery involved in COPI transport (Haga et al. 2009; Yang et al. 2008, 2011; Valente et al. 2012; our unpublished data).

Indeed, the release of CtBP1/BARS from Golgi membranes after stringent salt washes results in inhibition of COPI vesicle formation, which can be restored when recombinant CtBP1/BARS is added to the system (Yang et al. 2005). Similarly, the depletion or inhibition of CtBP1/BARS in vivo affects COPI-dependent transport of a KDELR chimera, with the increased levels of COPI-coated buds on Golgi cisternae indicating the critical role of CtBP1/BARS in the fission step (Yang et al. 2005). This role of CtBP1/BARS is suggested to be ARFGAP-binding dependent, and the interaction between CtBP1/BARS and ARFGAP supports COPI vesicle fission (Yang et al. 2005). As acyl-CoA has also been shown previously to be critical for the fission step (Ostermann et al. 1993), Hsu and coworkers defined an unexpected mechanism: palmitoyl-CoA participates in COPI vesicle formation as a cofactor for CtBP1/BARS in the regulation of its interaction with ARFGAP1 (Yang et al. 2005). Thus, as for PAK1-mediated phosphorylation of CtBP1/BARS, acyl-CoA binding to CtBP1/BARS is believed to induce the dimer to monomer conformational shift that is required for the ARFGAP interaction, and thus for COPI vesicle fission. Conversely, NAD(H) binding induces dimer formation, with the consequent inhibition of the ARFGAP interaction, which in turn reduces COPI vesicle release (Yang et al. 2005; Nardini et al. 2003).

However, how does CtBP1/BARS drive COPI-coated vesicle fission? CtBP1/BARS has been shown to deform membranes that are enriched in PA (Yang et al. 2008). Indeed, CtBP1/BARS binds PA, and like other characterised fission factors, it has been shown to induce liposome tubulation (Yang et al. 2008). It has been proposed that PA produced via PLD2 is the critical lipid required for COPI-coated vesicle fission. Depletion or inhibition of PLD2 blocks COPI transport and induces the accumulation of buds with constricted necks, which indicates that PLD2 activity is required for COPI buds to become vesicles. CtBP1/BARS accumulates at the neck of these buds, where it functions at the later stages of vesicle formation. The PA that is generated by PLD2 is proposed to recruit CtBP1/BARS onto the membranes and then to cooperate with CtBP1/BARS in the generation of the membrane curvature that leads to COPI-coated vesicle fission (Yang et al. 2008). However, the role of PA in this fission process is more complex. More recent studies have shown that in addition to PLD2, LPAATγ is the PA-generating enzyme that is required for CtBP1/BARS-dependent COPI vesicle formation (Yang et al. 2011). But how do these two pools of PLD2-produced and LPAATγ-produced PA act in the fission process? Evidence suggests that LPAATγ, which is a Golgi-resident enzyme (Schmidt and Brown 2009), regulates the release of COPI buds as vesicles by promoting the constriction of the COPI bud neck; conversely, the phospholipase A_2 cPLA2α (which catalyses the removal of an acyl group and the formation of lysolipids) inhibits this fission of COPI buds, converting them into tubules. The conversion of phosphatidylcholine to PA by PLD2 completes the COPI bud constriction that is initiated by LPAATγ, to reach the final step of COPI vesicle fission (Yang et al. 2011). These findings might explain the original evidence of an acyltransferase role in COPI-coated vesicle formation (Pfanner et al. 1989; Yang et al. 2005).

We and others have previously proposed that the observed fission-inducing property of CtBP1/BARS on isolated Golgi membranes was dependent on an intrinsic LPAAT activity, which would promote the incorporation of the acyl group of acyl-CoA into LPA to form PA, followed by membrane fission (Weigert et al. 1999; Shemesh et al. 2003; Kooijman et al. 2003; Scales and Scheller 1999; Schmidt and Brown 2009; Schmidt et al. 1999). However, this LPAAT activity was later shown to be associated with, rather than intrinsic to, CtBP1/BARS (Gallop et al. 2005). Indeed, the minimal domain of CtBP1/BARS that is sufficient for COPI-coated vesicle fission

does not incorporate this activity (Yang et al. 2005), which suggests that an LPAAT might be in a complex with CtBP1/BARS and have a role in fission. This is an interesting aspect that needs to be further investigated on the basis of the emerging role of the different LPAATs at the Golgi and in membrane trafficking (Yang et al. 2011; Schmidt and Brown 2009).

What are the common factors in the CtBP1/BARS-fission machineries?

There are some common factors in the fission machineries that are driven by CtBP1/BARS at the Golgi complex and at the plasma membrane. It is interesting to note that 14-3-3γ binds the monomeric fission-active form of CtBP1/BARS, and under these conditions, it is involved not only in PGC formation, but also in the CtBP1/BARS-dependent COPI and macropinocytosis pathways (Valente et al. 2012). Thus, it is possible that 14-3-3γ functions as a positive regulator of CtBP1/BARS by stabilising its monomeric, phosphorylated, fission-driven, active conformation (a well-known function for the 14-3-3 proteins; Hausser et al. 2006). This might, in turn, facilitate fission, either by binding new partners in an assembling of the fission complex (as shown by Valente et al. 2012), or by exposing hydrophobic residues that might then induce membrane curvature.

In line with this hypothesis, it has been recently proposed that proximity to the membrane might facilitate conformational changes of 14-3-3γ, which would be compatible with an opening of this protein upon interaction with the membrane, for exposure to the bound phosphorylated peptides/proteins (Bustad et al. 2011).

ARF1 is another common player that controls the assembly and disassembly of the vesicle coat machinery in the early *cis*-Golgi (Yang et al. 2005; Beck et al. 2011; Moss and Vaughan 1998), promotes PGC formation (Godi et al. 2004), is required for macropinocytosis (Hasegawa et al. 2012; Haga et al. 2009), and modulates lipid-metabolising enzymes, including PLD (Donaldson and Jackson 2011). Moreover, CtBP1/BARS stimulates PLD synergistically with ARF1, which in turn is required for agonist-induced macropinocytosis (Haga et al. 2009). However, the PA generated locally by PLD (or the PA metabolite, diacylglycerol) has a key function in all of the membrane fission events controlled by CtBP1/BARS (Yang et al. 2008; Haga et al. 2009; Siddhanta et al. 2000).

Finally, it is interesting to note that similar to CtBP1/BARS, its plant homologue ANGUSTIFOLIA has recently been proposed to have a role both in membrane trafficking (Minamisawa et al. 2011), and in transcriptional regulation (Gachomo et al. 2013). Like CtBP1/BARS, ANGUSTIFOLIA localises on the TGN, and its mutation results in an increasing number of snowman-shaped and large-sized vesicles around the TGN, which suggests a role in the biogenesis of these intracellular transport carriers (Minamisawa et al. 2011). Two PLD proteins in plants have been identified as 14-3-3 interactors (Chang et al. 2009), which is consistent with phospholipase/14-3-3 interactions in animals (Andoh et al. 1998). Additionally, as well as CtBP1/BARS, 14-3-3 has been shown to bind PA generated from PLD (Testerink et al. 2004; Roth et al. 1994; Yang et al. 2008), which is a signalling molecule in plants (Testerink and Munnik 2005). This interconnection of 14-3-3, PA, CtBP1/BARS and PLD in plants raises the question of whether they are in the same CtBP1/BARS-mediated fission complex as in animals, and how they assemble together and induce membrane fission.

The similarities between the fission processes in animal and plant cells point at conserved mechanisms controlling membrane traffic and dynamics in different physiological processes. While the fission complexes discussed above are approaching a good level of understanding for their molecular and mechanistic aspects, other questions are emerging and need to be addressed. In particular, the elucidation of the signalling leading to the different fission complexes' assembly and action as well as the interplay with the cell cytoskeleton will give information relevant not only to our knowledge but also to developing appropriate pharmacological manipulation.

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