

CELL BIOLOGY

Molecular mechanism of CtBP1-S/BARS-driven membrane fission and its cellular control by metabolic ligands

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CtBP1-S/BARS (C-terminal binding protein 1-S/Brefeldin A ADP-Ribosylation Substrate) is a moonlighting protein with key roles in membrane trafficking and gene regulation. We show that CtBP1-S/BARS couples enzymatic lipid remodeling [lysophosphatidic acid (LPA)-to-phosphatidic acid (PA) conversion] with membrane deformation to drive fission and that this activity is directly controlled by metabolic ligands. CtBP1-S/BARS exists as a monomer or dimer. The monomeric, acyl-CoA-bound form drives membrane fission by coupling acyltransferase-dependent LPA-to-PA conversion with amphipathic helix insertion into PA-enriched membranes. Under metabolic stress, reduced nicotinamide adenine dinucleotide (NADH) binding triggers dimerization and structural rearrangements that disable fission, enabling the binding of transcription factors regulating apoptosis and energy metabolism. This NADH/acetyl-CoA competition likely coordinates trafficking shutdown with gene expression programs through a single conformational change. In intact cells, increased NADH promotes nuclear accumulation of dimeric/tetrameric CtBP1-S/BARS, whereas elevated acyl-CoA favors the cytosolic, membrane-associated monomer, indicating that cofactor availability determines protein function under physiological and stress conditions. This work reveals the structural basis for integrating membrane transport with transcriptional control, demonstrating how evolution embeds distinct cellular functions into a unified molecular pathway.

INTRODUCTION

CtBP1-S/BARS is a moonlighting protein with dual functionality in the cytosol and nucleus (1–3). The name CtBP (C-terminal binding protein) refers to its role in transcriptional regulation of survival, energy, and lipid metabolism programs (4, 5), while BARS (Brefeldin A ADP-Ribosylation Substrate) refers to its function in intracellular membrane transport, specifically in membrane fission (6–8).

Structurally, CtBP1-S/BARS is a hydroxyacid dehydrogenase (5) that lost enzymatic activity during evolution and acquired regulatory functions in major cellular processes. It consists of a nucleotide-binding domain (NBD) that contains a NAD(H)-binding Rossmann fold (9), a substrate-binding domain (SBD), and an unstructured C-terminal domain (CTD) (10). In mammals, the CtBP family is encoded by two genes, CtBP1 and CtBP2. CtBP1 produces two closely

related splice variants: CtBP1-L (long) and CtBP1-S/BARS (short) (1), the latter being the isoform referred to here as BARS.

A central property of BARS is its ability to switch between its two cellular roles in response to the metabolic state (11). This switch is driven by a conformational change between the monomeric and dimeric forms of BARS. The monomeric, acyl-CoA-bound form is active in membrane fission and transport (12, 13), while the dimeric, reduced nicotinamide adenine dinucleotide (NADH)-bound form disengages from the fission machinery and translocates to the nucleus, where it regulates gene expression programs involved in apoptosis, lipid and energy metabolism, and other stress-adaptive pathways (9, 14–17). Because acyl-CoA and NADH vary depending on metabolic states and compete for binding to the Rossmann fold of BARS, their relative levels determine BARS monomeric or dimeric conformation and hence function. Notably, the BARS switch has been shown to support cell survival during severe energy stress (11). In contrast, the CtBP2 paralog lacks cytosolic functions and operates exclusively in the nucleus as a transcriptional corepressor, targeting many of the same genes regulated by dimeric nuclear BARS (18, 19).

The BARS-dependent fission machinery operates in a broad spectrum of transport routes, encompassing many known exocytic and endocytic pathways. These include Golgi-to-plasma membrane transport, intra-Golgi trafficking, retrograde transport from the Golgi to the endoplasmic reticulum (ER), parts of the endocytic pathway, a specialized secretion route for survival-related proteins, and additional trafficking segments (11–13, 20–24). BARS also mediates Golgi partitioning during mitosis (25–27). BARS-dependent fission is route specific and does not participate in dynamin-driven events such as clathrin-mediated endocytosis (28–30), or in fission steps mediated by other machineries, like SAR1-dependent ER-to-Golgi transport

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(31, 32). Because of these multiple roles, changes in efficiency of the BARS-dependent fission machinery can lead to global reconfiguration of the trafficking system (11).

Unusually, despite their biological relevance, the molecular machinery by which BARS drives membrane fission and the switch mechanism between trafficking and transcriptional functions have been investigated less extensively than other transport machineries and remain poorly understood. In this study, we used multiple complementary approaches, including reconstitution of membrane fission with defined components, various electron microscopy (EM) techniques, structural modeling, and quantitative fission assays, to define the minimal molecular machinery required for BARS-dependent fission and to elucidate the BARS switch mechanism, which appears to be structurally tightly linked to the BARS dual function.

Membrane fission can be driven by various mechanisms, including mechanical constriction, amphipathic helix (AH) insertion, lipid phase separation, and friction-induced cleavage (28, 33–38). Despite this diversity, the outcome is conserved: separation of continuous membrane compartments via molecular rearrangements in the bilayer, with scission occurring without content leakage (33, 39, 40). In the case of BARS, our previous work showed that fission during trans-Golgi network (TGN) to plasma membrane transport involves a protein complex comprising PI4KIII β , 14-3-3 γ , FAPP2, ARF, the kinases PKD and PAK, and the lysophosphatidic acid acyltransferase δ (LPAAT δ /AGPAT4) (12, 13). Within this complex, BARS activates LPAAT δ , promoting the conversion of lysophosphatidic acid (LPA) into phosphatidic acid (PA). This reaction is essential for fission (13, 41). However, how it leads to final membrane cleavage remains elusive. Moreover, other BARS-dependent fission processes have been studied and found to require BARS interaction with route-specific protein complexes and with different acyltransferases (e.g., LPAAT γ in COPI-dependent traffic) (23), presumably to adapt to the features of different cellular compartments. The common thread appears to be the enzymatic conversion of LPA to PA, suggesting that this reaction may constitute a conserved core of BARS-dependent fission. Nevertheless, the precise composition and operation mode of this core machinery has remained undefined until the present study.

To identify the minimal requirements for BARS-dependent fission, we reconstituted the process *in vitro* using purified BARS, LPAAT δ , acyl-CoA, and unilamellar liposomes. Fission proceeds through two interdependent steps. First, monomeric BARS engages LPAAT δ via specific surface interactions, activating PA synthesis from LPA at the membrane site. Second, BARS binds the newly synthesized PA through a specific arginine residue and inserts into the PA-enriched membrane via an AH located in its C-terminal region. This PA-guided shallow insertion induces membrane curvature, tubulation, and ultimately fission. Notably, this mechanism is unique in that BARS not only drives the local metabolic reaction, LPA-to-PA conversion, but also exploits the resulting lipid product to execute the mechanical scission event. Thus, BARS integrates enzymatic lipid remodeling with induction of membrane curvature into a minimal core module.

Building on these insights, we also analyzed the molecular logic underlying the metabolism-dependent functional switch of BARS. The switch mechanism appears to be embedded in the structural architecture of the BARS-dependent machinery itself. Under conditions of metabolic stress, such as hypoxia, NADH levels rise and bind the Rossmann fold of BARS, stabilizing its dimeric form (11). In this conformation, BARS no longer exposes the residues required for LPAAT δ activation, nor can it insert its AH into the membrane.

As a result, BARS disengages from its fission partners, inactivating several trafficking routes and inducing a global reconfiguration of the intracellular transport network. This remodeling supports cell survival under stress (11). Concurrently, dimeric BARS tetramerizes, binds transcription factors, and translocates into the nucleus, where it participates in the regulation of stress-adaptive gene expression programs, further promoting cellular resistance to energy stress.

The ability of BARS to switch between membrane transport and transcriptional functions in response to metabolic cues contributes not only to physiological adaptation but also to pathological processes, including tumor chemoresistance, metabolic syndrome, diabetes, and genetic neurological disorders (42–46). The Rossmann fold of BARS, which mediates this conformational switch, represents a druggable site. Our findings thus suggest a pharmacological opportunity: Targeting the BARS switch could provide a unique means to modulate membrane trafficking and genes controlling apoptosis and metabolic homeostasis to treat diseases characterized by imbalances in these systems.

RESULTS

Reconstitution of core BARS-dependent fission with minimal defined lipid and protein components

On the basis of previous studies in live-cell models, BARS-dependent fission is thought to require, in addition to BARS itself, the acyltransferase LPAAT and its substrates, acyl-CoA and LPA, which together generate PA and trigger membrane fission (13). However, additional components such as 14-3-3 γ , ARF, and PAK are involved in fission of post-Golgi carriers *in vivo*, though their role in the core machinery versus regulatory function remains unclear (12). Moreover, partially different partners are involved in other transport steps with BARS (11, 23, 24, 26, 47). To define the minimal core system, we used two artificial membrane platforms and two LPAAT isoforms: mammalian LPAAT δ and PlsC, the *Escherichia coli* acyltransferase (henceforth *EcLPAAT*) (48). Notably, *EcLPAAT* binds and is activated by BARS, promoting membrane fission similar to LPAAT δ (13). This interaction may reflect an evolutionarily conserved recognition mechanism between ancestral bacterial forms of mammalian acyltransferases and of BARS (see below).

Giant unilamellar vesicles (GUVs) are convenient tools to study membrane fission and tubulation (49–53). We first used GUVs [~ 20 μ m diameter; Golgi-membrane like lipid composition; (50) and Materials and Methods] to monitor membrane remodeling in real time via confocal video microscopy (see Materials and Methods and Fig. 1, A to E). GUVs were added with 1% LPA and a fluorescent lipid tracer; they remained stable under basal conditions (Fig. 1B). We then added BARS, oleoyl-CoA, and soluble *EcLPAAT* (see Materials and Methods): BARS or *EcLPAAT* alone with oleoyl-CoA had no effect (Fig. 1, C and D), but the full combination triggered the fission process (Fig. 1A). Membrane tubules (200 to 400 nm diameter) emerged from the GUV surface and elongated over ~ 2 min, developing evenly spaced constrictions (pearling) that led to fission and the release of spherical vesicles (Fig. 1A and movie S1). Tubulation, pearling, and vesicle release continued, gradually depleting the GUV surface area over ~ 2 hours. Instead, in the presence of NADH-bound BARS, neither vesicle tubulation nor activation of *EcLPAAT* was observed (Fig. 1E). In parallel, a PA increase was recorded under conditions identical to those used in the imaging analysis (Fig. 1, A to E), supporting the occurrence of bilayer tubulation and fission (Fig. 1F).

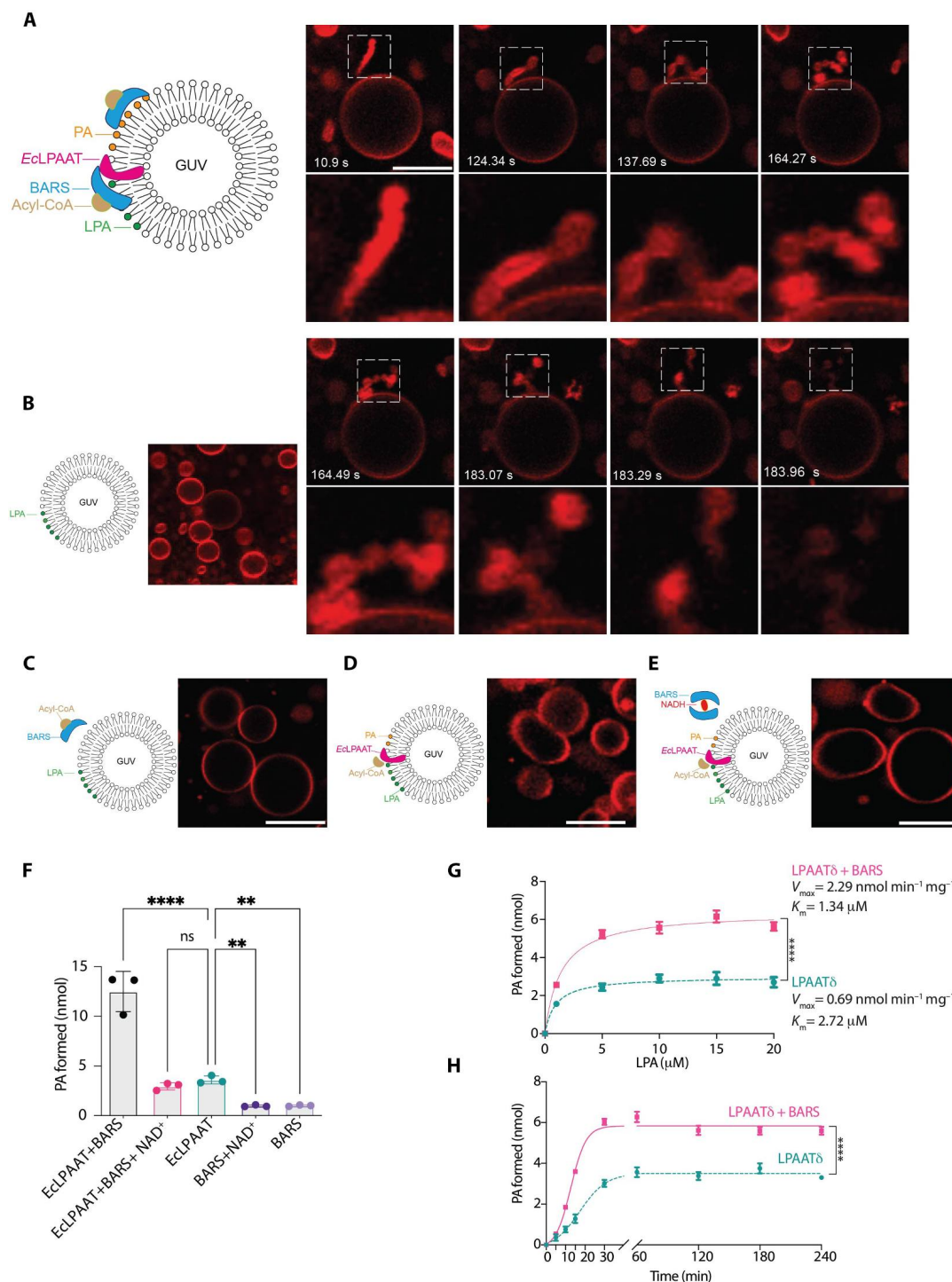


Fig. 1. BARS-induced membrane fission on GUVs and LUVs. (A to E) Time-lapse images and magnified views (white dashed boxes) of Rhodamine-PE-labeled GUVs under the indicated conditions (see Materials and Methods for GUVs preparation). For each panel, the schematic representation of the addition of BARS and/or Acyl-CoA and/or EcLPAAT is shown on the left. GUVs were incubated for 30 min with (A) BARS + *E. coli* LPAAT (EcLPAAT) + oleoyl-CoA, or (C) BARS + oleoyl-CoA, or (D) EcLPAAT + oleoyl-CoA, or (E) NADH-bound BARS + EcLPAAT + oleoyl-CoA. An image of GUVs recorded without the addition of any of the above compounds is shown in (B). Scale bars, 10 μ m. (F) Quantification of phosphatidic acid (PA) produced in the LPAAT activity assay including EcLPAAT and purified BARS, in the presence or absence of NADH (see Materials and Methods). Data are means $\pm$ SD of three independent experiments. **** $P \leq 0.0001$, ** $P \leq 0.01$ versus EcLPAAT alone (one-way ANOVA with Sidak's multiple comparisons test). (G) Dose response of PA production in the LPAAT assay (in LPAAT δ -containing LUVs, see Materials and Methods) with 1 to 20 μ M oleoyl-LPA and minus (●, blue dots) or plus (■, magenta dots) BARS. Kinetic values are indicated in the figure. (H) Time course of PA production in the LPAAT assay, conditions as in (G), including 5 μ M oleoyl-LPA, in the absence (●, blue dots) or presence (■, magenta dots) of BARS. Data are means of three independent experiments $\pm$ SD. **** $P \leq 0.0001$ versus LPAAT δ^{WT} -containing LUVs (one-way ANOVA with Sidak's multiple comparisons test).

In a second set of experiments, we used large unilamellar vesicles (LUVs, 160 to 240 nm in diameter) to use a system more amenable to *in vitro* assays and quantification of fission parameters. The LPAAT δ (a three-span transmembrane domain protein) was purified as detailed in Materials and Methods (fig. S1, A to C) and reconstituted into “Golgi-like LUVs” (49) containing 1% LPA. The dose response and time course of LPAAT δ enzymatic activity were evaluated in control or in BARS-added LUVs (Fig. 1, G and H, and fig. S1D). Negative staining EM (NS-EM) showed that the addition of BARS and oleoyl-CoA induced membrane fission with morphology similar to that seen in GUVs. Tubular profiles (~25 to 30 nm diameter) emerged from the LUVs, many displaying constrictions and pearling, and eventually generating vesicles of comparable diameter (Fig. 2A). A gradual decrease in the number and size of LUVs could be observed accompanied by a corresponding increase in vesicles of ~25 nm in diameter (Fig. 2, A and C). Smaller vesicles were not observed (table S1). The vesicle size and bilayer integrity were confirmed by cryo-EM (fig. S1E). Cryo-EM also showed that the BARS and/or LPAAT δ proteins could be visualized as nonbilayer amorphous bodies in the membrane of the LUVs (Fig. 2D).

These findings suggest that LUVs are gradually consumed (over ~2 hours) through tubule extrusion followed by vesiculation and that the fission process continues until the LUVs are converted into ~25-nm vesicles. This size likely represents the lower limit for vesicle formation under the lipid composition used.

Notably, the BARS fission system operates efficiently on membrane tubules of varying diameters, in both GUVs and LUVs, indicating that it functions without a need for rigid structural constraints.

To quantitatively assess BARS fission activity, we measured the reaction efficiency using a characterized lipid sedimentation assay (35). Following ultracentrifugation, LUVs and small vesicles were separated, and the fission index (vesiculation rate) was used to quantify the efficiency of fission as previously described (see Materials and Methods). When BARS and oleoyl-CoA were added to LUVs containing membrane-integrated LPAAT δ , ~25- to 30-nm vesicles accumulated in the supernatant, whereas BARS or LPAAT δ alone had no effect (Fig. 2E). These results confirm that a minimal system composed of liposomes, BARS, LPAAT δ , and its substrates, LPA and acyl-CoA, is necessary and sufficient to recapitulate the fission process observed *in vivo*.

BARS-dependent fission requires the activation of LPAAT δ to catalyze LPA-PA conversion

To evaluate the role of LPAAT δ in the fission reaction, we used a PA synthesis assay based on radiolabeled acyl-CoA incorporation into LUVs under the same conditions as the fission assay (as detailed in Materials and Methods). BARS-activated LPAAT δ converted ~1% LPA into PA within ~1 hour, yielding a final PA concentration of ~0.7%. This value derives from 6.30 nmol that was the amount of PA obtained under the experimental conditions (see Materials and Methods). The conversion in the absence of BARS was negligible. PA production is spatially restricted at fission sites, and therefore, the measured average PA increase underestimates the local concentration experienced by BARS.

Previous studies identified His⁹⁶, located in the conserved acyltransferase catalytic motif I (NHX₄D) of LPAAT δ , as a critical residue for catalysis (13), while Lys¹²³ and Lys¹²⁴ were predicted to be essential for LPAAT δ activity due to their potential interactions with the LPA phosphate group (41). To test the requirement for

enzymatic activity for fission, we generated a triple point mutant of LPAAT δ ^{H96A/K123A/K124A} (designated LPAAT δ ^{CI}, catalytically inactive) designed to inactivate its catalytic site. This mutant lost both basal and BARS-stimulated PA-production (fig. S2A), while retaining normal binding to BARS, as confirmed by co-immunoprecipitation (co-IP; fig. S2B).

Moreover, pharmacological inhibition of LPAAT δ with the established inhibitor CI-976 (50) as well as the expression of the LPAAT δ ^{CI} mutant strongly suppressed the fission reaction (Fig. 2, B, C, and E), confirming that LPAAT δ catalytic activity with LPA-PA conversion is essential for BARS-mediated membrane fission.

Because PA can be produced in cells via additional pathways (51, 52), we also asked whether PA synthesis via acyltransferase activity is required for membrane fission, or PA is sufficient to trigger the process regardless of its biosynthetic origin. To address this, we generated liposomes containing increasing concentrations of exogenous PA and tested the extent of BARS-induced fission. While no fission could be detected in the absence of PA, exogenously added PA did support fission (Fig. 3A). It is noteworthy that the added PA was much less “efficient” than the enzymatically synthesized one, as the same fission index (see the “Membrane fission assay” section) was achieved with 10% of the former (Fig. 3A) versus only ~0.7% of the latter “type” of PA (Fig. 2E) at identical BARS concentrations (see Discussion). This large difference presumably reflects the fact that LPA-to-PA conversion generates lipid packing defects that facilitate fission (see below) and/or that LPAAT δ generates PA near the BARS AH. Altogether, considering that LPAAT δ activity is strictly required for BARS-dependent fission in live cells, we conclude that enzymatic PA synthesis represents the dominant, if not exclusive, mechanism for fission under physiological conditions.

The morphology of fission intermediates was similar under both conditions, whether PA was supplied exogenously or generated enzymatically (Figs. 3, B and C, and 2A). The time course of the formation of small vesicles (~30 nm in diameter) in PA-enriched LUVs, with tubulation and constriction points (~10 to 26 nm in diameter) recapitulating the hallmarks of BARS-mediated fission is also shown (Fig. 3, B and C).

To further enrich BARS-mediated fission intermediates (that could be partially affected by NS-EM staining) and preserve their native state, we used EPON embedding and cryo-EM, respectively (Fig. 4, A and B). Of note, both approaches showed similar fission intermediates (tables S2 and S3) and revealed the presence of constriction points of about 10 to 11 nm (see table S4 for detailed values).

The range of cryo-TEM-detected membrane constrictions ranged from a minimal diameter of approximately 15 nm to a minimal diameter of 9 nm; no narrower structures were observed (Fig. 4C). This dimension likely represents the lower threshold below which membrane fission proceeds rapidly to completion (table S4).

We also assessed the BARS distribution across different membrane regions. We performed quantitative analysis using NS-EM coupled with immunogold labeling. BARS was found to be strongly enriched, by more than 100-fold, at vesicle necks and constriction sites compared to flat regions of the liposome membrane (see Fig. 4D, table S5, and Materials and Methods for details of the morphometric analysis and surface density calculation). Briefly, the extreme local concentration of BARS at sites of high membrane curvature is compatible with an enrichment in the hundreds of folds, as assessed by quantitative morphometric analysis (see Materials and Methods and table S6).

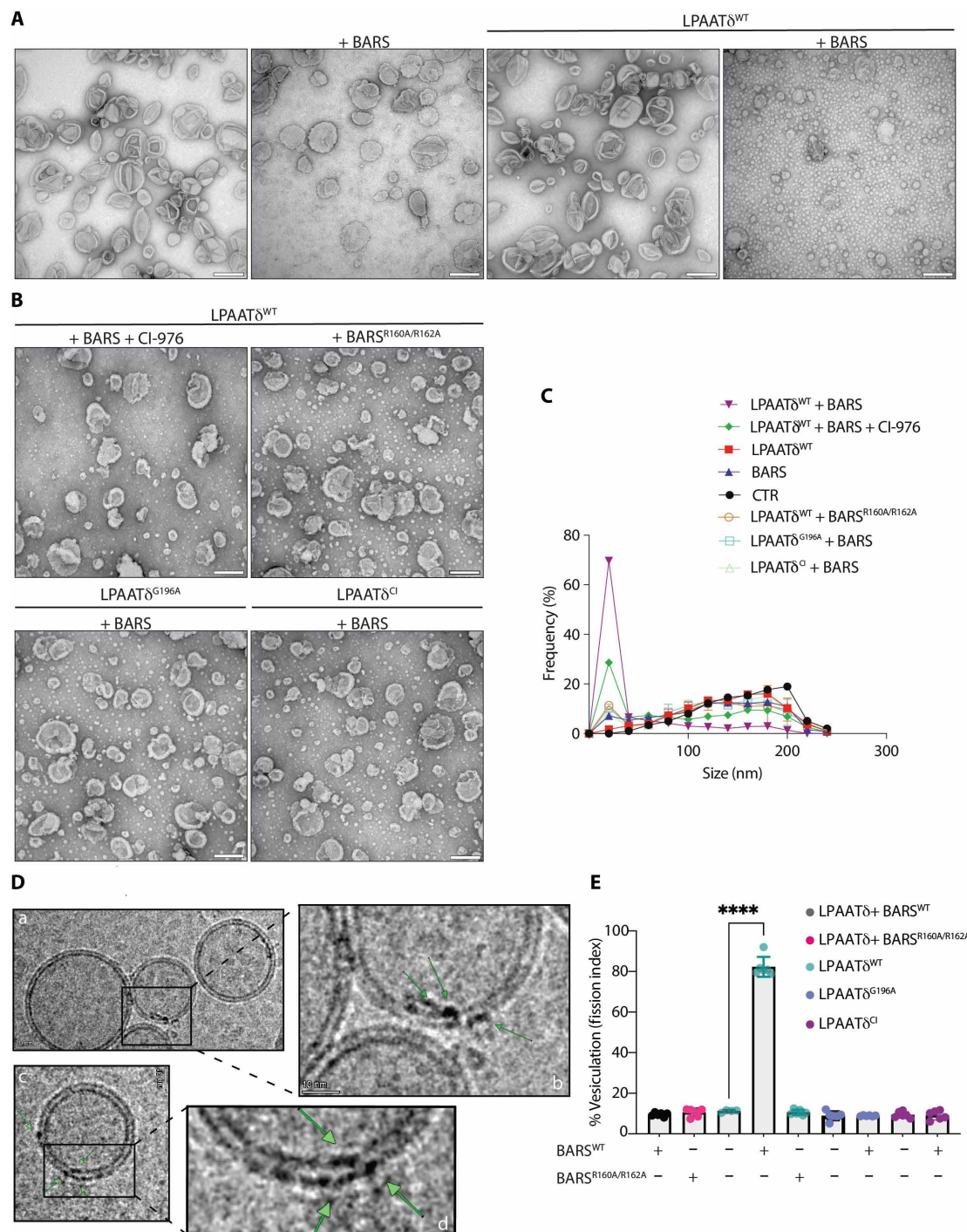


Fig. 2. Morphometric analysis of BARS- and LPAAT δ -induced fission in LUVs analyzed by NS- and cryo-EM. (A) Representative NS-EM of LUVs containing 1% LPA, in the presence of BARS without (left panels) or with (right panels) LPAAT δ^{WT} ; or (B) in the presence of LPAAT δ^{WT} and BARS plus the LPAAT δ inhibitor CI-976, or in the presence of the mutant BARS^{R160A/R162A} (top panels) or BARS and the mutant LPAAT δ^{G196A} , or BARS plus the inactive LPAAT δ^{CI} (bottom panel; see Materials and Methods). Scale bars, 200 nm. (C) Size distribution analysis (Frequency) of the vesicles formed in (A) and (B). The analysis included 100 liposomes in three different EM grids (see Materials and Methods). The color codes are indicated. (D) LUVs analyzed by cryo-EM (A and C) show the integrity of the bilayer and the association of the added proteins to it [BARS and LPAAT δ , magnified (B) and (D)] (see Materials and Methods). More than 50 vesicles were analyzed. Scale bars are indicated. (E) LUVs fission index, indicated as % of vesiculation, evaluated by lipid co-sedimentation assays (see Materials and Methods) under the conditions indicated in the figure by color coding.

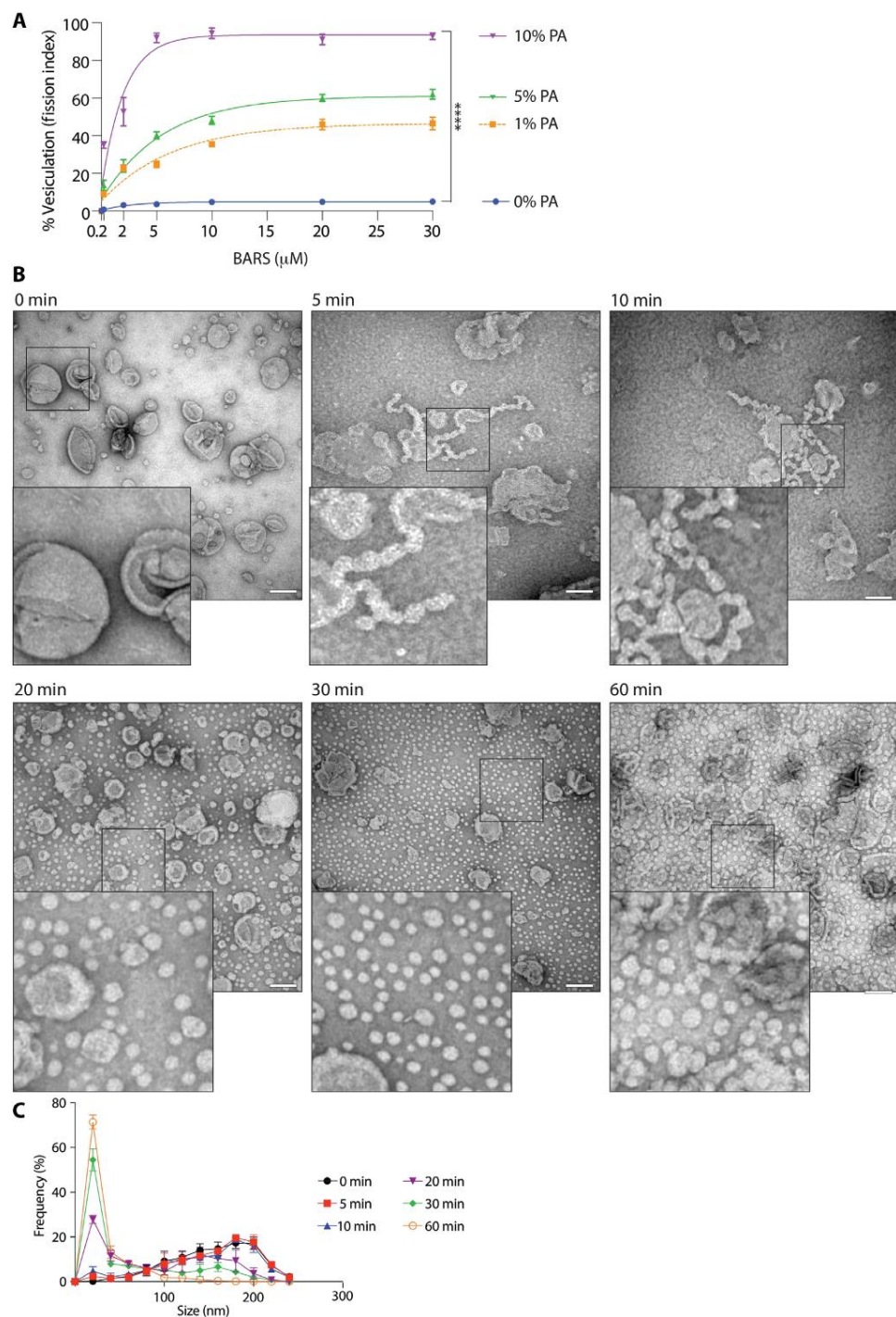


Fig. 3. BARS-induced fission in PA-enriched liposomes. (A) Dose response of fission induced by BARS at varying PA concentrations (0, 1, 5, and 10%; see Materials and Methods). **** $P \leq 0.0001$ for each BARS concentration versus 0% PA (two-way ANOVA Tukey's multiple comparisons test). (B) Representative negative-staining images: time course of the fission of LUVs containing 10% PA and added with 5 μ M BARS. Scale bars, 200 nm. (C) Size distribution analysis (Frequency) at different times as described in Fig. 2C. The color coding is indicated.

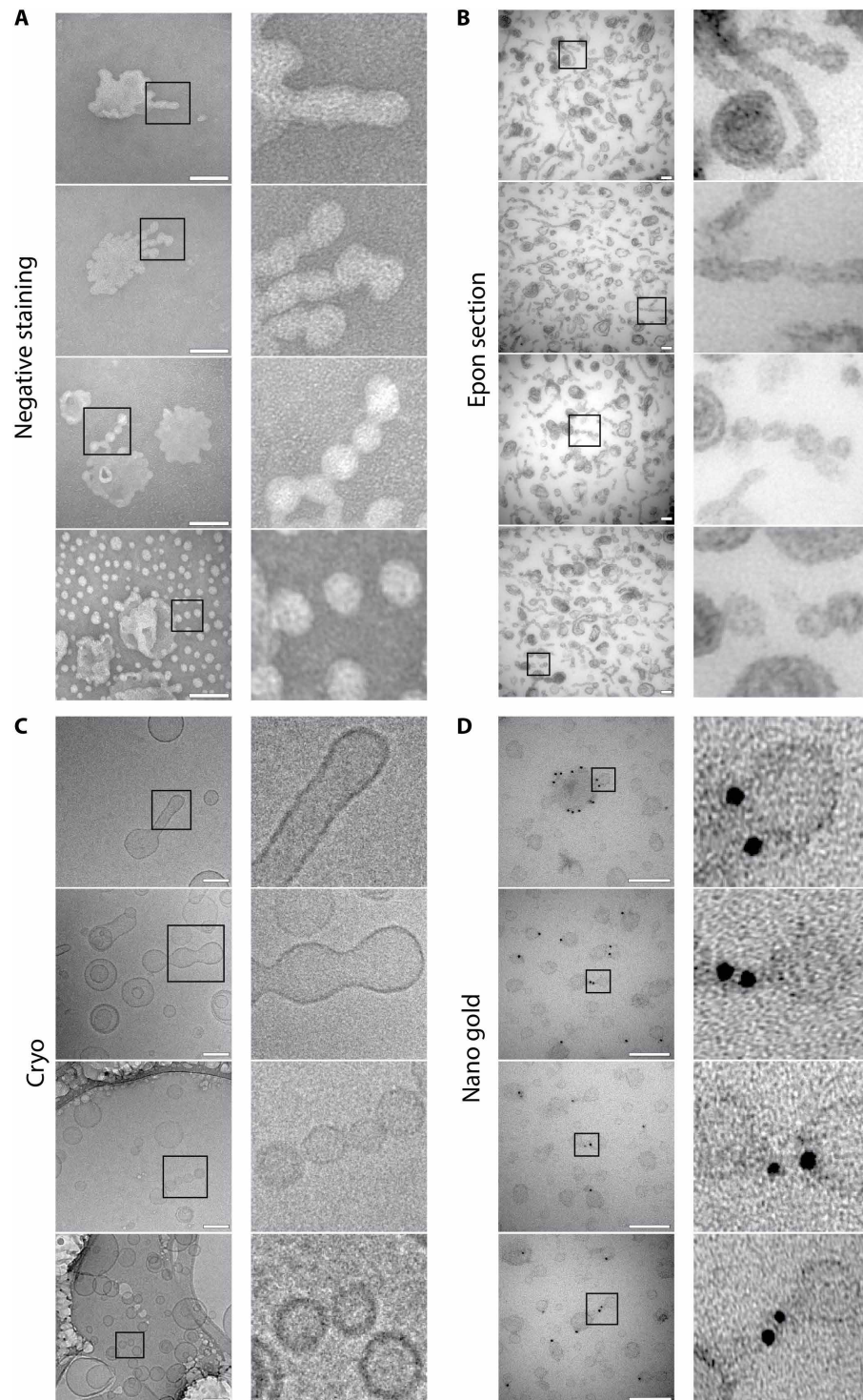


Fig. 4. Ultrastructural EM analysis of BARS-mediated fission steps. (A to D) Representative images of LUVs (including 10% PA and 5 μ M BARS^{WT}) by (A) negative staining, (B) Epon embedding, (C) cryo-TEM, (D) immunogold labeling with anti-BARS and Protein A-gold; magnified insets are shown on the right of each panel. See Materials and Methods for experimental details. Images are representative of more than three independent experiments. Scale bars, 100 nm.

Mechanism of LPAAT δ activation by BARS

To clarify the mechanism by which BARS activates LPAAT δ , we sought to identify their interacting surfaces. As a first step, we investigated the LPAAT δ residues potentially involved in binding BARS. Given that both human LPAAT δ and *E. coli* LPAAT bind BARS (13), we aligned the sequences of these two enzymes and searched for short stretches of high similarity, then introduced mutations in selected residues within those regions (see the Supplementary Materials and note S1). Mutation of Gly¹⁹⁶ in LPAAT δ abolished its interaction with BARS in both co-IP and pull-down assays (fig. S2, B and D), suggesting that this residue lies within the BARS-binding interface. We also identified several anionic residues near Gly¹⁹⁶ on the LPAAT δ surface and hypothesized that they might interact with regions in BARS we had noticed to contain couples of cationic residues (see the Supplementary Materials, note S2, and fig. S9). When Arg¹⁶⁰ and Arg¹⁶² in BARS were mutated to alanines (BARS^{R160A/R162A}), the mutant protein lost the ability to bind mammalian LPAAT δ (fig. S2, D and E). Together, these findings demonstrate that Gly¹⁹⁶ in LPAAT δ and Arg¹⁶⁰ and Arg¹⁶² in BARS are critical for the BARS-LPAAT δ interaction. The BARS^{R160A/R162A} mutant fails to bind wild-type LPAAT δ , and conversely, the LPAAT δ ^{G196A} mutant fails to bind wild-type BARS (fig. S2D).

We also tested the role of the above-defined BARS-LPAAT δ interaction in LPAAT activation by assaying PA production in LUVs. The BARS^{R160A/R162A} mutant failed to activate wild-type LPAAT δ , and the LPAAT δ ^{G196A} mutant could not be activated by BARS^{WT} (fig. S2F), indicating that the interaction between these specific residues is required also for PA synthesis activation. Furthermore, BARS mutants deficient in LPAAT δ binding and activation were unable to induce membrane fission in the same LUVs (Fig. 2, B, C, and E). In summary, the interaction between the BARS region containing Arg¹⁶⁰ and Arg¹⁶² and the LPAAT δ segment encompassing the Gly¹⁹⁶ is required for protein binding between the two proteins and for acyl-transferase activation and fission.

Mechanism of BARS-induced fission of PA-enriched membranes through shallow insertion of an AH guided by PA binding via Arg³³¹

We have so far established that PA synthesis by BARS and LPAAT δ drives membrane fission. However, the molecular steps leading from PA production to membrane scission remain unclear. We have examined the BARS features, in search for structural clues. BARS consists of an NBD and an SBD, with the CTD, also known as C-terminal region, being part of the SBD (9, 10, 21, 22) (see Fig. 5A). The NBD domain contains a NAD(H)-binding Rossmann fold (9, 53).

Using HELIQUEST (54) for segments with amphipathic properties in the BARS sequence, we identified a few candidates exhibiting varying hydrophobic moments. Among them, the segment spanning residues [Arg³²⁶-Asn³⁴¹] displayed a hydrophobic moment of 0.569, exceeding that of AHs from known fission-inducing proteins (Fig. 5B) (55–59). This value is sufficient to support membrane insertion despite the presence of a single hydrophilic residue on the hydrophobic face. We thus examined the possibility of interaction between the BARS AH and the membrane (see Materials and Methods and fig. S3). Although most of the AH-containing segment (i.e., AH and a few residues upstream and downstream) could be involved in shallow membrane insertion (35) with residues 310 to 329 already folded into an α helix, downstream residues appear unstructured (fig. S3A). The modeled helix displays a hydrophobic face composed of Ile³¹⁴,

Ala³²¹, Ile³²⁴, Ile³²⁸, Ile³³², Leu³³⁶, and Cys³³⁹ (fig. S3B), and a hydrophilic face that includes key charged residues such as Arg³²², Arg³²⁶, and Lys³³⁷. Membrane interaction promotes extension of the α helix from residues 310 to 341, yielding a longer AH capable of membrane insertion. It is known that protein segments capable of becoming AH often become completely helical upon membrane interaction. For instance, the AH segments of endophilin A1 (60, 61), α -synuclein (62), epsin (63), and influenza virus M2 (64) become completely helical (i.e., fold) upon membrane binding. They are referred to as membrane-induced AHs (55). To investigate this, we performed far-ultraviolet (UV) circular dichroism (CD) spectroscopy of full-length BARS showing that BARS undergoes measurable structural changes upon binding to LUVs. The CD spectra suggest that unstructured regions of BARS, including the predicted AH segment, adopt an α -helical conformation upon membrane interaction, with this folding enhanced in the presence of PA. In contrast, the BARS^{I332A/L336A} mutant fails to exhibit PA-dependent increases in helical content (Fig. 5C).

Also important, the model indicates that the AH remains rotationally flexible, allowing it to align parallel to the membrane surface for optimal insertion (fig. S3C). To assess the functional relevance of the identified AH, we performed site-directed mutagenesis targeting hydrophobic residues (see Materials and Methods). Large hydrophobic residues on the hydrophobic face of the AH were substituted with Ala to reduce the hydrophobic moment without disrupting the α -helical structure (54). Similar strategies have been shown to impair AH-mediated membrane interactions in other systems (56–59). We focused on Ile³²⁴, Ile³²⁸, Ile³³², and Leu³³⁶, all within or adjacent to the AH, and generated three double mutants: BARS^{I324A/I328A}, BARS^{I328A/I332A}, and BARS^{I332A/L336A} (Fig. 5, A and D). BARS^{I332A/L336A} failed to support membrane fission in multiple *in vitro* assays (Fig. 5, E to H, and fig. S4A), indicating that Ile³³² and Leu³³⁶ are critical for the fission activity of BARS.

Also of note was the presence of several positively charged residues in the AH located at an intermediate position between the hydrophilic and hydrophobic faces. These Arg residues appear to extend their positive charges toward the membrane surface, potentially enabling electrostatic interactions and hydrogen bonding with PA headgroups (fig. S4, B and C). To test this hypothesis, we generated point mutations replacing Arg³³¹ and Arg³²⁵ with tryptophan, which lacks a positive charge. The Arg³³¹Trp mutant was unable to trigger membrane fission *in vitro* and in intact cells (fig. S4, D to G), and the Arg³²⁵Trp mutant had a partial effect, confirming the critical role of the arginines.

To further assess the role of the AH, we tested a segment including the CTD of BARS and the AH peptide (Fig. 6). This segment induced membrane fission efficiently (Fig. 6, A to C). We also introduced mutations to increase the hydrophobicity of the AH, substituting Ile³³² and Leu³³⁶ with the bulkier and more hydrophobic Trp (65, 66). The mutations potentiated the fission-inducing capacity of the AH (Fig. 6A), confirming that the AH segment is involved in membrane fission and indicating that its activity correlates with its hydrophobic moment.

Overall, these findings support a model in which BARS-mediated fission requires hydrophobic interactions with membranes via the AH hydrophobic face as well as electrostatic PA binding via Arg³³¹, probably to favor the docking of the AH peptide to the target membrane and the optimal depth of membrane insertion (see Discussion). It is important to note here that these data prove that the BARS Arg³²⁶-Asn³⁴¹ helical segment is necessary for fission; however, they do not prove that it is sufficient. Other regions of the protein might have a complementary role in the fission process.

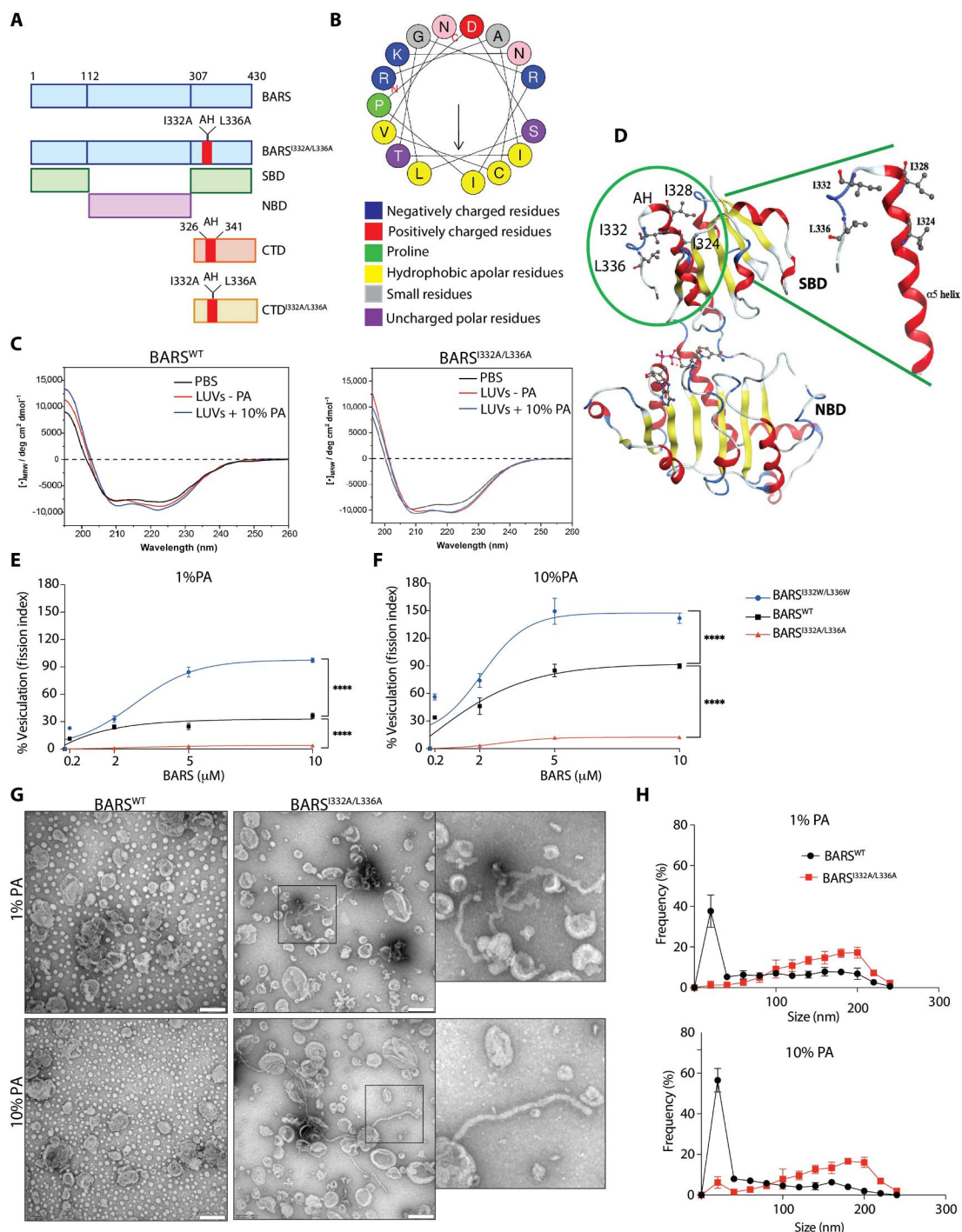


Fig. 5. Amphipathic helix insertion drives BARS-induced fission. (A) Schematic representation of BARS full-length (amino acids 1 to 430, light blue), SBD (amino acids 1 to 112 and 307 to 430, green), NBD (amino acids 113 to 306, magenta), C-terminal domain (CTD) (amino acids 307 to 430, orange), and CTD^{I332A/L336A} (amino acids 307 to 430, yellow). AH is represented as red bars. (B) Helical wheel of BARS segment (Arg³²⁶-Asn³⁴¹) with color-coded residues as indicated at the bottom of (B). (C) Far-UV CD spectra of BARS^{WT} and BARS^{I332A/L336A} mutant in LUV buffer alone (black line), in the presence of LUVs (red line) and in 10% PA-enriched LUVs (blue line) at a 200:1 lipid-to-protein mole ratio (see Materials and Methods). (D) Structure of full-length BARS showing domains and AH. Yellow ribbons: β strands; red helices: α helices; SBD: substrate-binding domain; NBD: nucleotide-binding domain; AH: amphipathic helix; green circle: details of BARS-AH; side-chain sticks (green): lipid binding sites (see the main text for details). (E and F) Dose response of the BARS^{WT}, BARS^{I332A/L336A}, and BARS^{I332W/L336W}-induced vesiculation as measured in fission assay and expressed as fission index in LUVs containing either 1% (E) or 10% (F) PA. The color coding is indicated on the right side of (F). **** $P \leq 0.0001$ for each BARS concentration versus BARS^{WT} (two-way ANOVA Tukey's multiple comparisons test). (G) Negative staining of LUVs containing either 1 or 10% PA and 5 μ M BARS^{WT} (left panels) or BARS^{I332A/L336A} (right panels). Magnified insets are shown on the right of the BARS^{I332A/L336A} panels. Scale bars, 200 nm. (H) Size distribution analysis (Frequency) of vesicles from (G). BARS^{WT}: full black dots; BARS^{I332A/L336A}: full red squares.

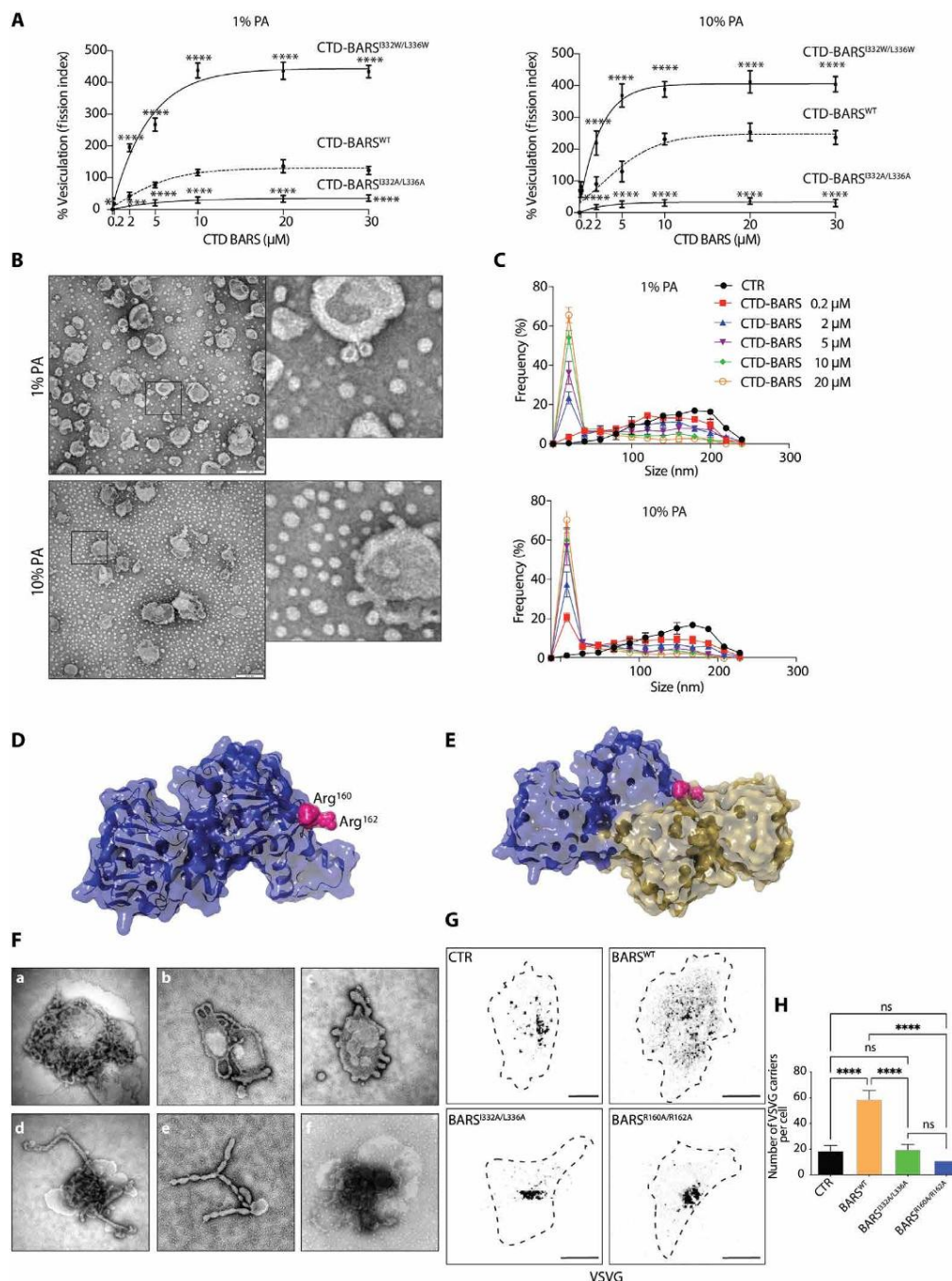


Fig. 6. BARS CTD is sufficient for membrane fission in vitro. (A) BARS CTD-induced membrane fission in vitro. Dose response of the fission index induced by CTD-BARS^{WT} or by BARS^{I332A/L336A} or BARS^{I332W/L336W} mutants (as indicated in the graphs) in LUVs formed with a 1% (left) or 10% (right) PA concentration. **P* ≤ 0.05, ****P* ≤ 0.001, and *****P* ≤ 0.0001 versus BARS^{WT} (two-way ANOVA Sidak's multiple comparisons test). (B) Images of NS-EM of LUVs formed with a 1% (top panel) or 10% (bottom panel) PA concentration incubated with CTD-BARS^{WT}. Magnified insets are shown on the right of the respective panels. Scale bars, 200 nm. (C) Quantification and vesicle size distribution (Frequency) in LUVs formed with 1% (upper graph) or 10% (lower graph) PA exposed to increasing CTD-BARS^{WT} concentrations; color coding and range of concentrations (0.2 to 20 μM) are indicated on the right of the upper graph in (C). (D and E) Surface models of BARS monomer (PDB ID 3GA0) and dimer (PDB ID 6V8A); Arg¹⁶⁰/Arg¹⁶² are highlighted in magenta and represent the LPAAT binding site (left model), which results hidden in the dimer (right model). (F) NS-EM of BARS-induced fission [(a) to (f)] on purified Golgi membranes isolated from KO-BARS HeLa cells incubated with BARS^{WT} (membrane preparation in Materials and Methods). Scale bars, 200 nm. (G) Confocal microscopy of KO-BARS HeLa infected with VSV and subjected to TGN-exit assay (see Materials and Methods). Cells microinjected or not (CTR) with recombinant BARS^{WT} or mutants defective in AH and LPAAT binding, BARS^{I332A/L336A}, or BARS^{R160A/R162A}, respectively (see panels), were processed for immunofluorescence detection with anti-VSVG-Cy3 antibody, to monitor the VSVG-containing carrier. Scale bars, 10 μm. (H) BARS^{WT}, unlike its mutants, rescues the impaired-carrier formation in BARS-KO HeLa cells. Quantification of the number of VSVG-positive carriers in (G). CTR: black column; BARS^{WT}: orange column; BARS^{I332A/L336A}: green column; BARS^{R160A/R162A}: blue column. *****P* ≤ 0.0001 versus CTR.

Arg³³¹ has been reported to be mutated to Trp in patients with a severe neurological HADDTS syndrome (Hypotonia, Ataxia, Developmental-Delay, and Tooth-enamel defects) (67–71). Given that BARS is essential for synaptic vesicle recycling following stimulation, a process crucial for maintaining synaptic function (72, 73), it is plausible that BARS-dependent fission contributes to the neurological symptoms observed in these patients (see Discussion).

Structural basis for the BARS switch between its membrane transport and transcription functions

As previously indicated, BARS has two alternative functions: one in the cytosol in membrane fission and transport, and another in the nucleus, in the transcriptional regulation of cellular programs such as apoptosis and energy/lipid metabolism (2, 11, 26, 53). The BARS paralog CtBP2 has only nuclear functions, which, to a large extent, overlap with those of BARS (18, 19), and, like BARS, exists in equilibrium between monomeric and dimeric states. Dimerization is strongly stabilized by NADH through tight packing of the two subunits, involving residue Trp³⁰⁷ (53, 74–77). In contrast, monomerization is promoted by acyl-CoAs, which compete with NADH for binding to the Rossmann fold due to structural similarity in the nucleotide-binding region (53, 78–80). Acyl-CoA-induced monomerization depends on the acyl chain length, as longer chains (e.g., oleoyl-CoA) cause steric hindrance protruding in the dimer interface (fig. S7, A to C) (78, 79). This is supported by experimental data on CtBP2 and by molecular docking simulations on BARS as well as by the structural identity of the Rossmann fold in the two paralogs (fig. S7, A to C) (74, 79–81). To obtain experimental data on the BARS transition between these two forms, we ran size-exclusion gel filtration experiments with purified protein (see Materials and Methods), which confirmed that acyl-CoA induces changes in the oligomerization state of BARS, from dimer to monomer (fig. S5A), whereas different concentrations of NADH caused a shift mainly to the tetrameric form, with a minimal dimeric form (fig. S5B). Under these conditions, the addition of NADH in fission assays blocked fission caused by BARS^{WT} (fig. S5C).

To clarify the effect of dimerization on BARS function, we used a monomeric BARS^{C123Y/N127R/R130A/R131A} mutant, which is unable to dimerize (82) (referred to as BARS^{Mono}, fig. S6A). These mutations prevented dimerization (fig. S6B) but did not affect the ability of BARS to interact with LPAAT δ in pull-down assays (fig. S6C), to activate LPAAT δ (fig. S6D), and to promote fission of PA-containing LUVs (fig. S6E). Altogether, these data confirm directly that only the monomeric form of BARS is competent for fission, and that the dimeric form, stabilized by NADH binding, is fission incompetent.

Thus, BARS is monomeric or dimeric depending on whether NADH or acyl-CoAs prevail in the competition for the Rossmann fold binding site, which in turn depends on cellular stress and metabolic states (11) (fig. S7D).

How does the monomer-dimer conformational change induce the switch between the two BARS functions? Analysis of the structural elements required for LPAAT δ activation and for AH insertion into PA-enriched membranes provides key insights. As noted, BARS activates LPAAT δ through the interaction of its Arg¹⁶⁰-Arg¹⁶² residues with the hydrophilic Gly¹⁹⁶-containing region on the LPAAT δ surface (see Fig. 6D). We find that in the monomeric structure, Arg¹⁶⁰-Arg¹⁶² are exposed on a surface loop and accessible for LPAAT δ binding (Fig. 6D). In contrast, in the dimeric state, the two arginines are engaged in dimerization interface contacts (Fig. 6E), rendering

them unavailable for LPAAT δ activation. As a result, dimerization prevents BARS-induced activation of LPAAT δ and PA synthesis.

Second, we compared the interaction between the BARS fission-inducing AH in monomeric or dimeric BARS and the PA-enriched membrane. Our model shows that, in monomers, the peptide is free to adopt the parallel orientation with respect to the membrane surface needed to insert optimally (fig. S3, A to C). In contrast, the structure of the dimer shows that the AH in each of the subunits is prevented from approaching the membrane due to steric hindrance from the other subunit, and this effect is even more pronounced in the tetrameric state (fig. S3, D and E). Thus, dimerization inhibits the BARS property to insert into the PA-enriched target membrane to initiate fission.

In summary, upon dimerization, BARS disengages from the fission machinery, losing its interaction with both LPAAT δ and the membrane. As a result, the functionality of the numerous BARS-dependent transport pathways is markedly reduced (see note S3). At the same time, BARS rapidly reorganizes into tetramers, exposing binding sites for transcription factors and acquiring the ability to translocate to the nucleus, where it regulates complex transcriptional programs in parallel with CtBP2 (83).

Experiments in intact cells validate the physiological relevance of the in vitro reconstitution data

Last, we validated the physiological relevance of the in vitro reconstitution using both isolated Golgi membranes and intact cells (3, 12, 13, 20). BARS induced fission on purified Golgi membranes derived from BARS-KO HeLa cells (see Materials and Methods), generating intermediates morphologically similar to those observed in the in vitro systems (Fig. 6F).

To assess this mechanism in living cells, we performed a Golgi-to-plasma membrane transport assay using the temperature-sensitive cargo vesicular stomatitis virus G protein (VSVG) (84). BARS-KO cells showed a marked reduction in carrier formation, with VSVG accumulating at the Golgi after release from the temperature block (Fig. 6, G and H). Microinjection of recombinant BARS^{WT} restored carrier formation, whereas BARS mutants (BARS^{I332A/L336A} and BARS^{R160A/R162A}) failed to rescue the phenotype, resulting in VSVG retention at the Golgi.

These results validate in living cells the mechanism defined in vitro and demonstrate that BARS-dependent fission requires both its structural determinants and the associated lipid remodeling pathway. Additional experimental details and extended analyses are provided in the Supplementary Materials (note S3).

Activation of the BARS functional switch by generating controlled metabolic imbalances

The conformational switch between monomeric and dimeric BARS, corresponding to the BARS roles in membrane fission versus transcriptional regulation, is governed by the competition between NADH and acyl-CoA for binding to the Rossmann fold, with metabolite levels reflecting the cellular metabolic state (11). To test how these metabolites influence the BARS switch in live cells, we used treatments designed to alter their intracellular levels. Oligomycin A, which inhibits mitochondrial respiration (85, 86), elevates NADH without inducing detectable toxicity over the experimental time frame (see Materials and Methods). We monitored its effects by tracking BARS translocation between cytosol and nucleus and by assessing the transport of the model cargo protein VSVG from the TGN to the plasma

membrane, a BARS-dependent step (see Materials and Methods and note S3) (12, 20). As predicted, oligomycin A caused BARS to translocate from the Golgi to the nucleus (Fig. 7A) and inhibited BARS-dependent VSVG export (Fig. 7B). In contrast, clathrin/dynamin-mediated endocytosis of transferrin (87, 88), a BARS-independent trafficking step (20), was unaffected (Fig. 7C). Concurrently, BARS association with its nuclear transcription factor client ZEB1 (89) increased markedly, as assessed by co-IP, suggesting that BARS had acquired a functional transcriptional role under high NADH conditions (Fig. 7D). Conversely, treatment with oleic acid, which is converted to oleoyl-CoA (90) in the cytosol, induced the opposite outcome: BARS relocated from the nucleus to the Golgi, and VSVG trafficking remained efficient (Fig. 7, B, E, and F). Last, sequential treatment with oligomycin A followed by oleic acid reversed the oligomycin-induced effects within minutes, restoring Golgi-localized BARS and reactivating VSVG transport (Fig. 7, G to I). Together, these findings support a model in which NADH and acyl-CoA act as metabolic signals that regulate the switch between functional state and cellular localizations of BARS, linking metabolic stress to intracellular membrane trafficking dynamics.

DISCUSSION

Our study defines a unified molecular mechanism of membrane fission in which lipid metabolism and membrane remodeling are directly coupled, and shows how this mechanism provides the structural basis for the metabolic control of BARS function. This coupling is biologically relevant because it coordinates two fundamental cellular processes—membrane trafficking and gene expression—that must be precisely balanced during metabolic stress to ensure cell survival. Below, we discuss some salient aspects of these observations.

Our reconstitution studies reveal that BARS-dependent fission operates through a different paradigm from established fission systems. Rather than relying on preexisting lipid pools maintained by upstream enzymes (35), BARS creates its own lipid cofactor through LPAAT δ activation and immediately exploits this newly synthesized PA for membrane insertion and scission. This self-contained mechanism provides clear advantages such as spatial specificity (fission occurs at sites of BARS-LPAAT δ interaction) and metabolic sensitivity (the process responds directly to acyl-CoA availability). The coupling of lipid enzymatic activity with fission dynamics represents a unique solution to the challenge of coordinating the activity of several segments of the membrane transport system with transcriptional control of metabolism.

Also notable is that this mechanistic foundation provides a structural logic for understanding BARS function switching capability. The monomer-dimer transition simultaneously controls both BARS functions through a simple structural logic. Dimerization driven by NADH binding creates two critical changes: The arginine residues essential for LPAAT δ activation become buried in the dimer interface, while steric hindrance prevents the AH from approaching membranes (fig. S3, D and E). This dual effect disengages BARS from the fission machinery while exposing transcription factor binding sites for nuclear function. The elegance of this switch lies in using the same structural elements, the arginines and AH, as both functional determinants for fission and regulatory switches for transcriptional control.

What determines the cellular concentrations of the main regulators of the BARS switch, NADH, and acyl-CoAs? Although their access

to the Rossmann fold may be modulated by regulatory mechanisms, such as acyl-CoA-binding proteins (91) or the AMP kinase, the levels of these metabolites remain the principal determinant of the two conformational states of BARS. Many physiological and pathological conditions can change their concentrations and hence induce the BARS switch. These include nutrient availability, mitochondrial activity, lipid flux, and redox balance (11, 44, 53, 77). NADH levels rise under conditions such as hypoxia, glycolytic dominance (as in the Warburg effect), or impaired oxidative phosphorylation (92), whereas acyl-CoA levels are influenced by lipid uptake, β -oxidation, and energy demand. Their relative abundance also depends on conditions such as hypoxia, obesity, diabetes, and cancer (42–46). Given that the BARS switch integrates its effects with those of several major regulators of metabolism and cell fate, including HIF1 α , AMPK, SREBP, and FOXO (17, 93, 94), the complexity of the system is such that a detailed analysis of the role of BARS requires quantitative models and is beyond the scope of this paper. However, certain pathological conditions provide interpretable entry points. For example, following DNA-damaging chemotherapy, tumor cells often experience metabolic collapse and activate proapoptotic signaling. In this context, elevated NADH levels stabilize dimeric BARS and CtBP2, creating transport conditions that enhance survival signaling, and leading to repression of apoptosis-related genes such as Bik, PUMA, and Noxa (95). This coordinated regulation can blunt the apoptotic response and promote tumor chemoresistance under stress.

The features of the reconstituted BARS-dependent fission mechanism align closely with those observed *in vivo*. Manipulations leading to changes in acyl-CoA and NADH levels have the effects expected based on our model on membrane trafficking and on the BARS nuclear-cytosolic translocation in living cells. Moreover, the requirement for monomeric BARS for active transport, the effects of mutating BARS Arg¹⁶⁰ and Arg¹⁶² (necessary for activating LPAAT δ), and the necessity for transport of the BARS hydrophobic residues in its AH as well as of Arg³³¹ and Arg³²⁶ for PA binding and membrane insertion, together, validate the physiological relevance of the *in vitro* model.

A remaining question is how LPA becomes available at the site of carrier formation in living cells. BARS has been shown to bind and activate phospholipase D (PLD) (72, 96), which generates PA from phosphatidylcholine. This PA can then be converted by phospholipase A2 (PLA2) into LPA (52), a lipid that preferentially accumulates in highly curved membranes such as forming tubules (97). Once enriched in precarrier tubules, LPA is rapidly reconverted into PA by LPAAT δ , activated locally by BARS, triggering fission. The curvature preferences of PLD and PLA2 support this spatially organized model: Both enzymes act primarily on flat or weakly curved regions (1, 98, 99), whereas LPAAT δ and BARS localize on tubular precarriers (13). This suggests a localized enzymatic cycle: PA is first synthesized on flat membranes, transformed into LPA, and then reconverted into PA specifically in curved carriers, where it drives deformation and scission. Underscoring the functional importance of this spatially restricted lipid remodeling loop, the pharmacological inhibition of PLD or PLA2 blocks the formation of BARS-dependent tubules. Naturally, in living cells, this mechanism operates within a broader regulatory network that controls the spatial and temporal dynamics of membrane fission (8, 100).

A final key question about the BARS switch is whether it exerts a greater influence on cellular responses through the changes in

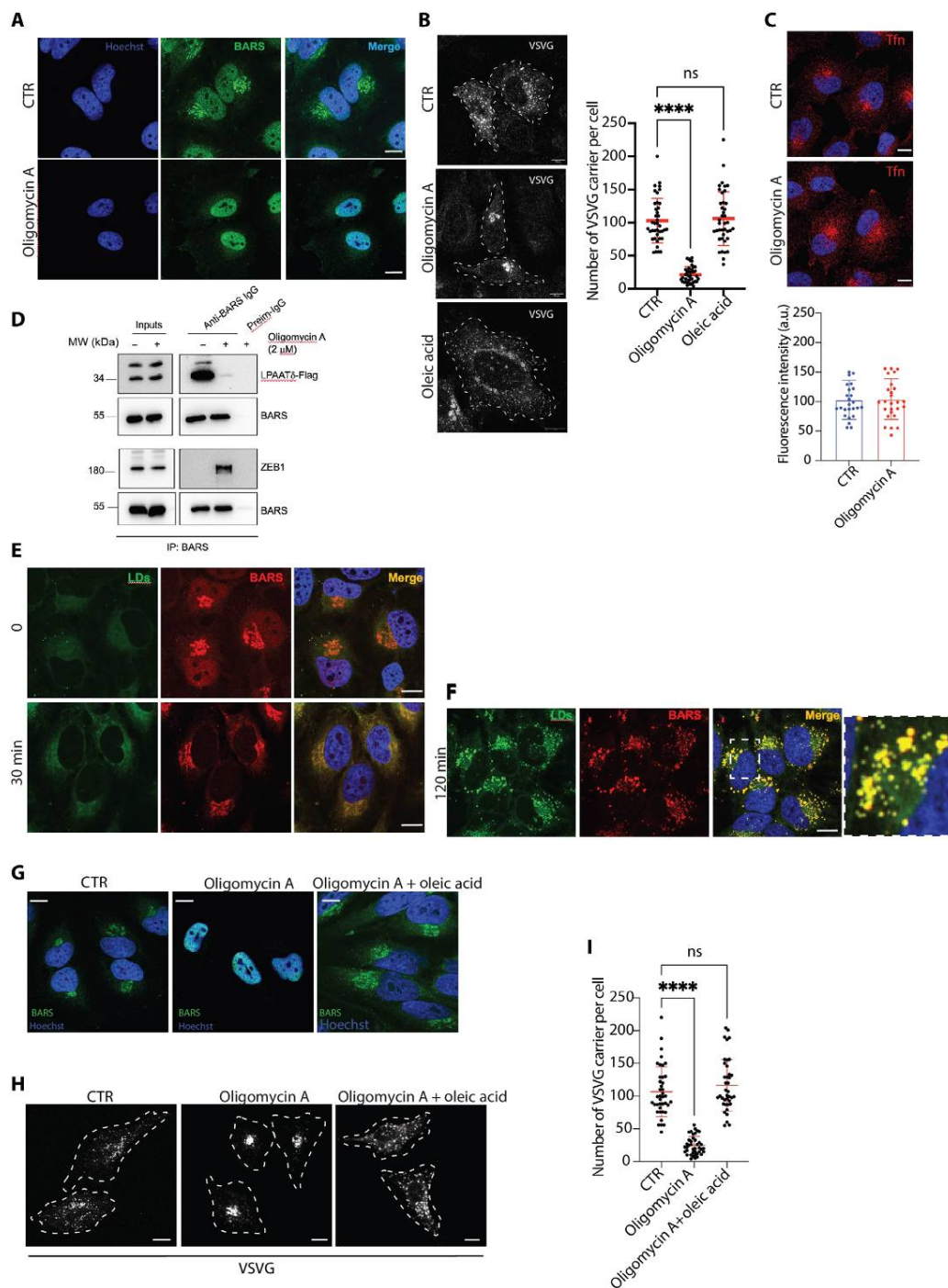


Fig. 7. The BARS monomer-oligomer switch regulates its dual function. (A) Confocal images of HeLa cells treated with DMSO (CTR) or with oligomycin A, stained with Hoechst 33342 (blue) and with anti-BARS antibody (endogenous BARS; green). Merged images are indicated. Scale bars, 10 μ m. (B) TGN-exit assay in VSV-infected HeLa cells treated with oligomycin A or with oleic acid; quantification of the number of VSVG-positive carriers per cell is indicated on the graph on the right of the panel. Scale bars, 10 μ m. **** P $\leq$ 0.0001 versus CTR (one-way ANOVA). (C) Transferrin (Tfn) internalization (Alexa-568-red signal) in HeLa cells with DMSO (CTR) or oligomycin A (see Materials and Methods). Quantification of internalized Tfn in CTR (blue dots) and oligomycin A (red dots) evaluated by fluorescence intensity is reported in the lower graph. (D) BARS immunoprecipitation (IP: BARS) of lysates from HeLa cells after oligomycin A treatment and coexpressing BARS and LPAAT6-Flag. Representative Western blotting (antibodies as indicated on the right of the blot) of total lysate (input) and immunoprecipitated proteins with preimmune-IgG (preim-IgG) or anti-BARS-IgG (as indicated). (E and F) HeLa cells treated with oleic acid at 0, 30, and 120 min, stained with BODIPY [lipid droplets (LDs), green], anti-BARS (endogenous BARS, red), and Hoechst (blue); inset in (F) shows a magnification of BARS and LD colocalization after 120 min of oleic acid stimulation. Scale bars, 10 μ m. (G) HeLa cells sequentially treated with oligomycin A and oleic acid, stained with Hoechst (blue) and anti-BARS antibody (endogenous BARS, green). Scale bars, 10 μ m. (H) TGN-exit assay in VSV-infected HeLa cells treated with oligomycin A or sequentially with oligomycin A and oleic acid; stained with anti-VSVG-Cy3. Scale bars, 10 μ m. (I) Quantification of the numbers of VSVG-positive carriers per cell from (H). **** P $\leq$ 0.0001 versus CTR (one-way ANOVA).

membrane trafficking or through transcriptional repression. We suggest that BARS' main innovation may lie in its ability to regulate membrane trafficking, particularly under stress conditions. BARS shares core transcriptional features with its nuclear paralog CtBP2, with a marked overlap in regulated gene networks, but uniquely contributes to cytoplasmic membrane trafficking, a function entirely absent in CtBP2. BARS, despite contributing to these transcriptional effects, is likely to operate primarily through the reconfiguration of the membrane transport system by modulating the transport rates of many pathways. In this model, BARS does not simply act as a partially redundant corepressor but rather integrates the membrane transport system into the cell's metabolic homeostasis and survival logic. The BARS switch, determined by NADH and acyl-CoA, occurs in parallel with the activation or inhibition of CtBP2 (79) functions and may complement CtBP2-driven transcriptional programs by ensuring that cells retain access to extracellular nutrients and mitogenic signals. This mechanism provides a direct link between metabolic state and membrane trafficking, enabling a single conformational change to coordinate distinct cellular functions.

More important, evidence suggests that under conditions such as hypoxia or oxidative stress, both normal and cancer cells can reconfigure their trafficking pathways to remodel their surface proteome, changing the composition of receptors and transporters to respond to environmental challenges (101). In this context, the BARS switch emerges as a yet uncharacterized major mechanism for adapting the membrane transport functions to physiological and pathological metabolic imbalances. At least six distinct segments of the intracellular transport network, and likely more, are BARS dependent and are partially but substantially inhibited under metabolic stress, resulting in large-scale reconfiguration of trafficking fluxes (11). Other major routes, including apical transport, clathrin-dependent endocytosis, and ER-to-Golgi trafficking, are dynamin dependent and likely respond differently to these stress signals (88, 102, 103). Collectively, these changes may substantially alter the regulatory and survival functions of the transport system. For example, under hypoxic stress, dynamin-dependent endocytosis of growth factor receptors proceeds normally, while BARS-dependent endosome-to-lysosome trafficking is markedly slowed (11). As a result, receptors such as EGFR accumulate in endosomes, where they continue to signal, promoting growth and survival during stress (11). Basolateral secretion of survival factors via the Golgin-245, which is BARS independent, also continues under stress. In contrast, the Golgin-97-dependent route, normally responsible for bulk transport, is BARS dependent and, when depleted of BARS, could likely contribute to energy conservation (20, 104, 105). Moreover, the slowing of BARS-dependent COPI-dependent retrograde trafficking may affect Golgi glycosylation, a modulator of many cellular functions (106–108). Altogether, the coordinated BARS-dependent remodeling of multiple trafficking routes can reshape the membrane landscape in major ways with broad functional consequences.

Several common human disorders, as well as rare genetic diseases, have been linked to CtBPs and BARS hyperactivation or loss of function (42, 80, 109). Among the latter, HADDTS (110) is particularly relevant to this study. It is caused by a point mutation Arg³³¹Trp of the cationic residue within the BARS AH, which we identify as essential for PA binding and membrane fission. The pathogenic effects of Arg³³¹Trp have been so far attributed to impaired transcriptional regulation (70). However, BARS plays a key role in synaptic vesicle recycling through its fission role (72, 73). Its dysfunction therefore

can disrupt vesicle-pool maintenance and neurotransmission. We now propose that, given the broad expression of BARS in the brain, the neurological symptoms of HADDTS may result, at least in part, from defective BARS-PA interactions and the consequently impaired intrasynaptic vesicle trafficking. The extent to which trafficking versus transcriptional defects drive this disease remains to be determined. Among the more common conditions linked to BARS dysfunction, the most studied are the metabolic syndrome, diabetes, and various cancers (42–46). BARS is frequently overexpressed in tumors and promotes tumor metabolic reprogramming, including the shift to aerobic glycolysis (Warburg effect), which supports growth and survival under hypoxic conditions (11, 111).

There is growing interest in pharmacological strategies targeting BARS (42, 81, 112, 113), including approaches from our group (83). Small molecules that modulate the BARS switch by binding its Rossmann fold offer promising therapeutic tools to reverse severe imbalances in membrane trafficking, signaling, and metabolism in disease. Targeting the BARS switch may offer a unique strategy to simultaneously modulate membrane trafficking and transcriptional programs in disease contexts characterized by metabolic imbalance.

MATERIALS AND METHODS

Plasmids

Generation of rat BARS constructs

To clone rat *BARS* coding sequence into the pcDNA3 mammalian expression vector, the rat *BARS* sequence was amplified with the primers Eco RI BARS fwd (5'-ATCCGGAATTCATGTCAGGCGTCCGACCTCC-3') and BARS Not I rev (5'-CATTCGCGGCCGCTACAACCTGGTCAGTCGTCATGGTC-3'). Then, polymerase chain reaction (PCR) fragment was digested with Eco RI and Not I restriction enzymes and cloned into pcDNA3 vector, previously digested with Eco RI and Not I restriction enzymes. The construct encoding the EYFP N-terminally tagged rat BARS protein was generated amplifying the rat *BARS* coding sequence with the primers Eco RI + BARS fwd (5'-ATCCGGAATTCATGTCAGGCGTCCGACCTCC-3') and BARS Sac II rev (5'-CATTCGCGGCCGCTACAACCTGGTCAGTCGTCATGGTC-3'). Then, PCR fragment was digested with Eco RI and Sac II restriction enzymes and cloned downstream to the EYFP protein into pEYFP-C1 vector, previously digested with Eco RI and Sac II restriction enzymes. The construct encoding the His N-terminally tagged rat BARS protein into pET-11D prokaryotic expression vector was generated by the restriction-free (RF) cloning procedure (114). Briefly, rat *BARS* coding sequence was amplified with primers pET-11D rat BARS fwd (5'-GATATACCATGGGC-CATCATCATCATCATCATATGTCAGGCGTCCGACCTCC-3') and rat BARS pET-11D rev (5'-CCTTTCGGGCTTTGTTAGCA-GCCGATCACTACAACCTGGTCAGTCGTCATGG-3'). Then, PCR fragment was run onto 1% agarose gel, purified by the QIAquick Gel Extraction Kit (Qiagen), according to the manufacturer's protocol, and used as a pair of primers in a linear amplification reaction around pET-11D circular plasmid.

The construct encoding the GST N-terminally tagged rat BARS CTD (amino acids 307 to 430) was generated amplifying the rat BARS CTD with the primers Eco RI BARS aa307 fwd (5'-ATCCGGAATTCATGTCAGTACAGTGAGCAGGCGTC-3') and BARS Not I rev (5'-CATTCGCGGCCGCTACAACCTGGTCAGTCGTCATGGTC-3'). Then, the fragment was digested with Eco RI and Not I restriction enzymes and cloned downstream to the GST protein into

pGEX-4T-1 vector, previously digested with Eco RI and Not I restriction enzymes. The sequences of all the constructs were checked by sequence analysis.

Generation of human *LPAAT8* constructs

The human *LPAAT8* pcDNA3 neo-Strep TEV 3XFlag C-terminal construct, encoding the human *LPAAT8* protein fused at its C-terminal end to streptavidin-binding peptide (SBP) followed by tobacco etch virus (TEV) protease cutting site and 3XFlag peptide, was generated by a two-step cloning procedure. First, the human *LPAAT8* coding sequence was cloned into the pcDNA3-neo-Strep_Flag_Cterm vector (Addgene no. 102645) by the RF cloning procedure. To this purpose, human *LPAAT8* sequence was amplified with primers pcDNA3 Strep Flag *LPAAT8* fwd (5'-GAGACCCAAGCTTGGTACCGAGCTCGGATCCATGGACCTCGCGGGACTGCTGAAG-3') and pcDNA3 Strep Flag *LPAAT8* rev (5'-CTCGAGCGGCCGCCAGTGTGATGGATATCTGTCATTTCAGTTTCTGCTTGCTGTC-3'). Then PCR fragment was run onto 1% agarose gel, purified by the QIAquick Gel Extraction Kit (Qiagen), according to the manufacturer's protocol, and used as a pair of primers in a linear amplification reaction around pcDNA3-neo-Strep_Flag_Cterm circular plasmid to generate the human *LPAAT8* pcDNA3-neo-Strep_Flag_Cterm construct. The RF reaction was performed as previously described (114); the only exception was the annealing temperature at 60°C. The second step was to insert the TEV protease cutting site between SBP sequence and the Flag tag, and in parallel to replace the Flag tag with the 3XFlag peptide by the RF cloning procedure. To this end, the TEV sequence fused to the 3XFlag peptide sequence was amplified with primers SBP TEV fwd (5'-GAGCACCACCCCCAGGGCAGCGCGAGCCGGAGAACTTGCTACTTCCAAGGCGAC-3') and Flag pcDNA strep rev (5'-GACATATAGAATAGGGCCCTCTAGAGTCTCATTATTTGTCGTCATCATCCTTATAG-3'). After purification on 1% agarose gel, PCR fragment was used as a pair of primers in a linear amplification reaction around *LPAAT8* pcDNA3-neo-Strep_Flag_Cterm circular plasmid to generate the final construct. The RF reaction was performed as previously described (114); the only exception was the annealing temperature at 50°C. In addition, the empty SBP-TEV-3XFlag vector was generated as a control to be used in the transfection experiments. To obtain the empty vector, the PCR fragment containing the TEV sequence fused to the 3XFlag peptide sequence was used as a pair of primers in a linear amplification reaction around the pcDNA3-neo-Strep_Flag_Cterm circular plasmid. The sequences of all the constructs were checked by sequence analysis.

Generation of rat *BARS* and human *LPAAT8* mutants

Rat *BARS* and human *LPAAT8* mutant constructs were generated by site-directed mutagenesis reactions. The oligonucleotide primers used for the mutagenesis reactions are listed in table S7. For the generation of human *LPAAT8*^{H96A/K123A/K124A} triple mutant, two subsequent mutagenesis reactions were performed: The first one introduced the H96A mutation into the *LPAAT8*^{WT} coding sequence, and the second one introduced the K123A and K124A mutations into the *LPAAT8*^{H96A} mutant coding sequence. PCRs were carried out in a 50-μl final volume including the following: 50 ng of DNA template, 200 μM of each dNTPs, 1× Pfu TurboCx Hotstart DNA Polymerase buffer (Agilent Technologies, no. 600414), 125 ng of each primer, 2% dimethyl sulfoxide (DMSO), and 2.5 U of Pfu TurboCx Hotstart DNA Polymerase (Agilent Technologies, no. 600414). PCR conditions were as follows: a single denaturation step (95°C, 5 min) was followed by 20 cycles of denaturation (95°C, 1 min), annealing (60°C, 1 min),

and elongation (68°C, the elongation time dependent on construct length and on the rate of DNA synthesis of DNA polymerase, which was 2.0 min/kb). Then, a final elongation step of 10 min at 72°C was carried out. The mutagenesis reactions products were treated with Dpn I endonuclease to digest the parental DNA template and transformed into TOP-10 chemical competent cells (Thermo Fisher Scientific, C404006). The sequences of all the constructs were checked by sequence analysis.

Cell culture and transient transfections

HeLa cells were from the European Collection of Authenticated Cell Cultures (Merck, Darmstadt, Germany). Cells were grown in minimum essential medium (MEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS), 100 μM MEM Non-Essential Amino Acids Solution, and 2 mM L-glutamine, at 37°C in a humidified 5% CO₂ atmosphere. The cultures were screened for *Mycoplasma* infection once a month. All cell culture reagents were from Thermo Fisher Scientific (Waltham, MA, USA). TransIT-LT1 Reagent (Mirus Bio) was used for transient transfection of plasmids encoding for *BARS* and *LPAAT8* wild-type or their mutants according to the manufacturer's instructions. Cells were then incubated 24 hours at 37°C and 5% CO₂ to allow the expression of constructs before their analysis. Only cells expressing moderate levels of the respective plasmids were used for the experimental analyses.

BARS-KO HeLa cell line generation

BARS knockout HeLa cells (henceforth, *BARS*-KO cells) were engineered using the CRISPR-Cas9 technique (115). Briefly, HeLa cells were plated in six-well plates (1.5 × 10⁵ per well) grown to 60 to 80% confluence and transfected with 1.5 μg of *CtBP1* CRISPR-Cas9 KO plasmid (Santa Cruz Biotechnology no. sc-400667) using TransIT-LT1 Reagent, according to the manufacturer's instructions. The *CtBP1* CRISPR-Cas9 KO plasmid consisted of a pool of three plasmids, each encoding the green fluorescent protein (GFP), Cas9 nuclease, and a target-specific 20-nt guide RNA (gRNA) (A, B, and C). After 48 hours, single-cell sorting was performed using a FACS Aria III cell sorter (BD Biosciences). All transfected cells were detached using trypsin buffer (0.25% trypsin + 1 mM EDTA) and resuspended in phosphate-buffered saline (PBS) + 1% FBS. Propidium iodide (0.5 μg/ml) was added to stain dead cells. Dead cells were excluded, and cells previously sorted, characterized by high GFP-expression (about 20%), were seeded into five 96-well plates (460 wells in total). Cells were then further cultured until colonies formed. Medium was replaced every 2 days. After 6 days, most colonies reached 80% confluence and were expanded to 24-well plates at a concentration of 2.0 × 10⁵ cells per well. Last, the cells were collected and analyzed by both PCR and Western blotting (fig. S8). Positive clones were amplified and frozen at low passages.

Genomic DNA preparation

Wild-type and *BARS*-KO HeLa cells were seeded into six-well plates and grown to 80% confluence. The cells were then washed with ice-cold PBS 1× and scraped into 500 μl of lysis buffer (50 mM tris-HCl, pH 8, 100 mM NaCl, 100 mM EDTA, and 1% SDS). After the incubation at 60°C for overnight, the lysates were mixed for 5 min at room temperature (RT) and 300 μl of 5 M NaCl was added to each sample. Samples were mixed and centrifuged at 16,000g for 10 min at RT to pellet the insoluble materials. The supernatants were collected, 330 μl of isopropanol was added to each supernatant, and the genomic DNA was precipitated by centrifugation at 16,000g for 10 min at RT. Then,

the genomic DNA pellet was washed with 70% ethanol and resuspended in sterile H₂O. The genomic DNA yield and quality were analyzed using a NanoDrop machine (Celbio).

PCR on genomic DNA

PCRs on genomic DNA from wild-type or BARS-KO HeLa cells were performed to amplify both the genomic DNA regions targets of the gRNAs and the genomic DNA regions of the putative off-targets of those gRNAs. The primers used in the PCRs are listed in table S7. PCR conditions were as follows: A single denaturation step (95°C, 5 min) was performed and followed by 30 cycles of denaturation (95°C, 1 min), annealing (55°C, 1 min), and elongation (68°C, 1 min). Then a final elongation step of 5 min at 72°C was performed. The PCR was carried out in a 50- μ l final volume including the following: 100 ng of genomic DNA template, 200 μ M of each dNTPs, 1 $\times$ PfuTurbo Cx Hotstart DNA Polymerase buffer, 0.2 μ M of each primer, 5% DMSO, and 2.5 U of PfuTurbo Cx Hotstart DNA Polymerase (Agilent Technologies). To verify the amplification of genomic DNA regions of interest, the PCR products were visualized on 1% agarose gel.

Molecular cloning of genomic DNA fragments

The genomic DNA fragments from the PCRs were run onto 1% agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen), according to the manufacturer's protocol. The DNA yield and quality were analyzed using a NanoDrop machine (Celbio). The PCR fragments were analyzed by DNA sequencing. The PCR fragment of interest was cloned into pUC19 vector, previously digested with Sma I restriction enzyme. The ligation reaction was performed in a 15- μ l final volume including the following components: 150 ng of pUC19 vector, PCR fragment (molar ratio 1:2 vector to fragment), 1 $\times$ T4 DNA Ligase Buffer, and 400 U of T4 DNA Ligase (NEB). The ligation reaction was incubated overnight at 16°C, then was transformed to chemical competent *E. coli* TOP-10 cells.

Protein purification

Recombinant purified His-BARS^{WT} and mutant proteins (BARS^{I332A/L336A}, BARS^{I332W/L336W}, and BARS^{R160A/R162A}) were produced as described previously (116). The His-BARS^{C123Y/N127R/R130A/R131A} (in short BARS^{Mono}) and His-BARS^{R331W} recombinant constructs were expressed in *E. coli* BL21(DE3) cells for 16 hours at 20°C with 0.1 mM isopropyl β -D-1-thiogalactopyranoside. After centrifugation, the pellets of wild-type and BARS mutants were resuspended in lysis buffer (50 mM tris, pH 8.0, 300 mM NaCl, 20% glycerol, 0.5 mM TCEP, 10 mM MgCl₂, and 20 mM imidazole), in the presence of DNaseI (10 μ g/ml), lysozyme (1 mg/ml), and protease inhibitor cocktail and left for 30 min at 4°C (rotating wheel) before sonication. After centrifugation, the supernatants were loaded onto a nickel-immobilized affinity chromatography column (1-ml His Trap FF column, Cytiva) and purified by Fast Protein Liquid Chromatography (FPLC) according to the manufacturer's instruction (Cytiva). Fractions containing His-BARS^{Mono} and His-BARS^{R331W} were pooled and dialyzed against 50 mM tris-HCl, pH 8.0, 300 mM NaCl, 10% glycerol, and 0.5 mM TCEP with a membrane cutoff (molecular weight cutoff) of 14,000.

Recombinant purified GST-CTD-BARS^{WT} and mutant proteins (CTD-BARS^{I332A/L336A} and CTD-BARS^{I332W/L336W}) were produced as described previously with modifications (116). The GST-tag was removed adding thrombin protease (GE Healthcare) at a concentration of 10 U/ml to the eluted proteins for 18 hours at 4°C. HiTrap Benzamidine FF column (GE Healthcare) was used to remove thrombin protease according to the manufacturer's instructions. The purified

CTD-BARS fractions were collected, concentrated using an Amicon ultra column (3-kDa cutoff, Merck, Darmstadt, Germany), and further purified by FPLC on a Sephacryl S-200 High-Resolution HiPrep 16/60 (Amersham Pharmacia) at a flow rate of 0.3 ml/min. The eluted fractions were collected and confirmed on 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by Coomassie staining.

His-EcLPAAT was purchased from CUSABIO (Wuhan, China, CSB-EP34083ENV). The purity of all recombinant proteins was confirmed by SDS-PAGE followed by Coomassie blue staining (fig. S10).

Size exclusion chromatography

HisTrap purified BARS^{WT} protein (200 μ g) or BARS^{Mono} mutant was applied to a Superdex 200-Increase 10/300 GL gel filtration column (Cytiva) equilibrated in gel filtration buffer (50 mM tris, pH 8.0, 300 mM NaCl, 10% glycerol, and 0.5 mM TCEP) and eluted at a flow rate of 0.5 ml/min on an Akta Purifier (GE Healthcare, Germany). The eluted protein was detected by monitoring absorbance at 280 nm. Alcohol dehydrogenase (158 kDa) and ovalbumin (44.5 kDa) were applied to the same column as molecular weight standards (Bio-Rad, Hercules, CA, USA).

Analysis of the oligomerization state of BARS: NADH treatment

One milligram (1.5 mg/ml) of purified BARS dialyzed in gel filtration buffer [25 mM tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 5% glycerol, and 1 mM dithiothreitol (DTT)] was incubated or not with 200 nM or 20 μ M or 100 μ M NADH (2 hours, 4°C, rotating wheel). The protein solution was then applied to a Sephacryl S-200 High Resolution HiPrep 16/60 (Amersham Pharmacia) gel filtration column equilibrated in gel filtration buffer. The eluted protein was detected by monitoring absorbance at 280 nm.

Analysis of the oligomerization state of BARS with oleoyl-CoA

BARS was purified as earlier with a change in the size exclusion chromatography (SEC) elution buffer (25 mM tris, pH 8.0, 300 mM NaCl, 1 mM EDTA, and 0.5 mM TCEP). Two hundred fifty microliters of concentrated BARS (5 mg/ml) was supplemented with 1 mM oleoyl-CoA and incubated for 90 min at 37°C. Another vial of BARS was treated the same way without the addition of oleoyl-CoA and used as a control. Both samples were analyzed using Akta Pure (GE Healthcare, Germany), a Superdex 200-Increase 10/300 GL gel filtration column, and the SEC elution buffer stated above.

Tandem affinity purification

LPAAT^{WT} and mutant proteins (LPAAT^{G196A} and LPAAT^{H96A/K123A/K124A}) were immunopurified by tandem affinity purification strategy. In detail, HeLa cells in 10-cm petri dishes were transiently transfected with 7 μ g of plasmid encoding LPAAT^{WT} SBP-3XFlag constructs and the empty SBP-3XFlag vector using TransIT-LT1 Reagent, according to the manufacturer's instructions. Twenty-four hours after transfection, the cells were collected, washed three times with PBS, harvested in the homogenization buffer (HB; 100 mM tris, pH 7.4, 150 mM NaCl, and 3 mM MgCl₂) supplemented with the protease inhibitor mixture, and homogenized (6 pulses, 30% amplitude; Branson Digital Sonifier). The lysate was centrifuged at 1000g for 10 min at 4°C, and the post-nuclear supernatant fraction was incubated with 200 μ l of Sepharose beads conjugated with streptavidin for 2 hours at 4°C (rotating wheel). The beads were washed extensively (five times) with 10 ml of wash buffer (100 mM tris, pH 7.4, 500 mM

NaCl, and 3 mM MgCl₂) followed by 5 ml of TEV cleavage buffer (100 mM Tris, pH 7.5, 500 mM NaCl, 1 mM DTT, and 0.5 mM EDTA). TEV enzyme (0.1 U/μl) was added to the beads and samples were incubated for 90 min at 4°C (rotating wheel). Eluted proteins were collected by centrifugation and further purified by incubation with 50 μl of anti-FLAG M2 agarose beads for 2 hours at 4°C (rotating wheel). The beads were then washed as described above and proteins were eluted with 3X Flag peptide (200 μg/ml; Merck, Darmstadt, Germany) in PBS for 30 min at 4°C (rotating wheel). The purity of the immunopurified LPAATδ was evaluated by 10% SDS-PAGE and Silver staining obtained by incubating gels in 50% methanol (15 min, twice) and 5% methanol (10 min), washing with H₂O, and treating with 10 μM DTT and 0.1% AgNO₃ (20 min). Gels were subsequently developed in 280 mM sodium carbonate with 0.02% formaldehyde. The reaction was stopped with citric acid (12 g/0.5 liters), followed by washing, incubation in 4% glycerol (45 min), and drying under vacuum.

Liposome preparation

Lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA): 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC); 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE); 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine sodium salt (DOPS); sphingomyelin (egg, chicken); cholesterol; L-α-phosphatidylinositol (liver, bovine) (sodium salt); 1-(9Z-octadecenyl)-2-hydroxy-*sn*-glycero-3-phosphate (ammonium salt) (LPA); L-α-phosphatidic acid (PA) (egg, chicken) (sodium salt); and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rhodamine-PE). All lipids were in chloroform, then diluted in a chloroform/methanol (2:1, v/v) solution and mixed in different molar ratios, which mimic mammalian Golgi membranes (49) supplemented with LPA (table S8). Multilamellar vesicles (MLVs) were prepared as described with modifications (49, 117). Briefly, lipids were dried in glass tubes and solvents were gently evaporated under N₂ stream. Tubes were placed under vacuum for 30 min to remove traces of solvents. The thin lipid film was then hydrated with prewarmed PBS at 50°C, and the tubes were placed in a water bath for 30 min at 50°C to allow the complete resuspension of the lipid film. After 30 to 60 s of vigorous vortexing, the liposome suspension was frozen and thawed 10 times in liquid nitrogen. The resulting dispersion of MLVs at 2 mM final concentration was extruded by passing 20 times through two polycarbonate membranes with a manual mini extruder (Avanti Polar Lipids) to generate LUVs. Polycarbonate membranes with different pore sizes were used to prepare sized liposomes. The experiments were performed with 200-nm liposomes, unless otherwise specified in the legends to the figures.

GUVs were prepared according to the electroformation method modified as described (118) in a special chamber that allows direct visualization under the microscope (119). Stock solutions of lipids [total lipid (0.2 mg/ml)] were prepared in a chloroform/methanol (2:1, v/v) solution. Rhodamine-PE 1% was added as a tracer to allow GUV membrane visualization by confocal microscopy. Then, 6 μl of the lipid stocks was added onto the surface of two platinum (Pt) electrodes located in a PRET-GUV 4 chamber supplied by Industrias Técnicas ITC (Bilbao, Spain), and solvent traces were removed by placing the chamber under high vacuum for 1 hour. The Pt electrodes were then covered with 400 μl of 10 mM Hepes and 150 mM NaCl, pH 7.4, previously equilibrated to 60°C. The Pt wires were connected to an electric wave generator (TG330 function generator; Thurlby

Thandar Instruments, Huntington, UK) under AC field conditions as follows: (i) frequency 500 Hz and amplitude 30 mV for 5 min; (ii) frequency 500 Hz and amplitude 300 mV for 20 min; and (iii) frequency 500 Hz and amplitude 900 mV for 90 min. Formed GUVs were incubated for 30 min with 0.4 μM BARS + 2 μM *E. coli* LPAAT (EcLPAAT) + 20 μM oleoyl-CoA or 0.4 μM BARS + 20 μM oleoyl-CoA or 2 μM EcLPAAT + 20 μM oleoyl-CoA or 0.4 μM NADH-bound BARS + 2 μM EcLPAAT + 20 μM oleoyl-CoA (Fig. 1). Images were acquired using a Zeiss-LSM 700 confocal microscope (Carl Zeiss, Göttingen, Germany).

Incorporation of LPAATδ into liposomes

Immunopurified LPAATδ^{WT} and mutants (LPAATδ^{G196A} or LPAATδ^{H96A/K123A/K124A}, in short LPAATδ^{CI}) were incorporated into liposomes using detergent free-mediated reconstitution as described previously (120) with modifications. DOPC-DOPE-DOPS-SM-Chol-PI-LPA-Rh-PE were combined at a molar ratio of 47:16:5:7:17:6:1:1. Lipids were dried under a stream of N₂ (see liposome preparation), then the lipid film formed was hydrated in 500 μl of 0.2 mM LPAATδ eluted in PBS to a final lipid concentration of 2 mM. The proteoliposome suspension was subjected to 10 freeze-thaw cycles with freezing in liquid nitrogen and thawing at 50°C in a water bath and then immediately extruded at RT through a 200-nm filter (Nuclepore, Whatman). After extrusion, the samples were kept for 30 min at RT and then used for the in vitro assays.

Membrane fission assay

Liposome co-sedimentation assays were performed as previously described (35) with modifications. Briefly, 10 μl of 2 mM LUVs (200 nm extruded; final concentration 0.4 mM) were incubated with proteins for 30 min at RT (unless otherwise indicated) in a total volume of 50 μl. Lipid compositions and protein concentrations are indicated in the figures and legends. The samples were centrifuged for 20 min at 4°C (250,000g; Beckmann Optima TL ultracentrifuge, rotor TLA-100). Supernatant and pellet fractions were separated; 10 μl of the total volume remained in the pellet fraction to avoid disruption of the pellet. Pellets were resuspended to obtain the same volume of the supernatant. Distribution of liposomes among the collected fractions was analyzed based on fluorescence intensity of Rhodamine-PE incorporated into the liposomes (in the presence of 0.5% Triton X-100 w/v) at 544/590 nm (ex/em) in a black 96-well microplate using the Cytation 3 (BioTek Instruments) plate reader. The ratio of the fluorescent signal in the supernatant and in the pellet was shown as percentage of fission index. In the figures, and unless otherwise indicated, fission index is shown as the mean of three independent experiments performed in hexaplicate. Error bars represent SD. One-way or two-way analysis of variance (ANOVA) test was used for statistical analysis.

Biochemical membrane fission assay: 2 μM recombinant BARS^{WT} or BARS^{I332A/L336A} proteins and LUVs (200 nm extruded) were incubated as indicated above. Then, following high-speed ultracentrifugation, input, resuspended pellets, and supernatants were analyzed by Western blotting.

In vitro acyltransferase assay

In vitro acyltransferase assays were carried out as previously described (13) with modifications. Purified LPAATδ^{WT} or LPAATδ^{G196A} or LPAATδ^{CI} (1.5 μg) was incorporated into Golgi-like LUVs (as described above) and incubated in the LPAAT reaction buffer [75 mM Tris, pH 7.4, 4 mM MgCl₂, 1 mM DTT, 4 mM NaF, bovine serum

albumin (BSA) fatty acid free (1 mg/ml), and 5 μM [^{14}C] oleoyl-CoA] in a final volume of 100 μl for 20 min at 37°C. Lipids were extracted by adding 450 μl of cold $\text{CHCl}_3/\text{CH}_3\text{OH}$ (2:1, v/v). After 30 min on ice, the samples were centrifuged at 10,000g for 5 min. The lower organic phase was dried under a stream of N_2 , resuspended in 50 μl of CHCl_3 , and spotted onto an oxalate-pretreated TLC plate (116) (Merck, Darmstadt, Germany). The lipids were separated by running the TLC plates with a mixture of $\text{CHCl}_3/\text{CH}_3\text{OH}/33\% \text{NH}_4\text{OH}/\text{H}_2\text{O}$ (54:42:2.9:9.1; v/v/v/v). The radiolabeled lipids were analyzed using a RITA TLC Analyser (Raytest, Germany) and quantified using GINA (Raytest, Germany) software analysis. For each analyzed sample, nanomoles of PA produced were calculated using dioleoyl [^{14}C] PA as an internal standard. PA levels are expressed in nanomoles (unless otherwise specified in the figure legends).

The above procedure was then conducted in the presence of 2 μg of His-BARS or 50 μM CI-976 or 100 ng of anti-LPAAT δ or anti-BARS blocking antibodies, and the samples were processed as described for PA production. The LPAAT δ^{WT} K_m and V_{max} parameters were determined in assays including 1.5 μg of immunopurified LPAAT δ^{WT} with Golgi-like LUVs (200 nm extruded), different concentrations of oleoyl-LPA (1, 5, 10, 15, and 20 μM), and 10 μM [^{14}C] oleoyl-CoA; or with 5 μM oleoyl-LPA and increasing concentrations of [^{14}C] oleoyl-CoA (1, 5, 10, 15, and 20 μM). The samples were then processed as described above for PA production. Time course of PA production in the LPAAT assay performed under the same conditions as above, using 5 μM oleoyl-LPA, in the absence or presence of 2 μg of His-BARS.

Quantification of PA was performed using 2 μM EcLPAAT and 0.4 μM purified BARS, with or without 100 μM NAD^+ , for 30 min at 37°C. The samples were then processed as described above. All the data are the mean of three independent experiments. One-way or two-way ANOVA test was used for statistical analysis. P values are indicated in the figures and corresponding legends.

PA synthesis catalyzed by LPAAT δ^{WT} was evaluated by TLC using radiolabeled [^{14}C] acyl-CoA incorporation into LUVs (2 mM total lipids equal to 900 nmol, including 1% LPA; 450 μl total volume), quantified using a RITA TLC analyzer. Conversion of LPA (1% of total lipids) into PA yielded a final PA content of 6.30 nmol (0.7% of total lipids) in the presence of BARS and 2.13 nmol (0.2% of total lipids) under control conditions. Values represent the mean of three independent experiments.

Immunoprecipitations and pull-down assays

BARS immunoprecipitations were performed as described previously (13). Where specified in the figures and corresponding legends, HeLa cells were treated with 2 μM oligomycin A for 2 hours. Immunoprecipitated proteins and 30 μg of total cell lysates were separated on 10% SDS-PAGE gels and analyzed by Western blotting.

Histidine pull-down assays were carried out as previously described (12), with modifications. His-BARS (2 μg ; wild type or mutants) were incubated with equimolar amount (1.5 μg) of immunopurified LPAAT δ (wild type or mutants) in His incubation buffer (50 mM tris-HCl, pH 8.0, 150 mM NaCl, 20 mM imidazole, 0.2% Triton X-100, and protease inhibitors) for 2 hours at 4°C (rotating wheel). Then, 50 μl of Ni-NTA agarose beads (Qiagen) was added, and the samples were incubated for 1 hour at 4°C (rotating wheel). The beads were then washed three times with incubation buffer by centrifugation at 700g for 5 min at 4°C and then twice with the same buffer without Triton X-100. Proteins were eluted with elution buffer

(50 mM tris-HCl, pH 8.0, 150 mM NaCl, and 250 mM imidazole, pH 8.0). Forty percent of the eluted proteins were separated by 10% SDS-PAGE and transferred onto nitrocellulose for Western blotting.

Lipid-protein pull-down assays were performed using lipid-coated beads (P-BOPA; Echelon Biosciences) according to the manufacturer's instructions with minor modifications. Briefly, 50 μl of PA-coated beads was incubated with 500 μg of total lysates from BARS-KO HeLa cells transfected with the indicated plasmids in a binding buffer [25 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM MgCl_2 , 1 mM EDTA, and 1% Igepal (Sigma-Aldrich)] for 3 hours at 4°C (rotating wheel). The beads were then washed five times, pelleted, and analyzed by 10% SDS-PAGE followed by Western blot. Beads without lipid were used as a negative control.

The Western blot membranes were blocked for 1 hour at RT in tris-buffered saline (TBS; Bio-Rad Laboratories, Hercules, CA, USA) with 0.05% Tween-20 (TBS-T; Merck, Darmstadt, Germany) containing 5% BSA (Merck, Darmstadt, Germany). The primary antibodies (see table S9) were diluted in blocking solution and incubated with membranes overnight at 4°C. After washing with TBS-T, the membranes were incubated with an appropriate horseradish peroxidase-conjugated secondary antibody diluted in 5% BSA in TBS-T for 30 min at RT. The membranes were washed extensively with TBS-T before chemiluminescent detection using the ECL Western Blotting Detection Reagents (Merck, Darmstadt, Germany; Cytiva, Marlborough, MA, USA) and x-ray film (Fujifilm, Tokyo, Japan). Densitometry analysis was performed using ImageJ2 software.

Transport protocols, immunofluorescence, and confocal microscopy

Immunofluorescence, TGN-exit assays of VSV-infected HeLa cells, and microinjections were carried out as previously reported (12, 13, 20). For the TGN-exit assay of VSVG, the cells were infected with VSV, then incubated for 2 hours at 40°C, followed by 2 hours at 20°C [with cycloheximide (100 $\mu\text{g ml}^{-1}$)] to accumulate VSVG in the Golgi complex. The cells were then shifted to 32°C (with continued cycloheximide) to release the TGN block and follow the arrival at the plasma membrane. The cells were fixed at 0 and 30 min after the shift at 32°C, incubated for 30 min at RT in blocking solution (0.5% bovine serum albumin, 50 mM NH_4Cl in PBS, pH 7.4, 0.1% saponin, and 0.02% sodium azide), and then labeled with the anti-VSVG-Cy3 antibody. To quantify VSVG-containing carriers, the TGN-exit assay was carried out in the presence of 0.5% tannic acid that inhibits fusion of the mature carriers to the plasma membrane and therefore leads to the accumulation of these carriers near the cell surface. The cells were then fixed and labeled with the anti-VSVG-Cy3 antibody. The fluorescent spots (0.5 to 2.0 μm) were then counted. All values are means $\pm$ SD from three independent experiments.

BARS was microinjected 1 hour after the beginning of the 20°C incubation in the VSVG transport assay, and after 1 hour of recovery, the cells were then processed for immunofluorescence staining. Oligomycin A (2 μM) or 200 μM oleic acid was added during the VSVG TGN-exit assay for 2 hours or 30 min, respectively, before and during the 32°C temperature-release block. The cells were fixed 30 min after the shift at 32°C and labeled with the anti-VSVG-Cy3 antibody.

For continuous transferrin uptake experiments, HeLa cells plated on glass coverslips were serum starved for 2 hours in serum-free growth medium and then incubated for 30 min with Alexa Fluor 568-conjugated Transferrin (50 $\mu\text{g/ml}$). Cells were then washed

with an acid solution (0.5 M NaCl–0.5% acetic acid), washed five times with ice-cold PBS, and processed for immunofluorescence staining. Cells were fixed in 4% paraformaldehyde for 10 min at RT, washed three times in PBS, and incubated for 30 min at RT in blocking solution (0.5% BSA, 50 mM NH₄Cl in PBS, pH 7.4, 0.1% saponin, and 0.02% sodium azide). Cells were subsequently incubated with the indicated antibodies diluted in blocking solution for 2 hours at RT, washed three times in PBS, and incubated with a fluorescent probe-conjugated secondary antibody (1:400 in blocking solution; see table S9) for 30 min at RT. Cells were then washed three times in PBS and twice in sterile water, mounted on coverslips and transferred on glass-microscope slides with Mowiol. Images were acquired using a Zeiss-LSM 980 confocal microscope (Carl Zeiss, Göttingen, Germany) equipped with 63 × 1.4 numerical aperture oil objective. Optical confocal sections were acquired at 1 Airy unit, with a resolution of 1.024 by 1.024 pixels, and exported as TIF files. The image analysis was performed using the open-source image processing software ImageJ2 (version 2.9.0, National Institutes of Health, Bethesda, MD). Quantifications of total cellular signal of 568-Tfn levels were performed using ImageJ software, as previously described (121).

Lipid droplet induction and labeling

To induce lipid droplet formation, HeLa cells were cultured in MEM supplemented with 200 μM oleic acid (Sigma-Aldrich) in 0.3% BSA (OA/BSA) for the indicated time points, as previously described in (122). Lipid droplets were visualized by incubating the cells in PBS with BODIPY 493/503 (10 μg/ml; Invitrogen) for 15 min following fixation, then analyzed with a Zeiss-LSM 980 confocal microscope (as above).

NS-EM experiments

NS-EM experiments were performed as previously described (123). First, 10 μl of purified Golgi membranes or LUVs (0.5 mM, containing PA or incorporated with 0.5 μM LPAAT^{WT} or its mutants) were incubated with 2 μM BARS^{WT} or mutants. The mixture was then spotted onto a clean sheet of parafilm in a 10-cm round petri dish. Then, an EM carbon-coated grid (Electron Microscopy Sciences, catalog no. FF100H-Ni) was placed to float on the drop (with the formvar-coated side facing the liquid) for 5 min at RT. Afterward, the grid was washed five times for 10 s on Milli-Q water drops (100 μl) and subsequently placed on 2% uranyl acetate solution (UA) (Electron Microscopy Sciences, catalog no. 22400-2) for 5, 10, and 15 s on the first, second, and third drop, respectively. Last, the excess of NS solution was removed by absorption on filter paper, and the grid was dried on air for 10 min.

Immunogold labeling of LUVs

LUVs (10 μl; 0.5 mM) were spotted on a clean sheet of parafilm layer in a 10-cm round petri dish. Then, an EM carbon-coated grid was placed to float on the drop (with the formvar-coated side facing the liquid) for 5 min at RT. Next, the grid was washed five times for 10 s on Milli-Q water drops (100 μl) and then placed for 5 min on a drop of blocking solution (1% BSA in PBS). Then, the grid was placed to float on a drop with primary antibody [in-house BARS antibody polyclonal anti-rabbit (0.4 μg/ml) diluted 1:20]. After washing the grid six times on six drops (200 μl) of PBS (total time, 10 min), the grid was placed on a drop (5 μl) of Protein A gold diluted 1:50 for 10 min. Further, the grid was washed six times on PBS and Milli-Q water drops (200 μl), respectively (total time, 5 min). Last, NS was

performed using a solution of methylamine vanadate (NanoVanTM, Nanoprobes.com) for 10 min, removing the excess of NS solution by absorption with filter paper and drying the grid on air before TEM visualization.

LUVs EPON embedding

LUVs (200 μl of 2 mM LUVs 200 nm extruded) were incubated with BARS for 30 min at RT in a total volume of 500 μl. Lipid compositions and protein concentrations are indicated in the figures and legends. The samples were centrifuged for 1 hour at 4°C (250,000g; Beckmann Optima TL ultracentrifuge, rotor TLA-100). After removing the supernatant, the liposome pellet was fixed with 1 ml of 4% paraformaldehyde and 2.5% glutaraldehyde solution in PBS, pH 7.4, and then washed three times with ice-cold MilliQ water for 5 min on ice. From this step, all the subsequent steps were performed blocking light on dark. In a fume hood, the liposome pellet was postfixed for 1 hour using 1% OsO₄ and subsequently washed three times with water. Afterward, the liposome pellet was stained en bloc with 2% UA for 1 hour on ice. After three washes with H₂O, the liposome pellet was dehydrated using increasing concentrations of ethanol for 6 min in each solution (30, 50, 70, 80, 90, and 100%) and further dehydrated in 100% acetone for 6 min at RT. Then, the liposome pellet was infiltrated with 25/50/75/100% EPON resin in acetone for 1/2/3/12 hours, respectively, and lastly embedded in 100% EPON at 60°C for 12 hours. Sections of 100 μm were cut using Ultramicrotome (Leica, EM UC6), inspected and imaged by a transmission electron microscope (TEM) TECNAI 12 G2 Spirit BioTwin (Thermo Fisher Scientific) and a MegaviewG2 CCD Camera (Olympus Soft Imaging Solutions GmbH, Germany).

Cryo-TEM

Cryo-TEM samples were prepared by plunge freezing into liquid N₂-cooled liquid ethane, using a VITROBOT mark IV FEL. Briefly, about 5 μl of LUVs suspension (0.2 mM) was spotted on glow-discharged lacey carbon grids. After 2 s, the samples were blotted for 2 s and plunged. The vitrified samples were transferred into a Gatan 626 cryo-holder and then imaged using a Philips CM200 FEG TEM equipped with a Temcam F224 HD 2 K × 2 K CCD Camera (TVIPS, Gilching, Germany). The images were analyzed using FIJI ImageJ.

Cryo-EM for vitrified sample preparation and image acquisition with a Falcon 4i detector

Five microliters of Golgi-like LUVs (200 nm extruded; incorporated with 0.5 μM LPAAT^{WT} and incubated with 2 μM BARS^{WT}) was applied to glow-discharged Quantifoil R 1.2/1.3 300 Mesh Cu grids (Quantifoil, Großlobichau, Germany) and was flash frozen in liquid ethane using a Vitrobot mark IV (Thermo Fisher Scientific, Waltham, MA, USA) set at 100% humidity and 4°C for the preparation chamber with a 2-min wait time and a 2-s blot time. Cryo-EM micrographs were acquired on a Glacios microscope (Thermo Fisher Scientific) operated at an accelerating voltage of 200 kV with a 50-μm C2 aperture at an indicated magnification of 79,000× corresponding to a pixel size of 1.5 Å in nanoprobe EFTEM mode with a total dose of 15 e/Å² and a defocus value of −2 μm. The direct electron detector was a Falcon 4i positioned post a Selectris energy filter (Thermo Fisher Scientific), operated in a zero-energy-loss mode with a slit width of 10 eV.

Images were acquired by using Smart EPU software (Thermo Fisher Scientific) and analyzed by using Velox software (Thermo

Fisher Scientific). To each image, the same low-pass filter was applied to enhance global contrast.

Golgi membrane purification

Golgi membranes were purified as previously described (123). Briefly, after culturing BARS-KO HeLa cells to 80% confluency in 5×15 cm round petri dishes, cells were detached using trypsin and washed twice in ice-cold HB (0.25 M sucrose and 10 mM tris-HCl, pH 7.4) supplemented with protease inhibitors (cOmplete, EDTA-free Protease Inhibitor Cocktail, Merck, catalog no. 5056489001). After centrifugation at 400g, cells were resuspended in 4 volumes of HB buffer and homogenized performing 10 to 12 passages in a cell homogenizer chamber with a 12- μ m clearance. The sucrose concentration of the cell lysate was adjusted to 1.4 M with the addition of 1 mM EDTA. A sucrose gradient was layered in an SW41 Ti tube (Beckman Coulter, catalog no. 344059) with different sucrose solutions according to the following order: 0.7 ml of 2 M, 1.4 ml of 1.6 M, 2.8 ml of cell lysate adjusted to 1.4 M, 4.2 ml of 1.2 M, and 2.8 ml of 0.8 M. After centrifugation at 90,000g at 4°C for 2.5 hours, the Golgi-enriched membrane fraction (~500 μ l) was collected at the interface between the 0.8 and 1.2 M sucrose solutions using a 1-ml syringe, aliquoted, and frozen in liquid nitrogen in 0.5-ml tubes stored at -80°C .

Morpho-functional analysis

Three EM grids were prepared for each condition in each single experiment. For NS-EM, the average diameter of liposomes and the size of BARS-mediated fission intermediates were quantified using iTEM, as previously described (123). For NS-EM, liposomes/vesicles were classified according to their average diameter; specifically, large LUVs and vesicles were defined as liposomes with an average diameter between 140 and 240 nm or below 40 nm, respectively. The “frequency” of each subpopulation of liposomes/vesicles was calculated as the percentage of the total amount of liposomes. All the data are the mean of three independent experiments. For EPON embedding and cryo-TEM experiments, the quantification of BARS-mediated fission intermediates (tubules, pearls, and vesicles) has been performed counting a total of 150 or 90 fission intermediates, respectively, from three independent experiments. The characterization of the morphology of BARS-induced constriction points has been performed considering a total of 50 constriction points from three independent experiments. For immunogold experiments, the quantification of the ratio between the number of nanogold particles on constriction points versus number of nanogold particles on LUVs has been performed calculating 10 ratios for each experiment considering three independent experiments. Details on surface density calculation are given in note S4.

Circular dichroism

Far-UV CD spectra were recorded by means of a JASCO J-715 spectropolarimeter (Jasco Corporation, Japan) in a 0.1-cm path length quartz cuvette as an average of three scans, at a fixed temperature of 25°C . The instrument parameters of 20-nm/min scan speed, 4-s response time, and 2-nm bandwidth were set. The protein samples were prepared in PBS buffer at pH 7.4 at a concentration of 1.5 μM in the absence and presence of Golgi-like LUVs at a total lipid concentration of 300 μM to obtain a lipid/protein ratio (L/P) = 200. For each sample, a background blank (buffer solution or lipid suspension) was subtracted. Measured values of ellipticity (Θ) were converted into the

ellipticity per amino acid residue and expressed as molar ellipticity $[\Theta]_{\text{MRW}}$ ($\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$) per residue.

Bioinformatic analysis

Sequence analysis

Protein sequences were obtained from the UniProt database (<https://uniprot.org>) [UniProt Consortium, 2023 (124)]: rat BARS (UniProt ID Q9Z2F5), human LPAAT γ (UniProt ID Q9NRZ7), human LPAAT δ (UniProt ID Q9NRZ5), human AGPAT6 (UniProt ID Q86UL3), human AGPAT8 (UniProt ID Q6UWP7), human LPAAT η (UniProt ID Q643R3), *E. coli* LPAAT (UniProt ID P26647), and *Thermotoga maritima* LPAAT (UniProt ID Q9X219). Sequence alignment was made using the Clustal Omega program available at <https://ebi.ac.uk/jdispatcher/msa/clustalo> (125).

AH prediction and helical diagram generation

BARS sequence was analyzed for putative amphipathic helices using HELIQUEST software available at <https://heliquet.ipmc.cnrs.fr/index.html> (54). This HELIQUEST software provides information about mean hydrophobicity, the hydrophobic moment HMom (126, 127), and the net charge for a given protein segment considered as helical. The parameter “Helix type” was set as: α . HELIQUEST was used to generate the helical wheel diagram shown in Fig. 5B.

Structural analysis

All protein structures have been obtained from the Protein Data Bank (PDB; www.pdb.org). To model the AH, the backbone dihedral angles (ϕ , ψ) of residues ranging from Arg³²⁶ to Asn³⁴¹ of the rat BARS structure (PDB 3GA0) have been modified accordingly to the α helix typical mean values (-60° , -45°). Before and after each introduced modification, all structures have been processed for energy refinement calculations using the Protein Preparation module embedded in Schrodinger Maestro suite (128), with standard settings. Energetic minimization has been performed with the MacroModel Minimization module (129) using OPLS3e as force field; a value of 100 of force constant was set as constraints on all backbone atoms; Polak-Ribier Conjugate Gradient was selected as the minimization method and the convergence threshold was set at 0.05.

Docking simulations for acyl-CoA binding study were carried out in a Schrodinger Maestro suite environment. The structure of CtBP1/BARS has been refined with the Protein Preparation module embedded in Maestro suite. Energetic minimization has been performed with the MacroModel Minimization module, using OPLS3e as force field; a value of 100 of force constant was set as constraints on all backbone atoms; Polak-Ribier Conjugate Gradient was selected as the minimization method and the convergence threshold was set at 0.05. The target box for docking simulations was defined with the Receptor Grid Generation module, as embedded in Maestro suite. Molecular docking calculations were performed with the Glide Ligand Docking module. Resulting complexes were followed by energetic minimization, as described before.

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 10.0 (GraphPad Software, Boston, MA, USA). Data are presented as mean $\pm$ SD from at least three independent experiments. Statistical significance was assessed using unpaired two-tailed Student's *t* test for comparisons between two groups. One-way ANOVA followed by Sidak's or Tukey's multiple comparisons test was used for comparisons involving one independent variable with more than two

groups. Two-way ANOVA followed by appropriate testing was used when two independent variables were analyzed simultaneously. For kinetic analyses, nonlinear regression was applied to determine enzymatic parameters, K_m and V_{max} . P value ≤ 0.05 was considered statistically significant. Significance is indicated in the figures as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and **** $P \leq 0.0001$.

Supplementary Materials

The PDF file includes:

Notes S1 to S4
Figs. S1 to S10
Legend for movie S1
Tables S1 to S9
References

Other Supplementary Material for this manuscript includes the following:

Movie S1

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Molecular mechanism of CtBP1-S/BARS–driven membrane fission and its cellular control by metabolic ligands

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