Golgi membrane fission requires the CtBP1-S/BARS-induced activation of lysophosphatidic acid acyltransferase δ

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Membrane fission is an essential cellular process by which continuous membranes split into separate parts. We have previously identified CtBP1-S/BARS (BARS) as a key component of a protein complex that is required for fission of several endomembranes, including basolateral post-Golgi transport carriers. Assembly of this complex occurs at the Golgi apparatus, where BARS binds to the phosphoinositide kinase PI4KIIIβ through a 14-3-3 δ dimer, as well as to ARF and the PKD and PAK kinases. We now report that, when incorporated into this complex, BARS binds to and activates a trans-Golgi lysophosphatidic acid (LPA) acyltransferase type δ (LPAATδ) that converts LPA into phosphatidic acid (PA); and that this reaction is essential for fission of the carriers. LPA and PA have unique biophysical properties, and their interconversion might facilitate the fission process either directly or indirectly (via recruitment of proteins that bind to PA, including BARS itself).
Membrane fission consists of a series of molecular rearrangements by which a tubular or neck-like bilayer joining two membranous compartments undergoes constriction and splits in two parts without leakage of contents. Fission is required for fundamental cellular processes such as the formation of transport vesicles during membrane traffic, organelle partitioning, cell division and in general for the maintenance of the compartmental organization of endomembranes. The mechanisms of fission have been studied intensely during the last decade, and multiple pathways leading to fission have been documented or proposed. The best characterized fission processes are based on constriction and destabilization of membranes by the mechano-enzyme dynamin, shallow membrane insertion of amphipathic protein domains and phase separation of lipid domains. Nevertheless, key aspects of the lipid rearrangements leading to membrane fission remain elusive, and further analysis is required.

We have identified the protein CIBP1-S/BARS (henceforth, BARS) as a key player in the fission of post-Golgi tubular/pliomorphic carrier, macropinosome, COPI-dependent transport vesicles and in the Golgi ribbon partitioning during mitosis. BARS (brefeldin A ADP-ribosylation substrate) is a member of the C-terminal-binding protein (CtBP) family, which evolutionarily derives from an ancestral dehydrogenase by gene duplication and functional differentiation into proteins involved in transcription, membrane transport, microtubule organization and synaptic transmission. BARS itself is a dual-function protein that controls fission in the cytoplasm and gene transcription in the nucleus. Structurally, BARS closely resembles the α-hydroxylated dehydrogenases and features a ‘classical’ NAD(H)-binding Rossman fold, which regulates the interconversion of BARS between a monomeric and a dimeric conformation depending on binding to NAD(H) and/or other ligands to the Rossman domain. This conversion is critical for function because BARS can drive fission as a monomer, while it is fission-incompetent as a dimer. The mechanism of action of BARS in fission has been studied mostly in the context of the process of basolateral post-Golgi carrier formation. Here BARS assembles into a complex that includes ARF, frequenin (also known as NCS-1), the phosphoinositide kinase PI4KIII, and PKD and PAK, and functions to couple the budding of carriers with fission. To induce fission, BARS must bind to 14–3-3γ through a phosphorylated serine (Ser147) in its dimerization domain. This conversion is critical for function because BARS can drive fission as a monomer, while it is fission-incompetent as a dimer.

We have previously proposed that BARS-dependent fission involves a lysophosphatidic acid (LPA) acyltransferase (LPAAT) activity, based on the following observations: an LPAAT activity is present in liver Golgi membranes which, on addition of suitable substrates, generates phosphatidic acid (PA); PA production induces, or correlates with, the fission of Golgi membranes; the addition of BARS to these Golgi membranes stimulates both PA production and membrane fission; treatments that inhibit the formation of monomeric fission-competent BARS inhibit both BARS-stimulated LPAAT activity and membrane fission. We have also shown that recombinant BARS is associated with a slow LPAAT activity (see below) which is inhibited by anti-BARS antibodies, and proposed that this activity is ascribable to BARS itself. This activity, however, was later shown not to be intrinsic to BARS. The simplest interpretation of these collective findings is that BARS binds to and stimulates an endogenous LPAAT, and that this reaction is involved in membrane fission.

Here we have examined this hypothesis. There are 11 known lysophospholipid acyltransferases (LPLATs), four of which have been cloned and shown to transfer fatty acids from acyl-CoA to the sn-2 position of LPA to form PA (LPAATα, β, γ and δ), while others have mixed specificities for LPA and glycerol phosphate. We find that (i) BARS interacts with LPAAT type δ (LPAATδ, also known as AGPAT4); (ii) this LPAAT localizes to the trans-Golgi and to post-Golgi carrier precursors at the trans-Golgi Network (TGN); (iii) the catalytic activity of LPAATδ is essential for Golgi fission carrier; (iv) BARS potently stimulates LPAATδ, and this stimulation is essential for carrier fission; (v) BARS needs to be incorporated into the PHKIIβ–14–3-3γ dimer–BARS complex to stimulate LPAATδ and induce fission. BARS thus appears to function as an adaptor/regulator protein that binds to and stimulates LPAATδ, to induce LPA–PA conversion and carrier fission. LPA and PA have unique biophysical properties that can markedly affect the organization of lipid bilayers. Their interconversion might facilitate the fission process either directly or through the action of PA-binding proteins, including BARS itself.

**Results**

**LPAATδ localizes at the Golgi and binds directly to BARS.** To examine whether BARS interacts with an LPAAT, we first sought to identify the LPAATs that localize to the Golgi, as most of the BARS-dependent fission reactions occur in this organelle. Here BARS assembles into a complex that includes ARF, frequenin (also known as NCS-1), the phosphoinositide kinase PI4KIII, and PKD and PAK, and functions to couple the budding of carriers with fission. To induce fission, BARS must bind to 14–3-3γ through a phosphorylated serine (Ser147) in its dimerization domain. This conversion is critical for function because BARS can drive fission as a monomer, while it is fission-incompetent as a dimer. The mechanism of action of BARS in fission has been studied mostly in the context of the process of basolateral post-Golgi carrier formation. Here BARS assembles into a complex that includes ARF, frequenin (also known as NCS-1), the phosphoinositide kinase PI4KIII, and PKD and PAK, and functions to couple the budding of carriers with fission. To induce fission, BARS must bind to 14–3-3γ through a phosphorylated serine (Ser147) in its dimerization domain. This conversion is critical for function because BARS can drive fission as a monomer, while it is fission-incompetent as a dimer. The mechanism of action of BARS in fission has been studied mostly in the context of the process of basolateral post-Golgi carrier formation. Here BARS assembles into a complex that includes ARF, frequenin (also known as NCS-1), the phosphoinositide kinase PI4KIII, and PKD and PAK, and functions to couple the budding of carriers with fission. To induce fission, BARS must bind to 14–3-3γ through a phosphorylated serine (Ser147) in its dimerization domain. This conversion is critical for function because BARS can drive fission as a monomer, while it is fission-incompetent as a dimer.

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**Results**

**LPAATδ localizes at the Golgi and binds directly to BARS.** To examine whether BARS interacts with an LPAAT, we first sought to identify the LPAATs that localize to the Golgi, as most of the BARS-dependent fission reactions occur in this organelle. We Flag-tagged and expressed the available mammalian LPAATs and inspected their localization by immunofluorescence microscopy. LPAATγ, LPAATδ and LPAATγ localized to both the Golgi and the endoplasmic reticulum (ER) (Fig. 1a), while LPAATβ and LPAATα localized to the ER and mitochondria, respectively (Supplementary Fig. 1; see also refs 30,31). We thus examined whether BARS interacts with the Golgi LPAATs by co-expressing BARS with each of these transferases and testing for co-immunoprecipitation of the two proteins. BARS co-precipitated with LPAATγ and LPAATδ (and vice versa), but not with LPAATγ (Fig. 1b,c). LPAATγ has been shown to reside at the cis-Golgi and regulate Golgi structure and retrograde transport to the ER33,34, while LPAATδ has no known Golgi-related function to date. We asked whether the LPAATδ location might be compatible with a role in post-Golgi traffic by immuno-electron microscopy of the Flag-tagged protein (Fig. 1d). Tagged LPAATδ localized preferentially in the trans-Golgi and in the TGN (Fig. 1d), and to a lesser extent in the ER in the cell periphery. We also examined the distribution of the endogenous protein using specific antibodies and immunofluorescence microscopy (Fig. 2). The endogenous LPAATδ showed a localization that was very similar but not identical to that of the tagged protein, in that it was visualized mostly in the elongating tubules emanating from the TGN (Fig. 2), and gave a weaker signal in the cell periphery compared with the tagged protein. Of note, while this manuscript was being revised, it was reported that LPAATδ localizes to the mitochondrial outer membrane in murine cells. This differs from our findings that LPAATδ localizes in the Golgi complex. However, cases of dual localization of the same transmembrane protein to the secretory pathway and to outer mitochondrial membrane have been reported several times. The mechanism of this dual localization is partially understood. We thus considered that LPAATδ might localize both to the Golgi and the mitochondria, but that only the Golgi LPAATδ can be stained using the low titre antibody conditions optimized for specificity in our study, while LPAATδ in mitochondria might require a higher antibody titre for detection. We find that this is indeed the case, in all of the cell types tested.
Moreover, both the Golgi and mitochondrial signals were abolished by LPAATδ depletion, that is, are specific (Supplementary Fig. 2c). We conclude that LPAATδ can localize both in the secretory pathway and to a lower extent in mitochondria in most or all cells, though we do not exclude that the LPAATδ distribution might be partially cell type-dependent.

We then asked whether BARS binds directly to LPAATδ by performing pull-down experiments with the two purified recombinant proteins. After coincubation, recombinant BARS by performing pull-down experiments with the two purified recombinant proteins. After coincubation, recombinant BARS

exposed at the protein surface (see models in ref. 30). BARS co-precipitated also with this fragment (Fig. 3a). These data indicate direct binding between the two proteins.

Finally, we examined whether LPAATδ binds selectively with the monomeric fission-competent form of BARS15,16. As noted, BARS shifts between monomeric and dimeric conformations depending on ligand binding to its Rossman fold. Dimerization is promoted by the binding either to NAD(H)19,26–28 or to the Brefeldin A–ADP-ribosylated conjugate (BAC), an ADP-ribosylated metabolite of brefeldin A, which locks BARS in the dimeric fission-inactive conformation29. Either BARS alone or BAC-bound BARS were added to lysates from cells overexpressing LPAATδ. BARS and LPAATδ interacted efficiently as judged from co-precipitation (see above), as expected, and this interaction was markedly reduced by the binding of BARS with BAC (Fig. 3b) (NAD(H) had similar effects, not shown), indicating that LPAATδ preferentially binds monomeric BARS.

We conclude that the LPAATδ is located in the elongating tubules emanating from the TGN (Fig. 2) and binds directly and selectively the monomeric fission-active form of BARS (Fig. 3).

**Figure 1 | BARS interacts with LPAATδ** (a) Representative confocal microscopy images of COS7 cells transected with Flag-tagged LPAATγ, LPAATδ and LPAATη, and fixed and processed for immunofluorescence with a monoclonal anti-Flag antibody (green) and with a polyclonal anti-TGN46 antibody (red; as indicated). Dotted lines indicate cell borders. (b) BARS immunoprecipitation (IP:BARS) of lysate from HeLa cells co-expressing BARS and LPAATγ–Flag, LPAATδ–Flag or LPAATη–Flag. Representative western blotting (antibodies as indicated) of total lysate (input) and immunoprecipitated proteins with preimmune-IgG (Preim-IgG) or anti-BARS-IgG (as indicated). IgGh, IgG heavy chain. (c) Immunoprecipitation with an anti-Flag antibody (IP:Flag) of lysate from HeLa cells co-transfected with BARS and LPAATγ–Flag, LPAATδ–Flag or the empty vector. Representative western blotting of total lysate (input) and Flag-immunoprecipitated proteins with an anti-Flag monoclonal antibody or the anti-BARS polyclonal antibody (as indicated). (d) Representative electron microscopy image of HeLa cells transfected with Flag-tagged LPAATδ for 24 h, and fixed and processed for cryo-immuno-electron microscopy with a monoclonal anti-Golgin-97 antibody (15-nm gold particles) and with a polyclonal anti-LPAATδ antibody (10-nm gold particles). Molecular weight standards (kDa) in (a) are indicated on the left of each panel. Data are representative of three independent experiments. Scale bars, 10 μm (a); 200 nm (d).
that the evolutionary ancestors of LPAATδ and BARS (probably an LPAAT and a \( \delta \)-hydroxyacid dehydrogenase, respectively)\(^{40}\), which are fundamental metabolic enzymes, were interactors in an ancient metabolic multi-enzyme complex\(^{43}\), and that this interaction was maintained through evolution in different functional contexts.

Regardless, these data explain our previous observations that recombinant BARS from \( E. \) \( \text{coli} \) is associated with an LPAAT activity\(^{7}\), which, as we now know, belongs to the \( E. \) \( \text{coli} \) enzyme. They also provides a potentially useful tool (the soluble bacterial enzyme) for \textit{in vitro} reconstitution of BARS-dependent fission.

\textbf{LPAATδ is required for post-Golgi carrier fission.} The TGN localization and the interaction of LPAATδ with BARS persuaded us to investigate the role of this LPAAT isoform in the BARS-dependent formation of post-Golgi carriers\(^{14}\). LPAATδ was silenced with specific small-interfering RNAs (siRNAs), and the formation of carriers, as well as the rate of transport to the

\textbf{Figure 2 | LPAATδ is a trans-Golgi-localized enzyme.} (a) Representative confocal microscopy image of HeLa cells at steady-state fixed and labelled with a polyclonal anti-LPAATδ antibody (endogenous LPAATδ; red), with a monoclonal anti-GM130 antibody (green) and with an anti-TGN46 antibody (blue). Inset, bottom: Magnification of Golgi area. (b) Line scan across the Golgi area (red line across the magnified image in a) indicates the colocalization of LPAATδ with TGN46. (c) Representative confocal microscopy images of COS7 cells at steady-state (top) or VSV-infected and subjected to the VSVG TGN-exit assay (bottom). Cells were fixed and labelled with a polyclonal anti-LPAATδ antibody (endogenous LPAATδ; green) and with an anti-TGN46 antibody (red; top) or a monoclonal anti-VSVG antibody (red; bottom). Inset, right: magnification of tubular carrier precursors. Scale bars, 10 \( \mu \text{m} \) (a, c).

\textbf{Figure 3 | BARS binds LPAATδ directly.} (a) Representative GST pull-down of equimolar amounts of GST, GST-LPAATδ (aa 152-251) and GST-LPAATδ (full length) purified recombinant proteins, for His-BARS, with unbound and eluted proteins analysed by western blotting (top, anti-BARS monoclonal antibody). GST fusion proteins were revealed by Ponceau staining (bottom). (b) Representative histidine pull-down for His or His-BARS beads of lysates from COS7 cells transfected with LPAATδ-Flag. Beads were treated with buffer alone (–) or with HPLC-purified BAC (BAC +) and, then incubated with the lysates. The eluted proteins were analysed by western blotting using a monoclonal anti-Flag antibody (top), with the pulled down His-BARS revealed by Ponceau staining (bottom). Molecular weight standards (kDa) are indicated on the left of each panel. Data are representative of three independent experiments.

\textbf{Figure 4 | BARS depletion reduces VSVG carriers.} (a) Effect on VSVG carriers of the depletion of LPAATδ and BARS. Abundance of VSVG carriers was measured by immunofluorescence microscopy and quantified by intensity analysis. Plotted are the mean intensity (arbitrary units) from representative confocal microscopy images of COS7 cells at steady-state (top), or VSV-infected and subjected to the VSVG TGN-exit assay (bottom). Bars indicate the standard error (s.e.m.) (n = 3). Spots show the distribution of these values. (b) Line scan across the Golgi area (red line across the magnified image in a) indicates the colocalization of LPAATδ with TGN46. (c) Representative histidine pull-down for His or His-BARS beads of lysates from COS7 cells transfected with LPAATδ-Flag. Beads were treated with buffer alone (–) or with HPLC-purified BAC (BAC +), and then incubated with the lysates. The eluted proteins were analysed by western blotting using a monoclonal anti-Flag antibody (top), with the pulled down His-BARS revealed by Ponceau staining (bottom). Molecular weight standards (kDa) are indicated on the left of each panel. Data are representative of three independent experiments.

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To determine whether the reduction in VSVG-containing carriers was due to inhibition of carrier budding or of fission, we monitored carrier formation in living cells expressing VSVG–green fluorescent protein (VSVG–GFP). While control
cells exhibited dynamic tubules that formed and underwent multiple fission events to generate a large number of free-moving carriers, which then fused with the plasma membrane. LPAATδ-depleted cells showed several long (>10 μm) tubular extensions that contained VSVG–GFP. These tubules represent carrier precursors that elongate out of the Golgi but do not detach to form mature transport intermediates14,15 (Supplementary Movies 1 and 2). When they (rarely) did detach, however, they could be seen to move towards, and fuse with, the plasma membrane (Supplementary Movie 2). This phenotype was similar to that induced by expressing BARS dominant-negative mutants or by depleting BARS14,15 or by depleting 14-3-3γ, a key GAPDH

### Graphs

#### Graph a

- **Non-targeting**
- **LPAATδ siRNAs**

0 min

| LPAATδ | kDa |
|--------|-----|
| 50     |
| 30     |

VSVG carriers per cell

#### Graph b

- **Non-targeting**
- **LPAATγ siRNAs**

0 min

| LPAATγ | kDa |
|--------|-----|
| 37     |
| 30     |

VSVG carriers per cell

#### Graph c

- **Non-targeting**
- **LPAATδ siRNAs**

0 min

- Permissive
- Non-permissive

#### Graph d

- **Non-targeting**
- **LPAATδ siRNAs**

0 min

| LDLRY18A–GFP (a.u.) | 0 min | 60 min |
|---------------------|-------|--------|
| 0.5                | 0.1   | 0.0    |

#### Graph e

- **Non-targeting**
- **LPAATδ siRNAs**

0 min

| p75–GFP | GM130 |
|---------|-------|
| 0.4     | 0.3   |

#### Graph f

- **Non-targeting**
- **LPAATδ siRNAs**

0 min

| HGH–FM–GFP | 30 min | 60 min |
|------------|--------|--------|
| 120        | 80     | 50     |
component of the PI4KIβ–14-3-3-γ dimer–BARS complex required for fission, or by expressing dominant-negative mutants of PKD16. Very similar effects were induced by microinjection of an affinity-purified antibody against LPAATδ (Supplementary Movie 3; see also Supplementary Fig. 5) and by the general LPAAT inhibitor CI-976 (ref. 44; Supplementary Movie 4).

These findings indicate an essential role for LPAATδ in the fission of tubular carriers exiting the Golgi complex.

We also determined the role of LPAATδ in other traffic steps. We first examined retrograde traffic from the Golgi to the ER (which is known to require LPAATγ)32,34, by monitoring the well-characterized retrograde transport marker VSVG-KDEL (a fusion of VSVG with the KDEL receptor)45. The transport of VSVG-KDEL was not affected by LPAATδ depletion (Fig. 4c).

Second, since BARS controls the fission of basolateral but not apical carriers14, we examined the role of LPAATδ in apical traffic (Fig. 4d) in contrast with the published report (Fig. 4d), but not that of p75 (Fig. 4e). Finally, we probed a soluble basolateral cargo, the stably expressed constitutively secreted GFP-tagged variant of the human growth hormone (hGH)48. Depletion of LPAATδ strongly inhibited export of hGH-FM–GFP from the Golgi to the plasma membrane (Fig. 4f). Therefore, like BARS, LPAATδ appears to be selectively required for the fission of basolateral carriers.

The LPAATδ activity is needed for post-Golgi carrier fission. To examine whether the enzymatic activity of LPAATδ49 is required for fission, we first set-up an assay to determine such activity. We prepared and incubated post-nuclear supernatants with the acyl donor [1,14C]-oleoyl-CoA and the acyl acceptor oleoyl-LPA, with [1,14C]-PA measured as the reaction product (see Methods and Fig. 5a). These are standard conditions used for LPAAT assays49,50 as attempts to purify the LPAAT enzymes result in activity loss49,50. Extracts from control cells showed an efficient LPAAT activity, which was suppressed by the general LPAAT inhibitor CI-976 (Fig. 5b)43,44. A difficulty with these extracts is that they contain multiple LPAATs. Therefore, we developed conditions to selectively determine the LPAATδ activity (Fig. 5a), based on suppressing or overexpressing this enzyme. Extracts from LPAATδ-depleted cells (Fig. 5a) or treatment of control extracts with a specific affinity-purified antibody against LPAATδ (Fig. 5b) (see Methods for antibody characterization) showed a reproducibly lower (≈25%) activity than in controls (Fig. 5a,b), confirming that LPAATδ is responsible for a fraction of the total LPAAT activity, and in line with the presence of other LPAATs such as the abundant glycerolipid synthetic enzymes LPAATγ and LPAATβ30,52. Extracts from LPAATδ-overexpressing cells showed a ≈40% increase in LPAAT activity over controls (Fig. 5a–c), and this increase was completely inhibited by antibodies against LPAATδ (Fig. 5b), down to the levels found in the absence of LPAATδ (Fig. 5a).

LPAATδ silencing or overexpression did not affect the cellular levels of other LPAATs (Supplementary Fig. 6). Similar data were obtained using [1,14C]-palmityl-CoA as acyl donor and arachidonoyl-LPA as acyl acceptor (extracts from LPAATδ-overexpressing cells showed a ≈45% increase in LPAAT activity over controls). Thus, we defined the LPAATδ-dependent activity (or LPAATδ activity) as the activity value of LPAATδ-overexpressing extracts (as measured using concentrations of substrates below the Km values, see below) minus the activity value of LPAATδ-depleted (or antibody-treated) extracts (Fig. 5a,b, see dashed line and Methods). The maximum reaction rate Vmax and Michaelis–Menten constant Km of this LPAATδ activity were 38 ± 3 nmol min−1 per mg protein and 58 ± 18 μM, respectively, for oleoyl-CoA, and 38 ± 1 nmol min−1 per mg protein and 29 ± 1 μM, respectively, for oleoyl-LPA (data are means ± s.d. of three independent experiments). These rates are comparable to those reported for LPAATγ53. Importantly, they are potentially sufficient, depending on substrate availability, to change the PA concentrations in the TGN rapidly and substantially.

Finally, we asked whether the LPAATδ catalytic activity is required for post-Golgi carrier fission. We generated a single-point mutant (LPAATδH96V) in the conserved acyltransferase catalytic site of LPAATδ (NHX,D)30,54. Overexpressed LPAATδH96V was devoid of LPAAT activity (Fig. 5c), confirming that LPAATδ is a canonical LPAAT family member30,54. We then depleted cells of LPAATδ, with the consequent inhibition of the post-Golgi transport of VSVG (see Fig. 4a and Supplementary Movie 2) and expressed either a siRNA-resistant variant of LPAATδ or of the catalytically dead LPAATδH96V mutant. Only the wild-type LPAATδ rescued carrier formation, while LPAATδH96V was completely inactive (Fig. 5d). These data indicate that the catalytic activity of LPAATδ, and hence most likely the formation of PA from LPA, is necessary for post-Golgi carrier formation.

Activation of LPAATδ by BARS is required for carrier fission. Since BARS and LPAATδ interact directly (Fig. 3a) and are required for post-Golgi carrier fission (see refs 14,15, and Supplementary Movies 2 and 3, respectively), we asked whether BARS regulates the enzymatic activity of LPAATδ, and whether this regulation is required for carrier fission. We first silenced...
BARS and measured the LPAATδ activity in cell extracts. BARS depletion abolished the LPAATδ activity (Fig. 6a). We then re-expressed BARS in BARS-silenced cells, using a siRNA-resistant replacement BARS construct (Supplementary Fig. 7a). This nearly completely restored the LPAATδ activity (Fig. 6a). As a specificity control, the above BARS manipulations did not affect the cellular levels of LPAATσ (Supplementary Fig. 7a) or of other LPAATs (Supplementary Fig. 7b). These results indicate that LPAATδ requires BARS to express its activity.

We then sought to manipulate the BARS levels acutely in 

_**vivo**_ lysates to exclude transcriptional or compensatory effects that might arise in siRNA-depletion experiments. We prepared extracts from LPAATδ-expressing and BARS-depleted cells, where LPAATδ is inactive (Fig. 6a, and added immunopurified BARS to the assay mixture to a final BARS concentration of 5 μg ml⁻¹ (comparable to the levels of endogenous BARS). Under these conditions, BARS completely restored the LPAATδ-dependent activity (Fig. 6b) (of note, the LPAAT activity associated with immunopurified BARS was quantitatively negligible). We also added BARS to control LPAATδ-expressing extracts. This treatment only slightly stimulated the LPAATδ-dependent activity (Fig. 6b, suggesting that endogenous BARS is sufficient to activate LPAATδ, at least in extracts from quiescent cells (that is, in cells not subjected to a traffic pulse; see below). As a further control, we used extracts from cells depleted of both BARS and...
LPAATδ (Fig. 6b). Here addition of immunopurified BARS had no effect on the LPAAT activity, suggesting that other LPAAT isoforms are not detectably stimulated by BARS, at least under our experimental conditions.

Further along this line, we sought to inhibit BARS by adding to the LPAAT assay mixture a characterized neutralizing affinity-purified anti-BARS antibody which, when microinjected into cells, inhibits carrier fission14,15,55. This antibody inhibited the LPAATδ-dependent activity, while preimmune-IgG addition had no effect (Fig. 6c). Moreover, a pre-treatment of the assay mixture with BAC, which locks BARS in its dimeric fission-incompetent conformation and inhibits the BARS-LPAATδ binding (see above and Fig. 3b)28, reduced the LPAATδ activity (Fig. 6d), supporting the role of BARS in LPAATδ activation and indicating that monomeric BARS is required for LPAATδ to express its enzymatic activity.

We further examined the relationship between LPAATδ activity, BARS and carrier fission by expressing suitable BARS mutants. Previously, we have characterized two single-point mutants, BARSΔ355A and BARSΔ147A that have dominant-negative effects on carrier fission in living cells14,15. We tested these mutants in the LPAATδ activity assay by co-expressing each of them with LPAATδ. Both nearly completely inhibited the LPAATδ-dependent activity (Fig. 6e), again without affecting LPAATδ expression levels (Supplementary Fig. 7c). As a control, we tested the effects of overexpressing wild-type BARS (of note, wild-type BARS and the dominant-negative mutants showed comparable expression levels in these experiments; see Supplementary Fig. 7c). Overexpressed BARS did not have significant effects on the LPAATδ activity in extracts from ‘quiescent’ cells (Fig. 6e), again suggesting that basal BARS levels are sufficient to support LPAATδ activity under these conditions (see above).

Figure 6 | BARS activates LPAATδ and this activation is required for post-Golgi carrier formation. Quantification of phosphatidic acid (PA) production in the LPAAT assay for post-nuclear supernatants from HeLa cells transfected with: (a) empty Flag-vector (Ctr) or LPAATδ-Flag (LPAATδ) and with BARS siRNAs for 48 h, and with the last 12 h with siRNA-resistant replacement BARS-YFP-encoding vector (BARS re-expression); (b) LPAATδ-Flag (LPAATδ) and/or with BARS siRNAs and/or LPAATδ siRNAs. Post-nuclear fractions were incubated with immunopurified BARS (Purified BARS) for 30 min at 25 °C before LPAAT assay (as indicated). (c) Quantification of PA production in the LPAAT assay for post-nuclear supernatants from HeLa cells transfected with empty Flag-vector (Ctr) or LPAATδ-Flag (LPAATδ) and with BARS siRNAs for 48 h. Post-nuclear fractions were incubated with HPLC-purified BAC (BAC) or with buffer alone (Buffer) for 30 min at 25 °C before LPAAT assay (as indicated). (e,f) Quantification of PA production in the LPAAT assay for post-nuclear supernatants from HeLa cells transfected with empty Flag-vector (Ctr) or LPAATδ-Flag (LPAATδ) and with BARS siRNAs for 48 h. Post-nuclear fractions were incubated with HPLC-purified BAC (BAC) or with buffer alone (Buffer) for 30 min at 25 °C before LPAAT assay (as indicated). (e) empty Flag-vector (Ctr) or LPAATδ-Flag (LPAATδ) for 48 h and the last 12 h with BARSΔ355Δ-YFP, BARSΔ147Δ-YFP or BARSΔ355Δ-YFP or BARSΔ147Δ-YFP (as indicated); (f) empty Flag-vector (Ctr) or LPAATδ-Flag (LPAATδ) for 48 h and the last 12 h with BARSΔ355Δ-YFP, BARSΔ147Δ-YFP or BARSΔ355Δ-YFP or BARSΔ147Δ-YFP (as indicated). Cells were infected with VSV, subjected to TGN-exit assay and post-nuclear fractionations were prepared 10 min after the shift to 32 °C temperature-release block. The dashed line indicates the level of endogenous LPAAT activity not associated with LPAATδ (see text for details). Data are means ± s.d. of three independent experiments. *P<0.05, **P<0.01, ***P<0.005 versus control (Student’s t-test).
overexpression of BARS stimulated the LPAAT\(\delta\) activity over control levels (Fig. 6f). Moreover, in these extracts, the expression of the fission-active BARS\(^{51,47,7}\) mutant that mimics the activatory phosphorylation of BARS on Ser\(147^{15,16}\) stimulated the LPAAT\(\delta\) activity to an even greater extent (Fig. 6f). These collective data are consistent with the idea that LPAAT\(\delta\) is activated during a traffic pulse and that this activation requires higher (local) levels of BARS than those present in quiescent cells. Testing this possibility further, we examined whether the interaction between BARS and LPAAT\(\delta\) might be enhanced during a pulse. Co-precipitation experiments indicate that BARS co-precipitates more efficiently with LPAAT\(\delta\) under pulse conditions (Supplementary Fig. 8).

We also tested the role of the PI4KIII\(\beta\)–14-3-3\(\gamma\) dimer–BARS complex in LPAAT\(\delta\) activation. As noted, within this complex, 14-3-3\(\gamma\) binds to phosphorylated Ser\(147\) in the BARS dimerization interface and is necessary for Golgi carrier fission\(^{15,16}\). The LPAAT\(\delta\) activity of cell extracts was markedly suppressed by 14-3-3\(\gamma\) depletion (Fig. 7a), while depletion of other 14-3-3 isoforms had no effect (Fig. 7b and see also Supplementary Fig. 9). Moreover, addition to cell extracts of a characterized affinity-purified anti-14-3-3\(\gamma\) antibody\(^{15}\) also suppressed the LPAAT\(\delta\) activity (Fig. 7c). These data indicate that 14-3-3\(\gamma\) is required for LPAAT\(\delta\) activity, presumably because it stabilizes BARS in its monomeric fission-competent conformation.

Finally, we repeated a few of the above experiments using a Golgi-membrane-enriched fraction (Supplementary Fig. 10a). Here the LPAAT\(\delta\) overexpression increased LPAAT activity by \(\approx60\%\) over controls (Supplementary Fig. 10b), while LPAAT\(\delta\)-depletion or treatment of the membranes with the anti-LPAAT\(\delta\) antibody (see also Fig. 5a,b) reduced the activity by \(\approx35\%\) compared with controls (Supplementary Fig. 10b,c), in line with the notion that Golgi membranes are enriched in LPAAT\(\delta\) over total cell extracts (compare with Fig. 5a). Also, as seen with total extracts (Fig. 6), the LPAAT\(\delta\) activity in Golgi membranes was nearly abolished by BARS depletion or inhibition by the anti-BARS antibody (Supplementary Fig. 10d).

In summary, a number of experimental results based on BARS silencing or overexpression, or on the use of BARS activatory or dominant-negative mutants and of anti-BARS antibodies and inhibitors, or on manipulations of the BARS-containing complex, converge towards the conclusion that the BARS-induced LPAAT\(\delta\) activation controls the fission of Golgi carriers. The stimulation of LPAAT\(\delta\) by BARS is potent, and appears to occur rapidly, most likely via the direct physical interaction between BARS and LPAAT\(\delta\) during assembly of the BARS protein complex that is required for carrier formation.

**The BARS–LPAAT\(\delta\) interaction occurs at the Golgi complex.**

The effects of BARS and LPAAT\(\delta\) on the fission of carriers emanating from the Golgi suggest that this BARS–LPAAT\(\delta\) interaction occurs at this organelle. To verify this notion, we first re-examined the Golgi localization of BARS and of the other components of the complex, 14-3-3\(\gamma\) and PI4KIII\(\beta\), focusing, in particular, on the carrier precursors elongating out of the Golgi, where fission takes place. Similar to LPAAT\(\delta\) (Fig. 2), these proteins were all seen to localize at the TGN (Fig. 8a) and on the VSVG-containing tubular carriers that form during synchronized exit from the TGN (Fig. 8b). Next, we directly examined whether the interaction between BARS and LPAAT\(\delta\) occurs at the Golgi using an approach based on Förster resonance energy transfer (FRET) and fluorescence lifetime imaging microscopy (FLIM).
This technique reveals the co-presence of suitable donor and acceptor fluorophores within the same complex (that is, at a distance of \( \leq 8 \) nm). We expressed E0GFP-BARS as the FRET donor, and tdTomato-LPAATδ as the acceptor, and the donor lifetime was measured to assess FRET under steady state and traffic pulse conditions. A comparison of the donor decay lifetime in control cells (cells not expressing the acceptor) and in cells co-expressing donor and acceptor showed in the latter case a marked reduction of donor lifetime at the Golgi (Fig. 8c,d), indicating that FRET, and hence interaction, between the fluorescent partners takes place (Fig. 8e). Moreover, the FRET signal at the Golgi markedly increased during a VSVG traffic
pulse (Fig. 8c–e), consistent with the enhanced BARS–LPAAT© interaction observed during a pulse (Supplementary Fig. 8). These results indicate that LPAAT© is in complex with BARS in vivo at the Golgi and that it colocalizes at the TGN with BARS, 14-3-3© and PI4KIII©, in agreement with the BARS–LPAAT© co-precipitation and interaction data (Figs 1 and 3 and Supplementary Fig. 8).

**Discussion**

The main finding of this study is that BARS induces fission of post-Golgi basolateral carriers by interacting directly with, and activating, the enzyme LPAAT©, a member of the acyltransferase family, which localizes at the TGN and converts LPA into PA. This indicates that the LPA–PA conversion plays a role in BARS-dependent fission. Notably, the metabolism of PA has been implicated in various aspects of membrane dynamics by other groups, albeit generally based on indirect evidence18,21,34,56–58.

Based on current and previous results, we propose the following working model for basolateral carrier formation. ARF initiates the process by recruiting and activating PI4KIII© at the Golgi. This produces a local increase in phosphatidylinositol 4-phosphate (PtdIns4P)19, which supports the budding of tubular carrier precursors, most likely through recruitment of PtdIns4P-binding proteins16. Carrier precursor budding is assisted by phospholipase A2 (PLA2) via production of positively curved liposyds, including LPA, which facilitate the bending of membranes into tubules20,23,40,61. Concomitantly, BARS assembles with 14-3-3© and PI4KIII© into a complex where 14-3-3© binds to the BARS dimerization surface, and thus keeps BARS in a monomeric fission-competent conformation15,16. BARS then activates LPAAT© at the TGN and on the elongating tubular carrier precursors, where the PLA2-generated LPA is converted into PA by LPAAT©, leading to carrier fission, in a process that might be aided by the property of BARS itself and ARF to insert into lipid membranes21,62.

LPA and PA have biophysical properties that may be relevant for fission. PA has a highly charged headgroup close to the glycerol backbone, a tendency to form intramolecular and intermolecular hydrogen bonds and segregate into microdomains. Because PA can act as a binding site for proteins bearing amphipathic/hydrophobic surfaces25, which can lead to fission through the shallow hydrophobic insertion mechanism11, its above properties might cause PA-binding proteins to insert into membranes at the optimal depth and extent for causing fission. Also of note is that BARS itself21 has been reported to bind PA. One can thus hypothesize that a runaway process might take place at the tubular precursor surface by which BARS-bound LPAAT© converts LPA into PA, causing more BARS molecules to be recruited to the membrane and bind to LPAAT©, and hence more LPA to be converted into PA (similar to the positive-feedback Sec7–Arf1 loop whereby Sec7 activates Arf1 to recruit more Arf1 to activate more Sec7 and so on)33, until the local concentrations of these molecules become high enough to induce fission. In addition, under physiological conditions LPA and PA have strongly positive and negative spontaneous curvatures, respectively25; thus the conversion from LPA to PA may generate negatively curved PA microdomains within overall positively curved membrane areas, which might lead to membrane destabilization. And, finally, the formation of PA might lead to other potential fission-related lipid-based mechanisms, such as the enzymatic conversion of PA into diacylglycerol27,64,65.

The precise role of the lipid and protein players considered in this study must now be defined. Most of the key components involved in BARS-dependent fission are available in pure form, and the possibility to reconstitute this fission pathway in artificial membranes using known components appears to be within reach.

**Methods**

**Plasmids and chemicals and recombinant proteins.** Human LPAAT cDNAs were from Imagenes GmbH (for subcloning and mutations, see Supplementary Table 1); tdTomato-C1 cloning vector was from Addgene; BARS-pCDNA3.—BARS—yellow fluorescent protein (BARS–YFP), BARS©147A–YFP, BARS©147D–YFP and BARS©355A–YFP were prepared as previously described14,15,17,18. EGFP was provided by L. Marchetti (Scuola Normale Superiore, Pisa, Italy); LDL©40,40,40–A©40 was provided by R. Polishchuk (TIGEM, Naples, Italy); the HeLa lG©- lG©–FM©–FM© expressing cell line was provided by A. Peden (the University of Sheffield, Sheffield, UK). CI-976 was from Tocris Bioscience, tunic acid and BFA from Fluka, protease inhibitors as Complete Mini EDTA-free tablets from Roche, cycloheximide, Protein A Sepharose, anti-FLAG M2 affinity gel antibody beads and 3 © 3-FLAG peptide from Sigma–Aldrich, AP21998 (D/D solubilizer) from Clontech, oleoyl-LPA from Avanti Polar Lipids, [14C]oleoyl-coenzyme A (specific activity, 60 mCi mmol−1) and dioleoyl [14C]-PA (specific activity, 140 mCi mmol−1) from PerkinElmer, and TRICH labelled dextran, FITC labelled dextran and MitoTracker Orange CM-H2TMRos from Molecular Probes. NAD+©, BAC and HeLa (CD38©) cells were described previously26,27. Ni-NTA agarose and glutathione Sepharose beads were from Amersham, and Protein A Gold was from Cell Microscopy Center (the University Medical Center Utrecht, the Netherlands). Recombinant purified GST and GST–BARS proteins were produced from E. coli XL1Blue transformed cells with pGEX©T1 or pGEX©T1-BARS cells, respectively, grown at 30°C before induction with 0.1 mM isopropyl-β-D-1-thiogalactopyranoside for 2 h at 37°C (described in detail in ref. 55). Full-length and partial GST–LPAAT© (aa 152–251) purified by in vitro wheat germ expression system were from Antibodies-Online© (catalogue number: AB1N1344584). His©E©LPAAT© (His©plC©) purified in vitro wheat germ expression system was from Cusabio (catalogue number: CSB©EP34083ENV). LPAAT© siRNAs (M©008620©0©0), LPA©7© siRNAs (M©009283©0©1) and BARS siRNAs (M©008609©0©2) were from Dharmacon.

**Antibodies.** All of the individually sourced antibodies for western blotting (WB), immunofluorescence and the LPAAT© assay were obtained and used as detailed in ref. 15, unless otherwise stated. The rabbit polyclonal anti-LPAAT© (ab188002; I©1:1,000) and anti-giantin (ab24586; I©1:5,000) antibodies (for WB use) were from Abcam; the mouse polyclonal anti-LPAAT© antibody (for microinjection and LPAAT© assay use) was from Abnova (H00056895©B01©P); the rabbit polyclonal anti-LPAAT© antibody (I©1:50 for IF use) was from Sigma–Aldrich (SAB4502436), the anti-GM130 monoclonal antibody (I©200 for IF use) was from BD
**Immunoprecipitations and pull-down assays.** HeLa cells in 10-cm Petri dishes were transiently transfected with 7 μg of each DNA (BARS-pCDNA3 and LPAAT5-Flag) using 42 μl TransIT-LT1 per dish. Twenty-four hours after transfection, the cells were washed three times with phosphate-buffered saline (PBS) and lysed using 1 ml lysis buffer/dish (25 mM Tris, pH 7.4, 150 mM NaCl, 5 μM EDTA, 5 mM MgCl2, 10 mM NaF, 40 mM β-glycerophosphate, 1 mM Na3VO4, 1 mM dithiothreitol) supplemented with 1% Triton X-100 and protease inhibitor mixture (30 min, 4 °C, shaking). The lysates were centrifuged (13,000g, 10 min, 4 °C), with the supernatants assayed for protein concentration (Bradford assay) and used fresh.

For BARS immunoprecipitation, 500 μg lysis protein from these HeLa cells was brought to 0.2% (v/v) Triton X-100 (final concentration), and incubated with 3 μg anti-BARS polyclonal antibody (overnight, 4 °C, shaking). Then 50 μl protein A Sepharose beads were added for a further 1 h of incubation (4 °C, shaking). For LPAAT5 immunoprecipitation, 1.2 mg lysis protein from the HeLa cells was brought to 0.2% (v/v) Triton X-100 (final concentration), and incubated with 40 μl anti-FLAG M2 affinity-gel-purified antibody (2 h, 4 °C, shaking). For BARS immunoprecipitation in the presence of Ecl-PAAT, 0.8 mg lysis protein from the COS7 cells was brought to 0.2% (v/v) Triton X-100 (final concentration) and incubated with 160 μg purified Ecl-PAAT (2 h, 4 °C, shaking) and then incubated with 3 μg anti-BARS polyclonal antibody (overnight, 4 °C, shaking). The immune complexes were collected by centrifugation (500g, 5 min, 4 °C). After three washes with lysis buffer with 0.2% Triton X-100, and twice with lysis buffer without Triton X-100, the bound protein was eluted from the protein A Sepharose beads or from anti-FLAG M2 affinity-gel-purified antibody by boiling (1 min) in 100 μl Laemmli sample buffer. Thirty micrograms of input and 70% of the eluted proteins separated by 10% SDS-PAGE, and subjected to WB via transfer to nitrocellulose membranes (Millipore).

For histidine pull-down from LPAAT5-Flag expressing cell lystate, His-BARS (20 μg) was incubated for 3 h at 37 °C with buffer alone (20 mM Tris, pH 7.4, 10 mM sucrose) or with 120 μM HPLC-purified RAC, to allow full activation of the enzyme to His-BARS. The reaction mixture was stopped on ice, and 1 mg lysis protein from LPAAT5-Flag expressing cells was incubated with each sample (2 h, 4 °C, shaking). Then, 30 μl Ni-NTA agarose beads were added, and the samples were incubated (1 h, 4 °C, shaking). The beads were then washed three times with lysis buffer at 80 °C (supplemented with 4 μM of each dNTPs, 1 μl Pfu buffer and 5 U Pfu Turbo Cx hotstart DNA polymerase, Agilent). The template DNA was purified using the Agilent StrataPrep PCR purification kit and used as the template in the second PCR for gene cloning. LPAAT5 was cloned into tdTomato-N1 vector, fused to the N-terminal of tdTomato sequence, and BARS was cloned into a pCMV-Flag vector. Full-length LPAAT were incubated with 0.4 μM of each dNTPs (including 100 ng of template DNA) at 32 °C for 1 h, followed by 5 min of centrifugation (700g, 5 min), and then twice with lysis buffer at 80 °C without Triton X-100 but supplemented with 20 mM imidazole. The bound protein was eluted from the Ni-NTA-agarose beads by boiling (1 min) in 100 μl Laemmli sample buffer (including 100 ng of template DNA). Thirty micrograms of input and 70% of the eluted proteins were separated by 10% SDS-PAGE, and subjected to WB via transfer to nitrocellulose membranes (Millipore).

**FLIM measurements.** The DNA coding for EGF-pBARS and LPAAT5-tdTomato were generated using the Restriction Free (RF) cloning procedure using the following two pairs of primers: 5′-cccttgctcaccatggtggcctgattcagtttctggttg-3′ and 5′-tttaaacgggccctctagagcactacaactggtcagtcgta-3′ for E0GFP–BARS and LPAAT respectively.

**Transport protocols and light and wide-field microscopy.** For the TGN-exit assay of VSVG, the cells were transfected with VSVG–GG–pCDNA3 DNA or VSVG with siRNAs for LPAAT and not other LPAATs by WB analysis (our unpublished data). For the TGN-exit assay of VSVG, the cells were transfected with p75–EGFP–pCDNA3 DNA or VSVG with siRNAs for LPAAT and not other LPAATs by WB analysis (our unpublished data). For the TGN-exit assay of VSVG, the cells were transfected with p75–EGFP–pCDNA3 DNA or VSVG with siRNAs for LPAAT and not other LPAATs by WB analysis (our unpublished data).
fixed cells, for ease and speed of acquisition of a statistically reliable number of samples. The donor lifetime values were extracted from the fitting of decay curves obtained from the donor emission (EGFP–BARS) in the Golgi area, and both global and pixel kinetic data analyses were computed by the commercially available software package SymPho Time, Version 5.3.2.2. Changes in donor lifetime due to PFR (0.008 s) assessed by comparing the donor lifetime in cells expressing only the donor with the lifetime in cells co-expressed with donor and acceptor and fixed either under steady-state condition or during a traffic pulse.

**Cryo-immunogold electron microscopy.** HeLa cells were transiently transfected with 8 μg plasmid DNA encoding Flag-LPAAT for 24 h (using TransIT-LT1) and then fixed with 2% formaldehyde and 0.2% glutaraldehyde in 100 mM phosphate buffer pH 7.4. The cells were pelleted by centrifugation, embedded in 12% gelatin, cooled on ice and cut into 1-mm² cubes at 4°C. The cubes were immersed in 2.3 mM sucrose (4°C) and postfixed in liquid nitrogen using NITAN FCM ultramicrotome. Thin sections (50 μm) were picked up in a mix of 2% m-cellose and 2.3 mM sucrose (1:1) and incubated with the rabbit anti-LPAAT (1:1:0) and the mouse anti-Golgin-97 (Life Technologies, catalogue number: A21270, 1:50) antibodies. Each of these incubations was followed by incubation with 1% glutaraldehyde for 30 min. Gold particles for anti-LPAAT were then 15 nm Gold particles for anti-LPAAT and embedded in m-cellose uranyl acetate. Electron microscopy images were acquired using FEI Tecnai-12 electron microscope.

**Transfections with siRNAs.** COS7 and HeLa cells were transfected with a non-targeting siRNA or with 100 nm of a Small Pool of LPAAT/M-009283 or LPAAT/M-008620 siRNAs, for 72 h (except for BARS and 14-3-3 siRNAs, where 100 nm of a Small Pool was used for 48 h, as it was previously described in ref. 15) using Lipofectamine 2000, according to manufacturer’s instructions. The efficiency of transfection and interference was assessed by WB. The treatment with Smart Pool siRNAs for LPAAT (M-008620) specifically reduces the endogenous protein levels of LPAAT and LPAAT (by WB), respectively, without affecting the levels of other tested LPAATs (our unpublished data). Alternatively, COS7 cells were transfected with the siRNAs (as above) in combination for the last 24 h with VSVG–CFP, VSVG–GFP, VSVG–ts045–KDELr-myc, LDRY18A–GFP (by WB), respectively, without affecting the levels of other tested LPAATs (our unpublished data). Alternatively, COS7 cells were transfected with the siRNAs (as above) in combination for the last 24 h with VSVG–CFP, VSVG–GFP, VSVG–ts045–KDELr-myc, LDRY18A–GFP (by WB), respectively, without affecting the levels of other tested LPAATs (our unpublished data).

**In vitro acyltransferase assay.** HeLa cells (1 × 10⁶) in 10-cm Petri dishes were transiently transfected with 8 μg plasmid DNA encoding Flag-LPAAT for 48 h (using TransIT-LT1). Alternatively, the HeLa cells were transfected with siRNAs (as above) in combination with Flag-LPAAT for 48 h (using Lipofectamine 2000). The cells were washed three times with PBS, harvested as 250 μl per dish in homogenization buffer (100 mM Tris, pH 7.4, 5 mM NaCl, 3 mM MgCl₂) supplemented with the protease inhibitor mixture, and homogenized (8 pulses, 30% amplitude; Branson Digital Sonifier). The lysate was centrifuged at 600g for 10 min at 4°C, and the post-nuclear supernatant fraction was used in the acyltransferase assay.

Golgí membranes from HeLa cells treated as described above were obtained as described previously⁶⁹, with some modifications. HeLa cells were washed with PBS, harvested by trypsinization and pelleted (400g, 5 min, 4°C). The cell pellets were resuspended in 1/4 vol. homogenization buffer (250 mM sucrose, 100 mM Tris, pH 7.4) and homogenized in a prechilled Dounce homogenizer. The cell suspension passed six times each direction (12 passes total) through the Balch homogenizer with a 7.988 mm diameter tungsten-carbid ball bearing (clearance, 12 μm) using constant manual pressure. Following homogenization, the samples (≈ 850 μl in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4) were adjusted to 1.4 M sucrose by addition of sucrose containing 3 mM MgCl₂ (7.5 μl of 3 M Tris-HCl pH 7.4), and then centrifuged for 24 h (using TransIT-LT1) with Flag-LPAAT (20) or Flag-LPAAT (a) (both encode an siRNA-resistant silent mutation), followed by infection with VSV for the TGN-exit assay.

**Size-exclusion chromatography.** For the fast protein liquid chromatography, 600 μg purified recombinant GST-BARS was applied to a MonoQ column (HR5/5 Pharmacia LKB) equilibrated with buffer PBS (4°C, flow rate, 0.3 ml min⁻¹), with 1 ml fractions collected using an AKTA Fast Protein Liquid Chromatography (FPLC) system. The eluted protein was detected by monitoring absorbance at 280 nm, and 10 μl of the collected fractions was separated on 10% SDS-PAGE gels and analyzed by silver staining. Eighty micrograms of each of these fractions was then subjected to the LPAAT assay.

**Statistical analysis.** Two-tailed Student’s t-tests were applied to the data. Significance is indicated as *P < 0.05, **P < 0.01 and ***P < 0.005.

**Data availability.** The authors declare that the data supporting the findings of this study are available within the article and its Supplementary Information files or are available from the corresponding authors upon request.

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**Author contributions**

A.P. and C.V. designed, carried out and analysed all of the experiments. L.L.G., A.F. and G.L. carried out immunofluorescence, immunoprecipitation and pull-down experiments. D. Circolo carried out in vitro acyltransferase assays and immunofluorescence experiments. G.T. carried out in situ acyltransferase assays, immunofluorescence and electron microscopy experiments. V.M.M. conceived and designed FLIM experiments and provided advice on data analysis. C.V. and F.F. carried out, with V.M.M., FLIM experiments. L.M. designed the strategy for constructs subcloning, purification and testing of them for FLIM experiments. M.A.Z. with C.V and A.F. have substantially contributed to the study of protein localization and commented on the manuscript. R.S.P. carried out time-lapse microscopy. C.V., D. Corda and A.L. conceived and supervised the project, discussed and analysed the data and co-wrote the manuscript.

**Additional information**

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