STARD3 mediates endoplasmic reticulum-to-endosome cholesterol transport at membrane contact sites

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Abstract

StAR-related lipid transfer domain-3 (STARD3) is a sterol-binding protein that creates endoplasmic reticulum (ER)–endosome contact sites. How this protein, at the crossroad between sterol uptake and synthesis pathways, impacts the intracellular distribution of this lipid was ill-defined. Here, by using in situ cholesterol labeling and quantification, we demonstrated that STARD3 induces cholesterol accumulation in endosomes at the expense of the plasma membrane. STARD3-mediated cholesterol routing depends both on its lipid transfer activity and its ability to create ER–endosome contacts. Corroborating this, in vitro reconstitution assays indicated that STARD3 and its ER-anchored partner, Vesicle-associated membrane protein-associated protein (VAP), assemble into a machine that allows a highly efficient transport of cholesterol within membrane contacts. Thus, STARD3 is a cholesterol transporter scaffolding ER–endosome contacts and modulating cellular cholesterol repartition by delivering cholesterol to endosomes.

Keywords cholesterol; endoplasmic reticulum; endosome; lipid transfer protein; membrane contact site

Subject Categories Membrane & Intracellular Transport

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Introduction

Eukaryotic cells are compartmentalized into discrete organelles, which allow a spatial division of labor. A universal feature of eukaryotic cells is that the various lipid species, and in particular sterols, are unevenly distributed between organelles (Chang et al., 2006; van Meer et al., 2008). In mammalian cells, cholesterol is highly enriched in the plasma membrane (PM), and intermediate concentrations are found in early endosomes and the trans-Golgi network (TGN), whereas the endoplasmic reticulum (ER) harbors the lowest level of cholesterol (Mesmin et al., 2013a). This precise distribution of cholesterol is ensured by vesicular and non-vesicular transport pathways (Holthuis & Levine, 2005; Holthuis & Menon, 2014). Of note, several members of the steroidogenic acute regulatory protein (StAR)-related lipid transfer (START) domain family are involved in non-vesicular sterol transport (Iaea et al., 2014). These proteins possess a conserved START domain of about 210 amino acids that serves to transfer lipids between membranes (Tsujishita & Hurley, 2000; Alpy & Tomasetto, 2005). Notably, this domain defines a hydrophobic cavity able to accommodate one lipid molecule and thereby serves as a “hydrophobic bridge” across the aqueous gap between donor and acceptor organelle membranes.

STARD3 is a cholesterol-specific START protein ubiquitously expressed and anchored at the membrane of late endosomes (Alpy et al., 2001). An early assumption was that STARD3 could act as a sterol transporter. It was legitimate in view of the sequence similarity between STARD3 and the founding member of the START protein family, StAR, alias STARD1. Indeed, STARD1 is essential for intracellular sterol transport in steroidogenic tissues as it conveys cholesterol to the mitochondria where it is metabolized into pregnenolone by the P450scc enzymatic complex (Clark et al., 1994; Clark & Stocco, 2014). For STARD3, despite numerous studies, it is not yet known whether this protein mediates a defined transport route for sterols. Because of its localization on the endosome surface, STARD3 was initially proposed to facilitate cholesterol exit from this organelle. However, this view was challenged by the fact that STARD3 overexpression does not increase sterol...
O-acyltransferase-mediated cholesterol esterification in the ER (Borthwick et al., 2010; Lapis et al., 2012) and does not rescue Niemann-Pick type C mutant cells, which accumulate cholesterol in endosomes (Alpy et al., 2001; Holtta-Vuori et al., 2005; Vanier, 2014). More confusingly, STARD3 expression was associated with higher cholesterol content in a number of distinct organelles such as endosomes, but also the PM and mitochondria (Alpy et al., 2001; Zhang et al., 2002; Holtta-Vuori et al., 2005; Charman et al., 2010; Lapis et al., 2012; Vassilev et al., 2015). Up until now, how STARD3 functions in cholesterol transport is still elusive.

Interestingly, the functional characterization of STARD3 revealed that it distinguishes itself from the other STARD domain proteins by the presence of a conserved amino-terminal domain named MENTAL (MLN64 NH2-terminal) that is shared with its paralog STARD3NL (alias MENTHO) (Alpy et al., 2002). The MENTAL domain has many functions: It anchors the protein in endosomal membranes; it exposes the STARD domain in the cytosol (Alpy et al., 2001); and it mediates homotypic as well as heterotypic interactions between STARD3 and STARD3NL (Alpy et al., 2005). Of note is that the MENTAL domain interacts with cholesterol in vivo (Alpy et al., 2005; Hulce et al., 2013) supporting the notion that this domain is a sterol reservoir. Along with this, we previously revealed that STARD3 has an FFAT (two phenylalanines in an acidic tract) motif (Loewen et al., 2003) located at the carboxy terminal end of the MENTAL domain and upstream of the START domain (Alpy et al., 2013). We then found that STARD3, anchored at late endosomes, uses this FFAT motif to interact with ER-localized VAP-A and VAP-B (vesicle-associated membrane protein-associated protein) proteins and create zones of close apposition between the ER network and endosomes (Alpy et al., 2013). Such an ability to create contact sites is reminiscent to what is known for CERT, alias STARD11. This protein is required for sphingomyelin (SM) synthesis by acting as a ceramide transporter, which shuttles this lipid between the ER and the Golgi apparatus (Hanada et al., 2003). Although the exact mechanism is not known, it is widely accepted that CERT ensures its function in ER–Golgi contacts by connecting the Golgi apparatus and the ER via its Pleckstrin Homology (PH) domain and its FFAT motif, respectively (Hanada, 2010). The characterization of CERT which showed the association of two distinct activities within the same protein, the ability to transport lipids, and to connect membranes, brought a novel paradigm to explain how lipid fluxes are guided inside cells (Munro, 2003).

By analogy with StAR and CERT, we speculated that STARD3 connects the ER and endosomes to pilot sterol transfer between these organelles. This prompted us to clarify STARD3 function by investigating how this protein modulates intracellular cholesterol distribution. Here, using a combination of cholesterol labeling and microscopy approaches and in vitro reconstitution, we provide evidence that STARD3 transports sterols from the ER to the endosome. Furthermore, we show that its activity depends both on STARD3’s ability to bind sterols and to create membrane contact sites. Finally, we show that STARD3 acts as a lipid transfer protein that redirects sterol to the endosome at the expense of the PM and favors membrane formation inside endosomes. Overall, our results describe a new pathway for sterol fluxes within eukaryotic cells and additionally provide data strengthening the concept that a reduced intermembrane distance at organelle contacts allows a highly efficient intermembrane transfer of lipid.

Results

STARD3 expression results in cholesterol accumulation in endosomes

Having previously established that STARD3 has an ER–endosome tethering activity and contains a sterol-binding domain (Alpy et al., 2013; Wilhelm et al., 2016), we surmised that the creation of extended ER–endosome contacts by STARD3 might significantly alter cholesterol distribution inside cells. To explore this possibility, we devised novel assays for quantifying free cholesterol, not only at the PM, but also inside cells, using two specific fluorescent probes: the D4 fragment of perfringolysin O (9 toxin, Clostridium perfringens) fused to GFP (GFP-D4) (Ohno-Iwashita et al., 2004; Abe et al., 2012) and the polyene macrolide filipin from Streptomyces filipinensis (Wüstner et al., 2012). We identified experimental conditions under which these two probes can be efficiently used to label sterols in the PM or in subcellular structures such as endosomes (Fig EV1). We then used these probes to compare sterol distribution in control and STARD3-expressing cells. Using GFP-D4, we first observed that in parental and in control HeLa cells (thereafter named HeLa/Ctrl), which both expressed very low amounts of STARD3 (Fig 1Ab and f), endosomes labeled by Lamp1 were not enriched in cholesterol (Fig 1Aa–h). In contrast, in HeLa cells that stably expressed STARD3 (HeLa/STARD3, Fig 2B), STARD3 localized in Lamp1-labeled endosomes, and these endosomes were also strongly labeled with the cholesterol probe (Fig 1Ai–l). In fact, most GFP-D4-positive vesicles were also positive for STARD3 and Lamp1 (Fig 1B, f), and GFP-D4 and STARD3 signals were correlated (Fig 1E). Since STARD3 is a protein from late endosomes (Alpy et al., 2001), we characterized STARD3-positive endosomes using a series of endosomal markers including EEA1, Rab7, CD63, and the lipid BMP. As shown in Fig EV2, STARD3 strongly co-localizes with all late endosome markers and not with the early endosomal protein EEA1 (Fig EV2A and B). This suggested that high STARD3 expression promotes free sterol accumulation in a STARD3-positive late endosomal compartment (Fig 1Ai–l). Moreover, as GFP-D4 binds only to membranes containing more than 35 mol% sterol (Ohno-Iwashita et al., 2004), this result indicated a massive accumulation in endosomes.

To substantiate this finding, we measured sterol accumulation by repeating the experiments using filipin as a cholesterol probe. Immunolabeling of Lamp1 together with filipin staining in control cells confirmed that endosomes contained low cholesterol amounts (Fig 1Ca–h). By contrast, in STARD3-expressing cells, Lamp1- and STARD3-positive endosomes accumulated free cholesterol (Fig 1Ci–l and D). In fact, most filipin-positive vesicles were also positive for Lamp1 and STARD3 (Fig 1D), and filipin and STARD3 signals were highly correlated (Fig 1E). Accordingly, filipin staining was strongly correlated with the late endosome marker Rab7 but not with the early endosome marker EEA1 (Fig EV2C). The two cholesterol probes filipin and GFP-D4 have different properties: While filipin binds to all free cholesterol, GFP-D4 only binds to cholesterol-rich membranes (Ohno-Iwashita et al., 2004). As anticipated, co-labeling experiments using filipin and GFP-D4 in HeLa/STARD3 cells showed that filipin labeled more endosomal vesicles than GFP-D4 (Fig EV2D and E), thus reflecting the heterogeneity of cholesterol accumulation levels between individual endosomes. To quantify
Figure 1.
in intracellular cholesterol, we set up an image analysis protocol based on a semi-automated image segmentation and quantitative analysis of intracellular filipin staining (Fig EV3), which allowed a systematic measure of intracellular sterol levels in many cells. As shown in Fig 1F, while control cells had little intracellular filipin staining, HeLa/STARD3 cells displayed ~4 times higher intracellular filipin staining. Jointly, these results indicated that STARD3 elicits a substantial accumulation of cholesterol in endosomes.

**STARD3 triggers cholesterol accumulation in endosomes and promotes internal membrane formation**

It has been widely assumed for lipid transfer proteins notably STARD3, that the ability to reduce intermembrane distance by tethering should facilitate lipid transfer between organelles (Loewen et al., 2003). Taking advantage of the unique phenotype observed in HeLa/STARD3 cells, we explored this question at the level of ER–endosome contact sites. We first assessed whether the buildup of cholesterol in endosomes by STARD3 was dependent on its sterol binding START domain, and then on its ability to form ER–endosome contacts.

First, we used a mutagenesis approach to generate STARD3 mutants, which are unable to bind sterols. Two mutants were created: A mutant lacking the entire START domain (STARD3 ΔSTART); and a mutant bearing two point mutations in the START domain (STARD3 M307R/N311D, herein referred to as MR/ND) described as being deficient in sterol binding (Tsujishita & Hurley, 2000; Holtta-Vuori et al., 2005) (Fig 2A). The sterol transport capacity of the START domain with the MR/ND mutation was assessed in vitro using liposomes (Fig EV4). Transfer was measured with a FRET assay using DHE, a fluorescent mimetic of cholesterol, and a second fluorescent lipid, DNS-PE (John et al., 2002; Moser von Fiseseck et al., 2015a). As the decrease in FRET between the two lipids is a function of DHE transport from the donor to the acceptor liposome, the amount of sterol that is transported can be quantified in real time. We found that the isolated START domain of STARD3 transfers DHE with an initial rate of ~2 molecules/min (Fig EV4), which is consistent with a previous study (Zhang et al., 2002). By contrast, the MR/ND mutant was totally devoid of sterol transfer activity (Fig EV4A and B). We then established stable cell lines expressing these mutants (HeLa/STARD3 ΔSTART and HeLa/STARD3 MR/ND) to study both ER–endosome tethering and in vivo endosomal cholesterol accumulation. Mutant and wild-type (WT) proteins were expressed at similar levels (Fig 2B). We observed that the STARD3 ΔSTART and MR/ND mutants, as they still possess the FFAT-like motif and the MENTAL domain (Fig 2A), kept their ability to generate ER–endosome contacts (Fig EV5). In situ staining using the GFP-D4 probe or filipin showed that intracellular accumulation of cholesterol in cells expressing the two sterol transfer-deficient mutants was highly decreased compared to that observed with WT STARD3 (Fig 2C and D). Moreover, quantitative image analysis of filipin staining of more than 100 cells indicated that sterol accumulation was lowered in endosomes bearing mutant STARD3 (Fig 1F).

Next, to directly study the contribution of membrane contact sites in the cholesterol accumulation phenotype, a STARD3 mutant lacking a functional FFAT motif was constructed. This mutant STARD3 F207A/Y208A (herein referred to as FA/YA) was obtained by alanine replacement of the two FFAT-like motif core aromatic residues (Fig 2A). A stable HeLa/STARD3 FA/YA cell line was generated (Fig 2B). As expected, this mutant was unable to make ER–endosome contacts (Fig EV5). Intracellular cholesterol levels and distribution were measured. We observed that the FA/YA mutant did not promote cholesterol accumulation in endosomes (Figs 2C and D, and 1F). This data supported the notion that the ability of STARD3 to scaffold ER–endosome contacts is essential for an efficient sterol transfer in endosomes.

To further substantiate this idea, we knocked down the VAP proteins, the partners of STARD3 (Alpy et al., 2013), implicated in ER–endosome tethering in HeLa cells expressing STARD3 (HeLa/STARD3) (Fig 3A). The proteins VAP-A and VAP-B were downregulated using two sets of small hairpin RNAs (shRNAs), targeting distinct sequences (Fig 3A). Compared to non-silenced cells (HeLa/STARD3/shCtrl), VAP-A and VAP-B levels were reduced by 80 and 30%, respectively, with one set of shRNAs (HeLa/STARD3/shVAPA/B-α), and 84 and 85%, respectively, with the other set (HeLa/STARD3/shVAPA/B-β) (Fig 3A). Compared with HeLa/STARD3 cells, HeLa/STARD3 silenced for VAP proteins did not
Figure 2. STARD3 needs a functional START domain and ER–endosome contacts to induce cholesterol accumulation in endosomes.

A Schematic representation of the different STARD3 mutants used in the study. The MENTAL domain in light blue contains 4 transmembrane helices (dark blue) and a FFAT motif (red); the START domain in pink contains two essential residues involved in cholesterol binding (M307 and N311). Positions of the epitopes recognized by the rabbit polyclonal pAbMLN64-Nt-1611 and pAbMLN64-Ct-605 antibodies are shown. Point mutation positions are labeled in black.

B Western blot analysis of STARD3 expression in the different cell lines. The expression of VAP proteins is unchanged; actin was used as a loading control.

C,D To mark cholesterol accumulation in endosomes, HeLa/Ctrl (a–c), HeLa/STARD3 ΔSTART (g–i), HeLa/STARD3 MR/ND (j–l), and HeLa/STARD3 FA/YA (m–o) were labeled with anti-Lamp1 antibodies (red), anti-STARD3 antibodies (magenta), and with the fluorescent cholesterol probe GFP-D4 (C: green) or filipin (D: Cyan Hot). Nuclei are stained in blue. Higher magnification (2.5×) images of the area outlined in white (a, d, g, j, m) are shown on the right. The GFP-D4 and STARD3 merged image (C) and the filipin and STARD3 merged image (D) are labeled Overlay.

Data information: Scale bars: 10 μm.
Figure 3. VAP protein knockdown abolishes STARD3-mediated cholesterol accumulation in endosomes.

A Western blot analysis of VAP-A and VAP-B expression in untreated (NT) HeLa/STARD3 cells or HeLa/STARD3 expressing a control shRNA (shCtrl) or two pairs of shRNAs targeting VAP-A and VAP-B (shVAP-A/B-α or shVAP-A/B-β). Actin was used as a loading control.

B HeLa cells (a–c), and HeLa/STARD3 cells expressing a control shRNA (shCtrl; d–f) or two pairs of shRNAs targeting VAP-A and VAP-B [shVAP-A/B-α (g–i) or shVAP-A/B-β (j–l)] were labeled with anti-STARD3 antibodies (magenta) and with the fluorescent cholesterol probe filipin (Cyan Hot). Merged images of filipin and STARD3 signals are shown in (c, f, i and l). Scale bars: 10 μm.

C Relative fluorescence intensity of intracellular filipin signal in HeLa, HeLa/STARD3/shCtrl, HeLa/STARD3/shVAP-A/B-α, and HeLa/STARD3/shVAP-A/B-β. n = 3 independent experiments. Total number of cells analyzed: HeLa: 84; HeLa/STARD3/shCtrl: 130; HeLa/STARD3/shVAP-A/B-α: 109; HeLa/STARD3/shVAP-A/B-β: 92. Number of cells analyzed per sample per experiment ≥ 25. Mean ± SD; ***P < 0.001, ANOVA with Tukey’s multiple comparison test.

D–G TEM images (a) of control HeLa cells (D), HeLa/STARD3 cells (E), or HeLa/STARD3 cells expressing a control shRNA (F) or a pair of shRNAs targeting VAP-A and VAP-B (G). Scale bars: 200 nm. Schematic representation (b) of images shown in (a); the ER, endosomes, and intraluminal membranes are in dark, light, and median gray, respectively. Mt: mitochondria; Nc: nucleus; PM: plasma membrane.

H Quantification by stereology of relative intraluminal membrane surface on TEM sections. Fifty (HeLa; HeLa/Ctrl; HeLa/STARD3) and 25 (HeLa/STARD3/shCtrl; HeLa/STARD3/shVAP-A/B-β) endosome sections were quantified. Mean ± SD; ***P < 0.001, Kruskal–Wallis with Dunn’s multiple comparison test.
accumulate cholesterol in endosomes as shown by in situ filipin labeling and quantification (Fig 3B and C).

To gain more insights into the biological role of STARD3 in endosomes, we characterized the ultrastructure of the endosomal compartment by transmission electron microscopy (TEM). TEM allows to get information on the inner organization of the endocytic compartment. In particular, it is known that endocytic vesicles enclose internal membranes termed intraluminal vesicles (ILVs) and multilamellar regions (Vacca et al, 2016). ILVs are small spherical structures of about 50 nm of diameter (Vacca et al, 2016). We thus addressed whether the increase in endosomal cholesterol resulting from the association between STARD3 and VAP impacts the inner membrane content of the endocytic compartment, by performing TEM analysis of control and HeLa/STARD3 cells (Fig 3D–H). We quantified internal membranes in all endocytic organelles containing at least an ILV, therefore including multivesicular bodies or endosomal carrier vesicles, and late endosomes, but excluding lysosomes. Compared to control cells, HeLa/STARD3 cells displayed endosomes with more internal membranes (Fig 3D, E and H). The overall morphology of the endocytic compartment was modified in STARD3 cells, notably MVB containing homogenous population of ILV were rarer, while pleomorphic late endosomes containing ILV and multilamellar or mixed multivesicular/multilamellar regions were frequently observed. This morphological alteration was consistent with our previous observations showing that STARD3 functions in organelle dynamics by altering the formation of endosomal tubules (Alpy et al., 2013). Strikingly, internal membrane quantification by stereology showed that compared to control cells, HeLa/STARD3 cells displayed endosomes with more inside membranes, namely ILVs or multilamellar or mixed multivesicular/multilamellar structures (Fig 3D, E and H). To understand the molecular mechanism involved in this process, we examined the ultrastructure of the endocytic compartment in VAP-silenced cells devoid of STARD3-mediated ER–endosome contacts (Fig 3F–H). While HeLa/STARD3/shCtrl cells also showed increased inner membrane content in endosomes, HeLa/STARD3/shVAPA/Bβ exhibited fewer inner membranes in the endosomes. To substantiate this result, we analyzed the endosome ultrastructure in cells expressing the STARD3 ΔSTART mutant (Fig EV5H), which is unable to bind sterol, and in cells expressing the STARD3 FA/YA mutant, which is unable to generate ER–endosome contacts (Fig EV5I). Compared to HeLa/STARD3 cells (Fig EV5G), which displayed endosomes with more internal membranes, HeLa/STARD3 ΔSTART and HeLa/STARD3 FA/YA cells possessed endosomes with internal membrane structures similar to control cells (Fig EV5J). These observations suggest that STARD3-mediated cholesterol transport provides building blocks for membrane formation within endocytic vesicles.

Overall, these results demonstrate that STARD3-mediated cholesterol buildup in endosomes directly depends on STARD3 START domain, and on its ability to scaffold ER–endosome contacts. At the ultrastructural level, STARD3 expression is associated with an alteration of the endosomal compartment with more inner membranes either in the form of ILVs or multivesicular/multilamellar structures. This increased formation of inner membranes is associated with STARD3’s ability to accumulate cholesterol in endosomes.

**STARD3 and VAP interaction couples tethering and sterol transfer between model organelles**

These in vivo experiments suggested that STARD3, with the help of VAP, autonomously connects the ER network to endosomes to pilot ER-to-endosome cholesterol transport. To fully demonstrate that STARD3 and VAP are necessary and sufficient to efficiently transfer sterols between these two organelles, we reconstituted in vitro the tethering complex using recombinant proteins and liposomes (Fig 4A).

To do so, we purified a shorter STARD3 protein, termed thereafter STD3, devoid of the transmembrane MENTAL domain but containing a FFAT motif and the START domain. To anchor this protein to the membrane, we introduced a cysteine residue at its N-terminal end (Fig EV4C and D) enabling its attachment to liposomes doped with thiol-reactive MPB-PE lipids. Thus, using this setting, the soluble part of STARD3 is positioned on liposomes like STARD3 at endosomes surface (Fig EV4E). In parallel, we purified VAPHis6 and the VAP(KD/MD)His6 mutant in which the C-terminal transmembrane region is substituted by a short His-tag. The double K94D/M96D mutation of VAP-A (herein named KD/MD) was shown to abolish FFAT binding (Kaiser et al., 2005). The His-tag enables the attachment of the soluble part of VAP to liposomes containing NTA-Ni2⁺ lipids in an orientation similar to that of VAP proteins on the ER (Fig EV4E) (Mesmin et al, 2013b).

Using flotation assays, we verified the attachment of the STD3 protein to a preparation of liposomes (Lα) containing 3 mol% of

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**Figure 4. Coupling of DHE transport by STARD3 with membrane tethering.**

A Description of the experimental strategy. For DLS experiments, Lα liposomes (endosome-like) are decorated with STD3 owing to covalent links with MPB-PE lipids, and mixed with Lp liposomes (ER-like) covered by VAPmem attached to DOGS-NTA-Ni2⁺. For DHE transport experiment, Lp liposomes also contain DHE and a dansylated lipid (DNS-PE). The transport of DHE from Lp to Lα liposomes is followed by FRET.

B Aggregation assays in real time. Liposomes (50 μM total lipids), decorated or not with STD3 (380 nM), were mixed with Lp liposomes (50 μM total lipids) naked or covered with VAPmem or VAP(KD/MD)His6 (380 nM). Aggregation was followed by DLS. Left panels: mean radius and polydispersity (shaded area) over time. Right panels: size distribution before (gray bars) and after (blue bars) the reaction. Please note that only the condition presented on the top graphs show increased polydispersity and mean radius over time.

C DHE transport assay. DOPC liposomes (62.5 μM total lipids, Lp) containing or not 3 mol% MPB-PE were mixed with STD3 (475 nM) After 5 min, Lp liposomes (DOPC/DOGS-NTA-Ni²⁺/DNS-PE/DHE 85:2:2:5/10 mol/mol, 62.5 μM total lipids), naked or covered with 500 nM VAPmem or VAP(KD/MD)His6, were added. FRET between DHE and DNS-PE in the Lp liposomes diminishes as DHE is transported to the Lα liposomes. The signal was converted into amount of DHE present in Lα liposomes (in mol %). Please note that a slow transport of DHE is observed without STD3 (gray line) due to spontaneous DHE transfer. The black and blue lines show the modest transfer activity of the untethered STD3. The magenta line show the rapid transfer activity of the tethered STD3.

D Initial DHE transport rate measured with STD3 in the presence or the absence of MPB-PE and functional VAP (475 nM). Mean ± SEM; n = 4 independent experiments; *p < 0.05, Mann–Whitney test.
Figure 4.
MPB-PE (Fig EV4F). We also assessed that VAPHis6 and its mutated form were recruited to a second type of liposomes (LB) containing 2 mol% DOGS-NTA-Ni²⁺ and, moreover, that cSTD3 was specifically recruited to these liposomes via the FFAT motif in the presence of VAPHis6 but not of VAP(KD/MD)His6 mutant (Fig EV4F).

Then, using dynamic light scattering (DLS), we monitored the tethering activity of STARD3 by recording the size of the particles formed in the presence of the two liposome populations (Fig 4A). In brief, cSTD3 was attached to LA liposomes that were then mixed with LB liposomes and VAPHis6. A rapid increase in the initial mean radius (98 nm on average) up to 600 nm (Fig 4B) was observed upon VAPHis6 addition. Size distribution analysis at the end of the kinetics was indicative of the formation of liposome aggregates (Drin et al, 2008) with high polydispersity (1.008 ± 0.836 nm) at the expense of free liposomes (98.2 ± 36.5 nm). This experiment was repeated with LA liposomes devoid of MPB-PE or LB liposomes either lacking VAP or covered with the VAP(KD/MD)His6 mutant. In all case, no aggregation was seen (Fig 4B). Jointly, these data indicated that the cytosolic part of STARD3 attached by its N-terminus to one membrane can tether a second membrane only when the latter bears VAP.

Using these established experimental conditions in which cSTD3 promoted contacts between LA and LB liposomes, we measured DHE transfer in real time by FRET (John et al, 2002; Moser von...
Filseck et al., 2015a). La liposomes including both DHE (10 mol%) and DNS-PE (2.5 mol%) and covered with VAPHis6 were added to La liposomes decorated with STD3. Strikingly, we observed a very fast DHE transport within the first seconds and DHE was fully equilibrated between liposome populations after a few minutes (Fig 4C, magenta curve). The initial DHE transport rate was $24.6 \pm 3.8$ DHE molecules/min per â€œSTD3 (mean ± SEM, $n = 3$, Fig 4D). In contrast, with La and La liposomes devoid of MBP-PE (the anchor for â€œSTD3 and VAP, respectively, the transport was twenty times slower (Fig 4C, black curve; Fig 4D, $1.1 \pm 0.54$ DHE molecules/min per â€œSTD3) and DHE distribution failed to reach equilibrium. Likewise, the transport was inefficient with La liposomes covered with VAP(KD/MD) (Fig 4C, blue curve; Fig 4D). Together, these data showed that STARD3 transfers sterols far more efficiently between two membranes while connecting them at the same time.

To substantiate the link between tethering and cholesterol transport, and to provide the biochemical basis for observations done in cells, we examined in vitro the activity of two â€œSTD3 mutants that are either unable to make membrane contacts or to transport cholesterol. To prevent the interaction with VAP, we generated a 7G mutant in which the FFAT motif is substituted by a stretch of glycine; to block sterol transport, we introduced an MR/ND mutation in â€œSTD3 (Fig EV4C and D). Each mutant can be attached to La liposomes doped with MBP-PE but, as expected, the â€œSTD3 7G failed to bind to VAP proteins at the surface of LB liposomes (Fig EV4F). Then, DLS assays showed that the 7G mutant failed to cause La and La aggregation (Fig 5A). Naturally, the MR/ND mutant START domain remained able to interact with VAP proteins and triggered aggregation (Fig 5A). In DHE transport assays, we found that both mutants had altered kinetics of sterol transport, with initial transport rates one order of magnitude lower than that of â€œSTD3 (Fig 5B and C). Of interest, the transport of DHE seen with the MR/ND mutant is likely due to a greater spontaneous DHE transfer resulting from membrane apposition (Fig EV4G). Of note, the transport of the 7G mutant (Fig 5B) is very similar to the transport kinetics of the soluble STARD3 protein (Fig EV4B).

To conclude, using a completely defined in vitro assay enabling us to mimic organelle contacts and measure sterol transport in real time, we show that STARD3 and VAP represent an autonomous molecular machine able to rapidly transfer sterols using membrane contacts.

**STARD3 expression does not activate transcriptional cholesterol sensing and favors ER cholesterol transport to endosomes**

Given that STARD3 expression promoted cholesterol accumulation in endosomes, a further step was to see whether this phenotype was associated with global changes in cellular sterol levels. To address this question, the total cellular cholesterol amount was quantified in lipid extracts originating from control cells, or cells expressing either WT STARD3 or MR/ND mutant STARD3. To ascertain the robustness of our dosage, cholesterol was also measured in lipid extracts of control cells treated with methyl-β-cyclodextrin (MβCD), a chemical known to remove cholesterol from the PM. As shown in Fig 6A, MβCD treatment resulted in the loss of about half of the total cholesterol in lipid extracts compared to untreated cells. In contrast, we found that the expression of STARD3 did not modify whole cholesterol levels compared to control cells or cells expressing MR/ND STARD3 (Fig 6A).

Thereafter, we examined the activation of the sterol regulatory element-binding proteins (SREBPs) in HeLa cells expressing or not STARD3. SREBPs are a family of transcription factors that maintain cellular lipid homeostasis by activating genes involved in cholesterol and fatty acids synthesis and uptake (Horton et al., 2002). SREBP inactive precursors are localized in ER membranes and under low sterol conditions, are activated by cleavage (Brown & Goldstein, 1999). We analyzed the activation of SREBP-2, which specifically controls genes involved in cholesterol metabolism (Horton et al., 2002). Under normal sterol conditions, no modification of SREBP-2 cleavage was observed in STARD3-expressing cells compared to control cells (Fig 6B). To study whether sterol sensing functions in STARD3 cells, we compared SREBP-2 processing between control and STARD3-expressing cells whose free cholesterol level was either lowered or increased. To activate the SREBP pathway by reducing cellular free cholesterol level, cells were grown in lipoprotein-depleted medium (LPDS) or treated with MβCD. We observed a similar increase in SREBP-2 cleavage in control and STARD3-expressing cells (Fig 6B). Next, we increased cellular cholesterol by adding exogenous cholesterol; again this treatment had a similar effect in all cell lines, SREBP-2 cleavage being maintained at a basal level both in STARD3-expressing and in control cells (Fig 6B). These experiments show that endosomal cholesterol accumulation driven by STARD3 did not activate the cholesterol sensor SREBP-2 and furthermore that cholesterol sensing was not altered in STARD3-expressing cells (Fig 6B). Moreover, SREBP-2 target genes (HMGCoA reductase, LDLR, and SREBF-2) expression analysis by RT-qPCR showed that STARD3-expressing cells responded to LDL addition in the culture medium similarly to control cells by lowering their level of expression (data not shown).

Last, we examined whether the pool of cholesterol accumulated in endosomes by STARD3 was originating from the LDL uptake or the neosynthesis pathway in the ER, both pathways providing cholesterol in HeLa cells (Ishitsuka et al., 2011). To test the contribution of the LDL pathway, cells were grown in lipoprotein-depleted medium (LPDS) containing medium for 48 h and cholesterol localization was analyzed with filipin (Fig 7A and B). In LPDS-treated cells, the cholesterol accumulation phenotype persisted (Fig 7D), indicating that the uptake pathway was not the main source of the cholesterol accumulated in endosomes, which therefore suggests that the ER is the major source. Next, to test the contribution of the cholesterol biosynthesis pathway, cells were treated with the cholesterol synthesis inhibitor mevinolin for 2 days and cholesterol localization was analyzed with filipin (Fig 7A-C). In control cells, no cholesterol accumulation was observed with and without inhibition. In HeLa/STARD3 cells, cholesterol accumulation was evident in normal culture conditions; contrastingly, in mevinolin treated cells, the cholesterol accumulation phenotype was markedly reduced (Fig 7E). Although intracellular cholesterol levels did not reach those of control cells, this experiment support the notion that the ER is the main source of the cholesterol accumulated in STARD3 endosomes.

Taken together, these data indicate that the cholesterol accumulation phenotype seen in STARD3-expressing cells is not caused by an increase in cholesterol synthesis and/or uptake by the endocytic
pathway. Rather they strongly suggest that STARD3 has the capacity to alter the intracellular cholesterol repartition by moving ER cholesterol toward endosomes.

**Figure 6.** STARD3-mediated cholesterol accumulation in endosomes does not alter cholesterol homeostasis.

A Total free cholesterol quantification after total lipid extraction in control cells and in cells expressing STARD3 or the lipid binding mutant form of STARD3 (STARD3 MR/ND) \((n = 3)\). Please note that control HeLa cells treated with MjCD have a significant depletion of total cholesterol \((n = 2)\). Mean ± SD; ANOVA with Tukey’s multiple comparison test.

B Western blot analysis of SREBP-2 activation in controls (HeLa, HeLa/empty vector) or STARD3-overexpressing HeLa cells. Cells were incubated for 2 h in DMEM culture medium supplemented with: 5% FCS (NT), 5% LPDS, 10 μM mevinolin (LPDS), 5% LPDS, 10 μM mevinolin, 1 mM MjCD (MjCD), 5% LPDS, 10 μM mevinolin, 500 μM cholesterol complexed to MjCD (MjCD-Chol). The proteasome inhibitor MG132 (10 μM) was present in all conditions. P = precursor form of SREBP-2; C = cleaved form of SREBP-2. Lower panel: WB quantification in which cleaved SREBP-2 is expressed as a fraction of total SREBP-2 \((P+C)\). Mean ± SD; \(n = 4\) independent experiments; **\(P < 0.01\), ***\(P < 0.001\), ANOVA with Tukey’s multiple comparison test.

Source data are available online for this figure.

**STARD3-VAP complex induces cholesterol accumulation in endosomes at the expense of the plasma membrane**

Among the different organelles, the PM contains most of cellular cholesterol (van Meer et al., 2008). Since our data pointed to a role of STARD3 in routing cholesterol toward endosomes, we reasoned that STARD3 might indirectly act on the PM cholesterol. We thus examined whether STARD3 alters cholesterol levels in the PM. To evaluate this possibility, we measured the sensitivity of cells expressing STARD3 to the amphoterin B toxin. This molecule is a polyene antibiotic, which binds PM cholesterol, creating non-selective ion pores that cause cell death (Kinsky, 1970). We observed that, compared to control cells, HeLa/STARD3 cells were less sensitive to amphoterin B treatment (Fig 8A). Conversely, cells expressing STARD3 mutants devoid of a functional START domain or unable to mediate ER–endosome contacts were as sensitive as control cells to the drug (Fig 8A). This experiment suggests that STARD3-mediated sterol transfer at ER–endosome contact sites is associated with a reduction in sterol abundance in the PM.

To determine more directly whether cholesterol accumulation in endosomes induced by STARD3 occurs concomitantly with a decrease of PM cholesterol, we used live cell membrane cholesterol staining with the GFP-D4 probe to image and quantify membrane cholesterol (fig EV1). Robust membrane cholesterol labeling using the D4 probe can be measured using flow cytometry (Iwamoto et al., 1997); compared with light microscopy, it offers the opportunity to evaluate this possibility, we measured the sensitivity of cells expressing STARD3 to the amphoterin B toxin. This molecule is a polyene antibiotic, which binds PM cholesterol, creating non-selective ion pores that cause cell death (Kinsky, 1970). We observed that, compared to control cells, HeLa/STARD3 cells were less sensitive to amphoterin B treatment (Fig 8A). Conversely, cells expressing STARD3 mutants devoid of a functional START domain or unable to mediate ER–endosome contacts were as sensitive as control cells to the drug (Fig 8A). This experiment suggests that STARD3-mediated sterol transfer at ER–endosome contact sites is associated with a reduction in sterol abundance in the PM.

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To further support the notion that STARD3-VAP ER–endosome contacts are modulating PM cholesterol levels, we prevented
Figure 7. The ER is the main source of cholesterol accumulated by STARD3 in endosomes.

A–C HeLa/Ctrl and HeLa/STARD3 cells were incubated in normal medium (A), LPDS-containing medium (B) or normal medium with 50 μM mevinolin and 100 μM mevalonate (C), for 48 h. Cholesterol accumulation in endosomes was detected by filipin staining (Cyan Hot) in endosomes identified by the presence of Lamp1 (red) and STARD3 (magenta). Nuclei were stained in blue. Merged image of filipin and STARD3 signals is shown in (d and h). The subpanels on the right are higher magnification (3.5×) images of the area outlined in white (a, e). The filipin and STARD3 merged image is labeled Overlay. Scale bars: 10 μm.

D, E Relative fluorescence intensity of intracellular filipin in HeLa/Ctrl and HeLa/STARD3 cells incubated or not in LPDS-containing medium (D) or treated or not with mevinolin and mevalonate (E). Mean ± SD; n = 5 (D) and n = 4 (E) independent experiments. **P < 0.01, ANOVA with Tukey’s multiple comparison test.
Figure 8. STARD3-mediated cholesterol accumulation in endosomes occurs at the expense of plasma membrane.

A Indirect analysis of plasma membrane cholesterol content. Cells were treated with amphotericin B (20 µg/ml; 4 h at 37°C), and cell viability was measured using a luminescent cell viability assay. Relative cell viability is represented on the graph. Mean ± SD; n: number of independent experiments; HeLa, HeLa/Ctrl, HeLa/STARD3: n = 5; HeLa/STARD3 ΔSTART; HeLa/STARD3 MR/ND; HeLa/STARD3 FA/YA: n = 4. **P < 0.01, ***P < 0.001, ANOVA with Tukey’s multiple comparison test.

B Plasma membrane cholesterol labeling using the GFP-D4 probe. Live cells were incubated with GFP-D4 probe (green) prior to fixation and nuclei staining (blue). Both signals are merged (a–f). Scale bar: 10 µm. (g) Quantification of mCherry-D4 membrane labeling by flow cytometry. Fluorescence mean intensities are compared with that of control cells set to one. Mean ± SD; n = 7 independent experiments; paired two-tailed t-test; ***P = 0.0006.

C Western blot analysis (a) of STARD3 in HeLa cells, HeLa cells expressing a control shRNA (shCtrl) and in HeLa cells expressing two shRNAs targeting STARD3 (shSTARD3-α and shSTARD3-β). Actin was used as a loading control. Plasma membrane cholesterol was labeled using the GFP-D4 probe (b) under normal cell culture conditions and under LPDS culture conditions (24-h treatment); flow cytometry quantification of the GFP-D4 staining is expressed as mean intensity with that of control cell in normal medium set to one. Mean ± SD; n = 9 independent experiments; **P < 0.01, ***P < 0.001, ANOVA with Tukey’s multiple comparison test.
ER-endosome contact formation by removing VAP proteins (Alpy et al., 2013). First, PM was labeled with GFP-D4 (Fig EV6A and B) in HeLa/STARD3 cells lacking VAP proteins. While HeLa/STARD3/shCtrl cells showed a reduction in GFP-D4 staining, VAP-silenced cells (HeLa/STARD3/shVAPA/B-α and HeLa/STARD3/shVAPA/B-β) had a staining similar to control cells, as shown by confocal imaging (Fig EV6A) and quantification by flow cytometry (Fig EV6B). Second, given that VAP protein reduction restores normal membrane cholesterol levels in STARD3 cells, we tested the sensitivity of HeLa/STARD3 cells lacking VAP proteins to amphotericin B treatment. While HeLa/STARD3/shCtrl cells were resistant to amphotericin B treatment, the silencing of VAP proteins in HeLa/STARD3 cells with two different shRNA (shVAPA/B-α and shVAPA/B-β) restored a sensitivity similar to control cells (Fig EV6C). Together, these experiments indicated that STARD3-mediated sterol transport toward endosomes is associated with a lowering of PM cholesterol content.

To substantiate the physiological relevance of these results, we knocked down STARD3 using two different shRNAs in control HeLa cells (Fig 8C). Compared to non-silenced cells (HeLa and HeLa/shCtrl), STARD3 was reduced to about 20% of endogenous level with shSTARD3-α and shSTARD3-β shRNAs (Fig 8Ca). PM cholesterol was stained on live cells with the GFP-D4 probe and quantified by flow cytometry (Fig 8Cb). Compared to control cells (HeLa/shCtrl), HeLa cells knocked down for STARD3 displayed an increased GFP-D4 staining at their surface (Fig 8Cb). Furthermore, to test the contribution of the LDL pathway, cells were grown in LPDS-containing medium during 24 h and PM cholesterol was labeled with GFP-D4. Under this condition, HeLa cells knocked down for STARD3 still displayed a higher membrane GFP-D4 staining than control cells, further supporting the hypothesis that the ER is the main source of cholesterol transported by STARD3. As the GFP-D4 binds cholesterol-enriched regions, these data suggest that STARD3 knockdown enriched regions, either increases cholesterol level in the PM and/or modulates the local concentration of cholesterol in the PM.

Collectively, these results indicate that the expression of STARD3 is associated with a reduction in PM cholesterol. The presence of a functional cholesterol transfer START domain and ER-endosome contact sites are both required to decrease cholesterol from the PM. These results support the notion that STARD3 pilots ER cholesterol to endosomes transfer while reducing ER to PM traffic.

**Discussion**

Previous reports were suggestive of the ability of STARD3 to promote cholesterol accumulation in endosomes (Alpy et al., 2001; Zhang et al., 2002; Holtta-Vuori et al., 2005; Liapis et al., 2012). In contrast, other studies argued that STARD3 functions in cholesterol transport to the mitochondria or to the PM (Charman et al., 2010; Vassilev et al., 2015). By using novel methods allowing the visualization and the quantification of intracellular cholesterol both in vitro and in vivo, our study provides new evidence that STARD3 acts on intracellular cholesterol repartition by mediating cholesterol transport from the ER to endosomes at the expense of the PM. Moreover, this study shows that STARD3 has a built-in capacity to efficiently transfer sterols between apposed membranes. Indeed, in vivo and in vitro data indicate that the mechanism of STARD3 action relies both on its ability to create contact sites between the ER and endosomes via the direct interaction with VAP proteins, and on the sterol exchange capacity of the START domain (Fig 9).
Sterol homeostasis is achieved by a number of molecular mechanisms governing the synthesis, uptake, storage, and efflux of this sterol. The PM, the ER, and endosomes are key organelles in this homeostasis (Maxfield & van Meer, 2010). Indeed, cholesterol is synthesized in the ER and trafficked to the PM or esterified by cholesterol acyl transferase in the ER to be stored as lipid droplets (Khor et al., 2013; Rogers et al., 2015). Beside this, the endocytic compartment controls cholesterol uptake via the LDL/LDL-R pathway (low density lipoprotein–receptor) that is followed by its cellular redistribution from endosomes (Brown & Goldstein, 1979). Consequently, the interface between the ER and endosomes is thought to represent a central crossroad between these two major pathways. While cholesterol exit from endosomes has been studied for many years (Ikonomov, 2008; Mesmin et al., 2013a), the ER-to-endosome sterol transport route was poorly documented. In this work, we propose that STARD3 localizes at this major interface to efficiently route ER cholesterol to endosome membranes. Interestingly, it has been recently reported that the endosomal oxysterol-binding protein ORP1L might exert a similar role but in a different context, namely in the case of membrane receptor traffic (Eden et al., 2016). After epidermal growth factor (EGF) treatment, its receptor (EGFR) is activated and then endocytosed. ER–endosome contacts are then formed and provide sites of interaction for the ER-localized phosphatase PTP1B with EGFR (Eden et al., 2010, 2016); PTP1B stops EGF signaling by both dephosphorylating the receptor and promoting EGF-stimulated ILV formation which sequester EGFR. In this context, ILVs formation involves the transport of cholesterol from the ER, which is mediated by the interaction between VAP and ORP1L.

The fact that the STARD3-VAP complex might be also involved in ER to endosome cholesterol transport but independently of ORP1L (Alpy et al., 2013) reinforces the notion that multiple distinct contact sites likely exist between endosomes and the ER network (Friedman et al., 2013; Eden et al., 2016). Distinct tethering complexes, as for example STARD3-VAP and ORP1L-VAP, which are formed under different cellular conditions, can share a similar function and transport sterols to endosomes. Besides Niemann-Pick C proteins (NPC2 and NPC1) which efflux cholesterol from endosomes, other proteins acting in contact sites might possibly be involved in sterol traffic in this direction (Vanier, 2014; Chu et al., 2015). The formation of MCSs provides a physical basis for inter-organelle communication and sterol exchange which will vary depending on the cell type and the cellular physiological state. This might explain the conflicting results found in different cell lines (Charman et al., 2010; Vassilev et al., 2015). In addition, one cannot exclude that upon regulatory mechanisms the same machinery could transport cholesterol in a bidirectional manner.

At the cellular level, cholesterol level needs to be constantly tuned (Steck & Lange, 2010). The regulation of cellular cholesterol occurs at the DNA level through the transcription factors from the SREBP family (Horton et al., 2002). SREBP-2 notably regulates the expression of genes involved in cholesterol synthesis and uptake in the ER and endosomes, respectively. One surprising finding of this study was the lack of SREBP-2 control over the cholesterol accumulation phenotype mediated by STARD3. This suggests that intracellular cholesterol levels can be modulated in discrete organelles without activation of the transcription machinery. Such a local regulation mode offers advantages in term of reactivity and precision, compared to the global regulation mode through transcription. This mode of regulation could also offer the advantage of sparing cellular energy by avoiding cholesterol synthesis and capture. The distribution of cholesterol mediated by STARD3 at MCS might represent an economic and fast way to modulate sterol content in specific subcellular territories such as in late endosomes internal membranes. It is increasingly clear that contact sites between organelles are zones of active non-vesicular lipid traffic. Notably, recent studies showed that members of the oxysterol-binding protein-related protein (ORP)/oxysterol-binding homology (Osh) family counter-exchange two lipid species within contact sites; OSBP in ER–Golgi contacts and ORP5/ORP8 in ER–PM contacts exchange PtdIns(4)P with sterols or PS, respectively (Mesmin et al., 2013b; Chung et al., 2015; Moser von Filsseck et al., 2015b). These proteins contain an OSBP-related domain (ORD) lipid-binding domain which, like the START domain, bears one lipid molecule and shields it from the hydrophilic medium through which it is transported (Raychowdhury & Prinz, 2010; Alpy & Tomasetto, 2014; Olkkonen, 2015). Nevertheless, ORD domains can bind two ligands alternately, PtdIns(4)P and another lipid such as a sterol or PS (de Saint-Jean et al., 2011; Maeda et al., 2013; Moser von Filsseck et al., 2015b). This dual binding specificity coupled to PtdIns(4)P gradient maintained by spatially distant enzymes at ER/Golgi or ER/PM interface drives lipid transport (Mesmin et al., 2013b; Drin et al., 2016). A membrane tethering ability appears to be essential for the lipid exchange activity of some ORP proteins such as ORP5 and ORP8 which connect the ER and the PM (Chung et al., 2015). Besides, cell experiments suggest that OSRP’s ability to transport sterols in exchange for PI4P relies on its lipid-binding domain but also on its tethering capacity (Mesmin et al., 2013b; Wang et al., 2014). Mechanistic data are yet lacking for the newly discovered sterol-binding proteins in yeast that have a dual ability to tether ER and PM and possess a START-like domain (Gatta et al., 2015; Murley et al., 2015). Our mechanistic approach, based notably on transport or tethering-deficient STARD3 mutants, shows that tethering distinct membranes can guide sterol transport.

In addition, we prove using a controlled in vitro system that STARD3 speeds up sterol transport only under tethering conditions. In this assay, the soluble parts of STARD3 and VAP were attached through chemical anchors to endosome-like or ER-like liposomes, respectively. This mediated an efficient connection between the two distinct liposome populations. Remarkably, we observed that tethering accelerated the sterol transfer rate by one order of magnitude. The kinetics calculated for the soluble and tethered protein was 2 and over 25 molecules of sterol/min per STARD3, respectively. Interestingly, the transport kinetics measured for the different soluble and isolated START domains fall into a similar range; for instance, STARD4 transports ~7 sterols/min (lae et al., 2015), whereas CERT/STARD11 transfers 4 ceramides/min (Hanada et al., 2003). The physiology tells a different kinetics, early work on STARD1 indicated that to promote an acute synthesis of steroids in an instance, STARD4 transports ~7 sterols/min (lae et al., 2015), whereas CERT/STARD11 transfers 4 ceramides/min (Hanada et al., 2003). The physiology tells a different kinetics, early work on STARD1 indicated that to promote an acute synthesis of steroids in
efficient exchange of many ligand molecules. The pool of accessible ligand, the vicinity of high-affinity acceptors such as metabolizing enzymes or lipids, will impose the rate and direction of transport.

A long-standing question is whether a shorter distance between membranes is an asset for an efficient lipid transport. Previous *in vitro* studies suggested that the activity of OSBP is increased when the protein is positioned between ER and Golgi-like membranes (Mesmin *et al.*, 2013b). Yet it remains difficult to prove that this higher activity solely arises from a reduced distance between membranes. Indeed, the anchoring points for OSBP on the ER (VAP) and the Golgi membrane (PtdIns(4)P), which control its tethering activity, play also an active role in its exchange activity. Here, our study showed that a simple connection of membranes, thus a shortened distance, seems critical for the activity of a lipid transporter. Interestingly, we were able to observe *in vitro* that spontaneous sterol transport seems faster when membranes are closer. This stated, it appears clear that the position of a functional sterol-binding domain is crucial to guarantee transport velocities compatible with cellular timescales.

To summarize, we report that STARD3, by making contact sites with VAP, modulates cholesterol traffic. Our results show that STARD3 and VAP make an autonomous molecular machine able to transfer cholesterol very rapidly and without energy, using a mechanism different from ORPs. Elucidating the molecular mechanism of STARD3 function provides new insights into the mechanism of sterol-binding domain is crucial to guarantee transport velocities compatible with cellular timescales.

**Materials and Methods**

**Cloning and constructs**

The retroviral expression vector for STARD3 (pQCXIP STARD3) was previously described (Alpy *et al.*, 2013). STARD3 mutants were constructed in the pQCXIP vector: The STARD3 ΔSTART (Q14849-1, residues 1–235), STARD3 M307R/N311D (referred to as MR/ND mutant), and STARD3 F207A/Y208A (referred to as FA/YA mutant) double mutants were constructed by site-directed mutagenesis.

STARD3 cDNA was subcloned into the retroviral pMXPIE vector (kind gift from Bernardo Reina San-Martin) to generate a bicistronic vector encoding STARD3 and the GFP.

A STARD3[196–445] fragment was PCR-amplified from an initial vector encoding STARD3 and the GFP. (kind gift from Bernardo Reina San-Martin) to generate a bicistronic vector encoding STARD3 and the GFP.

A human VAP-A [8–212] fragment was subcloned into pET-21b for expression as C-terminal His-tag proteins in *E. coli*. The position of the C-terminal His tag corresponds to the beginning of the C-terminal transmembrane region. As for STARD3, the soluble part of VAP construct should be positioned on DOGS-NTA-Ni²⁺ containing liposomes in a manner similar to the full-length VAP protein. The mutations and deletion/insertions of sequences were done using the QuiKchange kit (Stratagene).

To silence VAP proteins, we used the previously described shRNA expression vectors targeting VAP-A (target sequence: 5′-GCGAAATCCCATCGGATAGAAA-3′ or β: 5′-CCTTTAATGATACCGAAACAA-3′) or VAP-B (α: 5′-GCAGAGAATGATAAACCACAT-3′ or β: 5′-CCAGTTCTTGTGACTATGA-3′) (Alpy *et al.*, 2013). To generate shRNA expression vectors targeting STARD3 (target sequence α: 5′-GACCTGTCCCTGACTTCA-3′ or β: 5′-CGGCAAGACGTTATCCTGAA-3′), oligonucleotides were cloned into the pLKO.1 vector (Moffat *et al.*, 2006).

The expression vectors encoding the GFP-D4 and mCherry-D4 probes were previously described (Ohno-Iwashita *et al.*, 2004; Abe *et al.*, 2012).

All constructs were verified by DNA sequencing.

**Cell culture, transfections, and infections**

HeLa cells (American Type Culture Collection CCL-2) were maintained in DMEM with 5% fetal calf serum (FCS) and 40 μg/ml gentamycin. 293T (ATCC CRL-3216) cells were maintained in DMEM with 10% fetal calf serum (FCS), penicillin 100 UI/ml, and streptomycin 100 μg/ml. LPDS medium is composed of DMEM supplemented with 40 μg/ml gentamycin an 5% LPDS purified as described in Goldstein *et al.* (1983).

Cells were transfected using X-tremeGENE 9 DNA Transfection Reagent (Roche). For retroviral infection, pQCXIP vectors were co-transfected with pCL-Ampho vector (Imgenex) into 293T retroviral packaging cell line. Retroviral infections were used to generate HeLa/Control, HeLa/STARD3, HeLa/STARD3 ΔSTART, HeLa/STARD3 MR/ND, and HeLa/STARD3 FA/YA cell lines. The HeLa/Control cell line was obtained using the empty pQCXIP or pMXPEI plasmid.

For lentiviral infection, pLKO.1 vectors were co-transfected with three packaging plasmids pLP1, pLP2, and pLP/VSVG (Invitrogen) into the 293T cell line. Viral particles supplemented with 10 μg/ml polybrene and 20 mM Hepes were then incubated with HeLa cells. HeLa cells were then incubated with HeLa cells. HeLa cells were then incubated with HeLa cells.

HeLa cells were then incubated with HeLa cells.

**Antibody production**

The cSTARD3 recombinant protein was used to produce a mouse monoclonal antibody as described before (Moog-Lutz *et al.*, 1997). The 3G11 hybridoma, which recognizes STARD3 by Western blot and immunofluorescence, was used in this study.

**Immunofluorescence**

Immunofluorescence was used as previously described (Alpy *et al.*, 2002). Primary antibodies were rabbit anti-STARD3 pAbMLN64-NT-1611 (1:1,000; Alpy *et al.*, 2001), goat anti-VAP-A (1:500; K-15—Santa Cruz Biotechnology, sc-48698), mouse anti-Lamp1 H4A3
(1:50; DSHB), mouse anti-EEA1 (1:1,000; 610457 BD Biosciences), mouse anti-CD63 H5C6 (1:1,000; DSHB), mouse anti-BMP (1:10; 6C4 (Kobayashi et al, 1998), and rabbit anti-Rab7L1 (1:1,000, Abcam ab199644). Slides were mounted in ProLong Gold (Invitrogen). Observations were made with a confocal microscope (Leica SP8 UV, 63×, NA 1.4).

Colocalization analysis

Linescans were drawn using ImageJ software (plot profile function; https://imagej.nih.gov/ij/). Colocalization was visualized using the colocalization highlighter plugin for ImageJ.

Colocalization coefficients (Pearson correlation coefficient or Manders M1 and M2) were determined using the JACoP plugin (Bolte & Cordelieres, 2006) in Fiji software. For Pearson correlation coefficient determination, auto-thresholding was performed using the Costes method (Costes et al, 2004). For Manders colocalization coefficient determination, thresholding was performed using Yen algorithm (Fiji).

To quantify the co-labeling of filipin or GFP-D4-positive vesicles with Lamp1 or STARD3, an automatic threshold (Yen, Fiji) was determined manually using thresholded images.

Filipin staining and quantification

Cells were grown on glass coverslips. In order to allow a better visualization of intracellular cholesterol pools, cells were treated with 10 mM methyl-β-cyclodextrin (MβCD, Sigma) for 30 min at 37°C to remove cholesterol from the plasma membrane (Fig EV1). Cells were then fixed for 10 min at RT with 4% paraformaldehyde in phosphate-buffered saline (PBS). Cells were incubated with a solution of filipin (0.1 mg/ml, F-9765 Sigma) for 30 min. After washing and blocking in 1% bovine serum albumin (BSA) in PBS, cells were incubated with the primary antibodies. After three washes in PBS, cells were incubated for 1 h with Cy3- and Alexa488-conjugated secondary antibodies (Jackson ImmunoResearch and Invitrogen-Molecular Probes, respectively). After three washes, cells were re-incubated in the filipin solution for 30 min. Finally, after two washes, cells were counterstained with propidium iodide (PI, 0.35 μg/ml; Sigma) or with TO-PRO-3 iodide (1:3,000; ThermoFisher scientific) for 5 min. Slides were mounted in ProLong Gold (Invitrogen). Image acquisition was performed with a Leica SP8 UV (63×, NA 1.4) confocal microscope using the photon counting mode; a 355 nm optically pumped semiconductor laser (Coherent) was used to excite filipin. The Fiji software was used to quantify filipin fluorescence intensity (http://fiji.sc/). Cells were segmented based on the PI staining (Fig EV3). Filipin staining intensity in individual cells was measured after an intensity thresholding. The threshold value was determined in control cells as the 99th percentile of pixel intensity values.

GFP-D4 and mCherry-D4 production and staining

Production

Proteins were overexpressed overnight in E. coli BL21(DE3) at 16°C in the presence of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma). The cell pellet was lysed and sonicated in 50 ml of lysis buffer (50 mM Na2HPO4/NaH2PO4, 300 mM NaCl, 10 mM imidazole, pH 8.0) complemented with EDTA-free cOmplete protease inhibitor (Roche). The lysate was centrifuged at 10,000 g for 20 min at 4°C. The supernatant was incubated with 1.5 ml of His-Select Nickel Affinity Gel (Sigma). The resin was washed with lysis buffer. Bound proteins were eluted with elution buffer (20 mM NaH2PO4/NaH2PO4, 250 mM imidazole, pH 7.4). Eluted fraction was dialyzed into phosphate buffer (20 mM NaH2PO4/NaH2PO4 pH 7.4, EDTA-free cOmplete).

Plasma membrane staining

For confocal acquisitions, cells were grown on glass coverslips, washed with PBS, and incubated in GFP-D4 or mCherry-D4 solution (1:200 in 1× PBS, 1% BSA) for 30 min. After a rapid wash, cells were fixed with 4% paraformaldehyde in PBS and nuclei were counterstained with Hoescht-33258 dye. Slides were mounted in ProLong Gold (Invitrogen).

For flow cytometry analysis, 6.10^5 cells were plated in a T175 flask 24 h before the experiment. Cells were trypsinized and incubated in a solution of GFP-D4 or mCherry-D4 probe (1:200 or 1:1,000 in 1 PBS, 1% BSA) for 30 min at 37°C. Cells were then fixed for 10 min at RT with 2% paraformaldehyde in PBS and nuclei were counterstained with Hoescht-33258 dye. Slides were mounted in ProLong Gold (Invitrogen).

Intracellular staining

Cells were grown on glass coverslips, treated or not with 1 μg/ml of U18666A ((3β)-3-{2-(Diethylamino)ethoxy}androst-5-en-17-one, 662015 Calbiochem) during 1 h, fixed 10 min at RT with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), and permeabilized in a nitrogen bath for 30 s. After blocking in 1% BSA in PBS, cells were incubated at RT with 2% paraformaldehyde in PBS, centrifuged, and finally resuspended in PBS before FACS analysis (FACS caliber and LSRFortessa X-20, BD biosciences). At least 2.10^6 cells were analyzed in duplicate. For cells expressing the pMXPI vector, GFP-positive cells were gated (FlowJo) and the mean mCherry-D4 fluorescence was measured in this cell population.

GFP-D4 and filipin co-staining

Cells were firstly stained with the GFP-D4 probe as described above (intracellular staining protocol). After incubation with primary (anti-STARD3) and secondary antibodies, cells were re-incubated with filipin solution during 1 h at RT. After 1 wash, nuclei were counterstained with TO-PRO-3 iodide and slides were mounted.

Statin treatment

Cells were treated with 50 μM mevinolin (M-2147, Sigma) and 100 μM DL-mevalonolactone (SC-211365, Santa Cruz) diluted in
normal medium during 48 h at 37°C. Cells were washed, and filipin staining was performed as described above.

**SDS–PAGE and Western blot analysis**

SDS–PAGE and Western blot analysis were performed as previously described (Alpy et al, 2005) using the following antibodies: STARD3: pAbMLN64-Ct-605 (Moog-Lutz et al., 1997); pAbMLN64-Nt-1611 (Alpy et al., 2001); VAP-A: K-15 sc-48698 (Santa Cruz biotechnology); VAP-B: rabbit anti-VAP-B (Kabashi et al., 2013); SREBP-2 1C6 (sc-13552; Santa Cruz biotechnology); actin: ACT-2D7 (Euromedex).

**Lipids extraction and quantification**

One million cells were plated in 150-mm petri dishes 24 h before the experiment. Cells were treated with 10 mM methyl-β-cyclodextrin (MJCD, C4955 Sigma) for 30 min for the positive control. Cells were washed three times and scraped in 500 µl of PBS. Cells in PBS (500 µl) were mixed with 2 ml of EtOH/chloroform (2/1) solution in glass tubes. After 5 min of centrifugation at 1,400 g, the supernatant was transferred to a new tube. Then, 0.5 ml of 50 mM citric acid, 0.5 ml of PBS, and 1 ml of water, and 0.5 ml of chloroform were added. After 20 min of centrifugation at 1,400 g, the lower phase-containing lipids were transferred in a new glass tube and dried in a stream of nitrogen. For the lipids quantification, dried lipids were solubilized in EtOH 95% solution. Cholesterol quantification was performed with the Amplex Red cholesterol assay (A12216 Invitrogen), and phospholipids quantification was performed with LabAssay phospholipid (296-63801 Wako Chemicals) according to the manufacturer instructions.

**SREBP-2 processing**

HeLa cells transduced with the empty pMXPIE vector (containing an IRES-GFP) or the pMXPIE construct encoding STARD3 were sorted by flow cytometry (FACS Aria II BD biosciences). Three hundred thousand GFP-positive cells were seeded in a 6-well plate and maintained 24 h in culture. Cells were then incubated for 2 h at 37°C in DMEM medium with 5% of FCS (NT condition) or 5% LPDS, 10 mM methionine, 10 mM MG132 (Sigma), which was supplemented with 1 mM MJCD or 500 µM MJCD/cholesterol (C4951 Sigma) to deplete or load the cells with cholesterol, respectively. Cells were subsequently harvested at 0°C and lysed in 150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% Triton X-100 complemented with EDTA-free cOmplete protease inhibitor (Roche).

**Amphotericin B sensitivity assay**

Five thousand cells were plated in a 96-well plate (5,000 cells/well). Twenty-four hours later, cells were treated or not with 20 µg/ml amphotericin B (A8888; Sigma) during 4 h at 37°C. Cell viability was evaluated by the CellTiter Glo Luminescent Cell viability Assay (Promega), according to the manufacturer instructions.

**Protein expression and purification**

GST-cSTD3 and VAP[8-212]His6 were expressed in E. coli at 37°C for 3 h upon induction with 1 mM IPTG (at an optical density (OD) equal to 0.6 at λ = 600 nm). For GST-tagged proteins, all purification steps were conducted in 50 mM Tris, pH 7.4, 120 mM NaCl (TN buffer) with 2 mM DTT. For VAPHis6, a buffer containing 50 mM Tris, pH 7.5, 300 mM NaCl, 5 mM imidazole was used. All buffers were supplemented during the first purification steps with PMSF (1 mM), bestatin (10 µM), pepstatin (10 µM), phosphoramidon (10 µM), and protease inhibitor tablets (Roche). Cells were lysed by a French press, and the lysate was centrifuged at 200,000 g for 1 h.

For GST-cSTD3, the supernatant was applied to Glutathione Sepharose 4B beads. After three washing steps with TN buffer containing 2 mM DTT, the beads were incubated with thrombin at 4°C overnight to cleave the GST fusion and allow the release of cSTD3. The protein was recovered by centrifugation, and the beads were washed three times with TN buffer. The fractions were pooled and concentrated. All constructs contain an N-terminal GS sequence deriving from the thrombin cleavage site. Stock concentration was estimated with a BCA assay (Pierce).

For VAPHis6 constructs, the supernatant was mixed with NTA-Ni²⁺ Agarose beads (QIAGEN). After three washing steps, the proteins were eluted from the beads with buffer containing first 250 mM, then 500 mM imidazole.

The day of experiment, in order to attach cSTD3 covalently to MBP-PE-containing liposomes, 100 µl from stock protein (adjusted at ~100 µM) was applied onto an illustra NAP-10 column (GE Healthcare) and eluted with freshly degassed TN buffer according to manufacturer’s indications to remove DTT. The concentration of the eluted protein was determined with a BCA assay.

**Lipids**

DOPC (1,2-dioleyl-sn-glycero-3-phosphocholine), POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), DNS-PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N(5-dimethylamino-1-naphthalenesulfonyl)), NBD-PE (1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine-N-[N-(5-amino-1-carboxypentyl)iminodiacetic acid]succinyl), and 18:1/18:1 MBP-PE (1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine-N-[4-(p-maleimidophenyl)butyramide]) were purchased from Avanti Polar Lipids. Dehydroergosterol (DHE) was from Sigma-Aldrich.

The concentration of DHE in stock solution in methanol was determined by UV-spectroscopy using an extinction coefficient of 13,000 M⁻¹.cm⁻¹.

**Liposome preparation**

Lipids, stored in stock solutions in CHCl₃ or methanol, were mixed at the desired molar ratio. The solvent was removed in a rotary evaporator under vacuum. For lipid films containing DOGS-NTA-Ni²⁺ or MBP-PE, the mix was pre-warmed to 33°C for 5 min prior to drying. The films were hydrated in TN buffer to obtain a suspension of multilamellar liposomes. The suspensions were extruded through polycarbonate filters of 0.2 µm pore size using a mini-extruder (Avanti Polar Lipids). Liposomes were stored at 4°C and in the dark when containing light-sensitive lipids (DHE, DNS-PE, NBD-PE) and used within 2 days.
Dynamic light scattering measurements of liposome aggregation

All experiments were performed at 25°C in a Dynapro apparatus (Protein Solutions). The sample initially contained Lₐ liposomes (50 μM total lipids) in TN buffer freshly degassed in a small quartz cell (volume 20 μl). A first set of about 12 autocorrelation curves was acquired to measure the size distribution of initial liposome suspension. Then, cSTD3 construct (380 nM final concentration) was added manually and mixed thoroughly. After an incubation of 5 min, Lₐ liposomes were added (50 μM followed by the injection of VAPHIS (380 nM)). The kinetics of aggregation was followed by acquiring one autocorrelation curve every 10 s. At the end of the experiment, a set of 12 autocorrelation functions was acquired. The data were analyzed using two different algorithms provided by the Dynamics v6.1 software (Protein Solutions). During the aggregation process, the autocorrelation functions were fitted assuming that the size distribution is a simple Gaussian function. This mode, referred as the monomodal or cumulant algorithm, gives a mean radius, R, and the width (or polydispersity). The polydispersity is represented in the kinetics measurements by the shaded area coverage of the fluorimeter.

DHE transport assay

Experiments were carried out in a Shimadzu RF 5301-PC fluorimeter equipped with a cylindrical quartz cuvette. A suspension (570 μl) of Lₐ liposomes (62.5 μM total lipids final concentration) made of DOPC and containing 3 mol% MPB-PE was incubated with 475 nM cSTD3 at 37°C under constant stirring in TN buffer. After 5 min, 30 μl of Lₐ liposomes, containing 10 mol% DHE, 2.5 mol% DNS-PE, and 2 mol% DOGS-NTA-Ni²⁺ (62.5 μM total lipids final concentration), pre-incubated or not with VAPHIS (500 nM final concentration) was added. Lipid transport was measured by recording the DNS-PE signal at 525 nm (bandwidth 10 nm) upon DHE excitation at 310 nm (bandwidth 1.5 nm). The quantity of DHE transported from Lₐ to Lₐ membrane is expressed in term of mol% DHE in Lₐ liposomes. It is equal to 10 x ((F-F₀)/(F_max-F₀)) where F_max is the signal measured in the absence of STD3 upon the addition of Lₐ liposome and F₀ is the signal measured upon total DHE extraction by 10 mM methyl-β-cyclodextrin (Sigma). Liposomes and proteins were injected from stock solutions with Hamilton syringes through a guide in the cover of the fluorimeter.

Flotation experiments

Proteins (750 nM) were incubated with NBD-PE containing liposomes (750 μM total lipids) in 150 μl TN buffer at room temperature for 5 min. The suspension was adjusted to 30% sucrose by mixing 100 μl of a 75% (w/v) sucrose solution in HK buffer and overlaid with 200 μl HK containing 25% (w/v) sucrose and 50 μl sucrose-free HK. The sample was centrifuged at 240,000 g in a swing rotor (TLS 55 Beckmann) for 1 h. The bottom (250 μl), middle (150 μl), and top (100 μl) fractions were collected. The top fractions were analyzed by SDS-PAGE using Sypro Orange staining and a FUJI LAS-3000 fluorescence imaging system.

Electron microscopy and stereology

Electron microscopy was performed as previously described (Alpy et al., 2013). Briefly, cells grown on type I collagen-coated Aclar film were cryoprotected with a solution of 20% BSA in culture medium and immediately frozen at high pressure (EMPACT-2 Leica). Alternatively, cells grown on carbon-coated sapphire disks were cryoprotected with DMEM containing 10% FCS and frozen at high pressure (HPM 10 Abra Fluid AG). Samples were then freeze-substituted and embedded in Lowicryl HM20. Thin sections were collected on formvar–carbon-coated nickel slot grids and stained with uranyl acetate and lead citrate. Imaging was performed with a transmission electron microscope (Philips CM12) coupled to an Orius 1000 CCD camera (Gatan). Inner endosomal membranes quantification was performed by stereology (Mayhew, 1991). A virtual array of points separated by 31.6 nm was randomly placed on TEM images. The number of points over inner endosomal membranes from a given endosome section was divided by the total number of points over the endosome section to get an estimate of the volume to volume percentage of internal membranes per endosome.

Statistical analyses

Unless otherwise specified, statistical analyses were performed using the parametric test ANOVA. All conditions were compared by the Tukey’s multiple comparison test (Prism, Graphpad and Sigma-Plot, Systat software).

P-values < 0.05, < 0.01, and < 0.001 are identified with 1, 2, and 3 asterisks, respectively. ns: P > 0.05.

Expanded View for this article is available online.

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Author contributions
CT, FA, and GD conceived and supervised the project. LPW performed and 
analyzed all cell biology experiments, with the help of CW, CT, and FA. CD 
performed and analyzed all in vitro tethering/transport experiments. TK helped 
cholesterol labeling experiment design. BV performed gene expression analyses 
and some biochemical experiments. M-PC assisted in designing and interpret-
ing experiments. CT, FA, and GD, and LPW wrote the manuscript. All authors 
commented on the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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