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Membrane curvature catalyzes lipid droplet assembly

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Summary

Lipid droplet (LD) biogenesis begins in the endoplasmic reticulum (ER) bilayer, but how the ER topology impacts this process is unclear. An early step in LD formation is nucleation, wherein free neutral lipids, mainly triacylglycerols (TGs) and sterol esters (SEs), condense into a nascent LD. How this transition occurs is poorly known. Here, we found that LDs preferably assemble at ER tubules, with higher curvature than ER sheets, independently of the LD assembly protein seipin. Indeed, the critical TG concentration required for initiating LD assembly is lower at curved vs. flat membrane regions. In agreement with this finding, flat ER regions bear higher amounts of free TGs than tubular ones and present less LDs. By using an *in vitro* approach, we indeed discovered that the presence of free TGs in tubules is energetically unfavorable, leading to outflow of TGs to flat membrane regions or condensation into LDs. Accordingly, *in vitro* LD nucleation can be achieved by the sole increase of membrane curvature. In contrast to TGs, the presence of free SEs is favored at tubules and increasing SE levels is inhibitory to the curvature-induced nucleation of TG LDs. Finally, we found that seipin is enriched at ER tubules and controls the condensation process, preventing excessive tubule-induced nucleation. The absence of seipin provokes erratic LD nucleation events determined by the abundance of ER tubules. In summary, our data indicate that membrane curvature catalyzes LD assembly.

Introduction

Lipid droplets (LDs) are organelles at the center of cellular energy metabolism [1]. LDs consist of a neutral lipid (NL) oil core, mainly composed of triacylglycerols (TG) and/or sterol esters (SE), covered by a phospholipid (PL) monolayer containing proteins.

The biogenesis of LDs is triggered under various physiological conditions and proceeds via successive biochemical and biophysical reactions taking place at the endoplasmic reticulum (ER) [2,3]. During conditions favoring lipid storage, these biochemical reactions mediate NL synthesis. NL oil molecules are encapsulated between the ER bilayer leaflets due to their hydrophobicity, and diffuse within the ER. When a critical concentration of NLS is reached, the molecules phase separate from the PL bilayer and assemble into stabilized condensed structures [4]. This condensation and phase separation process, predicted by molecular dynamic simulations [5,6] is termed nucleation [7]. The NL condensate forms a nascent LD which grows and buds into the cytosol [3,8]. This emergence of a LD from the ER bilayer is influenced by several membrane physicochemical properties such as PL composition, tension and shape, as well as proteins bound to the surface of the forming LD [5,9]. For example, large membrane surface tension, altered by PLs or membrane shape, impede LD assembly and promote large LDs [5]; membrane curvature and the recruitment of proteins or PLs to the outer ER membrane leaflet, favor external LD emergence [9–11]. However, how these factors impact on LD nucleation, i.e. LD assembly initiation, remains poorly understood. This is because nucleation happens at nanometric scales, making it difficult to investigate.

NLS condense in a bilayer when they reach a critical concentration [5,6,12]. This concentration is coupled to an energy barrier that depends on factors such as protein-lipid and lipid-lipid interactions, and membrane topology such as curvature [7]. Topologically, the ER bilayer is made of sheets and tubules, whose relative abundance is affected by the cell type and phase [13]. Interestingly, LDs made of different NLS may arise at precise ER subregions [14–17]. It is currently unknown whether the ER topology and composition affect LD nucleation.

While many proteins affect the size and number of LDs, no single protein appears indispensable for LD formation. This is likely because NLS may behave as confined fluids that can spontaneously condense into droplets [7]. However, it is crucial for cells to control such a condensation process to regulate LD assembly. A key protein implicated in LD assembly is seipin [18,19]. It is an oligomeric ER membrane protein [20–22] whose dysfunction is linked to severe forms of lipodystrophy [23] and neurological diseases [24,25]. Seipin oligomers are motile in the ER and arrest at sites where LDs emerge [26,27]. At these sites, seipin is able to promote the growth of the newborn LDs and prevent their shrinkage by Ostwald ripening [7,28]. How seipin acts in this process is still unclear but it might transfer TGs into forming LDs [21,28] and/or modulate the local PL milieu [2,22,29]. In the absence of seipin, a phenotype of few supersized and many tiny LDs appears [18,26,27]. This tiny LD population is reduced by the concomitant depletion of Rab18 [28], another organizer of LD assembly [30].

Here, we found that the ER membrane curvature catalyzes LD assembly and LDs form at ER tubules. However, free TGs do not favor highly curved bilayers per se. Rather, curvature increases the chemical potential of TGs and upon increasing TG concentration in tubules, phase separation into LDs

is triggered. Seipin is enriched at tubules, controlling LD assembly and facilitating TG flux to LDs. By this mechanism, seipin keeps the free TG level in the ER below a critical nucleation concentration, thereby preventing uncontrolled nucleation. Indeed, without seipin, erratic nucleation events occur, controlled by the abundance of ER tubules.

Results

ER tubules are the sites of LD assembly and enriched in seipin

We previously reported that upon oleic acid (OA) administration to human A431 cells, LDs start to develop at ER domains marked by seipin [28]. Indeed, in these cells, nascent LDs, visualized by the LD targeted model peptide LiveDrop-mCherry, appear at sites marked by endogenously fluorescently tagged seipin (Figure 1A). Several studies have suggested ER tubules as the birthplace for LDs [31,32] but this has not been systematically investigated and the underlying principles remain unknown.

To investigate the relationship between LD formation and ER morphology, we utilized the machine-learning based image analysis software *ilastik* to discern ER sheets and tubules from Airyscan live cell images of the ER visualized by the fluorescent ER marker BFP-KDEL. Using this analysis, we found that the sites of LD formation marked by seipin were highly enriched at ER tubules (Figure 1A and 1B). Of note, our ER segmentation method recapitulated the previously documented enrichment of known ER subdomain marker proteins at sheets or tubules (Figure S1A-S1C) [33,34]. As a further validation of the analysis tool, we analyzed the ER morphology in control and Rab18 knockout (KO) cells. Rab18 KO has been reported to expand ER sheets at the expense of tubules in Cos7 cells [35] and a similar phenotype was found in A431 cells (Figure 1C). Since Rab18 KO strongly reversed the tubule-to-sheet ratio (Figure 1C), we examined the sites of LD formation under this condition as compared to WT. To this end, we stringently delipidated the cells and then induced LD biogenesis by addition of OA, imaging cells during the first 15 min of LD biogenesis. We analyzed the localization of LDs marked by the NL dye LD540 and LD-associated seipins. Nascent LDs were enriched at ER tubules in both WT and Rab18 KO cells, despite the higher fraction of sheets in Rab18 KO cells (Figure 1D and 1E).

As seipin can determine the site of LD formation [28] and LDs appeared to preferentially assemble at ER tubules, we investigated the localization of seipin in the ER. In live cell video recordings, seipin foci were highly motile in the ER, as previously reported [26,27]. Often, when travelling via a sheet-like region, seipin appeared to move along the edges of the sheet (Figure 1F), barely scanning the planar surface. Image analysis revealed seipin to be enriched at ER tubules (Figure 1G). This enrichment was also apparent in Rab18 KO cells (Figure 1G), even though these cells harbored less ER tubules (Figure 1C).

To further investigate the localization of seipin in relation to membrane curvature, we induced the formation of large intra-cellular ER-derived vesicles by adding a hypotonic culture medium to the cells (Figure 1H, 1I, and Figure S1E) [36,37]. We worked with Cos7 cells transfected with sec61 β -mCherry and mouse YFP-seipin, which colocalized (Figure 1H and Figure S1D). We observed the formation of numerous ER-vesicles of heterogeneous sizes, larger vesicles with lower curvature and smaller vesicles with higher curvature, decorated by sec61 β -mCherry (Figure 1H, 1I, and Figure S1E). We also captured situations where ER-vesicles co-existed with tubular structures (Figure S1F). In this system, we found that the YFP-seipin signal was more intense on smaller ER-vesicles (Figure 1I and Figure S1E) and tubules (Figure S1F) than on large ER-vesicles. By comparing the ratio of seipin to sec61 β signal, we observed an enrichment of seipin in smaller ER-vesicles (Figure 1J). Similar results

were obtained in HeLa cells (Figure S1G and S1H). These data suggest that seipin prefers regions of the ER with a higher curvature, and may act as a curvature sensor or be associated with one.

Overall, these data show that LD assembly is initiated at ER tubules, even when the tubular fraction is markedly decreased by Rab18 KO. Seipin is enriched at curved regions and may function as a nucleator of LDs at ER tubules.

LDs also nucleate at tubules in the absence of seipin

If seipin nucleates LDs at tubules, its depletion might alter this tubule-dependent nucleation of LDs. We thus investigated the sites of LD biogenesis in the absence of seipin. We used seipin-degron cells where seipin can be inducibly and rapidly depleted by the administration of indole-acetic-acid (IAA) [28]. IAA pre-treated seipin degron cells display dramatically altered LD formation, with a high number of tiny LDs and some supersized LDs appearing upon OA treatment [28]. Remarkably, even in the absence of seipin, nascent LDs were enriched at ER tubules (Figure 2A and 2B). In both the absence and presence of seipin, this enrichment at ER tubules was more evident for smaller, apparently younger LDs (Figure S2A and S2B).

To further study the contribution of curvature and seipin in LD nucleation, we took advantage of the heterogeneity of curvature in the ER-derived vesicles and probed LD assembly sites in this system. We used Cos7 cells transfected with mouse YFP-seipin, which were able to make LDs (Figure S2C). We next induced the formation of ER-vesicles and added the NL dye LipidTox. We then fed the cells with OA and red Bodipy-C₁₂ (Bpy-C₁₂) (1000:1), previously shown to be incorporated into NLs [26,38]. Accordingly, Bpy-C₁₂ became incorporated into new LipidTox-positive LDs (Figure 2C and S2D), indicating that the swollen cells were capable of *de novo* LD formation, albeit slightly less efficiently than normal cells (Figure S2E). Interestingly, almost all generated Bpy-C₁₂-positive LDs appeared near submicrometric seipin-positive vesicles (Figure 2C and 2D). We next performed similar experiments, transfecting the cells with YFP-seipin and sec61 β -mCherry (to mark the ER), and fed the cells with OA, using LipidTox to label the formed LDs. Upon this treatment, the majority of forming LDs were also found on submicrometric ER-vesicles (Figure 2E-2G, and Figure S2F) or in tubules co-existing with large ER-vesicles (Figure S2G). The density of formed LDs per seipin signal was about 4-5 times higher at smaller ER-vesicles than at larger ones (Figure 2H). Indeed, LDs rarely appeared at larger ER-vesicles (Figure 2C-2G and Figure S2F-S2G) even though they contained clear seipin signal (Figure 1I, Figure 2C-2E, 2I, and Figure S2F-S2G). Thus, curvature per se and/or proteins localized to ER tubules facilitate LD formation in both the absence and presence of seipin.

We previously reported that relocation of seipin to the nuclear envelope (NE) was sufficient to relocate LD biogenesis to this site [28]. As the NE is a relatively flat membrane, this seemed at odds with our above observations suggesting that membrane curvature facilitates LD assembly. However, the GFP-nanobody based confinement will trap seipin to both the NE as well as nearby ER tubules, and distinguishing between these two possibilities is not possible at the light microscopy resolution (Figure S2H). To investigate this further, we transfected cells with seipin trapped at the NE with the ER-LD marker peptide HPos and imaged LD formation in live cells. Interestingly, we found that also in this situation virtually all LDs formed in close proximity to nearby ER tubular structures (Figure S2I and

S2J). Thus, LDs forming in the seipin NE-trapped cells may initially form at ER tubules in close proximity to the NE.

In summary, LDs preferentially form at ER tubules independently of seipin or the abundance of sheets. Since there is so far no protein known whose sole inhibition aborts LD formation, we hypothesize that membrane curvature could be sufficient and mandatory to efficiently trigger LD assembly.

TGs are diffusive in ER membranes and their distribution and assembly in LDs is curvature-dependent

Since LDs were mainly appearing from submicrometric ER-vesicles, we thought that maybe larger vesicles were not capable of making NLs. To test this, we generated large ER-vesicles in Cos7 cells and added LipidTox. We then fed the cells with OA to drive NL production. We found an increase in LipidTox signal in the large ER-vesicles (Figure 3A and 3B), suggesting accumulation of NLs in the membrane. Indeed, in model membranes, increasing the concentration of TG increased LipidTox signal (Figure S3A). These data suggest that the large ER-vesicles are able to synthesize NLs.

We next used Bpy-C₁₂ to better monitor the distribution of NLs in ER-vesicles. Similarly to red Bpy-C₁₂, also green Bpy-C₁₂ was incorporated into newly formed LDs upon OA administration, in both WT and swollen cells (Figure S3B and S3C). In addition, a uniform Bpy-C₁₂ signal was observed in ER-vesicles (Figure 3C, 3D, and Figure S3D). Similarly to LipidTox, the Bpy-C₁₂ signal also increased over time, likely due to ongoing lipogenesis. This uniform Bpy-C₁₂ signal rapidly recovered following photobleaching of a fraction of the membrane (Figure 3E), suggesting that the generated fluorescent NLs were freely diffusive in the membrane of the vesicle. Whilst Bpy-C₁₂ is also known to be incorporated into PLs [26], the majority of the Bpy-C₁₂ signal at ER-vesicles under these conditions likely stems from NLs, as the fluorescence intensity of Bpy-C₁₂ was much lower in the plasma membrane (a PL bilayer devoid of NLs) than at the ER (a PL bilayer harboring NLs) (Figure S3E-S3I).

We next focused on the NL level relative to ER-vesicle size. The concentration of Bpy-C₁₂ signal was much higher in larger ER-vesicles compared to smaller ones, as measured by comparing Bpy-C₁₂ to sec61-mCherry signal (Figure 3F-3G). Large vesicles were thus not defective in NL synthesis as compared to smaller ones and instead appeared to harbor more NLs. However, as found in Figure 2, we again observed that most LDs formed at smaller, submicrometric ER-vesicles with lower Bpy-C₁₂ membrane signal, rather than at the larger ER-vesicles with higher Bpy-C₁₂ signal (Figure 3H, 3I, and Figure S3J). In line with this observation, HPos-positive LDs were also found on smaller vesicles (Figure S3K). These results suggest that despite a lower concentration of NL in the membrane, smaller ER-vesicles were more prone to support LD generation. This implies that the critical concentration of NLs to nucleate a LDs may be lower on curved membranes compared to flat ones.

In agreement with our above conclusion, the depletion of seipin and Pex30 in yeast leads to large onion ring-like ER membrane structures, low in curvature but full of NLs [39]. Analogously, our osmotic swelling treatment produced some cells with very similar appearing structures, rich in NLs but incapable of forming LDs (Figure 3J), despite the fact that all ER proteins are a priori present in these

membranes. In comparison, in cells where submicrometric ER membranes were generated, LDs could be formed (Figure 3K).

Altogether, our data support the model that NLs synthesized in the ER membrane are initially freely diffusive. At a critical concentration, they condense into nascent LDs, phase separating from the bilayer. Our results suggest that flat membranes can accommodate a higher amount of NLs than curved membranes, implying that the critical concentration for nucleating LDs in flat areas is higher. By sufficiently diminishing this critical NL concentration, membrane curvature assists seipin to locally decrease the energy barrier and control LD nucleation.

The presence of TG but not SE is unfavorable in tubules

Our hypothesis required proof that membrane curvature can modulate the distribution of TG molecules. We thus decided to study how NL distribution is altered by membrane curvature in a protein-free system. We employed the droplet-embedded vesicle system (DEV), composed of an artificial LD (aLD) embedded in a giant unilamellar vesicle (GUV) [40]. We made TG aLDs containing 0.5% of TG-NBD to report for TG localization. The GUVs were made of dioleoylphosphatidylcholine containing 0.5% Rhodamine-DOPE (Rh-PE) to mark membranes, and 0.5% of biotinylated PLs, enabling us to pull out tubules. When DEVs were made under these conditions, the aLD was in equilibrium with free TG molecules in the bilayer (Figure 4A), similarly as seen in the ER-vesicles in (Figure 3F). By using beads grafted with streptavidin and thereby binding biotinylated PLs, we pulled out tubules from the DEV and varied their radius by modulating the bilayer surface tension [41] (Figure 4B).

When the radius of the tube was decreased, we observed a clear reduction in the TG/PL ratio (Figure 4C). In other words, TG was more avidly excluded from the tubule to the flat membrane in comparison to PLs as curvature was increased (Figure 4D, 4E, and Figure S4A). To rule out artifacts induced by TG-NBD, we performed analogous experiments without TG-NBD, using Bodipy dye to label membrane hydrophobicity. The signal evolution of Bodipy with regards to curvature was similar to that of TG-NBD (Figure S4B and S4C). These data indicate that it is unfavorable for highly curved membranes to bear free TG molecules. Here, because the tubule is in equilibrium with a flat region, the TG molecules simply escape from tubules to the flat region where they seem to be of lower chemical potential, i.e. lower free energy per TG molecule. These findings are consistent with the TG distribution we previously found in ER-vesicles (Figure 3F, 3G, and 3J).

We next compared the behavior of TG vs. SE in this system. We used a TG solution containing ~ 29.5% SE, the maximum solubility of SE in TG that we could attain at room temperature, and 0.5% SE-NBD (Figure 4F, 4G, Figure S4D and S4E). Surprisingly, upon reduction of the tube radius, we found that the SE molecules remained on tubules and were relatively more enriched than PLs (Figure 4G). This is opposite to the behavior of TG alone (Figure 4E). To visualize TG localization in this TG-SE mixture, we performed similar experiments but switching SE-NBD to TG-NBD (Figure 4F, 4H, and Figure S4F). In this situation, we found that TG was still excluded from tubules (Figure 4H and Figure S4F). These data revealed that membrane curvature demixed the TG-SE mixture: TG

molecules were sorted out to flat regions while SE remained slightly enriched in tubules. Thus, TG and SE have different chemical potentials in tubules vs. sheets.

The difference between TG and SE behavior in regards to membrane curvature was unexpected. To further study whether their dynamics were also differently altered, we simultaneously bleached both NBD and Rhodamine signals, reporting for NLs and PLs, respectively. Photobleaching was performed on tubules (Figure 5A-5C) and at the flat apex region of the DEV (Figure S5A-S5C). On flat regions, we found that both TG and SE signals recovered faster than the PL signal, indicating that SE and TG were both more mobile than PLs (Figure 5D, Figure S5B and S5C). In contrast, in tubules TG was slightly less mobile than PLs, whilst SE still remained faster (Figure 5B-5D). Thus, relative to PL motility, curvature slows down TG but not SE.

Altogether, our above data revealed that the presence of independent TG molecules is unfavorable in curved regions. Furthermore, TG and SE have intrinsic static and dynamic behaviors discriminated by membrane curvature. They have different chemical potentials, energy per molecule, at flat and curved membrane areas. Thus, LD assembly mechanism likely depends on the NL composition.

Membrane curvature promotes TG LD nucleation *in vitro*

The presence of free TG molecules was more unfavorable in curved than flat regions (Figure 4E). Therefore, it could have been favorable for TG molecules to condense into droplets in tubules. However, TGs simply escaped from the tubule to the flat region, as they were part of a continuous system (Figure 4C). Under these static experimental conditions, there was no means by which to increase the concentration of TGs in tubules relative to PLs. In cells, ongoing lipogenesis guarantees that the concentration of TG in the ER membrane increases.

We reasoned that if we rapidly decreased the tube radius, faster than the NL diffusion time, we should be able to increase the local concentration of NL per PL in the tube. Starting from a tube diameter of around 200 nm, the radius was rapidly decreased by drastically increasing the DEV bilayer tension (Figure 5E). We observed nucleation events whereby TG molecules condensed in the tubule into lens-like or droplet shapes, as reported by TG-NBD or Bodipy signals (Figure 5E-5G and Figure S5D-S5H). The TG signal in these droplets was greater than that observed in the flat or tubular bilayer regions prior to nucleation (Figure 5F, Figure S5E, S5F and S5I), supporting the notion that the TG molecules in the tubules had condensed. The success rate of nucleation increased with the decrease of the radius of the tubule (Figure 5H), either because the local concentration increased more or the effect of curvature was more pronounced. The nucleation of these model LDs was most often observed when the tube diameter was decreased to around 35 nm (Figure 5H), in the range of ER tubules [42].

Finally, as we had observed differences between TG and SE behavior in regards to membrane curvature, we repeated the previous experiment with a TG/SE (70/30) mixture. The success rate of nucleation in the presence of SE was much lower than that for TG alone (Figure 5I). These results suggest that membrane curvature more efficiently promotes the nucleation and assembly of TG- rather than SE-containing LDs. This divergence very likely pertains to the difference

in chemical potential and mobility between the molecules (Figure 4G, 4H, and Figure 5D). TG, which diffused more slowly and was disfavored at tubules, was more efficiently assembled into droplets as the curvature was increased.

These data suggest that membrane curvature can solely mediate LD nucleation, especially of TG droplets. This is due to the higher chemical potential of TG in tubular regions, which is annihilated by the spontaneous condensation of TG molecules into droplets.

Modulating ER sheet-to-tubule ratio alters LD nucleation frequency in seipin deficient cells

Two main conclusions can be derived from our *in vitro* approach (Figures 5H and 5I). First, curvature increases the chemical potential of TG and thereby catalyzes LD assembly. Second, the presence of SE has an inhibitory contribution on the curvature-induced LD assembly. If our model is correct, modulating ER tubule-to-sheet ratio could affect the number of assembled LDs during NL synthesis, and this could be altered by SE levels. Since seipin interferes with LD nucleation, its presence might mask the contribution of membrane curvature. We therefore decided to work on a reductionist cell system lacking seipin, wherein cells generate a large amount of tiny LDs that fail to grow [26,27].

We first investigated the effect of increasing the amount of ER tubules. To this end, we stably transfected seipin degenon cells with select ER tubule generating proteins: Reticulon 4 [34], Atlastin 1 [43] or Rab10 [44]. We then acutely removed seipin and induced LD generation by OA. Seipin removal led to the drastic increase of the tiny nucleated LDs, which could be efficiently rescued by the concomitant overexpression of seipin (Figure 6A and 6B). Overexpression of the tubule generating proteins increased the number of LDs in this system (Figure 6A and 6B), suggesting that increasing tubules may enable higher number of LDs to be assembled.

We next studied the effect of increasing sheets at the expense of tubules. We previously observed that the number of *de novo* assembled LDs in seipin depleted cells is markedly reduced by Rab18 KO [28], which increases ER sheets (Figure 1C). This observation supports the notion that increasing sheets at the expense of tubules decreases nucleation events. To further investigate this, we depleted Rab10 with siRNAs or treated the cells with the microtubule polymerizing drug nocodazole, both of which induce ER sheet expansion [44,45]. These manipulations efficiently reduced the number of tiny nucleated LDs in seipin depleted cells (Figure 6C, 6D, Figure S6A and S6B). In the presence of seipin, increasing ER sheets only mildly reduced the number of assembled LDs (Figure S6C and S6D).

To investigate this phenomenon in more detail, we modulated the ER tubule-to-sheet ratio in seipin deficient cells and imaged them live during 45-75 min after OA administration. We modulated the sheet-to-tubule ratios by transient overexpression of mCherry-tagged ER shaping proteins CLIMP-63 (increasing sheets) or REEP-5 (increasing tubules), or soluble mCherry as control. We imaged peripheral ER regions with varying ER morphologies and found that regions of cells with higher ratios of tubules versus sheets typically had more LDs (Figure 6E and 6F). Overall, these data suggest that the number of LDs generated in seipin deficient cells is linked to the ER-sheet-to-tubule ratio, with an increase in sheets lowering the number of LDs.

Our *in vitro* data suggested that SE have an inhibitory contribution to tubule-induced LD nucleation (Figure 5I). To test this hypothesis, we treated stringently starved cells with either 200 μ M OA in the presence of SOAT1 inhibitor, to induce TG-enriched LDs, or 200 μ M cholesterol-cyclodextrin in the presence of DGAT1 and DGAT2 inhibitors, to induce SE-enriched LDs, and imaged live nascent LDs. Despite equimolar lipid loading, OA induced a much higher number of LDs compared to cholesterol (Figure 6G and Figure S6E), which agrees with our prediction, although we cannot rule out differences in efficiencies of the enzymes involved. Nascent SE-LDs were also more prevalent at ER tubules than sheets, albeit with a slight preference for sheets in comparison with TG-LDs (Figure 6H and 6I). Finally, the number of LD assembly events in seipin-depleted cells could be increased by a concomitant treatment of cells with OA and SOAT1 inhibitor, i.e. by increasing the TG/SE ratio (Figure 6J). These observations are well in line with our *in vitro* data that TG LDs are more efficiently assembled than SE LDs and that SE has an inhibitory effect on LD nucleation.

Altogether, these data suggest that the number of TG LD assembly events is determined by the abundance of ER tubules in the absence of seipin and that seipin controls the number of LD assembly sites.

Discussion

Here, we studied the initial step of LD assembly, nucleation, which is the condensation of freely diffusing NL molecules. This process occurs when an energy barrier is crossed (Figure 7A) and this barrier is associated with a critical concentration of NLs [7]. Our findings suggest that membrane curvature decreases this energy barrier and the associated critical concentration (Figure 7A). This happens because curvature particularly increases the chemical potential of a TG molecule. In this thermodynamic scenario, this increase of potential reduces the energy gap to the nucleation barrier. By doing so, membrane curvature catalyzes LD nucleation at tubules (Figure 7A).

The molecular mechanism by which membrane curvature increases the chemical potential of TG is unknown. Nonetheless, one of the major effects of curvature on membranes is to space PLs and to expose their hydrophobic core to water (Figure 7B). Thus, TG molecules might be more exposed to water molecules in a bilayer with higher curvature. To minimize this contact, TGs can escape from highly curved regions by diffusing to less curved ones, or by clustering and condensing into droplets (Figure 7A-B). Indeed, the latter situation minimizes the total free energy, similarly as when oil droplets merge to minimize the total oil/water contact area.

There may be several reasons for the unexpected difference between TG and SE behavior in regards to membrane curvature. The difference in TG and SE chemistry should lead to a priori different interaction profiles with molecules in their environment, impacting their tendency to condense in membranes. In addition, these NLs may adopt different conformations in tubules. One could imagine the possibility that SE remains parallel to PLs while TG would be perpendicular to them, or vice versa. Such organization would influence their contact with water molecules and with PLs, and hence their propensity to condense.

The thermodynamic framework underlining phase separation is well documented, and we have previously described it in the context of LD assembly [7]. It predicts that before the critical concentration for nucleation is reached, TG clusters appear and resorb rapidly, at a frequency increasing with the concentration of free TG (Figure 7A). The formation of such clusters would slow down TG diffusion, in line with our results indicating that membrane curvature reduces TG diffusion (Figure 5B and 5D). When the TG concentration reaches a critical nucleation concentration, larger clusters will transit into nascent LDs (Figure 7A). On flat membranes, this critical concentration is about 3-5% of free TG to PLs [6,12]. On curved membranes, this value is expected to be smaller (Figure 7A). Consequently, membrane curvature should increase the frequency of appearance of unstable TG clusters, and hence the likelihood that they transit into stable, nascent LDs (Figure 7C). Such spontaneous process would lead to uncontrolled LD assembly, sporadically triggered at and by tubules.

Because seipin can dictate where LDs form [28] and seipin controls the number of LD assembly events on tubules (this study), it has to act very early to aid some clusters to jump the energy hill and transit into nascent LDs (Figure 7A and 7C). Indeed, recent results show that the seipin/promethin complex forms a LD assembly complex copurifying with TG molecules [46]. Our data support that seipin would foster some TG clusters and controls their transition into stable, nascent LDs (Figure 7C). Concretely, seipin might diffuse and detect TG clusters in tubules and/or the clusters

might directly appear at seipin oligomers, since favorable protein-lipid interactions can trigger phase separation [7]. In any case, by controlling the transition of some TG clusters into nascent LDs, seipin guarantees that only seipin-positive TG clusters get stabilized and grow. As these nascent LDs grow, the local concentration of TG in the tubule decreases, inhibiting LD assembly close to seipin defined sites [28]. In the absence of seipin, LD assembly is more dominated by curvature and much more TG clusters will transit into nascent LDs, controlled by the abundance of ER tubules (Figure 6 and Figure 7C).

Interestingly, seipin collaborating proteins, from plant to human cells, seem to be membrane curvature sensors or inducers. These include the membrane shaping protein PEX30/MCTP2 [31,39], promethin, Ldo16/45 [47,48] and LDIP [49]. The induction of local curvature by these proteins could facilitate nucleation and trigger LD assembly at specific sites determined by seipin. Indeed, the concomitant depletion of Pex30 and seipin did not alter TG synthesis but rather their packaging into LDs, with TG molecules remaining in large onion-like multilamellar ER vesicles [39], similarly as observed in Figure 3J. These structures are of almost zero curvature and are unable to make LDs. LDs did not efficiently form on large ER-vesicles (Figure 2 and Figure 3) that contained abundant levels of seipin, suggesting that seipin activity in LD assembly relies on membrane curvature (Figure 7C).

In conclusion, we unveiled how NLs are distributed in membranes and found that membrane curvature catalyzes their assembly into LDs, in cooperation with seipin. Our results shed light on the role of ER topology on LD biogenesis and pave the way for further studies addressing how curvature is coupled to the function of seipin and other proteins regulating LD biogenesis.

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Author contribution

A.R.T., E.I., V.S. and A.S. designed experiments; A.S. performed *in vitro* and intra-cellular vesicle experiments, with the help of M.O. and A.C.; V.T.S. performed experiments in A431 cells with the help of S.L. and X.Z; A.R.T., E.I., V.S. and A.S. analyzed data and wrote the manuscript.

Declaration of interests

The authors declare no competing interests.

Figure legends

Figure 1: LDs are generated at ER tubules where seipin is enriched

A) A431 cells with seipin tagged endogenously with GFPx7 (end-seipin-GFPx7) and stably expressing BFP-KDEL and LiveDrop-mCherry were delipidated (1 d LPDS) and imaged live with Airyscan microscopy, starting 50 s after OA loading. ER tubules and sheets were segmented as described in Materials and methods. Insets show an example of an emerging LD, circle denotes ROI marked by seipin, used for analysis in B.

B) Analysis of nascent LD formation sites from A, the distribution of segmented ER pixels at LD-associated seipin foci prior to LiveDrop accumulation was analyzed and compared to overall pixel distribution of sheets/tubules in the same cells. Bars: mean +/- SEM, n= 24 LDs, 6 cells, 2 experiments.

C) WT and Rab 18 KO cells stably expressing BFP-KDEL cells were imaged live, the ER was segmented and analyzed. Bars: mean +/- SEM, n= 61 ROIs from 61 cells/genotype, 3 experiments.

D) A431 WT and Rab18 KO cells with seipin tagged endogenously with SNAPf-tag and stably expressing BFP-KDEL were delipidated (3 d LPDS, 18 h DGATi) and seipin was stained with SIR647 4-6 h prior to imaging. After DGATi washout, OA, SOAT1 inhibitor and LD540 were added to the cells and they were imaged live 0-15 min after OA addition.

E) Analysis of D. The distribution of segmented ER pixels at LD-associated seipin foci was compared to overall pixel distribution of sheets/tubules in the same cells. Bars: mean +/- SEM, n= 25-38 ROIs from 25-38 cells, 2-3 experiments (>800 LD-associated seipins/genotype).

F) End-seipin-GFPx7 cells stably expressing BFP-KDEL were imaged live. Seipin is enriched at ER tubules and when moving through sheet regions (insets) prefers the edges of sheets.

G) Analysis of D. The distribution of segmented ER pixels at each seipin ROI was compared to overall pixel distribution of sheets/tubules in the same cells. Bars: mean +/- SEM, n= 25-38 ROIs from 25-38 cells, 2-3 experiments (> 9000 seipin foci/genotype).

H) Schematic representation of the ER-vesicles formation with swollen cells. Left: Cos7 cell expressing sec61 β -mCherry and YFP-seipin was imaged live by confocal microscopy. Right: Cos7 cell expressing sec61 β -mCherry and YFP-seipin was imaged live 10 min after ER-vesicle formation.

I) Cos7 cells expressing sec61 β -mCherry and YFP-seipin were imaged live 10 min after ER-vesicle formation. Submicrometric ER-vesicles (red arrows) display brighter YFP-seipin signal than larger vesicles (blue arrows). Line scan over two ER-vesicles (yellow line) displays brighter signal for the small vesicle.

J) Analysis of I. The fluorescence intensity ratio of YFP-seipin and sec61 β -mCherry is plotted relative to vesicle size, indicating seipin is enriched on submicrometric vesicles, n= 69 vesicles, 3 experiments. See also Figure S1.

Figure 2: LDs nucleate at ER tubules in the absence of seipin and not at large vesicles in the presence of seipin

- A) Seipin degran cells were treated and imaged as in Figure 1D. Seipin was depleted by adding IAA to the cells 18 h prior to imaging.
- B) Analysis of A. The distribution of segmented ER pixels at nascent LDs was compared to overall pixel distribution of sheets/tubules in the same cells. Bars: mean +/- SEM, n= 44-47 ROIs from 44-47 cells, 2 experiments (> 1000 LDs/group).
- C) Swollen Cos7 cells were imaged before and after OA and Bpy-C₁₂ addition. Note Bpy-C₁₂ and LipidTox colocalization in LDs.
- D) ER-vesicles were induced in Cos7 cells expressing YFP-seipin and sec61β-mCherry and imaged 15 min after OA addition. Note submicrometric ER-vesicles apposed to LDs.
- E) ER-vesicles were induced in Cos7 cells expressing YFP-seipin and sec61β-mCherry and imaged 15 min after OA addition.
- F) Analysis of D and E. LD number per ER-vesicle size in swollen cells overexpressing seipin. Mean +/- SEM; n = 1230 LDs, 4 experiments.
- G) Analysis of D and E. LD number per ER-vesicle size in F is normalized to sec61β signal, defined as the number of LDs per ER area.
- H) Analysis of D and E. Number of LDs normalized to seipin signal per ER-vesicle size, defined as the number of LDs per seipin signal.
- I) Analysis of D and E. Seipin intensity per ER-vesicle, normalized to sec61β signal, defined as seipin signal per ER area.
- See also Figure S2.

Figure 3: Neutral lipid and LD distribution in ER-vesicles.

- A) ER-vesicles were induced in Cos7 cells in the presence of LipidTox and OA is added 16 min later. The LipidTox signal in an ER-vesicle before and 15 min after OA addition is shown, with the membrane intensity profile displayed below the images.
- B) Analysis of A. Mean +/- SEM; n = 14 vesicles, 4 cells, 2 experiments.
- C) Bpy-C₁₂ signal in Cos7 cell ER-vesicles 20 min and 35 min after OA addition. Line scan shows an increase in signal.
- D) Analysis of B. Mean +/- SEM; n = 22 vesicles, 5 cells, 2 experiments.
- E) Rapid Bpy-C₁₂ signal recovery in a partly bleached large ER-vesicle; arrowhead indicates the bleached area. The recovery signal was normalized to that of non-bleached regions.
- F) ER-vesicles induced in Cos7 cells expressing sec61β-mCherry were imaged 15 min after OA and Bpy-C₁₂ addition. Bpy-C₁₂ signal intensity is higher in large micrometric-sized vesicles (blue arrowhead) than in submicrometric vesicles (red arrowhead).
- G) Analysis of F, mean +/- SEM; n = 15-38 vesicles, 3 cells, 2 experiments.
- H) ER-vesicles induced in Cos7 cells expressing sec61β- mCherry were imaged 15 min after OA and Bpy-C₁₂ addition. Two planes of a single cell are shown. Most forming LDs are localized on submicrometric membranes.
- I) Analysis of H. Number of LDs per ER-vesicle size. Mean +/- SEM; n = 3268 LDs, 4 experiments.

J) A swollen Cos7 cell, expressing sec61 β -mCherry and imaged 30 min after OA and Bpy-C₁₂ addition exhibits large onion ring-like ER membrane structures full of NLs.

K) Example of another cell treated as in J, that has formed submicrometric ER structures and was able to assemble LDs.

See also Figure S3.

Figure 4: The presence of TG but not SE is unfavorable in tubules

A) Illustration of the DEV system consisting of GUVs mixed with aLDs. An exemplary DEV is shown. Rh-PE marks the membranes; TG-NBD marks the TG phase. The signal at the line scans (yellow lines) is shown.

B) A nanotube is pulled from a DEV by using streptavidin coated micro-beads bound to biotinylated PLs on the DEV. Increasing the bilayer tension with a micropipette decreases the tube radius.

C) Nanotubes with 60 nm and 20 nm diameters are pulled from a DEV. Line scans on the tubes indicate that TG-NBD signal intensity is reduced relative to Rh-PE signal as the tube radius is decreased.

D) Definition of the TG enrichment index in the tube.

E) Analysis of C. Plot of TG enrichment relative to the nanotube radius, r_{tube} .

F) Schematic description of different oil molecules used in G and H. The green squares represent NBD.

G) Nanotubes are pulled from DEVs as in C, but the DEVs are made with aLDs containing TG, SE and SE-NBD as indicated (w/w). Analysis of the SE signal relative to tube radius is shown.

H) Same experiment as in G but with TG-NBD as indicated (w/w). Analysis of the TG signal relative to tube radius is shown.

See also Figure S4.

Figure 5: Membrane curvature more efficiently triggers the assembly of *in vitro* TG than SE LDs

A) Diagram of the FRAP region where both Rh – PE and TG –NBD or SE – NBD are bleached in the entire nanotube.

B) Representative time sequence of Rh-PE and TG-NBD recovery following photobleaching. The normalized fluorescence intensity evolution of the bleach region is shown with its fitting curve. Experiment was repeated three times.

C) Representative time sequence of Rh-PE and SE-NBD recovery following photobleaching. The normalized fluorescence intensity evolution of the bleach region is shown with its fitting curve. Experiment was repeated three times.

D) Analysis of B and C. Plot of the average recovery half time of Rh-PE, TG-NBD and SE-NBD (normalized by Rh – PE) as a function of membrane curvature. For examples of photobleaching recovery in flat membranes, see Figure S5.

E) A nanotube is pulled from a DEV and the bilayer was submitted to a sudden increase of surface tension to drastically increase tube curvature. An artificial TG LD is nucleated following this manipulation. The arrowhead shows the nucleated TG aLD shown in the inset.

F) Smoothened image of the nucleated aLD from the inset in E is shown. For analysis of signal ratio, the intensity of the nucleated aLD signal is divided by the tube or the GUV intensity prior to nucleation. Bars: mean \pm SEM, n = 9.

G) An additional example of a nucleation event in a nanotube is shown with using TG only, labelled by Bodipy which was added to the medium. Signal profiles along the tube of Rh-PE and Bodipy are shown before and after nucleation.

H) Distribution of the nucleation events is reported against the final nanotube radius. Bars: mean \pm measurement uncertainty, n = 9.

I) Frequency of nucleation events observed relative to aLD NL composition. For TG, n = 9 nucleation events out of 36 trials; for TG-SE, n = 1 nucleation event out of 19 trials.

See also Figure S5.

Figure 6: Modulation of sheet/tubule ratio alters LD assembly

A) ER tubule proteins were stably overexpressed in seipin degron cells. Cells were delipidated for 3 d, seipin was depleted by IAA treatment for 18 h and LD biogenesis was induced by OA 1 h treatment. Cells were fixed, stained with LD540 and DAPI and imaged by widefield microscopy. Maximum intensity projections of deconvolved z-stacks. The insets show tiny LDs, brightness is adjusted to display them more clearly.

B) Analysis of A. Bars: mean \pm SEM, n = 167-549 cells/group, 2 experiments.

C) Seipin degron cells were treated with control or Rab18 siRNA for 3 d in LPDS including 18 h IAA treatment to deplete seipin. Cells were then treated with OA for 1 h, fixed and stained as in A. The insets show tiny LDs, brightness is adjusted to display them more clearly.

D) Analysis of C. Bars: mean \pm SEM, n = 344-353 cells/group, 2 experiments.

E) Seipin degron cells stably expressing BFP-KDEL were transfected with indicated plasmids for 2 d, including 1 d delipidation (LPDS, DGATi) and IAA treatment. After DGATi washout, cells were imaged live with Airyscan microscopy starting 45-75 min after OA addition in the presence of LipidTox Green.

F) Analysis of E. Peripheral ER ROIs were segmented for sheets and tubules using the BFP-KDEL channel and the number of LDs plotted (normalized to total ER area in each ROI) relative to ER morphology, n = 172 ROIs from 172 cells, 2 experiments.

G-I) Cells were treated imaged and analyzed as in Figure 1D-E. Bars: mean \pm SEM, n = 39 cells/group, 3 experiments (428-1095 LD-associated seipins). The data for TG LDs is same as in Figure 1E WT LDs. In H, the distribution of segmented ER pixels at LD-associated seipin foci was compared to the overall pixel distribution of sheets/tubules in the same cells, in I the distribution of segmented ER pixels at LD-associated seipin foci is shown without normalization to overall ER pixel distribution.

J) Seipin degron cells were delipidated for 3 d (including 18 h with DGATi) and treated with OA with or without SOAT inhibitor for 1 h, fixed, stained and analyzed for LD sizes as in A. Bars: mean \pm SEM, n = 308-368 cells/group, 2 experiments.

See also Figure S6.

Figure 7: Model for the role of curvature and seipin in triggering LD assembly

A) Top panel: illustration of the energy profile of phase transitions in the context of LD assembly. Curvature decreases the critical concentration and the energy barrier for nucleation. (Bottom panel: before the critical nucleation concentration, c^* , short-lived TG clusters appear (msec). Above this concentration, nascent droplets form.

B) Proposed mechanism by which curvature favors TG clustering, by exposing the hydrophobic core to water molecules.

C) Without seipin, many TG clusters are triggered at tubules and transit into nascent droplets (bottom). Seipin stabilizes clusters in tubules, TGs favorably move to these seipin-stabilized clusters, which will turn into nascent LDs, fewer in number than without seipin.

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