StAR-related lipid transfer domain 11 (STARD11)-mediated ceramide transport mediates extracellular vesicle biogenesis

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Keywords: exosome, endosome, endoplasmic reticulum, microvesicle, palmitate, lipotoxicity, lipid metabolism, sphingosine, vesicle trafficking, lipid secretion

ABSTRACT
Extracellular vesicles are important carriers of cellular materials and have critical roles in cell-to-cell communication in both health and disease. Ceramides are implicated in extracellular vesicle biogenesis, yet the cellular machinery that mediates the formation of ceramide-enriched extracellular vesicles remains unknown. We demonstrate here that the ceramide transport protein, StAR-related lipid transfer domain 11 (STARD11), mediates the release of palmitate-stimulated extracellular vesicles having features consistent with exosomes. Using palmitate as a model of lipotoxic diseases and as a substrate for ceramide biosynthesis in human and murine liver cell lines and primary mouse hepatocytes, we found that STARD11-deficient cells release fewer extracellular vesicles. Moreover, STARD11 reciprocally regulated exosome ceramide enrichment and cellular ceramide depletion. We further observed that in STARD11-knockout cells, intracellular ceramide accumulates and that this apparent inability to transfer cellular ceramide into extracellular vesicles reduces cellular viability. Using endogenous markers, we uncovered structural and functional co-localization of the endoplasmic reticulum (ER), STARD11, and multivesicular bodies. This co-localization increased following palmitate treatment, suggesting a functional association that may mediate ceramide trafficking from the ER to the multivesicular body. However, the size and number of multivesicular bodies were comparable in wildtype and STARD11-knockout cells. In conclusion, we propose a model of how STARD11 mediates ceramide trafficking in palmitate-treated cells and stimulates exosome biogenesis.

INTRODUCTION
Extracellular vesicles are an important carrier of cell-derived information with emerging roles in cell-to-cell communication in health and disease(1-4). Extracellular vesicles can be classified into exosomes, which arise from the multivesicular body, microvesicles derived from the plasma membrane, and apoptotic bodies formed by dying cells(5). Ceramides are known to play a role, in particular, in the formation of multivesicular body-derived exosomes(6). We have recently demonstrated that palmitate, a
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saturated fatty acid, elevated in the liver and in the circulation in obesity-associated disorders including fatty liver disease, stimulates the release of extracellular vesicles(7,8). Palmitate-stimulated extracellular vesicles are enriched in ceramide, and their release depends on the de novo synthesis of ceramide from exogenously supplied palmitate(8). De novo ceramide synthesis occurs at the endoplasmic reticulum (ER)(9), and exosomes are formed as intraluminal vesicles (ILVs) of the multivesicular body (MVB)(10). However, it is not known how palmitate-stimulated ceramide is transported from the ER to the MVB to form ILVs which give rise to exosomes upon their release from cells.

Ubiquitin-dependent cargo sorting of proteins to form ILVs requires the endosomal sorting complex required for transport (ESCRT) machinery, such that RNA silencing of ESCRT machinery components yields fewer and morphologically abnormal MVBs; yet, neither the formation of ILVs nor MVBs is fully inhibited, indicating that ILVs can be generated by cellular processes in addition to the ESCRT machinery(11). For example, the sorting of other cargoes, such as the melanosomal protein Pmel17, remains unaffected in the absence of ESCRT components(12). Furthermore, lipids are also implicated in the formation of ILVs. The phospholipid, lysobisphosphatidic acid (LBPA), can induce the formation of multivesicular liposomes in cell-free systems that resemble the MVB; although this lipid is found in vivo, its role in the formation of ILVs remains unexplored(13-32). The sphingolipid ceramide is implicated in the formation of ILVs and release of exosomes, such that inhibition of ceramide biosynthesis decreases exosome release(6,8). Thus, accumulated observations indicate that MVB biogenesis is diverse and multiple mechanisms contribute to cargo packaging into the ILVs of MVBs and their release as exosomes.

The de novo biosynthesis of ceramide occurs at the ER(9). Newly formed ceramide is transported from the ER to the Golgi by non-vesicular transport mediated by ceramide transport protein (CERT), also known as StAR related lipid transfer domain (STARD) 11, an evolutionarily conserved member of the STARD family of lipid transporting proteins with high substrate specificity(14-17). The role of STARD11 in the formation of exosomes that are formed in a ceramide-dependent manner remains unknown.

Lipotoxicity of the saturated free fatty acid palmitate is a robust model for examining signaling pathways activated in lipotoxic hepatocytes, a cellular model germane to nonalcoholic fatty liver disease(19). In this regard, we have previously demonstrated that palmitate treatment leads to the release of extracellular vesicles from hepatocytes, and that significant extracellular vesicle release occurs before the onset of palmitate-induced apoptosis(8). Furthermore, we have demonstrated that these vesicles are enriched in ceramide and dependent on the de novo synthesis of ceramide. However, the cellular trafficking machinery that mediates ceramide transport to form extracellular vesicles remains unknown. Herein, we report that in the absence of STARD11 palmitate-induced extracellular vesicle release is attenuated; correspondingly, when cells are unable to release ceramide containing extracellular vesicles, intracellular ceramide content increases with deleterious consequences. Palmitate-stimulated extracellular vesicles display markers consistent with exosomes. We demonstrate structural and functional colocalization of the ER, STARD11 and the MVB; this colocalization is increased by palmitate treatment. Thus we have demonstrated that the ceramide transport protein STARD11 mediates the release of lipotoxic extracellular vesicles, likely via transport of newly synthesized ceramide from the ER to the MVB.

RESULTS

STARD11 is expressed in hepatocytes and mediates palmitate-induced extracellular vesicle release.

Prolonged palmitate treatment induces hepatocyte apoptosis(19,20); therefore, we designed our experimental conditions to collect conditioned media for extracellular vesicle isolation following 16 hours of treatment prior to the onset of significant palmitate-induced apoptosis which occurred following 24 hours of palmitate treatment as measured biochemically by caspase 3/7 activity (Supporting Information Figure 1A). Moreover, we confirmed that palmitate-induced extracellular vesicle release was...
preserved in the presence of the caspase inhibitors Z-VAD-FMK and IDN-6556 (Supporting Information Figure 1B), and thus, was not due to apoptotic bodies, which cannot be formed in the presence of caspase inhibitors(21). We further excluded a contribution from protein aggregates to palmitate-stimulated extracellular vesicles by floating extracellular vesicles in an iodixanol gradient, as previously described by Kowal et al(22). Palmitate-stimulated extracellular vesicles were isolated from fractions 3, 5 and 6 (Supporting Information Figure 1C).

Having established conditions for extracellular vesicle isolation, we next probed the hepatocyte expression of STARD11 in human and murine hepatocellular cell lines and primary mouse hepatocytes. STARD11 protein and mRNA were easily detected in human hepatoma cell lines, HepG2 and Huh7, immortalized mouse hepatocytes (IMH), mouse hepatoma cell line Hepa1-6, and primary mouse hepatocytes (Supporting Information Figure 1D-F). To determine the biologic significance of STARD11 in the release of palmitate-stimulated extracellular vesicles, also referred to as lipotoxic extracellular vesicles, we asked if the release of these lipotoxic extracellular vesicles was dependent on STARD11 in mouse and human hepatocytes (Figure 1A and B, respectively), in several distinct knockout clones. We verified the deletion of STARD11 by western blotting (inset Figure 1A and B). Lipid loading can stimulate lipoprotein secretion; therefore, we interrogated our cell line for apolipoproteins A1, B100, and B48 which are requisite for the secretion of HDL and VLDL particles, respectively. We could not detect these lipoproteins in our cell line and in palmitate-stimulated extracellular vesicles excluding a contribution of co-isolated HDL particles and VLDL particles to our extracellular vesicle counts (Supporting Information Figure 1G).

Oleate, a monounsaturated fatty acid, is the second most physiologically abundant fatty acid in humans(7). Unlike palmitate, oleate is not a direct substrate for the formation of ceramides(23). Therefore, we asked if oleate treatment led to extracellular vesicle release. We found no increase in extracellular vesicle release in oleate treated wildtype cells, and similar responses in STARD11-/- cells (Figure 1A and B). Thus, STARD11 is requisite for the release of palmitate-stimulated lipotoxic extracellular vesicles. We did not observe any changes in the size distribution of extracellular vesicles from STARD11-/- cells in comparison with wildtype cells in either mouse or human hepatocytes (Figure 1C and D). We next analyzed basal extracellular vesicle release in control and STARD11-/- cells. We found that basal extracellular vesicle release was comparable in wildtype and STARD11-/- mouse (Figure 1E) and human (Figure 1F) hepatocytes. Altogether these data demonstrate that STARD11 mediates extracellular vesicle release specifically following palmitate treatment. This is likely due to de novo synthesis of ceramide from palmitate, as previously reported by us(8), and possibly due to the trafficking of palmitate-stimulated ceramide to the MVB.

**Extracellular vesicle and whole cell ceramide are reciprocally related in STARD11 knockout cells.**

As the release of palmitate-stimulated extracellular vesicles is dependent on ceramide biosynthesis as previously shown by us(8), and confirmed (Supporting Information Figure 2A), we asked if a defect in ceramide transport affects extracellular vesicle-ceramide content. We reasoned that extracellular vesicles derived from wildtype cells would be ceramide-enriched, whereas, extracellular vesicles derived from STARD11-/- cells, which lack ceramide transport protein, would not. To demonstrate that STARD11 is required for the biosynthesis of ceramide-enriched extracellular vesicles, we measured extracellular vesicle ceramide by tandem mass spectrometry. Consistent with our previous data(8), palmitate treatment led to the release of ceramide-enriched extracellular vesicles (Figure 2A). However, extracellular vesicles derived from palmitate-treated STARD11-/- cells were not enriched in ceramide. We found no increase in ceramide in oleate-stimulated extracellular vesicles (Figure 2B). Palmitate-treatment led to an
increase in whole cell ceramide in wildtype cells (Figure 2C). The increase in whole cell ceramide levels was significantly greater in STARD11-/- cells (Figure 2C). Oleate-treatment did not increase whole cell ceramide levels in wildtype or STARD11-/- cells (Figure 2D). These data suggest that STARD11-mediated trafficking of palmitate-stimulated ceramide is an important pathway for the cellular export of ceramide as STARD11-/- cells accumulate ceramide intracellularly.

**STARD11-mediated ceramide transport mitigates palmitate-induced cytotoxicity.**

Having demonstrated ceramide accumulation in STARD11-/- cells, we next asked whether ceramide accumulation is toxic for cells. As discussed above, we found no increase in cell death or decrease in cell viability following 16 hours of treatment with palmitate to correspond to the time that conditioned media was collected for extracellular vesicle isolation. However, a longer duration of palmitate treatment led to a greater increase in cell death and a reduction in cell viability in STARD11-/- cells compared to wildtype cells following 20 hours of palmitate treatment, consistent with our previous publications which have demonstrated a significant time-dependence of palmitate-induced hepatocyte apoptosis (Supporting Information Figure 2B and C) (19,20). These data suggest that the ability to exclude ceramide from hepatocytes partially mitigates palmitate-induced toxicity.

**Palmitate-stimulated extracellular vesicles have features of exosomes.**

Small extracellular vesicles derived from multivesicular bodies are known to express an array of markers, suggestive of their cellular origin within the MVB(22,24). These markers include Alix, syntenin and tumor susceptibility gene 101 (TSG101), which have been demonstrated to be markers of small extracellular vesicles arising from the MVB, i.e., exosomes(22,24). We interrogated palmitate-stimulated EVs for the presence of Alix, syntenin and TSG101, and found that these markers were enriched in palmitate-stimulated extracellular vesicles from wildtype cells suggesting that these extracellular vesicles are exosomes arising within the MVB (Figure 3). In STARD11-/- cells basal levels of Alix, syntenin and TSG101 were comparable to wildtype cells and there was no increase in these exosome markers following palmitate treatment. We also observed an increase in CD81 in extracellular vesicles from palmitate-treated wildtype cells and not in STARD11-/- cells. Whole cell levels of Alix, syntenin and TSG101 were unchanged following palmitate treatment.

STARD11 is a membrane bound protein which is known to bind with vesicle associated membrane protein-associated protein (VAP) in the ER membrane with its FFAT motif and phosphatidylinositol-4 monophosphate in the Golgi membrane with its N-terminal pleckstrin homology (PH) domain(25,26). However, it is known to also bind phosphatidylinositol-3 monophosphate which is enriched in endosome membranes(27). In this location, it could be internalized as a component of the ILV membrane. To address this question, we first asked whether STARD11 is a part of the extracellular vesicle cargo. As demonstrated in the western blot analysis, we did not detect STARD11 on isolated extracellular vesicles, both basally and in palmitate-stimulated extracellular vesicles (Figure 3).

**STARD11 colocalizes with ER and MVB.**

We asked whether STARD11 associates with the membrane compartment where ceramide synthesis occurs (the ER) and the MVB, where exosomes are formed. As previously reported by others(29), we observed MVBs within 30 nm of the ER membrane suggestive of functional colocalization (Supporting Information Figure 3A). To confirm these observations, we used immunofluorescence microscopy in mouse hepatocyte cell line transfected with GFP tagged STARD11 (STARD11-GFP) and CD63 tagged with mCherry (CD63-mCherry) (Figure 4A). We found that STARD11 colocalized with the MVB. Next, we performed immunofluorescence for the ER marker KDEL, an ER localization motif(30), in a mouse hepatocyte cell line transfected with STARD11-GFP. We found that STARD11 colocalized with the ER. We also confirmed that the ER and MVB were in close proximity in cells transfected with CD63-mCherry to label the MVB and KDEL immunofluorescence to label the ER. We confirmed this colocalization in human hepatoma cell line, Huh7 (Figure 4B).
Palmitate increases the colocalization of STARD11 with the MVB.

Having demonstrated a structural colocalization between the ER, MVB and STARD11, we next asked whether STARD11 association with the MVB increases following palmitate treatment. We first validated the specificity of the STARD11 antibody (Supporting Information Figure 3B). Next, as demonstrated in Figure 5A and B, we found that there was significant colocalization of endogenous STARD11 with the MVB, which increased significantly further following palmitate treatment (Figure 5B). To confirm these findings we utilized the fluorescent sphingolipid N-Rh-PE which efficiently labels MVBs and has been used to study MVB trafficking(31,32). Mouse hepatocyte cell line transfected with CD63 tagged with GFP (CD63-GFP) was labeled with N-Rh-PE. Both N-Rh-PE and CD63 labeled the same cellular compartment as demonstrated in Figure 5C. Next, as demonstrated in Figure 5D and E, we found that there was significant colocalization of STARD11 with the N-Rh-PE labeled MVB, which increased significantly further following palmitate treatment. Notably, total protein and mRNA levels of STARD11 were unchanged in palmitate-treated hepatocytes (Supporting Information Figure 1D-F). We did not observe an increase in STARD11 and Golgi colocalization (Figure 5F and G). The increased association of STARD11 with MVBs following palmitate treatment demonstrates an increase in functional association between the ceramide transport protein STARD11 and the exosome formation compartment, the MVB, and likely mediates increased ceramide flux to the MVB, though we cannot exclude indirect trafficking of ceramide from the ER to the Golgi to the MVB.

To examine the association between the ER, the MVB and STARD11 using endogenous markers, we used triple immunofluorescence microscopy in a mouse hepatocyte cell line with antibodies against protein disulfide isomerase (PDI) to label the ER, CD63 to label the endosome and STARD11, which was detected in the cytosol and the nucleus, consistent with its known distribution. We found that STARD11 colocalized with both the ER and the MVB (Figure 6A), as previously observed with the fluorescently tagged proteins. We quantified the colocalization of the endogenous markers and found that STARD11 colocalization with the MVB and ER increased following palmitate treatment as did ER and MVB colocalization (Figure 6B). To further confirm in situ proximity between the ER and MVB, we performed a proximity ligation assay using antibodies against KDEL to label the ER and CD63 to label the MVB in palmitate-treated cells. There was a significant increase in the ER-MVB proximity as measured by the proximity ligation assay in palmitate-treated cells (Figure 6C and D). These colocalization data support an increase in functional association between the ER, STARD11 and the MVB following palmitate treatment.

MVB formation is unchanged in STARD11-/- cells.

To determine if an MVB formation defect in STARD11-/- cells is the mechanistic basis of a reduction in palmitate-induced extracellular vesicle release, we assessed MVB size and number in wildtype and STARD11-/- cells. We used the sphingolipid N-Rh-PE to label MVBs as previously described by others(32,33) followed by super resolution structured illumination microscopy and also electron microscopy to quantify the size and the number of MVBs. When quantified by this approach the size, morphology and number of MVBs was unchanged between WT and STARD11-/- basally and following palmitate treatment (Supporting Information Figure 3C-F), suggesting that MVB formation is unchanged in STARD11-/- cells. Processes that determine MVB trafficking into one of two fates either targeted to the plasma membrane for release or to the lysosomes for degradation remain incompletely understood. We asked whether STARD11, and hence ceramide enrichment, may contribute to endocytic trafficking. Therefore, we examined the endocytic fate of N-Rh-PE labeled MVBs over time in live cells and focused on dissecting whether they were headed to lysosomes, labeled with LysoSensor Green. The colocalization of the MVBs and the lysosomes increased significantly in palmitate treated STARD11-/- cells (Figure 7A and B).

DISCUSSION
Our present study describes a mechanism for the release of ceramide enriched extracellular vesicles in palmitate-treated cells, a cell-based model for hepatocyte lipotoxicity. We report that: i) STARD11 is abundant in hepatocytes; ii) STARD11 mediates the release of palmitate-stimulated ceramide-enriched extracellular vesicles; iii) STARD11 colocalizes to the ER and to the MVB; and iv) STARD11-MVB colocalization and ER-MVB colocalization increases in palmitate-treated hepatocytes. These data suggest that STARD11-mediated ceramide transport to the MVB may lead to the formation of ceramide-enriched extracellular vesicles in palmitate-treated cells. Given the known physiologic role of STARD11 in transporting ceramide, which is the predominant cellular ceramide transport pathway, these data suggest that the inter-compartmental transport of palmitate-stimulated ceramide by STARD11 from the ER leads to the formation of small extracellular vesicles that have features of exosomes. These data are germane to the liver lipotoxicity syndrome observed in human fatty liver disease.

STARD11 is conserved in humans and mice(34). It is a splice variant of collagen type IV alpha-3 binding protein (COL4A3BP) gene product lacking 26 amino acids encoded by exon 11, in comparison with the longer isoform which is designated CERTL. STARD11 protein contains a C-terminal START domain which mediates ceramide transport, an N-terminal PH domain that binds to phosphoinositide in the Golgi membrane. A middle region contains an FFAT (diphenylalanine [FF] in an acidic tract) motif that targets STARD11 to VAP on the outer surface of the ER membrane(35). Our data support a role for STARD11 in mediating ER to MVB ceramide trafficking leading to the formation of ILVs. This may occur indirectly via trafficking to the Golgi and then to the MVB or may be a result of direct trafficking from the ER to the MVB. Though we see an increase in ER-MVB colocalization following palmitate treatment, whether truly functional membrane contact sites are formed and necessary for ceramide transport in this context is a question for future studies. We did not detect STARD11 on exosomes, suggesting that it is not an integral part of the MVB membrane. Regardless of the trafficking pathway STARD11 mediates the release of palmitate-stimulated extracellular vesicles.

In cells that lack STARD11 we found no decrease in basal extracellular vesicle release. However, palmitate-stimulated extracellular vesicles, which have features consistent with exosomes, were reduced. Thus, we conclude that STARD11 plays a role specifically in the release of palmitate-stimulated exosome formation. Consistent with a reduction in the release of ceramide enriched extracellular vesicles, STARD11/-/- cells demonstrated an intracellular accumulation of ceramide which correlated with a reduction in cell viability. These data suggest that the release of ceramide via exosomes may be a pathway to mitigate palmitate-induced toxicity. As we were preparing this manuscript, Obata et al. published a manuscript demonstrating unloading of ceramide from endothelial cells via extracellular vesicle efflux mechanisms(36). Given our expertise in hepatocyte lipaapoptosis(19,20), we were careful in selecting cell culture conditions where apoptosis is not significantly increased in order to try to understand events that occur in viable cells. Additionally, to confirm that we were not mistaking apoptotic bodies for exosomes we confirmed that palmitate-induced extracellular vesicle release was preserved in the presence of caspase inhibitors, which are known to inhibit the release of apoptotic bodies(21).

We found that STARD11 was abundant in human and mouse hepatocytes and mouse liver. Though levels of STARD11 did not change with lipotoxic (palmitate) treatment of cells, we noted that the compartmental distribution of STARD11 was altered in palmitate-treated cells such that there was an increase in its association with the MVB following palmitate treatment. We observed no increase in STARD11 and Golgi colocalization in palmitate-treated cells. Furthermore, we observed an increase in ER-MVB colocalization by immunofluorescence microscopy and by the proximity ligation assay in palmitate-treated cells, suggesting an increase in functional association between the ER and the MVB under palmitate treatment. Recent studies have shown phosphorylation-dependent regulation of STARD11 contact with the ER membrane protein VAP, such that phosphorylation at serine 315
markedly enhanced STARD11-VAP interactions(37). Palmitate is a well-recognized activator of the stress kinase c-jun N-terminal kinase and other cytosolic and plasma membrane kinases(19). Whether, palmitate induces a phosphorylation-dependent increase in STARD11 interactions at the MVB is a question we will address in future experiments. We found that though there were no significant decreases in MVB size and number in STARD11-/- cells.

In conclusion, this is the first report describing the importance of the ceramide transport protein, STARD11, also known as CERT in the formation of ceramide-enriched extracellular vesicles. Our data support a model wherein STARD11 promotes the efficient transfer of palmitate-stimulated ceramide from the ER to the MVB where it drives the formation of ILVs. Elucidation of this fundamental pathway provides a potential target to modulate the release of extracellular vesicles in lipotoxic conditions. STARD11 may be important in other diseases associated with ceramide-dependent extracellular vesicles, a testable hypothesis.

EXPERIMENTAL PROCEDURES

Cells.

Immortalized mouse hepatocyte (IMH) cell-lines derived from IRE1α wildtype mice were a kind of gift of Dr. Randal Kaufman. IMH, Hepa1-6, Huh7 and HepG2 were cultured in Dulbecco’s Modified Eagles Media (DMEM, Life Technologies) containing glucose (4.5 g/L), penicillin (100 U/mL), streptomycin (100 µg/mL) and 10% fetal bovine serum (FBS). Primary mouse hepatocytes were isolated by collagenase perfusion followed by percoll purification and cultured as previously described by us(38). Greater than 95% viable isolated primary hepatocytes were plated in Waymouth’s medium supplemented with 10% FBS, penicillin and streptomycin, as above.

Extracellular vesicle isolation and characterization.

Cells were grown to 90% confluency on 150 mm tissue culture dishes. Cells were washed twice with phosphate-buffered saline (PBS) to eliminate FBS-derived extracellular vesicles. Then cells were treated with either 400 µM palmitate (PA), 400 µM oleate (OA) or vehicle for 16 hours in growth medium supplemented with 5% extracellular vesicle-depleted FBS, which was prepared by overnight centrifugation at 100,000 × g at 4°C according to standard protocols(39) and 1% bovine serum albumin. The optimal concentration and duration for cellular extracellular vesicle release prior to the onset of apoptosis in IMH and Huh7 was decided as previously described(8). Differential ultracentrifugation was used to sequentially isolate small extracellular vesicles, which we have referred to simply as extracellular vesicles throughout. Briefly, collected cell culture conditioned medium was depleted of cells and cellular debris initially by low-speed centrifugations at 2,000 × g for 20 minutes and 10,000 × g for 40 minutes. This was followed by ultracentrifugation at 100,000 × g for 90 minutes to pellet extracellular vesicles. The pellets were washed in PBS and centrifuged again 100,000 × g for 90 minutes. The obtained extracellular vesicle pellets were resuspended in PBS or lysed in RIPA buffer for downstream experiments. For each experimental condition, isolated extracellular vesicles were normalized to cell number, and expressed relative to the vehicle treated condition, unless indicated otherwise. The size distribution and concentration of isolated extracellular vesicles was assessed by nanoparticle tracking analysis as previously described by us(8). Briefly, extracellular vesicle samples were diluted in PBS at a range of concentrations between 2E+08 – 8E+08 particles/mL. Each sample was perfused through the sample chamber at a constant rate of 25 µL/min using a syringe pump. The light scatter and Brownian motion of each sample of nanoparticles was recorded at least three times, 30 s each at constant room temperature (22.5°C); particle tracks were analyzed by NanoSight software to measure the concentration of the particles (particles/mL) and size (in nanometer). For each experimental condition, EVs isolated from equal number of cells were expressed relative to the vehicle treated condition. For basal EV release, EV numbers were normalized to cell numbers.

Caspase 3/7 assay.
Activation of caspase 3/7 is the biochemical hallmark of apoptosis (19). For caspase 3/7 assay, 30,000/cm² IMH cells were cultured in 96 well plates. 48 hours later cells were first pretreated with 10 μM ZVAD-FMK (SantaCruz Biotechnology, Dallas, TX) or IDN-6556 (MedKoo Biosciences, Morrisville, NC) for 1 h followed by 400 μM palmitate or vehicle in the absence or presence of caspase inhibitors for 16 hours or 24 hours. Equal volume of DMSO was used as control. After 16 or 24 hours of treatment, respectively, apoptotic cell death was measured by Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega, Madison, WI) following manufacturer’s protocol.

**Extracellular vesicles in caspase inhibitor-treated cells.**

IMH cells were cultured in 10 cm dishes. Approximately 90% confluent cells were treated with 10 μM of ZVAD-FMK (SantaCruz Biotechnology, Dallas, TX) or IDN-6556 (MedKoo Biosciences, Morrisville, NC) for 1 h. Equal volume of DMSO was used as control. Before palmitate treatment, cells were washed twice with PBS and then the medium was changed to assay medium supplemented with palmitate or vehicle, as above, and caspase inhibitors (10 μM of ZVAD-FMK or IDN-6556). After 16 h, media was recovered and sequential low-speed centrifugation was performed as described above to isolate extracellular vesicles. The extracellular vesicle pellet was resuspended in 100 μL of PBS and subjected for nanoparticle tracking analysis. For each experimental condition, isolated extracellular vesicles were normalized to cell number and expressed relative to the vehicle-treated condition.

**De novo ceramide synthesis inhibitor.**

IMH cells were treated with vehicle or palmitate, with or without 10 μM serine palmitoyltransferase-1inhibitor (Myriocin, Cayman Chemicals, Ann Arbor, MI) for 16 hours. Extracellular vesicles were isolated and analyzed by nanoparticle tracking analysis, described above.

**Iodixanol gradient separation of extracellular vesicles.**

Extracellular vesicles isolated by ultracentrifugation from palmitate or vehicle treated IMH cells (~200 million) were diluted in 1.5 mL buffer containing: 0.25 M sucrose, 10 mM Tris pH 8.0, 1 mM EDTA (pH 7.4). The solution was mixed 1:1 with 60% (wt/vol) stock solution of iodixanol (OptiPrep, Stemcell Technologies, Cambridge, MA). Then 3 mL of extracellular vesicle suspension was transferred to a SW55Ti rotor tube. A 40% iodixanol working solution was prepared [40% (wt/vol) iodixanol, 0.25 M sucrose, 10 mM Tris pH 8.0, 1 mM EDTA, pH 7.4] and used to prepare 20% and 10% (wt/vol) iodixanol solutions. Next to make the gradient, 1.3 mL 20% (wt/vol) iodixanol and 1.2 mL 10% iodixanol were successively layered on top of the 3 mL of vesicle suspension and tubes were centrifuged for 1 h at 4°C at 350,000 × g in SW55Ti; 10 fractions of 490 μL were collected from the top of the tube. Density was assessed from 5 μL of each fraction using a refractometer. Remaining fractions were diluted with 4 mL PBS and centrifuged for 30 min at 100,000 × g in the same rotor. These concentrated fractions were resuspended in 30 μL of PBS and subjected to nanoparticle tracking analysis. For each experimental condition, isolated extracellular vesicles were normalized to cell number and expressed relative to the vehicle-treated condition. This method was based on the protocol described by Kowal et al.(22).

**Lipidomics.**

Ceramides were measured using tandem mass spectrometry at Mayo Clinic Metabolomics Core Laboratory, as previously described by us(8). Extracellular vesicles isolated from equal numbers of cells treated with either vehicle, oleate or palmitate were used to quantify changes in extracellular vesicle ceramides. Cell pellet ceramides were normalized to protein content.

**Western blotting.**

Treated cells were collected by scraping and lysed in RIPA buffer (50 mM Tris HCl, 1% NP-40, 0.1% SDS, 150 mM NaCl, 1 mM EDTA and 0.5% sodium deoxycholate) with protease and phosphatase inhibitors. The protein concentrations were determined by the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of whole cell lysate or protein
isolated from extracellular vesicles derived from equal number of cells were loaded onto Criterion 4-15% or 12.5% Tris-HCl gel (Bio-Rad Laboratories, Hercules, CA) and electro-transferred to PVDF membrane. The membranes were blocked with blocking buffer (5% nonfat milk in TBS-tween) for 1 hour at room temperature. Membranes were incubated with the following primary antibodies overnight: anti-Alix (#2171, Cell Signaling); anti-STARD11 (15191-1-AP, Proteintech); anti-TSG101 (ab125011) and anti-Sytntenin (ab19903) from Abcam; anti-ApoA1 (sc30089), anti-Actin (sc1615), anti-CD81 (sc166029) from Santa Cruz Biotechnology; anti-ApoB (MABS2046) and anti-GAPDH (MAB374) from EMD Millipore Corp. Antigen bound primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies against mouse, rabbit or goat (Santa Cruz Biotechnology). Proteins of interest were visualized with enhanced chemiluminescence reagents (Amersham. Arlington Heights, IL) and HyBlot CL film (Denville Scientific Inc. Metuchen, NJ). Films were scanned using a Cannon LiDe 110 scanner, converted to grayscale in Adobe Photoshop CC 2015, and brightness and contrast adjusted equally across entire images.

**Reverse transcription PCR and quantitative real time PCR.**

Total RNA was isolated by Quick-RNA MiniPrep kit (Zymo Research. Irvine, CA). Quantity and quality of RNA were assessed with a NanoDrop ND1000 (ThermoScientific, Waltham, MA), reverse transcription was performed with the iScript cDNA synthesis kit (Bio-Rad). The PCR reactions were performed in the Tetrad Thermal Cycler (MJ Research Inc. Watertown, MA) using e2TAK DNA polymerase (TaKaRa Biotechnology Inc. Japan). The PCR-amplified fragments were analyzed with the QIAxcel Advanced System (QIAGEN). PCR primers used for mouse STARD11 were as follows: forward 5’-AGTGCTCTTGACGTGTGTCAG-3’ and reverse 5’-ACCGTTGAGCAATTTGCATCA-3’. PCR primers used for human STARD11 were as follows: forward 5’-AGTGCCTCTTGAGCAATTTGCATCA-3’ and reverse 5’-ATCCCTGAACCCATGAACTGA-3’. Primers for mouse hypoxanthine guanine phosphoribosyl transferase (HPRT) (forward 5’-TCAGTCAACGGGGAGATAA-3’ and reverse 5’-GGGGCTGTACTGCTTAACCAG-3’) and human actin (forward 5’-CATGTACGTTGCTATCCAGGC-3’ and reverse 5’-CTCCCTTAATGTCACGCGAT-3’) were used as controls.

**Immunofluorescence.**

Cells were grown on sterile glass coverslips in 6 well plates. Cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min or fixed and permeabilized with ice cold methanol for 15 min, and blocked with 5% BSA / 0.1% glycine in PBS for 30 min at room temperature. After incubating with anti-CD63 (MBS438072, MyBioSource), anti-STARD11 (PA5-28797, Thermo Fisher Scientific), anti-syntaxin6 (ab12370, Abcam), or anti-KDEL (ab50601, Abcam and sc-58774, Santa Cruz Biotechnology) antibodies diluted in blocking buffer overnight at 4˚C, cells were washed three times with PBS, and incubated with fluorescent secondary antibodies (Alexa Fluor 350, 488 or 594, Thermo Fisher Scientific) for 1 hour at room temperature protected from light. For triple immunofluorescence anti-CD63 (564221, BD Pharmingen), anti-STARD11, and anti-protein disulfide isomerase (PDI), MA3019, Thermo Fisher Scientific) were utilized. Then after washing with PBS, coverslips were mounted in Prolong AntiFade (Life Technologies) on a clean glass slide.

**Labeling MVBs with Fluorescent Lipid.**

The fluorescent phospholipid analog N-lissamine rhodamine B 1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine (N-Rh-PE) was obtained from Thermo Fisher Scientific. Cells were labeled at 4˚C with 5 μM N-Rh-PE in serum free DMEM for 1 hour. After this incubation period, the medium was removed and cells were washed with cold serum free DMEM to remove excess unbound lipids. After the addition of treatment medium, labelled cells were incubated for 1 hour and then fixed with 4% paraformaldehyde for observation by confocal microscopy.
**Proximity Ligation Assay.**

Cells were cultured on glass coverslips, fixed and permeabilized with ice cold methanol. The cells were incubated with anti-CD63 (sc15363, Santa Cruz Biotechnology) and anti-KDEL (sc58774, Santa Cruz Biotechnology) antibodies, and processed according to the manufacture’s protocol (Duolink PLA, Sigma). Images were observed by confocal microscope.

**Labeling lysosome with LysoSensor Green.**

Cells were labeled with 1 μM LysoSensor Green (Thermo Fisher Scientific) into growth medium for 30 minutes in the incubator. Cells were washed with PBS and then fixed.

**Cell Transfection.**

For DNA transfection, IMH cells and Huh7 cells were co-transfected with GFP-tagged STARD11 (gift from Dr. Hanada), mCherry-tagged CD63 (gift from Dr. McNiven), CD-63-pEGFP (gift from Dr. Paul Luzio, Addgene plasmid # 62964) using Lipofectamine LTX reagent (Invitrogen, Carlsbad, CA) following the manufacture’s protocol. In some experiments, transfected cells were labeled with N-Rh-PE, or used for immunofluorescence.

**Confocal microscopy.**

Fluorescence was observed with an LSM 780 confocal microscope (ZEISS) and a Plan-Apochromat 40x/1.4 Oil DIC M27 objective (ZEISS). A 405 nm laser was used for the blue channel. A 488 nm laser was used for the green channel, and a 561 nm or a 594 nm laser was used for the red channel. Images were taken at room temperature. ZEN 2.3 lite software (ZEISS) was used for acquiring images. Image J (National Institutes of Health) was used to analyze the raw images. The quantification shows the Pearson coefficients of colocalization. To avoid artifacts from peri-nuclear crowding fluorescence pixel intensity was measured in the entire observed field rather than sub-cellular regions of interest. 10 - 20 random fields in each group were selected for quantification and statistical analysis.

**Electron Microscopy.**

Vehicle treated IMH cells were fixed in Trump’s solution (4% formaldehyde + 0.1% glutaraldehyde in 0.1M phosphate buffer). Palmitate-treated cells were fixed in 3% glutaraldehyde and 0.1 M sodium cacodylate buffer. These cells were then processed for electron microscopy analysis using standard procedures and observed with a JEOL 1400 Plus transmission electron microscope (JEOL USA, Peabody, MA) at 80kV.

**Super-Resolution Microscopy.**

IMH cells were treated with vehicle or 400 μM palmitate for 6 hours, then labelled with N-Rh-PE. For imaging of MVBs of IMH cells, cells were incubated in treatment medium for 1 hour, and then fixed with 4% paraformaldehyde. Structure illumination microscopy (SIM) images were captured using an Elyra PS.1 microscope (ZEISS) and a Plan-Apochromat 63x/1.4 Oil DIC M27 objective (ZEISS) at room temperature.

**CRISPR/Cas9 gene editing.**

The online tool at (http://crispr.mit.edu) was used to design the single guide RNA spanning the mouse STARD11 (sgRNA 5’-CACCGCGGCTGCAGCTTCATGT-3’) and human STARD11 (sgRNA 5’-CCGGGAATTCACAGCTTGCGGACA-3’, 5’-CCGGTAATCAGAGCTGGAACTCGT-3’). Annealed oligonucleotides of sgRNA were used to generate a lentivirus following subcloning into the lentCRISPRv1 plasmid (Addgene), and transfected into HEK293T cells using Lipofectamine LTX reagent, as previously described by us(8). Virus was harvested at 48 hours after transfection and IMH cells were infected with STARD11 targeting lentivirus in the presence of 8 μg/mL polybrene. Infected cells were selected under 4 µg/mL blasticidin (InvivoGen) selection pressure. STARD11 knockout cell lines in Huh7 cells were created by Guide-it CRISPR/Cas9 system (TaKaRa Biotechnology Inc. Japan) according to the manufacture’s instruction.

**Quantitation of apoptosis and cytotoxicity.**

IMH cells were stained with 4’,6-diamidino-2-phenylindole (DAPI) for 5 min and visualized using fluorescent microscope to count number of apoptotic nuclei. IMH wildtype and STARD11-/- cells were plated in 96-well plates.
Cells were then treated with 400 μM palmitate. After 16 hours and 20 hours of treatment, cell viability was measured by WST-1 assay (Sigma-Aldrich) per manufacturer’s instructions.

Statistical and data analyses.

Data represented as mean ± S.E.M. from three or more experiments unless indicated. Two-tailed student’s t-test was used for comparing groups. Statistical analyses were performed in GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA). A p value of <0.05 was considered significant.

ACKNOWLEDGEMENTS
The authors are grateful to Dr. Gregory J. Gores for critical review of the manuscript; Dr. Kentaro Hanada for the gift of GFP-hCERT, CD63-mCherry was a gift from Dr. Mark McNiven, and CD63-GFP was from Addgene. We are also grateful to Dr. David Katzmann and Dr. Brian Davies for useful discussions and insight into MVB formation, and Ms. Courtney Hoover for outstanding administrative support.

CONFLICT OF INTEREST
The authors declare that they have no conflicts of interest with the contents of this article.

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FOOTNOTES

Funding was provided in part by NIH grant DK111378 (H.M.), by Gilead Sciences Research Scholars Program in Liver Disease, the Palumbo Foundation, and the Strickland Career Development Award (H.M.), the optical microscopy core of the Mayo Clinic Center for Cell Signaling (P30DK084567), and the Mayo Clinic Metabolomics Core (U24DK100469, UL1TR000135).

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ABBREVIATIONS

The abbreviations used are: CERT, ceramide transport protein; COL4A3BP, collagen type IV alpha-3 binding protein; ESCRT, endosomal sorting complex required for transport; EV, extracellular vesicle; FFAT, diphenylalanines in an acidic tract; HPRT, hypoxanthine guanine phosphoribosyl transferase; ILV, intraluminal vesicle; IMH, immortalized mouse hepatocyte; LBPA, lysobisphosphatidic acid; LPC, Lysophosphatidylcholine; MVB, multivesicular body; N-Rh-PE, N-lissamine rhodamine B 1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine; OA, oleic acid; PA, palmitate; PH, pleckstrin homology; SIM, Structure illumination microscopy; STARD, StAR related lipid transfer domain; TSG101, tumor susceptibility gene 101; VAP, vesicle associated membrane protein-associated protein.
Figure 1. Palmitate-induced EV release is STARD11 dependent. (A) EV release in immortalized mouse hepatocyte (IMH) cell line from wildtype (WT) and two STARD11 knock out (STARD11-/-) clones treated with vehicle or 400 μM, oleate (OA) or palmitate (PA), each for 16 hours. The inset western blot confirms the deletion of STARD11 in both clone A and clone B. (B) EV release in human hepatocyte cell line, Huh7, WT and STARD11-/- clones treated with vehicle or 400 μM OA or PA, each for 16 hours. The inset western blot confirms the deletion of STARD11 in both clone A and clone B. (C, D) Size of EVs from IMH and Huh7 WT and STARD11-/- cells measured by nanoparticle tracking analysis. (E, F) Basal EV release per cell in WT and STARD11-/- IMH and Huh7 cells. **, P < 0.01, ns, not significant. All error bars are SEM. These data were obtained from three or more independent experiments.
Figure 2. EV ceramide enrichment is STARD11 dependent. (A, B) EVs were isolated from immortalized mouse hepatocyte cell line from wildtype (WT) and STARD11 knock out (STARD11-/-) cells treated with vehicle, 400 μM palmitate (PA) or oleic acid (OA) for 16 hours. The C16:0 ceramide content of EVs was measured by LC-MS/MS. (C, D) The C16:0 ceramide was measured in whole cell pellets under the same condition as above, and normalized to protein content. *, P < 0.05, **, P < 0.01, ns, not significant. All error bars are SEM. These data were obtained from three or more independent experiments.
Figure 3. Palmitate-stimulated EVs are consistent with exosomes. (A) EVs isolated from equal number of cells and whole cell lysates from immortalized mouse hepatocyte cell line were analyzed by western blot. Alix, TSG101, syntenin and CD81 were used as small EV markers. GAPDH was used as a loading control. (B) These expression levels were quantified by densitometry. *, P < 0.05, **, P < 0.01, ns, not significant. All error bars are SEM. These data were obtained from three or more independent experiments.
Figure 4. STARD11 is colocalized with ER and MVB. (A, B) Immortalized mouse hepatocyte (IMH) (A) and Huh7 (B) were co-transfected with GFP tagged-STARD11 and mCherry-tagged CD63 to label MVB (top panel). Cells were transfected with GFP tagged-STARD11 and were then labeled with anti-KDEL antibody by immunofluorescence for ER marker (middle panel). Cells were transfected with mCherry-tagged CD63 and were then labeled with anti-KDEL antibody by immunofluorescence (bottom panel). Inset depicts a higher magnification of the selected region. The scale bar indicates 10 μm.
Figure 5. The colocalization of STARD11 and MVB is upregulated by palmitate. (A) Immortalized mouse hepatocyte (IMH) cells were treated with vehicle or 300 μM palmitate (PA) for 1 hour. After
treatment, cells were double-labeled with anti-CD63 antibody (red) and anti-STARD11 antibody (green) by immunofluorescence and observed by confocal microscopy. The scale bar indicates 10 μm. (B) The Pearson correlation coefficients of colocalization between CD63 and STARD11 were analyzed by Image J software in 20 random fields for each condition. (C) IMH cells were transfected with GFP-tagged CD63 and then labeled with N-Rh-PE to label MVB. The scale bar indicates 10 μm. (D) IMH cells were transfected with GFP-tagged STARD11 and then labeled with N-Rh-PE. Cells were treated with vehicle or PA as above. After treatment, cells were fixed, and observed by confocal microscopy. (E) The Pearson correlation coefficients of colocalization between STARD11-GFP and N-Rh-PE were analyzed by Image J software in 10 random fields for each condition. The scale bar indicates 20 μm. (F) After treatment as above, IMH cells were double labeled with anti-Syntaxin6 (trans-Golgi network marker, green) and anti-STARD11 (red). (G) The Pearson correlation coefficients of colocalization between Syntaxin6 and STARD11 were analyzed by Image J software in 20 random fields for each condition. The scale bar indicates 10 μm. **, P < 0.01, ns, not significant. All error bars are SEM.
Figure 6. Palmitate upregulates ER-MVB colocalization. (A) Immortalized mouse hepatocyte (IMH) cells were triple-labeled with anti-PDI antibody (ER marker, red), anti-CD63 antibody (MVB marker, pink) and anti-STARD11 antibody (green) after treatment with vehicle or 300 μM palmitate (PA) for 1
hour. The scale bars indicate 10 μm. (B) The Pearson correlation coefficients of colocalization between CD63 and STARD11, PDI and STARD11, PDI and CD63 were analyzed by Image J software in 30 random fields for each condition. (C) Proximity ligation assay (PLA) showed the interaction between anti-KDEL (ER marker) and anti-CD63 (MVB marker) antibodies as red fluorescent signals in IMH cells treated with vehicle or 300 μM PA. The scale bars indicate 10 μm. (D) The total number of PLA signals per cell was quantified in more than 50 cells for each condition per experiment. *, P < 0.05, ***, P < 0.001. All error bars are SEM. These data were obtained from three independent experiments.
Figure 7. Palmitate treatment increases the colocalization of the MVB and lysosome in STARD11-/cells. (A) Immortalized mouse hepatocyte (IMH) wildtype (WT) cells and IMH-STARD11-/cells were labeled with N-Rh-PE. Cells were treated with 300 μM palmitate (PA) for 1 hour or 2 hours. They were also labeled with 1 μM LysoSensor Green and then fixed. (B) The Pearson correlation coefficients of colocalization between N-Rh-PE and LysoSensor Green were analyzed in 10 random fields for each condition using Image J software. The scale bar indicates 10 μm. **, P < 0.01, ns, not significant. All error bars are SEM.
StAR-related lipid transfer domain 11 (STARD11)-mediated ceramide transport mediates extracellular vesicle biogenesis
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J. Biol. Chem. published online August 23, 2018

Access the most updated version of this article at doi: 10.1074/jbc.RA118.002587

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