MLN64 Mediates Mobilization of Lysosomal Cholesterol to Steroidogenic Mitochondria

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SUMMARY

This study demonstrates that the START domain containing protein, MLN64, participates in intracellular cholesterol trafficking. Analysis of the intracellular itinerary of MLN64 and MLN64 mutants tagged with green fluorescent protein (GFP) showed that the N-terminal transmembrane domains mediate endocytosis of MLN64 from the plasma membrane to late endocytic compartments. MLN64 constitutively traffics via dynamic NPC1-containing late endosomal tubules in normal cells; this dynamic movement was inhibited in cholesterol-loaded cells and MLN64 is trapped at the periphery of cholesterol-laden lysosomes. The MLN64 START domain stimulated free cholesterol transfer from donor to acceptor mitochondrial membranes and enhanced steroidogenesis by placental mitochondria. Expression of a truncated form of MLN64 (ΔSTART-MLN64), which contains N-terminal transmembrane domains but lacks the START domain, caused free cholesterol accumulation in lysosomes and inhibited late endocytic dynamics. The ΔSTART-MLN64 dominant negative protein was located at the surface of the cholesterol-laden lysosomes. This dominant negative mutant suppressed steroidogenesis in COS cells expressing the mitochondrial cholesterol side-chain cleavage system. We conclude that MLN64 participates in mobilization and utilization of lysosomal cholesterol by virtue of the START domain’s role in cholesterol transport.
INTRODUCTION

Cells obtain cholesterol by either low-density lipoprotein (LDL)-receptor mediated endocytosis of LDL or de novo synthesis in the endoplasmic reticulum (1). Endocytosed cholesterol moves to the late endocytic compartment and subsequently modulates homeostatic responses via SREBP-SCAP located in the endoplasmic reticulum (2). Mutations in NPC1 and NPC2 cause Niemann-Pick Type C (NPC) disease, characterized by a phenotype of excessive cholesterol accumulation in lysosomes (3, 4). Although NPC1 and NPC2 are required for egress of lysosomal cholesterol, the mechanism by which cholesterol is removed from the late endocytic pathway, a dynamic tubular network that communicates with lysosomes, is not known.

The mysteries of lysosomal cholesterol transport began to be unraveled when the gene responsible for the majority of cases of NPC disease, NPC1, was cloned (3). NPC1, a protein with a sterol-sensing domain, spans the surface membrane of the late endosomal compartment and cellular uptake of low-density lipoproteins promotes enrichment of NPC1 in late endosomes (5, 6, 7). The sterol-sensing domain was shown to be critical for cholesterol transfer, confirmed by the findings based on site-directed mutagenesis and protein trafficking analysis of NPC1 protein (5, 6, 7). NPC1 was recently suggested to be a permease that may be involved in material transfer across the endosomal/lysosomal limiting membrane (8). The critical role of another protein, HE1, in clearing cholesterol out of lysosomes was established when it was discovered that the HE1 gene is mutated in the second and less frequent form of Niemann-Pick Type C disease and is, therefore, the NPC2 gene. HE1 encodes a soluble cholesterol-binding protein (4, 9).

Recently, MLN64, a protein that contains a C-terminal StAR-related lipid transfer (START) domain was found to reside in late endosomes and lysosomes (10, 11, 12). The N-terminus of MLN64 consists of a 6 aa leader sequence and four anchoring transmembrane domains (10). The START domain binds cholesterol and can stimulate sterol transfer between membranes (10, 13, 14). The topology of MLN64 in late endosomal membranes predicts that the START domain projects into the cytoplasm where it is positioned to effect cholesterol transfer to opposing membranes (12). The location of a START domain protein in late endocytic pathway with NPC1 and HE1 suggests that MLN64 is a component of the
machinery that facilitates cholesterol egress from lysosomes. To investigate this possibility, we elucidated the trafficking, in living cells, of wild type and mutant MLN64 proteins fused with green fluorescent protein (GFP). We found that a truncated MLN64 without the START domain (ΔSTART-MLN64) is a dominant negative mutant. The dominant negative action of this mutant caused extensive cholesterol accumulation in CHO cells and COS-7 cells accompanied by inhibition of late endosomal tubular trafficking, similar to the NPC phenotype that is caused by loss of function of NPC1 and NPC2 (3, 4, 7). This dominant negative mutant also inhibited steroidogenesis in cells. An *in vitro* assay provided evidence that the START domain mediates cholesterol transfer between membranes and stimulates mitochondrial metabolism of cholesterol into steroid hormones. These findings strongly suggest that MLN64 plays a role in maintenance of intracellular cholesterol homeostasis.
EXPERIMENTAL PROCEDURES

Construction of expression plasmids for MLN64-GFP, MLN64-CFP, ΔSTART-MLN64-GFP, START-MLN64-GFP, and NPC1-YFP

The human MLN64 cDNA has been previously described (10). MLN64 cDNA encoding either full length or specific domains of the MLN64 protein were fused to the green fluorescent protein (pEGFP-N2; Clontech Laboratories Inc, Palo Alto, CA). All of the cDNAs used in making these fluorescent fusion proteins were generated by PCR using either Pfu or Taq DNA polymerases. The PCR primer pair used for the MLN64-GFP (forward, ATTAGAATTCTAGCACACAAGCTGCCCAGGGAG; reverse, TAATGTCGACTGATGCGCTGTCGCAGGGAAAG) spanned the cDNA encoding for amino acids 1-436 of the human MLN64 protein. The MLN64-CFP was constructed by replacing the GFP of MLN64-GFP with CFP from pECFP-N2 (Clontech). The START domain for MLN64 (amino acids 216-445) was also PCR amplified and fused to the GFP vector. A shared forward PCR primer (ATTAGAATTCTAGCACACATGGGGTCAGCTGCCCAGGGAAAG) for generation of the GFP construct was used with the START-MLN64-GFP specific reverse primer (TAATGTCGACTGATGCGCTGTCGCAGGGAAAG). The ΔSTART-MLN64-GFP fusion protein was generated by restriction digestion with Xmn1 and Sma1, gel-isolation of the vector DNA plus cDNA encoding amino acids 1-225 followed by ligation of the blunt ends to form the ΔSTART-MLN64-GFP construct. All constructs were sequenced and compared to the human MLN64 cDNA sequence (GenBank Accession Numbers NM 006438; NM 006445, respectively). The human StAR-RFP fusion protein was constructed with the RFP tag on the C-terminus of the full-length coding sequence pDsRed1 N-1 vector (Clontech). The human NPC1-YFP fusion protein was constructed by inserting NPC1 together with the six-histidine linker from vector pNES6 (7) into pEYFP-N1 (Clontech) in-frame with the N-terminal of YFP. The function of NPC1-YFP was confirmed by analyzing cholesterol clearance in CT60 cells using filipin staining (data not shown). The F2 expression plasmid, which encodes a fusion protein consisting of the P450 side chain cleavage enzyme and its electron donor (adrenodoxin and adrenodoxin reductase) partners (15) was generously provided by Dr. Walter L. Miller, University of California, San Francisco. An
expression plasmid for murine type II 3β-hydroxysteroid dehydrogenase (3βHSD) was kindly provided by Dr. Anita Payne, Stanford University. Plasmids for transfection were prepared using the Qiagen Maxiprep system.

Cell culture and transfection

For assays of steroidogenic activity (16) of the GFP fusion constructs, COS-1 were cultured in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% fetal calf serum and 50 µg gentamycin/ml in 5% CO$_2$-95% air at 37 ºC for cell propagation and plating. These cells were obtained from the American Type Culture Collection (Rockville, MD). COS-1 cells were plated at 30,000 cells/well in 12 well plates, respectively, on day 0. On day 1, cells were transfected with FuGENE6 (Roche/Boehringer Mannheim, Indianapolis, IN) plus plasmid DNA in the presence of serum as described in the manufacturer’s protocol. Cells were co-transfected overnight with 250 ng of each of the 3βHSD and F2 expression plasmids and 500 ng of the GFP fusion vectors. On day 2, medium was changed to DMEM containing 10% fetal calf serum. Some cultures were incubated with 22(R)-hydroxycholesterol, a soluble steroidogenic substrate, to assess maximal hormone production (10). After 24 h, medium was collected and frozen at –20 ºC until steroid analysis (See Progesterone Assays), and cells were harvested for protein determinations.

To examine utilization of LDL-derived cholesterol, human LDL was reconstituted with [3H]cholesterol linoleate by the method of Krieger et al. (17). COS-1 cells transfected with the various MLN64 constructs and the steroidogenic enzymes as described above were cultured in medium supplemented with 10% lipoprotein deficient serum and 50 µg protein (70 µg cholesterol)/ml reconstituted LDL (0.85 x 10$^5$ dpm/µg cholesterol). The culture medium was collected 40 h later and radiolabeled progesterone was isolated as described by Soto et al. (18).

Wild type CHO cells, CT60 CHO cells, and COS-7 cells were used for microscopic analysis. Cells were maintained in Ham’s F12 medium (CHO cell lines) or McCoy’s medium (COS-7 cells and fibroblasts) containing 10% fetal bovine serum (FBS, HyClone, Logan, UT). Before transfection, cells were seeded in LabTek chambered coverslips and grown for 20 h to achieve approximately 60% confluence before transfection. All the cell lines were transfected using FuGENE6 according to manufacturer’s instructions.
with some modification. For each chamber of the 2-chamber coverslips, cells were incubated with mixtures containing DNA 1 µg and FuGENE6 reagent 4 µl in 400 µl Ham’s F12 medium for 5 h followed by culture in 10% FBS containing Ham’s F12 medium. Transfected cells were either imaged as live cells at different times of expression of the transfected cDNAs, or as fixed cells (in 3% paraformaldehyde for 30 min) stained with 50mg/ml filipin (Polysciences, Warrington, PA) to examine lysosomal cholesterol content (7). For live cell imaging, cells were grown on chambered coverslips (LabTek) and temperature was maintained using a combination of a heating stage and an Objective Heater System (Bioptechs, Butler, PA).

**Progesterone assays**

Media samples from transfected cells were assayed using Progesterone Coat-A-Count tubes and reagents (Diagonistic Products Corporation, Los Angeles, CA) as described by the manufacturer. Statistical tests were performed using the JMP 3.1.5 computer program (SAS Institute Inc., Cary, NC). One-way analysis of variance (ANOVA) was used to analyze the effect of the expression plasmids on steroidogenic activity. Tukey-Kramer mean separation tests were performed for comparison between the means with P<0.05 taken as the level of significance.

**Immunoblotting**

Western blotting was used to confirm expression of the GFP-fusion proteins and the coupling of GFP to the MLN64 sequences. The rabbit MLN64 antiserum was generated against recombinant MLN64 START domain as a 6xHis fusion protein (see below). An antiserum to GFP was obtained from Roche. Western blotting was carried out as described by Watari et al (10).

**Production of recombinant MLN64 START domain and assay of biological activity**

The human MLN64 START domain (amino acid residues 216-445) was expressed in *E.coli* as a 6xHis tag protein with the tag on the START domain C-terminus using a pET vector and the protein purified using previously described methods (13, 14). The recombinant protein was used to generate rabbit anti-MLN64 antibodies. It was also tested for sterol transfer activity as described by Kallen et al (14) in an assay that measures transfer of [14C] cholesterol from unilamellar egg phosphatidylcholine to heat-treated liver mitochondria acceptor membranes. Preliminary studies demonstrated that steroidogenic mitochondria from bovine corpus luteum or human placenta also served as equivalently effective acceptor membranes for
START domain stimulated sterol transfer. Transfer of \[^{3}H\] triolein incorporated into the liposomes was measured to correct for liposome fusion or trapping. The sterol transfer activity of recombinant MLN64 START domain was compared to that of sterol carrier protein 2 (SCP2).

Preparations enriched in syncytiotrophoblast mitochondria were prepared from human term placenta as described by Martinez et al. (19). The mitochondria were incubated in 120 mM KCl, 10 mM MOPS, 0.5 EGTA, 10 mM isocitrate, 0.1% BSA, 0.4 µg aprotinin/0.1 ml, 1 µM leupeptin, pH 7.4 with or without recombinant proteins (19). Reactions were terminated by addition of methanol and progesterone was assayed as described above. Progesterone present at zero time was subtracted from values obtained at various time points of incubation.

**Pharmacological perturbations**

Wild type CHO cells were treated with progesterone (10 µg/ml) plus LDL (100 µg/ml) to induce cholesterol accumulation in lysosomes (7, 21). To assess the effect of microtubule disruption on MLN64-GFP trafficking, cells were cultured in 10% FBS-containing medium with 10 µM nocodazole (Sigma) for up to 2 h at 37°C.

**Labeling endocytic pathway in living cells**

Late endocytic compartments (late endosomes and lysosomes) were labeled by incubating cells with fluorescent dextran (5, 7). Cells were cultured at 37°C, in media containing 1 mg/ml rhodamine-dextran (Dextran, tetramethylrhodamine, 70,000 MW, lysine fixable, Molecular Probes, Eugene, OR) for 1 h of uptake plus 1 h chase (2 h of uptake/chase time), or 17 h of uptake plus 1 h chase (18 h of uptake/chase time) before live cell imaging.

**Immunostaining**

Cells were fixed with 3% paraformaldehyde in PBS for 30 min to 1 h, washed in PBS, and immunostained with mouse monoclonal anti-C terminal MLN64 antibody (10) at 1:500 dilution and the secondary antibody, Alexa Fluor 568 conjugated goat anti-mouse IgG (Molecular Probes) at 1:100 dilution.
Confocal microscopy and time-lapse imaging

Confocal images were collected using an LSM410 confocal microscope system equipped with a krypton-argon Omnichrome laser with excitation wavelengths of 488 nm and 568 nm for GFP and rhodamine (and Alexa Fluor 568), respectively (Carl Zeiss Inc., Thornwood, NY), and an argon ion laser (Coherent, Santa Clara, CA) with excitation wavelength of 351 nm for filipin. Emissions were collected with a C-apochromat 63x 1.2NA water immersion objective (Zeiss) using a 514-540 nm band-pass filter for GFP, a 590 nm long-pass filter for rhodamine and DsRed RFP, and a 400-435 band-pass filter for filipin.

Time-lapse images were captured using a cooled CCD camera (CoolSNAP HQ Monochrome, Photometrics, Tucson, AZ), with a Sony Interline high quantum efficiency chip and custom macros provided by BioVision Technologies. The camera was attached to an Axiovert 35 inverted microscope (Zeiss) with a plan Apochromat 100x 1.4NA oil immersion objective (Zeiss) or a C-apochromat 63x 1.2NA water immersion objective (Zeiss). The excitation was from a 100w Hg-Arc lamp (Atto Instruments Inc., Rockville, MD) with standard fluorescein and rhodamine optics for GFP and RFP, respectively. For CFP and YFP, exciters 410-440 and 487-502 nm, dichroic 505DCXR, and emitter HQ530-580 nm were used. For dual color time-lapse imaging of CFP/YFP or GFP/RFP in co-transfected cells, cells were imaged at 20ºC to slow late endosomal tubular movement. CFP and YFP, or GFP and RFP, were excited and collected sequentially with a time interval of zero s to minimize time lag during switching channels. IPLab software system (Scanalytics, Fairfax, VA) and custom scripts provided by BioVision Technologies were used for image capture, processing, animation, and export to QuickTime movies. Adobe Photoshop 5.0 was also used for image processing (Adobe Systems Inc., San Jose, CA).
RESULTS

**Characterization of the expression of wild type and mutant MLN64-GFP fusion proteins**

The fusion protein constructs, MLN64-GFP, START-MLN64-GFP and ΔSTART-MLN64-GFP, used in this study are shown schematically in Figure 1. Western blotting using anti-GFP or anti-MLN64 antibodies demonstrated that GFP is attached to MLN64 (Fig. 1, A and B), ΔSTART-MLN64 (Fig. 1C) and START-MLN64 (Fig. 1, D and E). We detected a low molecular weight band in cells transfected with MLN64-GFP with anti-GFP antibody (Fig. 1B). This band was not found either in cells at early times after transfection with MLN64-GFP (15 h) when the MLN64-GFP was found mainly on the plasma membrane (see below), or in cells transfected with ΔSTART-MLN64-GFP or START-MLN64-GFP. This GFP-positive protein is probably derived from post-translational proteolysis in which GFP is cleaved from the MLN64 protein. We do not yet have an antibody recognizing the MLN64 N-terminus. However, in extracts of cells transfected with ΔSTART-MLN64 the anti-GFP antibody detected a single protein of the expected molecular weight of the mutant-GFP fusion protein. Similarly, analysis of cells transfected with the START-MLN64-GFP expression plasmid revealed a single MLN64- and GFP-positive protein of the anticipated molecular weight of the fusion protein. We obtained similar results when the plasmids were introduced into either COS or CHO cells.

Although MLN64 has been localized to late endosomes (12), the path that it takes to reach this destination has not been identified. To study the biosynthetic pathway of MLN64, we followed the expression of MLN64-GFP in living CT60 CHO cells that have mutations in the *NPC1* gene and thus display a cholesterol trafficking defect (Fig. 2). Twelve to seventeen hours after transfection, we found MLN64-GFP was mainly associated with the plasma membrane (Fig. 2A). Very little MLN64-GFP fluorescence was present in cholesterol-laden lysosomes that were labeled by rhodamine dextran uptaken by the cells for 17 h before transfection (data not shown) (7). Seventy-two hours after transfection, MLN64-GFP was mainly present as ring-shaped structures (Fig. 2B) recognized by immunostaining with anti-C-terminal MLN64 antibody (Fig. 2C). Filipin staining confirmed that MLN64-GFP was at the
periphery of cholesterol-loaded lysosomes (data not shown). These observations confirm that MLN64-GFP travels first to the plasma membrane before being targeted to the late endocytic compartment. In wild type CHO cells MLN64-GFP followed an identical biosynthetic pathway to the plasma membrane (data not shown) and then to the late endocytic pathway (see below), as in CT60 cells.

The ΔSTART-MLN64-GFP followed a biosynthetic trafficking pathway similar to that of wild-type MLN64, first to the plasma membrane (Fig. 2D) then to the cholesterol-laden lysosomes (Fig. 2E), showing that ΔSTART-MLN64-GFP contains the essential signals for plasma membrane localization and lysosomal targeting similar to newly synthesized MLN64. ΔSTART-MLN64-GFP is not recognized by the anti-C terminal MLN64 antibody (Fig. 2F). START-MLN64-GFP, the mutant lacking the N-terminal domains, was present only in a cytoplasmic and nuclear pattern 16-72 h after transfection, further substantiating the role of the N-terminal domain in directing MLN64 to the plasma membrane and then to the late endocytic compartment (see below). ΔSTART-MLN64-GFP (Fig. 2G) and MLN64 (Fig. 2H) co-localized at the periphery of cholesterol-loaded lysosomes in CT60 cells co-transfected with ΔSTART-MLN64-GFP and non-tagged MLN64. The non-tagged MLN64 was identified with the anti-C terminal MLN64 antibody. The fact that ΔSTART-MLN64 and MLN64 follow an identical biosynthetic trafficking pathway and locate together at the surface of cholesterol-containing compartments provides morphological evidence that the mutant MLN64 interferes with endogenous MLN64’s role in cholesterol mobilization from lysosomes (see later in results).

**MLN64 constitutively traffics within the cholesterol regulated, dynamic late endosomal tubulovesicular network**

We used WT CHO cells transiently transfected with MLN64-GFP or stably expressing MLN64-GFP. MLN64-GFP was found in late endosomal tubulo-vesicular compartments that constitutively traffic in a microtubule-dependent manner (movie 1, 2). The dynamic late endosomal tubular movement is inhibited by nocodazole (10 µM), which disrupts the microtubular network. Similar to previous findings on NPC1 trafficking (7), within 8 min after nocodazole treatment, the MLN64-GFP-containing endosomal compartments ceased to bud tubules and gathered in the perinuclear region, showing little movement (data not shown).
Analysis of living cells cotransfected with MLN64-CFP (Fig. 3, A and C) and NPC1-YFP (Fig. 3, B and D) confirmed that MLN64 colocalizes and traffics in the same dynamic late endosomal tubules as NPC1 (movie 3). As we predicted MLN64 containing late endosomal trafficking is sensitive to its cholesterol content. In WT CHO cells transfected with MLN64-GFP and exposed to LDL and progesterone, MLN64-GFP (Fig. 3E) was localized at the periphery of lysosomes (Fig. 3F) similar to the location of NPC1 (7, 21). Late endosomal tubules were rarely observed in the progesterone plus LDL treated cells. Late endosomal tubular trafficking was also not observed in cholesterol laden NPC2 and CT60 cells transfected with MLN64-GFP (data not shown). These data show that both MLN64 and NPC1 traffic in late endosomal tubules and the tubular mobility is inhibited when cells accumulate endocytosed cholesterol.

We examined the potential for the endosomal tubules to come into close proximity with mitochondria by imaging cells co-transfected with MLN64-GFP and StAR-RFP, which labels the mitochondrial matrix (movie 4). We noted parallel alignment of late endosomal tubules with mitochondria in living cells. The late endosomal tubules extending from vesicles increase the surface area of the late endocytic compartment and provide more sites for potential close association with mitochondria where cholesterol transfer could be effected. Because MLN64-GFP was not anticipated to enter into the mitochondrial matrix, close approximation, but not overlap of the green and red signals was expected as shown in movie 4.

The MLN64 START domain transfers cholesterol from sterol-rich donor liposomes to acceptor mitochondrial membranes and stimulates progestin synthesis by placental mitochondria

To document that the MLN64 START domain can indeed function as a sterol transfer protein, we tested the ability of recombinant MLN64 START domain to promote the movement of [14C] cholesterol from sterol-rich phosphatidylcholine unilamellar liposomes to acceptor membranes (heat-treated mitochondria) in an established assay system that is corrected for sterol transfer resulting from fusion or trapping of liposomes with acceptor membranes (14). The recombinant MLN64 START domain stimulated cholesterol transfer from liposomes in a dose- and time-dependent manner (Fig. 4). The sterol transfer activity of 1 mM recombinant MLN64 START domain was approximately 82% of that of 1 mM sterol carrier protein 2 (SCP2), a well-characterized lipid transfer protein. Recombinant MLN64 START domain
(1 µM) also stimulated progestin synthesis by isolated human placental mitochondria by approximately 2-fold after 40 min of incubation, whereas an irrelevant recombinant protein, the extra domain A of human fibronectin also bearing a 6xHis tag (22) had no effect on steroidogenesis.

**The START domain deleted MLN64 (ΔSTART-MLN64) induces cholesterol accumulation in lysosomes and inhibits steroidogenesis activity in transfected cells.**

Comparison of late endosomal dynamics in wild type CHO cells and COS7 cells transfected with MLN64-GFP and ΔSTART-MLN64-GFP, resulted in finding the dominant negative effects caused by ΔSTART-MLN64-GFP expression. Unlike MLN64-GFP which is present in rapidly moving late endosomal tubules (Fig. 5, A and D, and movie 1), ΔSTART-MLN64-GFP is present as immobile ring-shaped structures in the perinuclear region of WT CHO cells 48-72 h after transfection (Fig. 5, G and J, and Fig. 6, A and B). Time-lapse movies of ΔSTART-MLN64-GFP-expressing living cells show no late endosomal tubular trafficking and immobile ΔSTART-MLN64-GFP-containing ring-shaped compartments in the perinuclear region of the cell (movie 5). The dynamic contact and parallel alignment of mitochondria with late endosomal tubules, present in MLN64-GFP transfected cells, were not present in cells co-transfected with ΔSTART-MLN64-GFP and StAR-RFP. The StAR-RFP enters mitochondria and labels the matrix (movie 6).

Endocytosis assays of WT CHO cells transfected with ΔSTART-MLN64-GFP revealed an alteration in the lysosomal filling of the endocytosed fluid phase marker, dextran (Fig. 5). NPC1-GFP-containing late endosomes were shown previously to load with fluorescent dextran in 2 h of uptake/chase time (5,7). In WT CHO cells MLN64 (Fig. 5A) containing late endosomes loaded with dextran (Fig. 5B) in 2 h of uptake/chase time (5C, merged image of 5A and 5B). Co-localization of MLN64-GFP (Fig. 5D) and endocytosed dextran (Fig. 5E) is shown in 18 h of uptake/chase time (Fig. 5F, merged image of 5D and 5E). In comparison, in WT CHO cells transfected with ΔSTART-MLN64-GFP and incubated with fluorescent dextran in 2 h of uptake/chase time, ΔSTART-MLN64-GFP and dextran were in separate compartments (Fig. 5I, merged image of 5G and 5H). Dextran (Fig. 5H) was not present in the ΔSTART-MLN64-GFP-containing ring-shaped structures (Fig. 5G). After 18 h of uptake/chase time, dextran (Fig. 5K) was present only associated with ΔSTART-MLN64-GFP containing rings (Fig. 5F, merged image of
This delayed dextran loading of the late endocytic compartment indicates that 1) ΔSTART-MLN64-GFP acts at the latest stage of endocytic pathway; 2) expression of ΔSTART-MLN64-GFP shifts the late endocytic pathway from a dynamic tubular reticulum to separate and immobile compartments; 3) the alteration of late endocytic compartments impairs the fusion capability of late endocytic compartments with earlier endocytic compartments, a phenomenon similar to a novel NPC phenotype revealed by the previous cell fusion study (7).

Because of the altered late endocytic pathway, as described above in both WT CHO cells and COS-7 cells transfected with ΔSTART-MLN64-GFP, we examined cholesterol accumulation in these cells that exhibit a phenocopy of lysosomal storage diseases. Interestingly but not suprisingly, filipin staining showed that ΔSTART-MLN64-GFP expression resulted in extensively cholesterol accumulation in the abnormally enlarged lysosomes. ΔSTART-MLN64-GFP-containing ring-shaped structures (Fig. 6A and inset) are at the periphery of cholesterol-laden lysosomes (Fig. 6B and inset), similar to the location of ΔSTART-MLN64 and MLN64 in transfected CT60 cells. Neither wild-type MLN64-GFP (Fig. 6C), nor START-MLN64-GFP (Fig. 6E), nor GFP expression (Fig. 6G) induced cholesterol accumulation in lysosomes (Fig. 6, D, F and H). In wild-type CHO cells (as well as COS-7 cells, see below), wild-type MLN64-GFP was located in late endocytic compartments throughout the cytoplasm (Fig. 6C), while the START-MLN64-GFP (Fig. 6E) and GFP (Fig. 6G) were present in a cytoplasmic and nuclear pattern 12 h to 3 days after transfection.

As shown in Fig. 7, the dominant negative effect of ΔSTART-MLN64-GFP was also found in COS cells. COS-7 cells expressing MLN64-GFP (Fig. 7, A, C and D, and movie 7) were similar to wild-type CHO cells expressing MLN64-GFP; and COS-7 cells expressing ΔSTART-MLN64-GFP (Fig. 7, B and E, and movie 8) were similar to wild-type CHO cells expressing ΔSTART-MLN64-GFP. The ΔSTART-MLN64-GFP mutant caused cholesterol accumulation in lysosomes (Fig 7F and inset, and movie 8) and inhibited trafficking of late endocytic tubules in COS-7 cells, whereas the START-MLN64-GFP mutant (Fig. 7G) was located to the nucleus and cytoplasm and did not induce cellular cholesterol accumulation in COS-7 cells.
To explore the biochemical consequences of expressing ΔSTART-MLN64-GFP, we examined the effects of co-expression of the GFP constructs on progesterone production in COS cells transfected with plasmids encoding the steroidogenic machinery. To correct for efficiency of expression of the co-transfected steroidogenic enzymes, we normalized the progesterone production by dividing it with the progesterone production of companion cells incubated in the presence of 22-OH-cholesterol, a substrate that readily enters into mitochondria. This hydroxycholesterol substrate supports maximal steroidogenic activity, limited only by the expression of the cholesterol side-chain cleavage system, and thus provides an index of the transfection efficiency of the steroidogenic enzymes. We have used this normalization method routinely in our structure-function studies of the StAR protein (10,16). Any adverse effects of the co-transfected GFP constructs on expression of the steroidogenic enzymes would be reflected in reduced conversion of 22-OH-cholesterol into progesterone. Cells transfected with the empty GFP vector produced equivalent amounts of progesterone as cells transfected with ΔSTART-MLN64-GFP (5.68 ng/dish vs. 5.99 ng/dish, respectively) (Table 1). However, the COS-1 cells transfected with the empty-GFP vector produced approximately 40% less progesterone than cells transfected with the other GFP constructs when incubated with 22-OH-cholesterol, demonstrating reduced total steroidogenic potential. The normalized progesterone production data as well as the relative steroidogenic activity (with the empty GFP vector results arbitrarily set to a value of 1.0) revealed a significant inhibitory effect of the ΔSTART-MLN64-GFP on steroidogenesis. The MLN64-GFP construct caused a modest but not significant increase in normalized progesterone production (1.3-fold above the empty GFP vector), whereas the START-MLN64-GFP construct increased normalized progesterone production more than 3.7-fold, a finding consistent with our previous observations (10). The ability of the START-MLN64-GFP to increase steroid production indicates that fusion of GFP to the C-terminus does not abrogate the sterol transfer activity of the START domain.

To examine the effects of ΔSTART-MLN64-GFP on the utilization of LDL-derived cholesterol, we incubated transfected COS-1 cells with LDL reconstituted with [3H] cholesteryl linoleate and assessed the formation of radiolabeled progesterone. The formation of radiolabeled progesterone by cells transfected with ΔSTART-MLN64-GFP (1944 ± 422 dpm/culture dish, mean ± S.D., N=3) was significantly less (P<0.01) than cells transfected with either the GFP empty vector (6111 ± 888 dpm/culture) or MLN64-GFP...
(13323 ± 2821 dpm/culture). The significantly reduced steroidogenic activity indicates that expression of ΔSTART-MLN64-GFP impairs the utilization of the lysosomal pool of cholesterol for steroidogenesis.
DISCUSSION

The present study demonstrates that: 1) MLN64 resides in the NPC1-containing dynamic late endosomal reticulum; 2) overexpression of the transmembrane domain of MLN64 (ΔSTART-MLN64) induces cholesterol accumulation in lysosomes and inhibits utilization of lysosomal cholesterol by mitochondria for steroidogenesis; 3) the START domain of MLN64 transfers cholesterol between membranes and stimulates mitochondrial steroidogenesis in vitro.

**MLN64 is a component of the dynamic late endosomal tubular reticulum and the dominant negative MLN64 mutant causes Niemann-Pick C phenocopy**

Studies on living cells show that MLN64 is a component of a dynamic late endocytic reticulum that is composed of rapidly trafficking late endosomal tubules (5, 7). Co-localization of MLN64 and NPC1 in the dextran-labeled late endocytic tubulo-vesicular compartments indicates that these two membrane-spanning proteins could function in similar membrane microdomains. Previous somatic cell fusion study revealed that these late endocytic compartments normally undergo rapid content exchange including membrane and fluid phase contents (7). MLN64 molecules may be rapidly equilibrated in the late endocytic pathway through these dynamic tubular movements that facilitate interactions between MLN64 and other components, such as NPC1 and HE1 in the late endosomal reticulum.

Visualizing the dynamics of the late endocytic pathway led to finding the dominant negative MLN64 mutant. The ΔSTART-MLN64-GFP contains all of the predicted transmembrane domains of MLN64 but lacks the START domain at the C-terminal half of the MLN64 protein. This N-terminal mutant is sufficient to mediate the biosynthetic trafficking of MLN64 from its site of synthesis, via the plasma membrane, to its final destination in the late endocytic compartment. ΔSTART-MLN64 not only follows the identical biosynthetic pathway as wild type MLN64, but also co-localizes with wild type MLN64 at the periphery of cholesterol-laden lysosomes. We had shown previously that cholesterol accumulation retards late endocytic tubular formation and mobility (7). While analyzing trafficking of MLN64 mutants, we noted that the morphology, distribution and movement of endocytic compartments were dramatically...
altered in wild type CHO cells and COS-7 cells expressing ΔSTART-MLN64-GFP. In these cells ΔSTART-MLN64-GFP containing ring-shaped structures aggregated in the perinuclear region, and showed limited saltatory movement. Fluorescent filipin cytochemistry showed accumulation of cholesterol in lysosomes in ΔSTART-MLN64-GFP transfected cells and the mutant protein is present associated with periphery of these cholesterol-laden lysosomes. This mutant MLN64 induced lysosomal cholesterol lipidosis mimics the NPC phenotype.

The finding that ΔSTART-MLN64 colocalizes with wild type MLN64 and over-expression of ΔSTART-MLN64 results in cholesterol accumulation in lysosomes suggests that endogenous MLN64 facilitates egress of cholesterol out of these lysosomes. The ability of truncation mutants consisting of the transmembrane domains to prevent the function of endogenous wild-type proteins involved in cholesterol metabolism has also been reported in the case of sterol response-element binding protein cleavage activating protein (23). The dominant negative effect caused by ΔSTART-MLN64 also suggest that another unknown protein may promote MLN64’s function and that the overexpressed ΔSTART-MLN64 interacts with this component, thus blocking the endogenous MLN64 function. The identification of the factor that interacts with MLN64 may lead to the further understanding of protein-protein interactions in the late endocytic compartments as well as cholesterol trafficking. Among the potential MLN64 interacting proteins are NPC1 and HE1, both of which are involved in cholesterol trafficking and homeostasis in the late endocytic compartment. Alternatively, MLN64 may function as a homodimer and the truncation mutant may prevent dimerization of endogenous MLN64.

**The START domain of MLN64 mediates utilization of LDL-derived lysosomal cholesterol by steroidogenic mitochondria**

The START domain of MLN64 shares high (35%) sequence identity within the START domain in the StAR protein, which is abundant in steroidogenic cells (24). The START domains of both MLN64 and StAR are similar in their abilities to bind cholesterol and stimulate steroidogenesis (10, 13). StAR is known to mobilize cholesterol from the cholesterol-rich outer mitochondrial membrane to the inner mitochondrial chamber where the first step in steroidogenesis occurs, conversion of cholesterol to pregnenolone (25). In this study, biochemical analysis demonstrates that steroidogenesis by mitochondria is inhibited when
blockage of cholesterol trafficking out of lysosomes by ΔSTART-MLN64 occurs. Our data also shows that the START domain in MLN64 can transfer cholesterol from donor liposomes to acceptor mitochondrial membranes and stimulate progestin synthesis by placental mitochondria, suggesting that MLN64 effects cholesterol egress out of lysosomes and mediates utilization of lysosomal cholesterol by mitochondria. The approximately 40% inhibition of progesterone production in COS cells transfected with ΔSTART-MLN64-GFP compared to that observed with MLN64-GFP is consistent with the 50% reduction in progesterone secretion by granulosa treated with drugs that produce the Niemann-Pick type C phenotype of lysosomal cholesterol storage (25). The inhibition of utilization of LDL-derived cholesterol for steroidogenesis was, however, more profound (68% inhibition compared to empty vector-GFP and 85% inhibition compared to MLN64-GFP), indicating a key role for MLN64 in the trafficking of LDL-derived cholesterol. Collectively, these findings indicate that a portion of the steroidogenic pool of cholesterol moves through the late endosomal system prior to reaching mitochondria where the first committed step in steroid hormone biosynthesis takes place.

The MLN64-containing compartment may deliver cholesterol substrate to mitochondria in steroidogenic cells, perhaps through transient interactions of the MLN64 START domain associated with the dynamic late endosomal compartment with the outer mitochondrial membranes. In support of this hypothesis, using dual color time-lapse imaging techniques, we found that the MLN64-GFP containing late endosomal tubules align parallel to StAR-RFP labeled mitochondria and transiently contact mitochondria, providing evidence for spatial interaction between two sterol-related intracellular organelles (movie 4). Because we have observed fragments of MLN64 containing the START domain in placental cell extracts (10) and also in placental mitochondrial preparations (F. Martinez, L.K. Christenson & J.F. Strauss, III unpublished observations), it is possible that the cleaved START domain transports cholesterol from the late endosomes/lysosomes to the mitochondria. The START domain may also promote movement of cholesterol from the outer to the inner mitochondrial membrane where the cholesterol side-chain cleavage enzyme resides. This is believed to be the process stimulated by StAR (25). However, the human placenta does not express StAR (16) and we have previously postulated that MLN64 subserves this role of StAR in the human placenta (10). The present observations are fully consistent with this notion. The fact that exogenous MLN64 START domain augments mitochondrial steroidogenesis indicates that whatever
endogenous MLN64 is present in the mitochondrial preparations is not sufficient to drive maximal hormone production. This raises the possibility that regulation of MLN64 levels contributes to the control of placental steroidogenesis. Based on our observations on the effects of the ΔSTART-MLN64-GFP construct on accumulation of free cholesterol and impairment of steroidogenesis, we conclude that MLN64 is directly involved in lysosomal cholesterol mobilization. MLN64 may have an additional role in steroidogenic cells where it mediates delivery of substrate to the mitochondrial cholesterol side-chain cleavage system.
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FOOTNOTES

1 The abbreviations used are: NPC, Niemann-Pick Type C; NPC1, Niemann-Pick Type C1 protein; StAR, steroidogenic acute regulatory protein; CHO, Chinese hamster ovary; LDL, low-density lipoprotein; GFP, green fluorescent protein; 3βHSD, 3β-hydroxysteroid dehydrogenase; DMEM, Dulbecco’s minimal essential medium; CCD, charged-coupled device
FIGURE LEGENDS

Figure 1. Western blot analysis of GFP-fusion protein constructs.

A schematic representation of each of the fusion protein constructs is shown. Extracts of COS cells transfected with the indicated plasmids were prepared 48 h after transfection and subjected to Western blotting using an anti-human MLN64 START domain antibody or anti-GFP antibody (5 blots, A-E). Blot A and B show that MLN64-GFP was detected by using anti-MLN64 or anti-GFP antibodies at predicted molecular mass 78 kDa (51 + 27 kDa). GFP was detected by anti-GFP antibody at 27 kDa (B) and was not detected by anti-MLN64 antibody (A). A small band was detected by anti-GFP antibody from cells transfected with MLN64-GFP (B-discussed in results). Blot C shows ΔSTART-MLN64-GFP was detected as a single band at molecular mass 54 kDa (27 + 27 kDa) by using anti-GFP antibody. Blot D and E show START-MLN64-GFP was detected by using anti-MLN64 and anti-GFP antibodies as a single band of 55 kDa (28 + 27 kDa).

Figure 2. Similar biosynthetic trafficking of MLN64-GFP and ΔSTART-MLN64-GFP in cholesterol-laden CT60 CHO cells.

CT60 CHO cells (NPC1 mutant) transfected with MLN64-GFP (A-C), ΔSTART-MLN64-GFP (D-F) or co-transfected with non-tagged MLN64 and ΔSTART-MLN64-GFP (G-H). Twenty-four h after transfection, MLN64-GFP was found mainly on the plasma membrane and in associated small vesicles (green in A). 72 h after transfection with MLN64-GFP (green in B) CT60 cells were stained with anti-MLN64 monoclonal antibody (red in C). MLN64-GFP and anti-MLN64 co-localize at the surface of cholesterol laden-lysosomes. The GFP tagged truncated MLN64 (ΔSTART-MLN64-GFP), containing the N-terminal transmembrane domains of MLN64, was also delivered to the plasma membrane (green in D) at early time of transfection (16 h after transfection) and internalized from plasma membrane to cholesterol-laden lysosomes (green in E) at later times of transfection (72 h). ΔSTART-MLN64-GFP is not stained by the anti-MLN64 antibody (F) because it lacks the antigenic START domain. The ΔSTART-MLN64-GFP (green in G) was found to be co-localized with wild type MLN64 (red in H, stained with anti-MLN64 antibody) at the surface of cholesterol-laden lysosomes 72 h after transfection. These results show that wild
type MLN64 and ΔSTART-MLN64-GFP mutant traffic together in the same cellular organelles. All images are confocal. *Bars* for A and D = 10 µm; for B, C, E, F, G and H = 5 µm.

**Figure 3. MLN64 traffics with NPC1 in late endosomal tubules that are sensitive to cholesterol.**

Wild type CHO cells co-transfected with MLN64-CFP and NPC1-YFP for 96 h (A,B) or 48 h (C,-D). MLN64-CFP is present in tubulo-vesicular structures (green in A and C) that also contain NPC1-YFP (red, in B and D). Time-lapse movies that show late endosomal tubular trafficking of MLN64-GFP transiently expressed in wild type CHO cells at 37 ºC (movie 1) or stably expressed in wild type CHO cells at 20ºC (movie 2) and trafficking of both MLN64-CFP and NPC1-YPF in late endosomal tubules (movie 3) at 20ºC are available as supplementary data in www.jbc.org. Wild type CHO cells transfected with MLN64-GFP for 48 h and incubated with LDL in the presence of progesterone for 24 h to cause cholesterol loading of lysosomes (E,F). Cells were fixed and stained with filipin. MLN64-GFP is present as ring-shaped structures (green rings in E) at the surface of enlarged cholesterol loaded lysosomes (blue with filipin staining in F). Mobile late endosomal tubules are rarely present in these cholesterol-laden cells. A-D were obtained with CCD camera. E,F are confocal. *Bars* = 10 µm.

**Fig. 4. The MLN64 START domain has cholesterol transfer activity and stimulates steroid synthesis by human placental mitochondria.**

A. Time course of recombinant MLN64 START domain (1 µM) stimulated cholesterol transfer from sterol-rich liposomes to acceptor membranes. Values are means of triplicate incubations. B. Dose response of recombinant MLN64 START domain-stimulated sterol transfer during a 20 min incubation. Values are means of triplicate incubations. C. Comparison of the sterol transfer activity of recombinant MLN64 START domain (1 µM) and SCP2 (1 µM) during a 20 min incubation. D. Recombinant MLN64 START domain (1 µM) but not recombinant fibronectin extra domain A (1 µM) stimulates progesterone synthesis by isolated human placental mitochondria. Values are means ± S.D. from triplicate incubations.
Figure 5. In comparison to MLN64-GFP, expression of ΔSTART-MLN64-GFP causes formation of enlarged late endocytic compartments with delayed dextran filling in wild type CHO cells.

Wild type CHO cells transfected with MLN64-GFP (A-F) or ΔSTART-MLN64-GFP (G-L) and subsequently incubated with rhodamine-dextran in 2 h of uptake/chase time (A-C, G-I) or 18 h of uptake/chase time (D-F, J-L), and imaged at the same time 72 h after transfection. MLN64-GFP is present in tubulo-vesicular structures (green, A,D) that are filled with dextran in 2 h of uptake/chase time (red in B) and 18 h of uptake/chase time (red in E). (C, F) Merges of A and B, D and E show colocalization of MLN64 and dextran. However, ΔSTART-MLN64-GFP is present in the wild type CHO cells as ring-shaped structures (green, G). Many of these ring-shaped structures do not contain core endocytosed rhodamine-dextran (red in H & I). After incubation of cells with dextran in 18 h of uptake/chase time, all ΔSTART-MLN64-GFP containing compartments (green in J) are filled with dextran (red, K). Merge of G and H shows little colocalization of ΔSTART-MLN64-GFP with dextran in 18h of uptake/chase time (I). Merge of J and K shows association of ΔSTART-MLN64-GFP with the surface of dextran containing vesicles (L). All images were obtained from live cells with confocal double fluorescence scanning. Bars = 5 µm.

Figure 6. Expression of ΔSTART-MLN64-GFP causes dominant negative effect on transfer of cholesterol out of lysosomes and inhibits late endosomal tubular trafficking in wild type CHO cells.

Wild type CHO cells (A-H), transfected with ΔSTART-MLN64-GFP (A, B), MLN64-GFP (C, D), START-MLN64-GFP (E, F), and GFP (G, H) for 48 h and stained with filipin (B, D, F, H). Cholesterol accumulates in ΔSTART-MLN64-GFP expressing cells in the perinuclear region (blue, B) along with ΔSTART-MLN64-GFP (green, A). Note as a control, the adjacent non-ΔSTART-MLN64-GFP expressing cells do not accumulate cholesterol (A & B). Cholesterol (inset of B) accumulates in lysosomes with ΔSTART-MLN64-GFP at their peripheries (inset of A). However, wild type MLN64-GFP (green, C), START-MLN64-GFP (green, E), and GFP expression (green, G) do not induce cholesterol accumulation in lysosomes (blue, D, F,H, respectively). MLN64-GFP is located in the late endosomal compartment (green, C) whereas the START-MLN64-GFP (green, E) and GFP (green, G) are located in the cytoplasm and nuclei. All images are confocal. Time-lapse movie shows that ΔSTART-MLN64-GFP was present as
ring-shaped structures in the perinuclear region of the cells but not in mobile tubules (movie 5). The movie is available as supplementary data in www.jbc.org. Bars for A-H = 10 µm; for insets of A and B = 2 µm

Figure 7. Expression of ΔSTART-MLN64-GFP causes dominant negative effect on transfer of cholesterol out of lysosomes and inhibits late endosomal tubular trafficking in COS-7 cells.

The location of MLN64-GFP (A, C), ΔSTART-MLN64-GFP (B, E), or START-MLN64-GFP (G) in COS-7 cells is similar to that in wild type CHO cells. ΔSTART-MLN64-GFP expression (green, E) induces lysosomal cholesterol accumulation (blue, F) in COS-7 cells. Cholesterol is in the core of lysosomes (inset of F) with ΔSTART-MLN64-GFP at the lysosomal surface (insets of E and B). Adjacent non-ΔSTART-MLN64-GFP expressing cells do not accumulate cholesterol in lysosomes (E, F). As controls, MLN64-GFP (green, C) and START-MLN64-GFP (green, G) did not induce cholesterol accumulation (blue, D, H) in COS-7 cells. Living COS-7 cells transfected with MLN64-GFP (green, A) and ΔSTART-MLN64-GFP (green, B) and cultured in 10% FBS containing medium for 72 h were imaged with a CCD camera. Cells were imaged at 20 °C to slow the movement of the late endocytic compartment. Time-lapse movies show MLN64-GFP traffics in tubules and small vesicles throughout COS-7 cells (Movie 7). However, ΔSTART-MLN64-GFP was present as ring-shaped structures in the perinuclear region of the cell but not in mobile tubules (Movie 8). Time-lapse movies are available as supplementary data in www.jbc.org. A and B were obtained with CCD camera. C-H are confocal. Bars for A-H = 10 µm; for insets in B = 5 µm, in E and F = 2 µm.
Table I

The ΔSTART-MLN64-GFP Construct Inhibits Steroidogenesis.

| Construct         | Progesterone Production ng/dish | Normalized Activity | Relative Activity |
|-------------------|---------------------------------|---------------------|-------------------|
|                   | -                               | +                   |                   |
| Empty GFP vector  | 5.68 ± 0.73                     | 127.5 ± 22.4        | 0.048 ± 0.007     | 1<sup>a</sup>    |
| MLN64-GFP         | 11.13 ± 1.67                    | 200.7 ± 36.1        | 0.064 ± 0.012     | 1.30 ± 0.15<sup>a</sup> |
| START-MLN64-GFP   | 28.48 ± 4.4                     | 222.7 ± 47.0        | 0.137 ± 0.013     | 3.05 ± 0.28<sup>b</sup> |
| ΔSTART-MLN64-GFP  | 5.99 ± 0.94                     | 207.5 ± 32.6        | 0.029 ± 0.004     | 0.63 ± 0.09<sup>c</sup> |

COS-1 cells were transfected with plasmids expressing the human cholesterol side-chain cleavage and 3β-HSD enzymes and the indicated plasmids. Cells were cultured in the absence (-) or presence (+) of 22R-hydroxycholesterol (5 µg/ml). Progesterone production was determined by radioimmunoassay. Values present are means ± S.E. from 8 separate experiments in which each treatment group contained triplicate cultures. Relative steroidogenic activities of the constructs are calculated normalizing progesterone production to total cholesterol side-chain cleavage and 3β-HSD activity as determined in the presence of 22R-hydroxycholesterol. The normalized data was expressed relative to the empty GFP vector control to determine relative activity. Normalized data were analyzed for statistical significance by Mann-Whitney U test. Values with a different superscript letter are significantly different from all others in the column (P<0.02).

Online Movie Legends:

QuickTime movies corresponding to Figs. 3, 6 and 7 are presented and available at http://www.jbc.org.

Movie 1: Dynamics of MLN64-GFP containing late endosomal tubules in wild-type CHO cell 72h after transfection with MLN64-GFP. Imaged at 37º C and 1 frame/s. 30 frames. Shown as 10x real time. 100x objective. Bar = 10 µm.
Movie 2: Dynamics of MLN64-GFP containing late endosomal tubules in wild-type CHO cells stably expressing MLN64-GFP. Imaged at 20°C and 1 frame/s. 50 frames. Shown as 10x real time. 63x objective. Bar = 10 µm.

Movie 3: Dynamics of MLN64-CFP (green) and NPC1-YFP (red) containing late endosomal tubules in a wild-type CHO cell 48 h after co-transfection. Co-localization of the 2 proteins appears yellow. Imaged at 20°C and 1 frame/s. 20 frames. Shown as 10x real time. 100x objective. Bar = 10 µm.

Movie 4: Dynamics of MLN64-GFP (green) containing late endosomal tubules and StAR-RFP (red) labeled mitochondria in wild type CHO cells 14 days after co-transfection with MLN64-GFP and StAR-RFP. Note the dramatic parallel alignment of late endosomal tubules with mitochondria. Late endosomal tubules extending from vesicles increase the surface area of the late endocytic compartment and provide more sites for potential interaction with mitochondria. Cells were imaged at 20°C to slow movement of late endocytic compartments. Taken at 1 frame/s. 30 frames. Shown as 10x real time. 63x objective. Bar = 10 µm.

Movie 5: Dominant negative effect of ΔSTART-MLN64-GFP in wild-type CHO cells 72 h after transfection with ΔSTART-MLN64-GFP. Gathered in the perinuclear region of the cells are large, cholesterol-laden lysosomes with ΔSTART-MLN64-GFP-containing ring-shaped structures at their peripheries. Late endosomal tubules are not present in these cholesterol-loaded cells. Imaged at 37°C and 1 frame/s. 40 frames. Shown as 10x real time. 100x objective. Bar = 10 µm.

Movie 6: Loss of dynamic late endosomal tubule-mediated contact between late endocytic compartment and mitochondria in wild type CHO cells 14 days after co-transfection with ΔSTART-MLN64-GFP (green) and StAR-RFP (red). Note the ΔSTART-MLN64-GFP labeled late endocytic compartment is present as immobile ring-shaped structures and endocytic tubules are not present. Cells were imaged at 20°C and 1 frame/s. 30 frames. Shown as 10x real time. 63x objective. Bar = 10 µm.

Movie 7: Dynamics of MLN64-GFP-containing late endosomal tubules in COS-7 cells 72 h after transfection with MLN64-GFP. Imaged at 20°C and 1 frame/s. 28 frames. Shown as 10x real time. 100x objective. Bar = 10 µm.
Movie 8: Dominant negative effect of ΔSTART-MLN64-GFP in COS-7 cells 72 h after transfection with ΔSTART-MLN64-GFP. Gathered in the perinuclear region of the cells are enlarged, cholesterol-laden lysosomes with ΔSTART-MLN64-GFP-containing ring-shaped structures at their peripheries. Late endosomal tubules are not present in these cholesterol-loaded cells. Imaged at 20°C and 1 frame/s. 30 frames. Shown as 10x real time. 100x objective. Bar = 10µm.
Fig 1: Western blot analysis of GFP-fusion protein constructs

| Plasmid Construct |
|-------------------|
| Empty vector     |
| MLN64-GFP         |
| START-MLN64-GFP   |
| ΔSTART-MLN64-GFP  |

A B C D E

Anti-MLN64 Anti-GFP Anti-GFP Anti-MLN64 Anti-GFP
Fig 2: Similar Biosynthetic Trafficking of MLN64-GFP and △START-MLN64-GFP in Cholesterol-Laden CT60 CHO Cells
Fig 3: MLN64 traffics with NPC1 in late endosomal tubules that are sensitive to cholesterol in WT CHO cells
Fig 4: The MLN64 START Domain Has Cholesterol Transfer Activity and Stimulates Steroid Synthesis by Human Placental Mitochondria
Fig 5: In Comparison to MLN64-GFP, Expression of ΔSTART-MLN64-GFP Causes Formation of Enlarged Late Endocytic Compartments with Delayed Dextran Filling in WT CHO Cells
Fig 6: ΔSTART-MLN64-GFP Causes Accumulation of Cholesterol in Lysosomes in WT CHO Cells.
Fig 7: Late Endosomal Tubular Trafficking is Inhibited in Cholesterol Laden COS 7 Cells Expressing ΔSTART-MLN64-GFP
Movie 1: Dynamics of MLN64-GFP containing late endosomal tubules in wild-type CHO cells

Movie 2: Dynamics of MLN64-GFP containing late endosomal tubules in a wild-type CHO cell stable clone
Movie 3: Dynamics of MLN64-CFP (green) and NPC1-YFP (red) containing late endosomal tubules in wild-type CHO cell 48 hr after transfection

Movie 4: Dynamics of MLN64-GFP (green) containing late endosomal tubules and StAR-RFP (red) labeled mitochondria in cotransfected wild type CHO cells
Movie 5: Dominant negative effect of ΔSTART-MLN64-GFP in wild-type CHO cells

Movie 6: Loss of contact between ΔSTART-MLN64-GFP (green) containing late endocytic compartment and StAR-RFP (red) labeled mitochondria in cotransfected wild type CHO cells
Movie 7: Dynamics of MLN64-GFP containing late endosomal tubules in Cos-7 cells
48 hr after transfection

Movie 8: Dominant negative effect of ΔSTART-MLN64-GFP in Cos-7 cells
MLN64 mediates mobilization of lysosomal cholesterol to steroidogenic mitochondria
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