Materials and Methods

Cell line, siRNAs, Reagents, and Plasmids – The rat hepatoma cell line, McArdle-RH7777 (McA), was purchased from American Type Culture Collection (ATCC) and maintained at 37°C in DMEM (Cellgro, Mediatech) with 10% fetal bovine serum (GemCell, Gemini), 10% horse serum, 0.1 mg/ml L-glutamine, penicillin and streptomycin. KLHL12-specific siRNA and control siRNA were obtained from Dharmacon (Thermo Scientific). A FLAG-tagged KLHL12 expression plasmid (pcDNA3.1-KLHL12) was a kind gift from Dr. Randall T. Moon (University of Washington). An anti-rat apoB100-specific antibody (designated 24.05) used for immunoprecipitation and immunofluorescence studies was kindly provided by Dr. Janet Sparks (University of Rochester). CellLight Golgi-RFP and ER-RFP BacMam 2.0 reagent dyes were from Life Technologies (C10593, C10591). Sheep anti-rat albumin antibody was purchased from Bethyl Laboratories (Montgomery, TX), and an anti-sheep Alexa Fluor 568 antibody (A-21099) used in the immunofluorescence studies was from Life Technologies. 3-methyladenine (3-MA) was purchased from Sigma. Anti-GAPDH antibody was from Santa Cruz Biotechnology, Inc. Anti-FLAG antibody was purchased from Sigma. Anti-COPII polyclonal antibody was from Thermo Scientific. Anti-KLHL12 polyclonal antibody was obtained from Proteintech and from ProMab Biotechnologies. DAPI was purchased from Life Technologies. Fibronectin (0.1%) was purchased from Sigma. The CellLight® ER-RFP and Golgi-RFP BacMam 2.0 system and plasmids were purchased from Invitrogen.

KLHL12 knock-down – Rat KLHL12-specific siRNA and non-specific control siRNA were purchased from Dharmacon (Thermo Scientific). The sequences of ON-TARGETplus SMARTpool siRNA for KLHL12 are: GGAGAUAUGAUUUACGUUU, GAUGGAAGCAGGCGUCAUA, AGUGGUGGCCAGCGGCAUA, and

GGACUAAUGUUACGCCUAU. siRNA transfection was performed as previously described[1] Briefly, cells were seeded at 30–40 % confluency, and on the next day the cells were transfected with siRNA at 20 nM using DharmaFECT 4 transfection reagent (Dharmacon, Thermo Scientific) according to the manufacturer's instruction. 48 h post transfection, cells were subject to the experimental protocols described below. The knockdown of KLHL12 was confirmed by qPCR and Western blotting.

Quantitative Real-Time PCR and Western blotting –The forward and reverse primers for KLHL12 are: CTTGATGGGCGGCATTATG and AGGATGGACTTGGCGTGAGT, respectively. In brief, total RNA was extracted by TRIzol reagent (Invitrogen, Life technologies) and cDNA was synthesized by the Verso cDNA Synthesis Kit (Thermo Scientific). PCR reactions were prepared with Absolute Blue qPCR SYBR Green mix (Thermo Scientific), performed and analyzed using the Applied Biosystem qPCR system. Methods for Western blot analysis have been previously described[1], and the images of the KLHL12 and GAPDH bands were obtained by the LI-COR system and analyzed with Image Studio software.

Effects of KLHL12 under and over-expression on apoB100 recovery and density distribution – For KLHL12 knockdown experiments, McA cells were transfected with KLHL12-specific siRNA or control siRNA (Dharmacon) as above. 48 h after transfection, the cells were incubated in methionine/cysteine-free media containing 0.5% FBS and 0.5% horse serum for 1 h with either bovine serum albumin (BSA) as a control or 0.6 mM oleic acid/bovine serum albumin (OA/BSA) complexes. ^{35}S-methionine/cysteine was then added, and incubation was continued for another 3 h to radiolabel apoB100 to steady state. ApoB100 was then immunoprecipitated from cell lysates and the collected media, the immunoprecipitates were

resolved by SDS-PAGE, and the intensities of the radiolabeled apoB100 bands were quantified by phosphorimager analysis as described before[1].

For KLHL12 overexpression experiments, McA cells were transfected with the FLAG-tagged KLHL12 plasmid (pcDNA3.1-KLHL12) or an empty vector (pcDNA3.1-KLHL12) as a control for 48 h using Fugene6 (Roche), followed by a 1 h incubation in the presence or absence of the 0.6 mM OA/BSA complexes and a 3 h incubation with ^{35}S-methionine/cysteine. The secreted and intracellular apoB100 levels were analyzed as described above. An increase in KLHL12 expression level was confirmed by quantitative qPCR and Western blot analysis.

The density distribution of the apoB100-VLDL species recovered from the conditioned media in the steady state labeling experiments was determined by NaBr/KBr density gradient fractionation, and the collected fractions were analyzed after apoB100 was immunoprecipitated. The proteins were resolved by SDS-PAGE and detected by phosphorimager analysis.

ER microsome and Golgi membrane apoB100 distribution analysis – Purified ER microsomes and Golgi membranes were isolated as described[2]. Briefly, McA cells were cultured and treated as above, and apoB100 radiolabeled to steady state. At the conclusion of labeling, cells were washed with PBS twice before being harvested by scraping into 2.5 ml of homogenization buffer (10 mM Hepes, pH 7.4, 250 mM sucrose, 0.5 mM DTT, 1x EDTA-free protease-inhibitor cocktail, 20 U/ml RNAse inhibitor, 200 μM BHT, 2.5 mg/ml trypsin inhibitor, 2 mM $MgCl_2$). Cells were then homogenized by nitrogen decompression in a cell disruption bomb (500 psi/15 min). The homogenates were centrifuged at 1,900g for 10 min to obtain the post-nuclear supernatant. The supernatant (2.3 ml) was then loaded on top of sucrose layers (20%, 30%, 35%, 40%, 45%, 50%, and 56%) and centrifuged at 100,000Xg for 18 h at 4°C to separate ER microsomes and Golgi membranes. After centrifugation, samples were obtained

from the top to the bottom of the gradient. The distribution patterns of the subcellular compartment markers (Golgi: mannosidase II; ER: calnexin) were determined by mannosidase II enzyme assay (below) and Western blotting, respectively. ApoB100 was immunoprecipitated from each fraction containing Golgi membranes or ER microsomes, and the immunoprecipitates were resolved by SDS-PAGE. The intensities of the radiolabeled apoB100 bands were quantified by phosphorimager analysis.

Mannosidase II enzyme assay – Each sample (4 µl) was mixed in 40 mM sucrose, 1.4 mM Hepes pH 7.4, protease inhibitors, 70 µM DTT with 1.0 mM 4-methylumbelliferyl-α-D-mannoside dissolved in 1X Dubecco's PBS (with $MgCl_2$, $CaCl_2$), pH 7.4, 0.1% Triton X-100, and incubated at 37 °C for 30 min. To stop the reaction, 180 µl stop reagent (0.5 M glycine, 0.5 M Na_2CO_3) was added to each well. The fluorescence was then read at 360 nm excitation and 465 nm emission within 30 min.

Confocal microscopy – In one series of experiments, McA cells were grown on fibronectin labtek slides and fixed in paraformaldehyde before they were incubated with primary antibodies (directed against COPII, KLHL12, or rat apoB100) at 4 ºC overnight, followed by Alexa-labelled secondary antibodies (Invitrogen) for 1 h. Nuclei were stained with DAPI. Images were taken on a Leica TCS SP5 II confocal microscope with a 63X objective and analyzed with ImageJ software. To quantitate the co-localization of KLHL12 with apoB100 or albumin, the areas of the cells positive (i.e., red) for either protein and for both KLHL12 with either protein (yellow) were determined, and the data expressed for either protein as yellow area/red area. At least 10 cells/each protein were used for this analysis.

In another series of experiments, the ER or Golgi was visualized using the CellLight® ER-RFP and Golgi-RFP plasmids and the BacMam 2.0 transduction system per the

manufacturer's instructions (Invitrogen). After transducing either plasmid overnight, McA cells were treated as in the first series of experiments, but using only the primary antibody to KLHL12 (and the secondary antibody) for immunostaining analysis.

Statistics – Analysis of data was done with GraphPad Prism 6 (GraphPad Software, Inc. La Jolla, CA). Results were expressed as means ± SEM and compared using Student's *t* test, except that the density gradient results were expressed as arbitrary units (AU) of the apoB100 recovered from each fraction. $p < 0.05$ was considered statistically significant.

References:

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