A Regulator of Secretory Vesicle Size, Kelch-Like Protein 12, Facilitates the Secretion of Apolipoprotein B100 and Very-Low-Density Lipoproteins—Brief Report

Chutikarn Butkinaree,* Liang Guo,* Bhama Ramkhelawon, Amarylis Wanschel, Jeffrey L. Brodsky, Kathryn J. Moore, Edward A. Fisher

Objective—One of the major risk factors for atherosclerosis is the plasma level of low-density lipoprotein (LDL), which is a product of very-low-density lipoprotein (VLDL). Hepatic apolipoprotein B100 (apoB100) is the essential component that provides structural stability to VLDL particles. Newly translated apoB100 is partially lipidated in the endoplasmic reticulum (ER), forming nascent apoB100-VLDL particles. These particles are further modified to form fully mature VLDLs in the Golgi apparatus. Therefore, the transport of nascent VLDL from the ER to the Golgi represents a critical step during VLDL maturation and secretion and in regulating serum LDL cholesterol levels. Our previous studies showed that apoB100 exits the ER in coat complex II vesicles (COPII), but the cohort of related factors that control trafficking is poorly defined.

Approach and Results—Expression levels of Kelch-like protein 12 (KLHL12), an adaptor protein known to assist COPII–dependent transport of procollagen, were manipulated by using a KLHL12-specific small interfering RNA and a KLHL12 expression plasmid in the rat hepatoma cell line, McArdle RH7777. KLHL12 knockdown decreased the secreted and intracellular pools of apoB100, an effect that was attenuated in the presence of an autophagy inhibitor. KLHL12 knockdown also significantly reduced secretion of the most lipidated apoB100-VLDL species and led to the accumulation of apoB100 in the ER. Consistent with these data, KLHL12 overexpression increased apoB100 recovery and apoB100-VLDL secretion. Images obtained from confocal microscopy revealed colocalization of apoB100 and KLHL12, further supporting a direct link between KLHL12 function and VLDL trafficking from the ER.

Conclusions—KLHL12 plays a critical role in facilitating the ER exit and secretion of apoB100-VLDL particles, suggesting that KLHL12 modulation would influence plasma lipid levels. (Arterioscler Thromb Vasc Biol. 2014;34:251-254.)

Key Words: apolipoproteins B ■ COP-coated vesicles ■ KLHL12 ■ lipoproteins, VLDL

Apolipoprotein B100 (ApoB100) normally associates with very-low-density lipoprotein (VLDL) particles in mammalian liver. The trafficking of nascent apoB100-containing VLDL (apoB100-VLDL) from the endoplasmic reticulum (ER) to the Golgi is essential for the final maturation and secretion of these particles. We previously showed that nascent apoB100-VLDL particles require coat complex II (COPII) for ER-Golgi transport; however, the apoB100-VLDL transport vesicles had physical properties distinct from those containing other secretory proteins. Notably, apoB100-VLDL transport vesicles were larger in diameter and denser than those transporting typical cargo proteins. It has been speculated that large cargo molecules, such as VLDL, require specialized factors to promote their packaging and exit from the ER. In fact, a recent study by Jin et al. established that Kelch-like protein 12 (KLHL12), a substrate-specific adaptor protein in the Cullin3-based ubiquitin ligase complex, facilitates the transport of procollagen fibers from the ER to the Golgi apparatus. Like VLDL, procollagen is much larger than nearly every other secretory cargo and is unable to fit inside the confines of a typical COPII vesicle. To overcome this hurdle, KLHL12 binds to a COPII component, Sec31, triggering its monoubiquitinilation, which leads to the assembly of larger vesicle coats. Here, we show that KLHL12 also facilitates the transport and secretion of apoB100-containing VLDL particles in a model of mammalian liver lipoprotein metabolism, rat hepatoma McArdle RH7777 (McA) cells.

Materials and Methods

Materials and Methods are available in the online-only Supplement.
Results and Discussion
To determine whether KLHL12 plays a role in apoB100-VLDL trafficking and secretion, we first asked whether manipulating KLHL12 expression impacted apoB100 levels and the secretion of mature VLDL particles. KLHL12 in McA cells was depleted by transfecting cells with a KLHL12-specific small interfering RNA, and the levels of secreted and intracellular apoB100 were measured under steady-state radiolabeling conditions. A significant reduction of KLHL12 expression (confirmed by quantitative polymerase chain reaction and by Western blotting; Figures I and II in the online-only Data Supplement, respectively) in the presence of oleic acid/BSA complexes resulted in >50% decreases in the levels of secreted and intracellular apoB100 (Figure 1A and 1B). The effect of KLHL12 knockdown was specific to apoB100 because secretion of the constitutive hepatic protein albumin was unaltered (Figure III in the online-only Data Supplement). This result provides further support that KLHL12 facilitates the transport of atypical, large COPII cargo. Data from density gradient fractionation showed that knockdown of KLHL12 had a profound effect on the secretion of the most lipilated species of apoB100, namely VLDL1 (Figure 1C). To further support a role of KLHL12 during the ER-to-Golgi trafficking of apoB100-VLDL, apoB100 levels were measured in ER microsomes and Golgi membranes isolated from KLHL12 silenced McA cells. The ratio of apoB100 recovery from ER to that from the Golgi apparatus increased by 60% in KLHL12-depleted cells compared with that of the controls (Figure 1D; the ER and Golgi markers distributions are shown in the Figure IV in the online-only Data Supplement). This shift suggested a decrease in VLDL vesicular transport and a concomitant accumulation in the ER. Overall, then, the collective data strongly imply that the trafficking of apoB100 from the ER to Golgi, where VLDL fully matures,11-13 was compromised when KLHL12 expression was reduced.

Opposite results were seen in McA cells overexpressing KLHL12 (Figure 1E and 1F; the overexpression was confirmed by Western blotting as shown in the Figure V in the online-only Data Supplement). In this case, the levels of secreted apoB100 were markedly increased, and there was a concomitant increase in the population of secreted VLDL1 (Figure VI in the online-only Data Supplement).
Data Supplement). In contrast, there was little effect on albumin secretion (Figure VII in the online-only Data Supplement), indicating the specificity of action of KLHL12 overexpression. These overexpression data suggest that the native level of KLHL12 is limiting for apoB100-VLDL trafficking in McA cells.

Although the focus of this report is on apoB100 (the sole form produced by human liver), rodent hepatic cells also produce apoB48. Limited analyses of the effects of KLHL12 knockdown and overexpression on apoB48 were also performed. On KLHL12 knockdown, there was also decreased recovery of apoB48, but to a lesser degree than for apoB100; in addition, there were minimal effects when KLHL12 was overexpressed (Figure VIII in the online-only Data Supplement). These results most likely reflect the smaller proportion of apoB48 (approximately one third that of apoB100) that can be assembled into VLDL (eg, Fisher et al14), which would be expected to decrease the reliance on KLHL12 for the ER export of apoB48-containing lipoproteins.

In the experiments presented above, the McA cells were incubated with oleic acid/BSA complexes. This treatment augments apoB100 lipidation and secretion but has no effect on the preferential association of apoB100 with VLDL particles. In the absence of exogenous fatty acids, the low level of endogenous lipid synthesis can result in an increase in the amount of apoB100 destroyed by the ER-associated degradation pathway, but of the surviving molecules, most would be secreted in association with VLDL (reviewed in Brodsky and Fisher15 and Ginsberg and Fisher16). Under these conditions, KLHL12 again modulated apoB100 secretion (Figure 2A and 2B) because the amount of apoB100 decreased on KLHL12 knockdown (primarily in the VLDL density fractions; data not shown) and increased on KLHL12 overexpression (Figure IX in the online-only Data Supplement).

Our laboratory and others have shown that apoB100 can be degraded by either the proteasome-mediated ER-associated degradation or by autophagy after ER exit (reviewed in Brodsky and Fisher15). To define the fate of apoB100 after KLHL12 knockdown, we first focused on the role of autophagy, given that it is highly unlikely that an assembled apoB100-VLDL particle could retro-translocate from the ER lumen and gain

Figure 2. Kelch-like protein 12 (KLHL12) regulates apolipoprotein B100 (apoB100) metabolism when lipids are primarily endogenously synthesized and colocalizes with apoB100 in the secretory pathway. McArdle RH7777 (McA) cells were transfected with control or KLHL12-specific small interfering RNA (siRNA) without oleic acid (OA)/BSA complexes. Forty-eight hours later, cells were incubated with 35S-methionine and cysteine for 3 hours to radiolabel apoB100 to steady state. Secreted (A) and intracellular (B) levels of radiolabeled apoB100 were decreased similar to that observed in Figure 1. C, McA cells were radiolabeled to steady state, and the incubation with the autophagy inhibitor 3-methyl adenine (3-MA) increased intracellular apoB100 recovery in KLHL12 siRNA-treated cells. D, Confocal indirect immunofluorescence images from McA cells probed with antibodies to apoB100 (red), coat complex II (COPII; red), or albumin (red), or KLHL12 (green) as indicated. Arrows in the insets indicate colocalization (yellow) of apoB100, COPII, or albumin with KLHL12. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; magnification, ×63). The inset is a ×10 zoom of the area of interest. E, Quantification of KLHL12 colocalization with either albumin or apoB100. A to C, Shown are the means, ±SEM, n=3. *P<0.05 and **P<0.01.
D, Shown are the results representative of 3 independent experiments. E, Same as A to C, but n≥10 cells analyzed for each protein.
access to the cytoplasmic proteasome, which must occur in ER-associated degradation. In contrast, autophagy is known to collect and destroy large substrates, even those that reside within cellular organelles (reviewed in Yang and Klionsky[17]). Consistent with this hypothesis, the autophagy inhibitor, 3-methyladenine, significantly increased the level of intracellular apoB100 in KLHL12 knockdown cells (Figure 2C).

Finally, we asked whether KLHL12 colocalizes with apoB100, which would provide additional support that this factor facilitates apoB100-VLDL secretion. By using a monoclonal antibody specific for rat apoB100, we observed that KLHL12 colocalized ≈8x more (P<0.01) with apoB100 than with albumin and that KLHL12 colocalized with COPII-positive vesicles (Figure 2D and 2E). Furthermore, the data also verified KLHL12 association with the ER and not with the Golgi (Figure X in the online-only Data Supplement). These colocalizations of KLHL12 are consistent with the subcellular distribution previously reported in the procollagen studies.7

In summary, our data demonstrate for the first time that KLHL12 plays a crucial role in delivering apoB100-VLDL particles to the Golgi apparatus for further maturation, an event that ultimately leads to the secretion of mature VLDL. Furthermore, KLHL12 appears to be a limiting factor that acts during ER-to-Golgi trafficking, at least in McA cells, because either increasing or decreasing KLHL12 levels had a significant effect on apoB100-VLDL secretion. We also find that autophagy is most likely responsible for the degradation of apoB100-containing particles that escape ER-associated degradation but do not progress to the Golgi. The atypical physical properties of the ER-derived secretory vesicles we previously described5 may have reflected not only a difference in physical structure relative to those vesicles carrying smaller cargo, but also the presence of additional proteins needed to form them, such as KLHL12 (this study) and others.14,16 Our next goal is to confirm the function of KLHL12 in vivo and to determine the molecular mechanism and relationships among KLHL12 and other factors required for apoB100-VLDL assembly and trafficking.

Acknowledgments
We thank Dr Janet Sparks (University of Rochester) for providing the rat apoB100-specific monoclonal antibody, Dr Randall Moon (University of Washington) for the gift of the KLHL12 expression plasmid, and Mr Hamza Sadhra (NYU) for assisting in some of the experiments.

Sources of Funding
This study was supported by National Institutes of Health grant HL58541 (to E.A. Fisher and J.L. Brodsky). L. Guo received support from postdoctoral fellowship 13POST16810071 from the American Heart Association.

Disclosures
None.

References
A Regulator of Secretory Vesicle Size, Kelch-Like Protein 12, Facilitates the Secretion of Apolipoprotein B100 and Very-Low-Density Lipoproteins—Brief Report

*Arterioscler Thromb Vasc Biol.* 2014;34:251-254; originally published online December 12, 2013;
doi: 10.1161/ATVBAHA.113.302728

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/34/2/251

Data Supplement (unedited) at:
http://atvb.ahajournals.org//subscriptions/

**Permissions**: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Arteriosclerosis, Thrombosis, and Vascular Biology* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

**Reprints**: Information about reprints can be found online at:
http://www.lww.com/reprints

**Subscriptions**: Information about subscribing to *Arteriosclerosis, Thrombosis, and Vascular Biology* is online at:
http://atvb.ahajournals.org//subscriptions/
Supplemental Figure I. KLHL12 mRNA abundance in McA cells transfected with control or KLHL12 siRNA. McA cells were transfected with 20 nM control or KLHL12 siRNA. 48 h post transfection, total RNA was extracted and mRNA levels of KLHL12 were measured by qPCR, and normalized to the levels of GAPDH. **p<0.01.
Supplemental Figure II. KLHL12 protein levels in McA cells transfected with control or KLHL12 siRNA. McA cells were transfected with 20 nM control or KLHL12 siRNA. 48 h post transfection, total cell lysate was harvested, and KLHL12 and GAPDH protein levels were measured by Western blotting. Ponceau Red staining was performed after protein transfer to indicate the total protein in each lane.
Supplemental Figure III. Effects of KLHL12 knockdown on albumin recovery from McA cells. McA cells were transfected with either scrambled (control) or KLHL12-specific siRNA or pcDNA3.1 vector (control) or pcDNA3.1-KLHL12. 48 h after transfection, cells were incubated with 0.6 mM OA/BSA complexes (or BSA as a control) for 1 h, followed by an additional 3 h incubation in which $^{35}$S-met/cys was added to the conditioned media. Total levels (sum of secreted and intracellular recoveries) of radiolabeled albumin in the conditioned media and cell lysates were normalized against total TCA-precipitable counts and total protein mass.
Supplemental Figure IV

Supplemental Figure IV. Mannosidase (Golgi-specific) and Calnexin (ER-specific) distributions in sucrose density gradient fractions of cell lysates. McA cells were transfected with 20 nM control or KLHL12 siRNA, and 48 h after transfection cells were harvested and ER microsomes and Golgi membranes were isolated and separated by sucrose gradient centrifugation. The fractional contents of mannosidase activity and calnexin were measured by enzymatic assay and Western blotting, respectively.
Supplemental Figure V. KLHL12 protein levels in McA cells transfected with a vector control, pcDNA3.1, or a FLAG-tagged pcDNA3.1-KLHL12 plasmid. McA cells were transfected with a pcDNA3.1 or FLAG-tagged pcDNA3.1-KLHL12 plasmid. 48 h post transfection, proteins were extracted from the cells, and KLHL12 and GAPDH protein levels were measured by Western blotting using anti-FLAG or anti-GAPDH antibodies.
Supplemental Figure VI. **Effects of KLHL12 over-expression on the density distribution of apoB100-lipoproteins secreted from McA cells.** McA cells treated and analyzed as in Figure 1C, except that instead of being knocked down, KLHL12 expression was increased by transfection with pcDNA3.1-KLHL12 (see Supplemental Figure V). Transfection with pcDNA3.1 was used as a control. The graph shows results representative of 3 independent experiments.
Supplemental Figure VII. Effects of KLHL12 over-expression on albumin recovery from McA cells. McA cells were transfected with either pcDNA3.1 vector (control) or pcDNA3.1-KLHL12. 48 h after transfection, cells were incubated with 0.6 mM OA/BSA complexes (or BSA as a control) for 1 h, followed by an additional 3 h incubation in which $^{35}$S-met/cys was added to the conditioned media. Total levels (sum of secreted and intracellular recoveries) of radiolabeled albumin in the conditioned media and cell lysates were normalized against total TCA-precipitable counts and total protein mass.
Supplemental Figure VIII. Effects of KLHL12 knockdown and overexpression on apoB48 in McA cells. McA cells were transfected with either a scrambled control or KLHL12-specific siRNA (A), or pcDNA3.1 vector or pcDNA3.1-KLHL12 (B). 48 h later, cells were incubated with 0.6 mM OA/BSA complexes (or BSA control) for 1 h, and then another 3 h after addition of $^{35}$S-met/cys to radiolabel apoB48 to steady state. Total levels (sum of secreted and intracellular recoveries) of radiolabeled apoB48 in the conditioned media and cell lysates were normalized against total TCA-precipitable counts and total protein mass. *p<0.05.
### Supplemental Figure IX

#### Total ApoB100 (- OA)

![Graph showing effect of over-expression of KLHL12 on the recovery of apoB100](image)

**Supplemental Figure IX.** Effect of over-expression of KLHL12 on the recovery of apoB100 from McA cells in the absence of oleic acid. McA cells were transfected with a KLHL12 expression vector as in Supplemental Fig. IV. Recovery of apoB100 in the absence of exogenous oleic acid was determined as in Supplemental Fig. III for albumin, but now using an apoB antibody for the immunoprecipitation. **p<0.01.**
Supplemental Figure X. KLHL12 (green) co-localizes not with the Golgi-RFP (red) marker (top panel), but with the ER-RFP (red) marker (bottom panel). Nuclei were stained with DAPI. Magnification is 63X. The inset is a ×10 digital zoom of the area of interest, with co-localization reflected by yellow signals (arrowheads). Shown are the results representative of 3 independent experiments.
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: GGAGAUAAUGAUUUACGUUU, GAUGGAAGCAGGCGUCAUA, AGUGGUGGCCACGCGCAUA, and
GGACUAAUGUUACGCCUAU. siRNA transfection was performed as previously described\textsuperscript{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\textsuperscript{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. \textsuperscript{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 MgCl₂, CaCl₂), 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 Na₂CO₃) 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 °C 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:**
