Supporting Information

Tarling and Edwards 10.1073/pnas.1113021108

SI Materials and Methods

Materials. FuGene 6 and complete protease inhibitor mixture were from Roche Applied Science; Ni^{2+}-NTA agarose beads from Qiagen; cholesterol, 7-ketocholesterol, mouse anti-His monoclonal antibody, and anti-FLAG M2 monoclonal antibody from Sigma; protein G PLUS-agarose, rabbit anti-PDI antibody and mouse anti–pan-cadherin antibody from Santa Cruz Biotechnology; ABCA1 and ABCG1 polyclonal antibodies from Novus Biologicals; Luciferase Reporter Assay system from Promega; CHO-K1 cells from ATCC (CCL-61); EZ-link Sulfo-NHS-SS Biotin and NeutrAvidin agarose beads from Thermo Scientific; 25-hydroxycholesterol, 27-hydroxycholesterol and 7-β-hydroxycholesterol from Avanti Polar Lipids; methyl-β-cyclodextrin and hydroxypropyl-β-cyclodextrin from Trappsol; ^{3}H-cholesterol (60–90 Ci/mmol) from American Radiolabeled Chemicals; anti-rabbit and anti-mouse HRP-conjugated secondary antibodies from BioRad; and 4× SDS sample loading buffer from Invitrogen.

Buffers. Buffer A contained 10 mM Hepes-KOH pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 250 mM sucrose, 1% Triton X-100, and 1 protease inhibitor mixture tablet. PBS^{++} contained 1× PBS supplemented with 0.02 mM $CaCl_2$ and 0.15 mM $MgCl_2$. Quenching buffer contained PBS^{++} supplemented with 100 mM glycine. Cell lysis buffer (5×) contained 0.5 M Tris-phosphate (pH 7.8), 1 M DTT, 0.1 M CDTA, 50% (vol/vol) glycerol, and 5% (vol/vol) Triton X-100.

Culture Medium. Medium A contained Kaighn's modification of Ham's F-12 medium supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin sulfate. Medium B comprised medium A supplemented with 10% FBS. Medium C comprised medium A supplemented with 0.2% BSA. Medium D was medium A supplemented with 5% LPDS, plus 5 μM simvastatin and 50 μM mevalonic acid. Medium E was DMEM supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin sulfate. Medium F comprised medium E supplemented with 10% FBS. Medium G comprised medium E supplemented with 5% LPDS, 5 μM simvastatin, and 50 μM mevalonic acid. Medium H comprised medium E supplemented with 0.2% BSA.

Plasmids. Full-length, untagged mouse ABCG1 (666 amino acids) was expressed in pcDNA3.1 under the control of a CMV promoter. Untagged ABCG1 containing single point mutations (alanine scanning) were generated by site-directed mutagenesis (QuikChange II XL kit; Stratagene). The coding regions of all ABCG1 alanine mutants were sequenced to confirm they contained no other mutations. For domain swapping experiments, full-length ABCG1 or ABCG2 containing a single COOH-terminal Flag epitope were cloned into pcDNA3.1. Fusion proteins were expressed from pcDNA3.1 and contained amino acids 1–409 of mouse ABCG1 fused to amino acids 394–655 of human ABCG2 (ABCG1-ABCG2), or amino acids 1–393 of human ABCG2 fused to amino acids 410–666 of mouse ABCG1 (ABCG2–ABCG1). These fusions proteins contained a single COOH-terminal Flag epitope. Where indicated, pcDNA3.1 contained wild-type mouse ABCG1 fused to either three tandem COOH-terminal Flag epitope tags or GFP. The cDNA for mouse Niemann-Pick type C-1 (NPC-1) (amino acids 1–1,277) was cloned into pEYFP-N1 to generate NPC-1 fused to YFP at the COOH terminus.

The following recombinant expression plasmids have been previously described by others elsewhere: pTK–herpes simplex virus (HSV)–SCAP–T7, encoding HSV fused to hamster SCAP under the control of the HSV-driven thymidine kinase (TK) promoter (1); pTK–INSIG-2–Myc, encoding human INSIG-2 fused to six tandem copies of a c-myc epitope tag driven by the HSV–TK promoter (2, 3); a sterol-sensitive luciferase reporter plasmid encoding a generic TATA box and three sterol response elements (SRE; –325 to –225) from the hamster 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase promoter fused into the luciferase pGL2 basic vector (pSynSRE) (4); pDsRed–Rab5 and pDsRed–Rab11 encoding human Rab5 and Rab11 fused to DsRed under the control of a CMV promoter (5). Detailed primer sequences are available upon request.

Preparation of Sterol/Methyl-β-Cyclodextrin Complexes. Cholesterol, and oxysterol derivatives of cholesterol, were complexed to cyclodextrin using a minor modification of the protocol described by Klein et al. (6). Briefly 10 mg of the indicated sterol (stock concentration 10 mg/mL) in 100% ethanol was added slowly to a stirred solution of 5% wt/vol methyl-β-cyclodextrin at 80 °C, until a clear solution was obtained. The resulting solution was lyophilized and the dried complex reconstituted in nanopure water to a sterol concentration of 2.5 mM.

Biotinylation of Cell Surface Proteins. Freshly isolated primary mouse peritoneal (thioglycollate-elicited) macrophages were allowed to adhere to six-well plates for 18 h in medium F. Cells were treated in medium G with or without 1 μM liver X receptor (LXR) agonist GW3965 for 24 h. Cells were washed in PBS^{++} and then incubated for 30 min on ice with 250 μM EZ-link Sulfo-NHS-SS Biotin (diluted in PBS^{++}). The cells were washed in PBS^{++} and the reaction was quenched for 30 min on ice in quenching buffer. Biotin-modified proteins were immunoprecipitated with NeutrAvidin streptavidin beads overnight at 4 °C. Biotin-modified proteins were collected by centrifugation at 5,000 × g for 5 min. Intracellular, unmodified proteins were collected from the supernatant of the 5,000 × g spin. The streptavidin beads were washed three times in PBS^{++} before proteins were removed from the beads by incubation at 42 °C for 20 min, in 2× SDS sample loading buffer supplemented with β-mercaptoethanol.

Immunoprecipitation of Protein Complexes. HEK293 cells were transfected in medium F with the indicated plasmids. Cells were lysed in buffer A. Proteins (100 μg) in buffer A were incubated overnight with anti-FLAG M2 antibody (used at 1:1,000) at 4 °C. Protein complexes were incubated with protein G-PLUS agarose beads at 4 °C for 6 h before centrifugation at 5,000 × g for 5 min. Beads were washed with buffer A. Protein complexes were retrieved by incubation at 42 °C for 20 min in 2× SDS sample buffer supplemented with β-mercaptoethanol.

Western Blot Analysis. From biotinylation studies, 5% of each fraction (total cell lysate, biotinylated proteins, unmodified intracellular proteins) was loaded and separated by SDS/PAGE. From immunoprecipitation studies, 20% of protein complexes were separated by SDS/PAGE. From overexpression and immunoprecipitation studies, cells were lysed in buffer A, and proteins (10 μg) were separated by SDS/PAGE.

Proteins were transferred to polyvinylidene difluoride membrane. Primary antibodies were diluted 1:1,000 (ABCA1, ABCG1, pan-cadherin, HA) or 1:5,000 (PDI, β-actin) in 1× TBS containing 0.1% Tween 20 and 5% nonfat milk. Immune complexes were detected with anti-rabbit (ABCA1, ABCG1, PDI) or anti-

mouse (β-actin, pan-cadherin, HA) HRP-conjugated secondary antibodies diluted 1:10,000.

Cell Transfection and Luciferase Reporter Assay. CHO-K1 cells were plated in medium B 1 d before transfection and cultured to reach 80% confluence. Medium B was replaced with medium A before transfection. Cells were transfected using FuGeneHD (Roche) according to manufacturer instructions. Each well was transfected with 100 ng pSynSRE plus 5 ng of each expression plasmid, and 50 ng β-galactosidase expression plasmid as a control to normalize for minor changes in transfection efficiency. After 5 h, the culture medium was replaced with specific treatment media, as indicated.

Cells were cultured for 24 h, washed twice with PBS, and lysed with 1× cell lysis buffer. The luminometer assays were performed using Promega luciferase reporter assay system according to instructions. The luciferase activity was measured using a Centro XS^3 LB 960 luminometer (Berthold Technologies).

Cellular Efflux of Cholesterol. HEK293 or CHO-K1 cells transfected with the indicated plasmids were incubated with 1 μCi/mL ^{3}H-cholesterol for 24 h in medium H. The media was removed, the cells washed and equilibrated for 24 h in medium H. To determine cellular cholesterol efflux, cells were incubated in fresh medium H in the presence or absence of 50 μg/mL HDL for 4 h. The radioactive content of the cells and media was determined as previously described (7). Cholesterol efflux was determined by dividing the radioactive content of the media by the sum of the radioactivity in the cells and the media. The basal efflux (% cholesterol that effluxes to medium H; ∼1–2%) was subtracted from the values obtained in the presence of HDL.

mRNA Quantification. Alveolar macrophages were isolated from bronchoalveolar lavage as previously described (8). Total RNA was isolated from alveolar macrophages using TRIzol (Invitrogen), and cDNA synthesized (Applied Biosystems). Quantitative real-time PCR was performed on a LightCycler 480 (Roche). mRNA expression was normalized to expression of 36B4. Detailed primer sequences are available upon request.

Statistics. Statistical analysis was performed using GraphPad Prism 5. For luciferase reporter assays and cholesterol efflux assays, statistical analysis was performed by one-way ANOVA, comparing ABCG1 (wild-type, mutant, or chimera) to control transfected cells. For luciferase reporter assays with CD:sterol complex treatment, statistical analysis was performed by two-way ANOVA.

1. Hua X, Nohturfft A, Goldstein JL, Brown MS (1996) Sterol resistance in CHO cells traced to point mutation in SREBP cleavage-activating protein. *Cell* 87:415–426.
2. Gong Y, et al. (2006) Sterol-regulated ubiquitination and degradation of Insig-1 creates a convergent mechanism for feedback control of cholesterol synthesis and uptake. *Cell Metab* 3:15–24.
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4. Dooley KA, Millinder S, Osborne TF (1998) Sterol regulation of 3-hydroxy-3-methylglutaryl-coenzyme A synthase gene through a direct interaction between sterol regulatory element binding protein and the trimeric CCAAT-binding factor/nuclear factor Y. *J Biol Chem* 273:1349–1356.
5. Choudhury A, et al. (2002) Rab proteins mediate Golgi transport of caveola-internalized glycosphingolipids and correct lipid trafficking in Niemann-Pick C cells. *J Clin Invest* 109:1541–1550.
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8. Baldán A, et al. (2006) Deletion of the transmembrane transporter ABCG1 results in progressive pulmonary lipidosis. *J Biol Chem* 281:29401–29410.

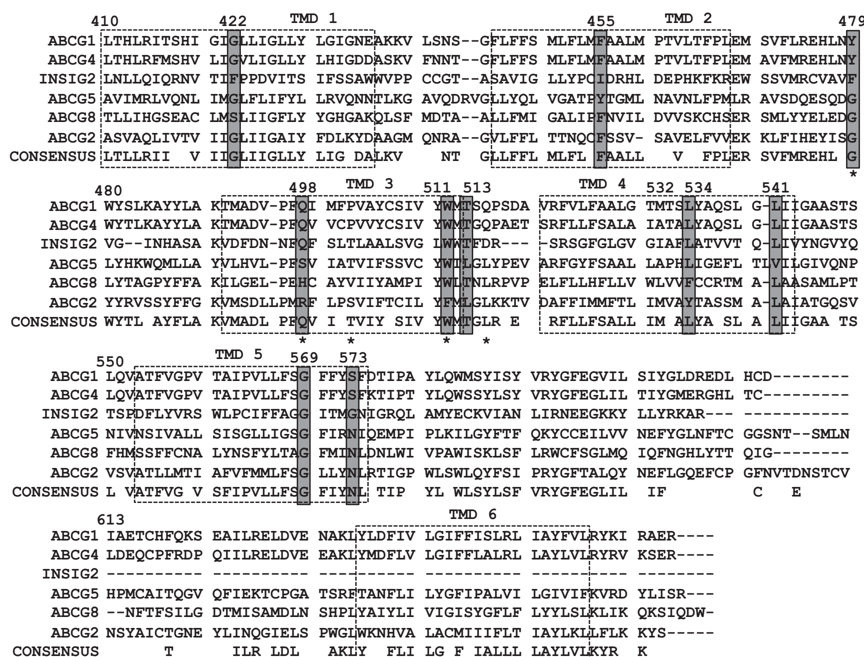

Fig. S1. Alignment of members of the murine ABCG family with human INSIG-2 identifies conserved residues. Amino acids are numbered based on the murine ABCG1 sequence. TM 1–6 are boxed (dashed lines). Individual boxed residues (shaded gray) indicate some level of conservation. *Identifies residues of INSIG-2 that when mutated resulted in loss of INSIG-2 function (1).

1. Radhakrishnan A, Ikeda Y, Kwon HJ, Brown MS, Goldstein JL (2007) Sterol-regulated transport of SREBPs from endoplasmic reticulum to Golgi: Oxysterols block transport by binding to Insig. *Proc Natl Acad Sci USA* 104:6511–6518.

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A.                410
                              TMD 1                                  TMD 2
Mouse ABCG1 TMD1-6 LTHLRITSHI GIGLLIGLLY LGIGNEAKKV LSNSGFLFFS MLFLMFAALM PTVLTFPLEM SVFLREHLNY
    Human NPC1 SSD LIPLVTTYII LFAYIYFSTR KID-----MV KSKWGLALAA VVTVLSSLLM SVGLCTLFGL TPTINGGEIF
    Human SCAP SSD VFTVVISYAI MFLYISLALG HMKSCRRLLV DSKVSLGIAG ILIVLSSVAC SLGVFSYIGL PLTLIVIEVI
    Human HMGR SSD IIILTITRCI AILYIYFQFQ NLR-----QL GSKYILGIAG LFTIFSSFVF STVVIHFLDK ELTGLN-EAL
                                                          *
                                        TMD 3                                  TMD 4
Mouse ABCG1 TMD1-6 WYSLKAYYLA KTMADVPFQI MFPVAYCSIV YWMTSQPSDA VRFVLFAALG TMTSLVAQSL GLLIGAASTS
    Human NPC1 SSD PYLVVVIGLE NVLVLTKSVV STP--VDLEV KLRIAQG--- ---------- ------LSSE SWSIMKNMAT
    Human SCAP SSD PFLVLAVGVD NIFILVQAYQ RDERLQGETL DQQLGRV--- ---------- ------LGEV APSMFLSSFS
    Human HMGR SSD PFFLLLIDLS RASTLAKFAL SSN--SQDEV RENIARG--- ---------- ------MAIL GPTFTLDALV
                   *
                               TMD 5
Mouse ABCG1 TMD1-6 LQVATFVGPV TAIPVLLFSG FFVSFDTIPA YLQWMSYISY VRYGFEGVIL
    Human NPC1 SSD ELGIILIGYF TLVPAIQEFQ LFAVVGLVSD FFLQMIFFTT VLS-------
    Human SCAP SSD ETVAFFLGAL SVMPAVHTFS LFAGLAVFID FLLQITQFVS LLGLDIKRQE
    Human HMGR SSD EQLVIGVGTM SGVRQLEIMC CFGCMSVLAN YFVFMTFFPA CV--------
                                                                   *

B.                 69
   Mouse ABCG1 NTD LPRRAAVNIE FKDLSYSVPE G--PWWKKKG YKTLLKGISG KFNSGELVAI MGPSGAGKST LMNILAGYRE
    Human NPC1 NTD CVWYGECGIA YGDKRYNCEY SGPPKPLPKD GYDLVQELCP GFFFGNVSLC CDVR-----Q LQTLKDNLQL
                                                                                   *

   Mouse ABCG1 NTD TGMKGAVLIN GMPRDLRCFR KVSCYIMQDD MLLPHLTVQE AMMVSAHLKL QEKDEGRREM VKEILTALGL
    Human NPC1 NTD PLQFLSWCPS CFYNLLNLFC ELTCSPRQSQ FLNVTATEDY VDPVTNQTKT NVKEL--CYY VGQSFANAMY
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Fig. S2. Comparison of transmembrane (TM) α-helices 1–6 of ABCG1 with reported sterol-sensing domains. The sequence corresponding to TM 1–6 of ABCG1 was aligned and compared with the previously reported sterol-sensing domain of HMG-CoA reductase, SCAP, and NPC-1.

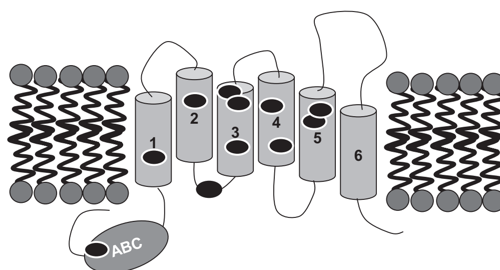

Fig. S3. Schematic of an ABCG1 monomer showing the approximate location of individual amino acid mutations (black ovals) within the six TM α-helices (1–6) or the Walker A motif.

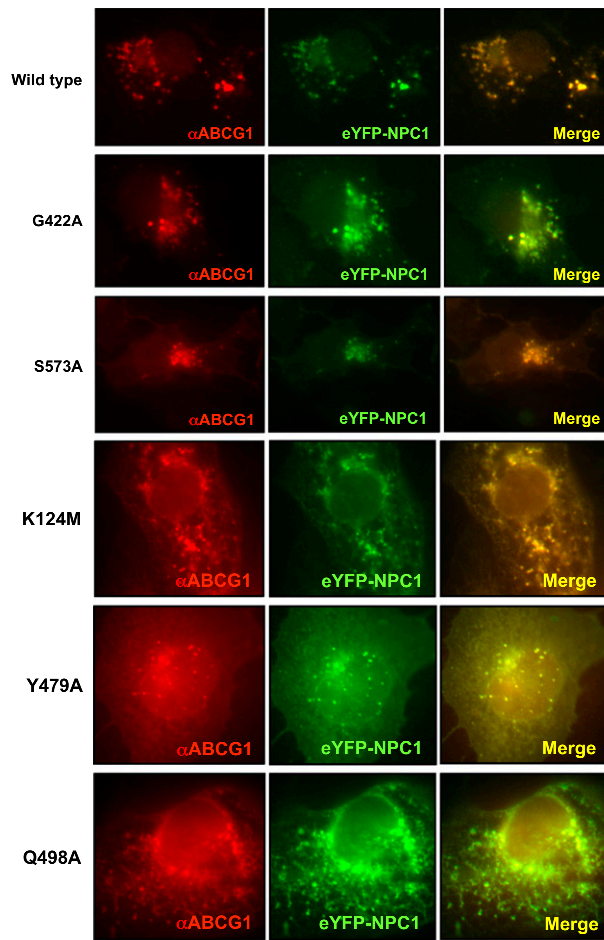

Fig. S4. Wild-type ABCG1 and ABCG1 containing inactivating alanine substitutions localize to similar intracellular vesicles. Cos-7 cells were cotransfected with plasmids encoding either untagged wild-type ABCG1 or the indicated mutant forms of ABCG1 and NPC-1–YFP. All images were taken at 63× magnification. Yellow dots indicate colocalization in the merged images.

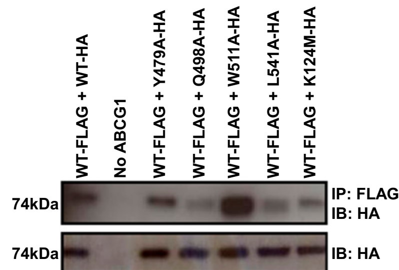

Fig. S5. Wild-type ABCG1 and ABCG1 containing alanine substitutions form homodimers. HEK293 cells were cotransfected with wild-type (WT) ABCG1–FLAG and either WT ABCG1–HA or the indicated mutant ABCG1–HA. Cells were lysed and protein samples (10 μg) were analyzed by Western blot (*Lower*, immunoblot, IB). Cell lysates were also treated with anti-FLAG and the immunoprecipitated (IP) protein complexes separated by SDS/PAGE, transferred to a membrane, and the membrane probed with anti-HA (*Upper*).

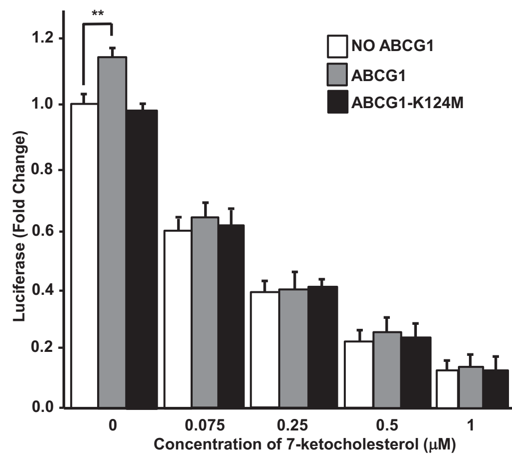

Fig. S6. ABCG1 does not attenuate 7-ketocholesterol–mediated repression of SREBP-2 processing. CHO-K1 cells were transfected with pSynSRE, an empty plasmid (open bars) or a plasmid encoding either wild-type ABCG1 or ABCG1–K124M. Following 5 h transfection, cells were treated with the indicated concentration of oxysterol for 24 h before determination of normalized luciferase activity. $^{**}P < 0.01$ vs. pSynSRE plus control.

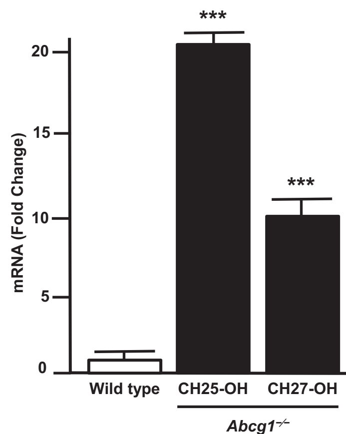

Fig. S7. Alveolar macrophages from *Abcg1*$^{-/-}$ mice express elevated levels of the cholesterol modifying enzymes, 25- and 27-hydroxylase. Alveolar macrophages were isolated from the lungs of wild-type and *Abcg1*$^{-/-}$ mice. mRNA levels of cholesterol 25-hydroxylase (CH25-OH) and cholesterol 27-hydroxylase (CH27-OH) were measured by quantitative real-time PCR. mRNA levels were normalized to 36B4, and values given as fold change relative to wild-type mice. $^{***}P < 0.001$ vs. WT macrophages.