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BASIC SCIENCE

TRAK2, a novel regulator of ABCA1 expression, cholesterol efflux and HDL biogenesis

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Aims The recent failures of HDL-raising therapies have underscored our incomplete understanding of HDL biology. Therefore there is an urgent need to comprehensively investigate HDL metabolism to enable the development of effective HDL-centric therapies. To identify novel regulators of HDL metabolism, we performed a joint analysis of human genetic, transcriptomic, and plasma HDL-cholesterol (HDL-C) concentration data and identified a novel association between trafficking protein, kinesin binding 2 (*TRAK2*) and HDL-C concentration. Here we characterize the molecular basis of the novel association between TRAK2 and HDL-cholesterol concentration.

Methods and results Analysis of lymphocyte transcriptomic data together with plasma HDL from the San Antonio Family Heart Study ($n = 1240$) revealed a significant negative correlation between *TRAK2* mRNA levels and HDL-C concentration, HDL particle diameter and HDL subspecies heterogeneity. TRAK2 siRNA-mediated knockdown significantly increased cholesterol efflux to apolipoprotein A-I and isolated HDL from human macrophage (THP-1) and liver (HepG2) cells by increasing the mRNA and protein expression of the cholesterol transporter ATP-binding cassette, sub-family A member 1 (ABCA1). The effect of TRAK2 knockdown on cholesterol efflux was abolished in the absence of ABCA1, indicating that TRAK2 functions in an ABCA1-dependent efflux pathway. TRAK2 knockdown significantly increased liver X receptor (LXR) binding at the *ABCA1* promoter, establishing TRAK2 as a regulator of LXR-mediated transcription of *ABCA1*.

Conclusion We show, for the first time, that TRAK2 is a novel regulator of LXR-mediated ABCA1 expression, cholesterol efflux, and HDL biogenesis. TRAK2 may therefore be an important target in the development of anti-atherosclerotic therapies.

Keywords Atherosclerosis • HDL • Cholesterol • Genetics • ABCA1 • TRAK2

Translational Perspective

Our work identifies TRAK2 as a novel regulator of HDL metabolism and cholesterol efflux. TRAK2 may be an important target in the development of anti-atherosclerotic therapies.

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† This article is dedicated to the late Associate Professor Jeremy Jowett.

Introduction

Studies into the regulation of HDL-cholesterol (HDL-C) levels have revealed that HDL metabolism is complex, and involving multiple pathways. The process of HDL biogenesis is mediated primarily by the liver and involves the secretion of apoA-I which stimulates cholesterol efflux through the ABCA1 cholesterol transporter.[1] While cholesterol efflux is integral to HDL biogenesis, it also enables removal of excess cholesterol from macrophages towards preventing development of atherosclerotic lesions.[2] The movement of cholesterol from peripheral tissues to the liver for clearance is termed reverse cholesterol transport (RCT), a pathway that represents a key atheroprotective function of HDL.[3] The liver X receptors (LXRs) are transcription factors that play a key role in regulation of RCT.[4] LXRs function as cholesterol sensors, and when cellular cholesterol content is high they activate the transcription of genes involved in cholesterol efflux, including *ABCA1* and *ABCG1*, to promote RCT.[5,6]

The well-established inverse correlation between HDL-C concentration and cardiovascular disease (CVD) led to development of the 'HDL hypothesis' which postulates that therapeutic elevation of HDL levels can reduce CVD risk.[7] The pursuit of efficacious HDL-centric therapy has proved difficult, with pharmacological attempts at raising HDL-C levels failing to reduce the incidence of cardiovascular events.[8] These results highlight that our current understanding of HDL metabolism is incomplete. In parallel, interest in pharmacological enhancement of other atheroprotective aspects of HDL biology has emerged, with particular attention on those that promote the role of HDL in RCT.[1,9] Therefore consolidation of the mechanistic basis of cholesterol efflux, HDL biogenesis, and broader HDL metabolism is required for the development of a successful HDL-centric therapy.

To identify novel regulators of HDL metabolism, we utilized an innovative approach of integrating large-scale human transcriptomic and genome-wide association (GWA) data to identify genes demonstrating a novel association with plasma HDL-C concentration.[10] Since a relationship with HDL-C concentration may reflect an effect on aspects of HDL biology including biogenesis and cholesterol efflux, this approach was anticipated to reveal targets that may also influence other HDL metrics of pharmacological interest. Our analysis identified a novel significant *in vivo* association between *trafficking protein, kinesin binding 2 (TRAK2)*, and plasma HDL-C concentration, thereby establishing *TRAK2* as a candidate gene for further investigation.[10] TRAK2 is a regulator of protein and organelle trafficking through its role as a kinesin and dynein binding protein, with its function in neuronal mitochondrial trafficking most thoroughly characterized.[11,12] As a role for TRAK2 in HDL or cholesterol metabolism had not been previously characterized, functional studies were undertaken to validate this novel association.

Here we provide data to establish a role for TRAK2 in HDL metabolism, demonstrating a negative *in vivo* correlation between *TRAK2* mRNA expression and HDL-C concentration, HDL diameter, and HDL subspecies heterogeneity. For the first time, we provide functional evidence of the mechanism underlying the association between TRAK2 and HDL-C, revealing that TRAK2 siRNA knockdown significantly promotes cholesterol efflux from human macrophage and liver cells by increasing LXR-mediated transcription of *ABCA1*. These data identify TRAK2 as an important contributor to HDL metabolism and function.

Methods

Detailed methods are available in the Supplementary material online.

Participants from the San Antonio Family Heart Study (SAFHS) ($n \approx 1400$), all provided informed consent and recruitment, lymphocyte isolation, and statistical analyses of genetic and transcriptomic data (available for 1240 participants) are previously described.[13] Separation of lipoprotein subfractions, and lipoprotein diameter, was determined by gradient gel electrophoresis.

Cellular investigations were performed on human Hepatocellular Carcinoma Cells (HepG2), Intestinal cells (FHs74Int), and Monocytes (THP-1) differentiated into macrophages. Knockdown of *TRAK2* and *ABCA1* gene expression in these cell lines was achieved following transfection with siRNA. Gene expression was analysed by real-time polymerase chain reaction, and calculated using the comparative C_t method with normalization to the housekeeping gene *ACTB*. Protein levels were measured using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

Cholesterol efflux was measured following a 2-h incubation with apoA-I (30 μg/ml) or HDL particles isolated from human plasma (40 μg/ml).

Measurement of LXR-α binding in the *ABCA1* promoter region was determined by chromatin immunoprecipitation (ChIP) assay. Significance was calculated using the 2-sided Student's *t*-test and Stouffer's *Z*-score method where a $P < 0.05$ was considered significant.

Results

TRAK2 mRNA expression is negatively correlated with human plasma HDL-cholesterol concentration, HDL particle diameter, and HDL subpopulation heterogeneity

We previously identified that *TRAK2* mRNA expression was significantly associated with human plasma HDL-C concentration,[10] however the nature of this association was unknown. Therefore we performed further analyses of the SAFHS gene expression data and identified a significant and negative correlation between *TRAK2* expression and plasma HDL-C concentration after adjustment for multiple covariates including age and sex ($\beta = -0.11$, $P = 1.15 \times 10^{-4}$). This significant association was largely genetic in origin (genetic correlation = -0.34, $P = 4.5 \times 10^{-4}$). Examination of *TRAK2* sequence data available from the SAFHS cohort to search for cis-acting variants influencing *TRAK2* expression identified two variants that were significantly associated with *TRAK2* mRNA levels and plasma HDL-C concentration (see Supplementary material online, *Table S3*, $P = 0.0062$ and $P = 0.013$), supporting the genetic basis of the association between *TRAK2* and HDL-C concentration. Examination of the tissue distribution of *TRAK2* in humans using publically available data from the Genotype-Tissue Expression project revealed that TRAK2 was most highly expressed in the brain, heart, arteries and thyroid (see Supplementary material online, *Figure S1A*).[14] In mice, across the selected tissues examined *Trak2* was most highly expressed in the spleen, bone marrow-derived macrophages and liver (see Supplementary material online, *Figure S1B*). Among human cell lines derived from tissues relevant to HDL-C metabolism, *TRAK2* was most highly expressed in hepatic cells (see Supplementary material online, *Figure S1C*).

Gradient gel electrophoresis studies on SAFHS plasma samples revealed a significant negative correlation between *TRAK2* expression and HDL particle diameter [excluding HDL1 β = -0.10 standard deviation units (SDU), $P = 7.2 \times 10^{-4}$; including HDL1 β = -0.10 SDU, $P = 7.6 \times 10^{-4}$] (*Table 1*). HDL particles are heterogeneous and can be separated into subpopulations by particle size (HDL2b, -2a, -3a, -3b, -3c).[15] Fractionation of HDL subpopulations from SAFHS participants revealed that *TRAK2* expression was significantly correlated with the level of HDL2b (β = -0.11 SDU, $P = 2.8 \times 10^{-4}$), HDL3a (β = 0.08 SDU, $P = 0.012$) and HDL3b (β = -0.11 SDU, $P = 1.6 \times 10^{-4}$) particles (*Table 1*). Overall, these data reveal that *TRAK2* expression is significantly correlated with multiple HDL parameters in humans, and that genetic variation influencing *TRAK2* expression may also contribute to the regulation of HDL-C concentration.

TRAK2 mediates cholesterol efflux to apoA-I and HDL in human macrophage cells

Cholesterol efflux from macrophages to HDL particles is an essential part of RCT and represents a key atheroprotective function of HDL.[2] We manipulated *TRAK2* gene expression using siRNA-mediated knockdown in human THP-1 human monocytes differentiated into macrophages and measured cholesterol efflux. Targeted knockdown of TRAK2 significantly reduced *TRAK2* gene expression in THP-1 macrophage cells by up to 90% relative to non-targeting control (NTGC) siRNA transfected cells (*Figure 1A*, $P < 0.00001$). TRAK2 knockdown significantly increased cholesterol efflux to apoA-I by 35% (*Figure 1B*, $P = 0.039$) and to isolated HDL by 20% (*Figure 1B*, $P = 0.0038$) relative to cells transfected with NTGC siRNA. These data validate our human *in vivo* data and suggest that conditions of low-TRAK2 expression may promote RCT.

TRAK2 is a key regulator of hepatic cholesterol efflux and HDL biogenesis

We then assessed whether TRAK2 was involved in the regulation of HDL biogenesis, a process mediated by cholesterol efflux to apoA-I.[1] Since the liver and intestine are the primary and secondary sites of HDL biogenesis respectively, the effect of TRAK2 knockdown on cholesterol efflux in human liver (HepG2) cells and human intestinal (FHs74Int) cells was investigated. Transfection of TRAK2 siRNA reduced *TRAK2* gene expression in HepG2 and FHs74INT cells by 80% and 90%, respectively (*Figure 1A*, $P < 0.00001$). TRAK2 knockdown in HepG2 cells significantly increased cholesterol efflux to apoA-I by 60% (*Figure 1B*, $P < 0.00001$), but had no significant effect on efflux to apoA-I in FHs74Int cells, relative to NTGC siRNA (*Figure 1B*, $P = 0.20$), suggesting that TRAK2's role in HDL biogenesis is primarily hepatic. TRAK2 knockdown did not increase efflux in the absence of a cholesterol acceptor (see Supplementary material online, *Figure S2*, $P = 0.80$), indicating that the significant effect of TRAK2 knockdown is not due to background passive cholesterol efflux. Further investigation demonstrated that TRAK2 knockdown significantly increased HDL production in HepG2 cells by 70% (*Figure 1C*; $P < 0.00001$); and increased the mRNA expression of *APOA1* in HepG2 cells by 25% relative to NTGC siRNA (*Figure 1D*, $P = 0.0026$). A non-significant trend to increase apoA-I protein levels and secretion was additionally observed (see Supplementary material online, *Figure S3*, $P = 0.38$ and $P = 0.080$, respectively), suggesting that TRAK2 may have broader effects on HDL biogenesis. Overexpression of TRAK2 using plasmid DNA showed a significant increase in *TRAK2* expression (see Supplementary material online, *Figure S4A*, $P = 0.0041$), however only a modest albeit significant decrease in cholesterol efflux was observed (see Supplementary material online, *Figure S4B*, $P = 0.029$).

We then investigated the effect of TRAK2 knockdown on efflux to isolated human HDL in HepG2 and FHs74Int cells. Although these cell types are not typically recognized as endogenous promoters of cholesterol efflux to HDL, it has been suggested that hepatic cells can utilize this pathway to release unwanted cholesterol.[16] We observed a significant 40% increase in cholesterol efflux to isolated HDL in HepG2 cells (*Figure 1B*, $P = 0.0026$), but TRAK2 knockdown had no significant effect on efflux to isolated HDL in FHs74Int cells (*Figure 1B*, $P = 0.063$), relative to NTGC siRNA. Overall, these results reveal a novel role for TRAK2 in HDL metabolism, and specifically as a novel regulator of cholesterol efflux and hepatic HDL biogenesis.

Table 1 The association of *TRAK2* mRNA with HDL particle diameter and HDL fractions

Trait	β standard deviation units	*P*-value
HDL diameter excluding HDL1	-0.101	0.00072[a]
HDL diameter including HDL1	-0.101	0.00076[a]
HDL1 (13.0–20.0 nm)	-0.026	0.42
HDL2b (9.7–12.9 nm)	-0.109	0.00028[a]
HDL2a (8.8–9.7 nm)	-0.015	0.63
HDL3a (8.2–8.8 nm)	0.079	0.012[a]
HDL3b (7.8–8.2 nm)	0.113	0.00016[a]
HDL3c (7.2–7.8 nm)	0.055	0.072

The particle size range of each HDL fraction is described.
[a]Traits significantly correlated with *TRAK2* mRNA ($P < 0.05$).

TRAK2 knockdown increases *ABCA1* mRNA and protein expression levels

ABCA1 mediates cholesterol efflux to apoA-I and HDL, while ABCG1 and SR-BI (encoded by *SCARB1*) only facilitate cholesterol efflux to HDL.[1] We therefore investigated whether these cholesterol transporters were mediating the significant effect of TRAK2 knockdown on cholesterol efflux. TRAK2 knockdown significantly increased *ABCA1* mRNA levels by 170% in HepG2 cells (*Figure 2A*, $P = 0.000036$) and by 70% in THP-1 macrophage cells (*Figure 2A*, $P = 0.012$) compared to controls, but had no significant effect on *ABCG1* expression (*Figure 2A*, $P = 0.85$ and $P = 0.30$, respectively). TRAK2 knockdown modestly but significantly reduced *SCARB1* gene expression in HepG2 and THP-1 macrophage cells (*Figure 2A*, $P = 0.00056$ and $P = 0.013$ respectively). Furthermore, we observed a significant 80% increase in ABCA1 protein levels (*Figure 2B*, and C $P = 0.046$), while there was no marked difference in ABCG1 or SR-BI protein abundance (*Figure 2B* and *C*). Overexpression of TRAK2 had no significant effect on *ABCA1* expression (see Supplementary material online, *Figure S4C*, $P = 0.066$). Taken together, these observations suggest that TRAK2 knockdown increases cholesterol efflux to

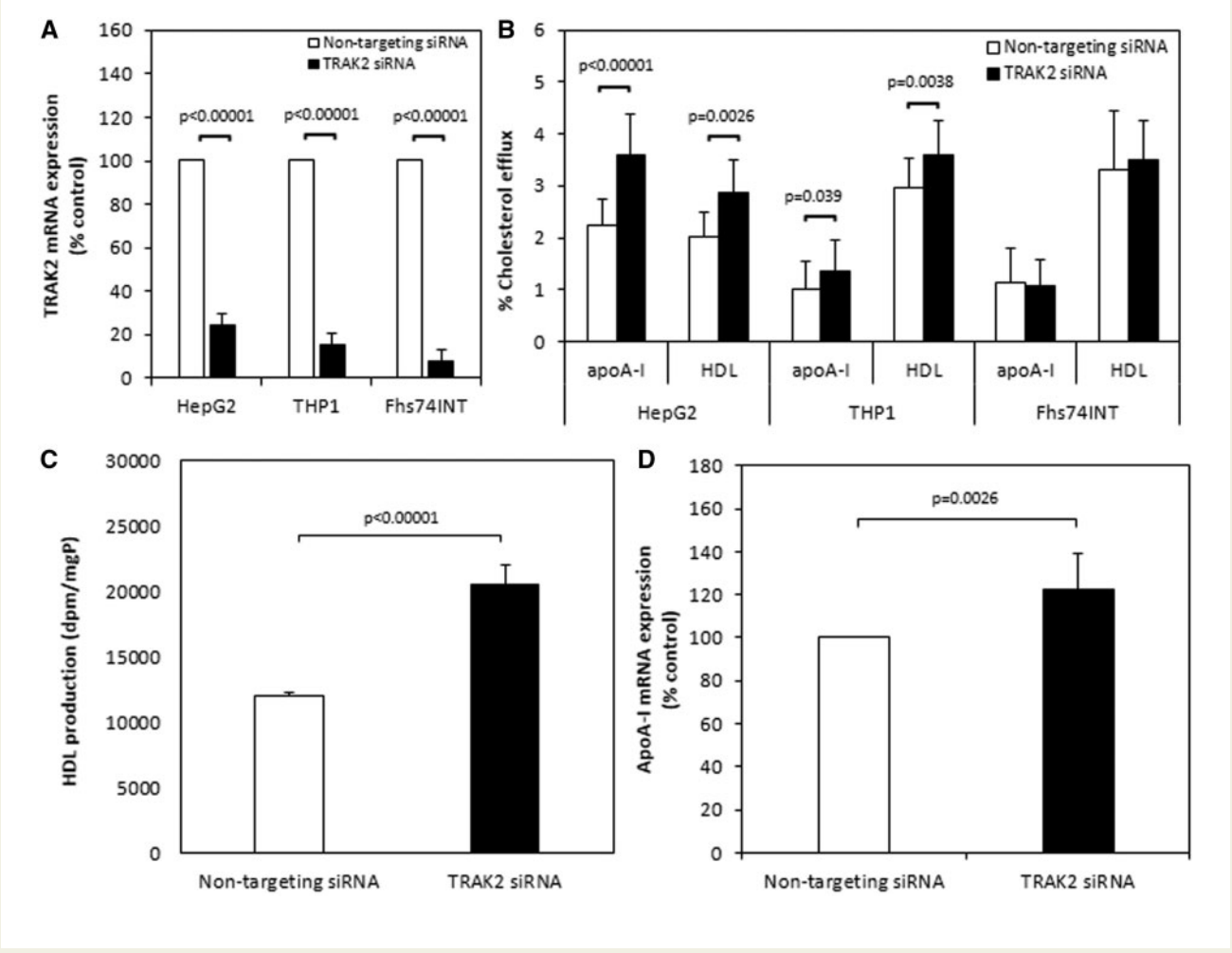

Figure 1 TRAK2 siRNA-mediated knockdown induces cholesterol efflux and apoA-I expression. (*A*) siRNA-mediated gene knockdown significantly decreased *TRAK2* expression in hepatic HepG2, THP-1 macrophage, and FHs74INT intestinal cells relative to non-targeting control (NTGC) siRNA; $n = 4$ independent experiments. (*B*) Cholesterol efflux to apoA-I and HDL was significantly increased following siRNA-mediated knockdown of TRAK2 in HepG2 and THP-1 macrophage cells, relative to NTGC siRNA. Transfection of FHs74INT cells with TRAK2 siRNA did not significantly affect cholesterol efflux; $n = 3$–4 independent experiments. (*C*) siRNA-mediated knockdown of TRAK2 significantly increased HDL production in HepG2 cells relative to NTGC siRNA; $n = 5$ biological replicates. (*D*) TRAK2 knockdown significantly increased *APOA1* mRNA expression relative to NTGC siRNA in HepG2 cells; $n = 5$ independent experiments. Data are represented as the mean ± standard deviation of *n*.

apoA-I and HDL by increasing the transcription and protein abundance of ABCA1.

TRAK2's effects on hepatic cholesterol efflux and HDL biogenesis are dependent on ABCA1

To investigate whether TRAK2 was regulating cholesterol efflux in an ABCA1-dependent pathway, we performed siRNA-mediated double knockdown of ABCA1 and TRAK2 to silence both genes simultaneously. A 75% suppression in *ABCA1* gene expression relative to NTGC siRNA was observed in HepG2 cells (*Figure 3A*, $P = 0.000078$), mirrored by a strong knockdown at the protein level (*Figure 3B*). Transfection of ABCA1 siRNA ablated cholesterol efflux to apoA-I (*Figure 3C*, $P < 0.00001$), and significantly reduced efflux to isolated HDL by 75% (*Figure 3C*, $P < 0.00001$). Cells transfected with both ABCA1 and TRAK2 siRNA displayed ablated cholesterol efflux relative to NTGC siRNA at levels comparable to cells transfected only with ABCA1 siRNA (*Figure 3C*, $P < 0.00001$). These results provide evidence that TRAK2 knockdown cannot increase cholesterol efflux in the absence of ABCA1. Knockdown of *ABCA1* expression had no significant effect on *TRAK2* mRNA levels (*Figure 3A*), suggesting that TRAK2 functions upstream of ABCA1 in this efflux pathway.

To further support that TRAK2 regulates an ABCA1-dependent cholesterol efflux pathway, we treated HepG2 cells with ABCA1 inhibitor Cyclosporine A (CsA),[17] and examined the effect on cholesterol efflux. CsA treatment significantly reduced ABCA1 expression by 75% relative to control (*Figure 4A*, $P = 0.00023$), ablated

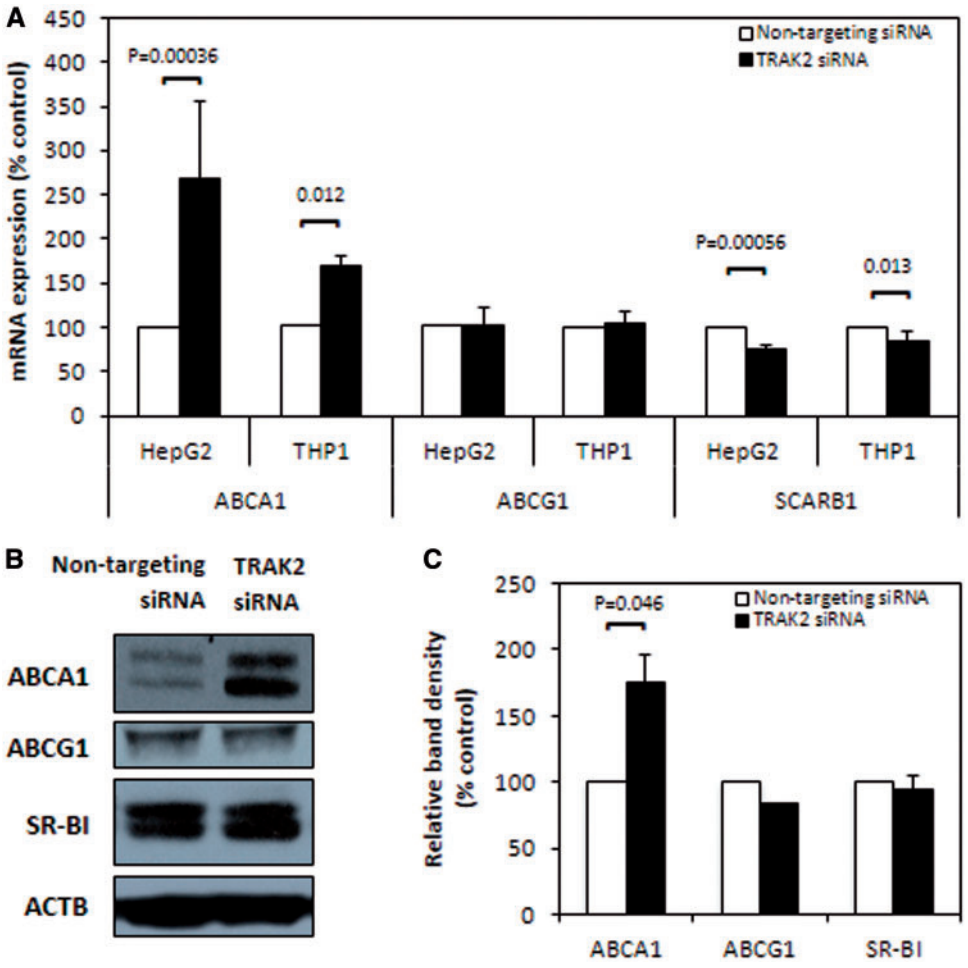

Figure 2 The effect of TRAK2 on ABCA1, ABCG1, and SR-BI expression. (*A*) TRAK2 knockdown in HepG2 and THP-1 macrophage cells significantly increased *ABCA1* mRNA expression, significantly decreased *SCARB1* mRNA levels, but had no significant effect on *ABCG1* mRNA expression levels relative to non-targeting control (NTGC) siRNA; $n = 4$. (*B*) siRNA-mediated knockdown of TRAK2 increased ABCA1 abundance but had no marked effect on ABCG1 or SR-BI protein levels relative to NTGC siRNA in HepG2 cells. (*C*) TRAK2 knockdown significantly increased ABCA1 protein levels. There was no significant difference in ABCG1 or SR-BI protein expression; $n = 2$. Data are represented as the mean ± standard deviation of *n* independent experiments.

cholesterol efflux to apoA-I (*Figure 4B*, $P < 0.00001$), and suppressed the effect of TRAK2 knockdown on cholesterol efflux (*Figure 4B*). However, TRAK2 knockdown was able to modestly but significantly increase cholesterol efflux during CsA treatment relative to the NTGC siRNA (*Figure 4B*, $P < 0.00001$), which was reproducible with a shorter treatment period (see Supplementary material online, *Figure S5*, $P = 0.0019$). This increase in efflux correlated with a significant increase in ABCA1 expression, where TRAK2 knockdown approximately doubled ABCA1 mRNA expression relative to NTGC siRNA during CsA treatment (*Figure 4B*, $P = 0.0032$). This suggests that TRAK2 knockdown is able to increase ABCA1 levels even in conditions of low-ABCA1 expression.

Given that cholesterol egress from late endosomes to the plasma membrane is required for efficient ABCA1-mediated cholesterol efflux,[18] we used U18666A to inhibit cholesterol movement from the late endosomal network and measured cholesterol efflux. Treatment with U18666A suppressed the effect of TRAK2 knockdown on cholesterol efflux (see Supplementary material online, *Figure S6A*), consistent with the role of TRAK2 in an ABCA1-dependent pathway. Interestingly, U18666A treatment doubled the increase in *ABCA1* expression elicited by TRAK2 knockdown relative to transfected cells without treatment (see Supplementary material online, *Figure S6B*, untreated cells $P = 0.000046$ and treated cells $P = 0.000048$), suggesting that the effect of TRAK2 knockdown on *ABCA1* expression is influenced by cholesterol accumulation in the late endosomal network. TRAK2 has been previously characterized as a regulator of endosomal trafficking.[19] We therefore utilized immunofluorescence confocal microscopy to determine whether TRAK2 knockdown also influenced the localization of early and late endosomes in HepG2 cells, but no discernible changes in endosomal

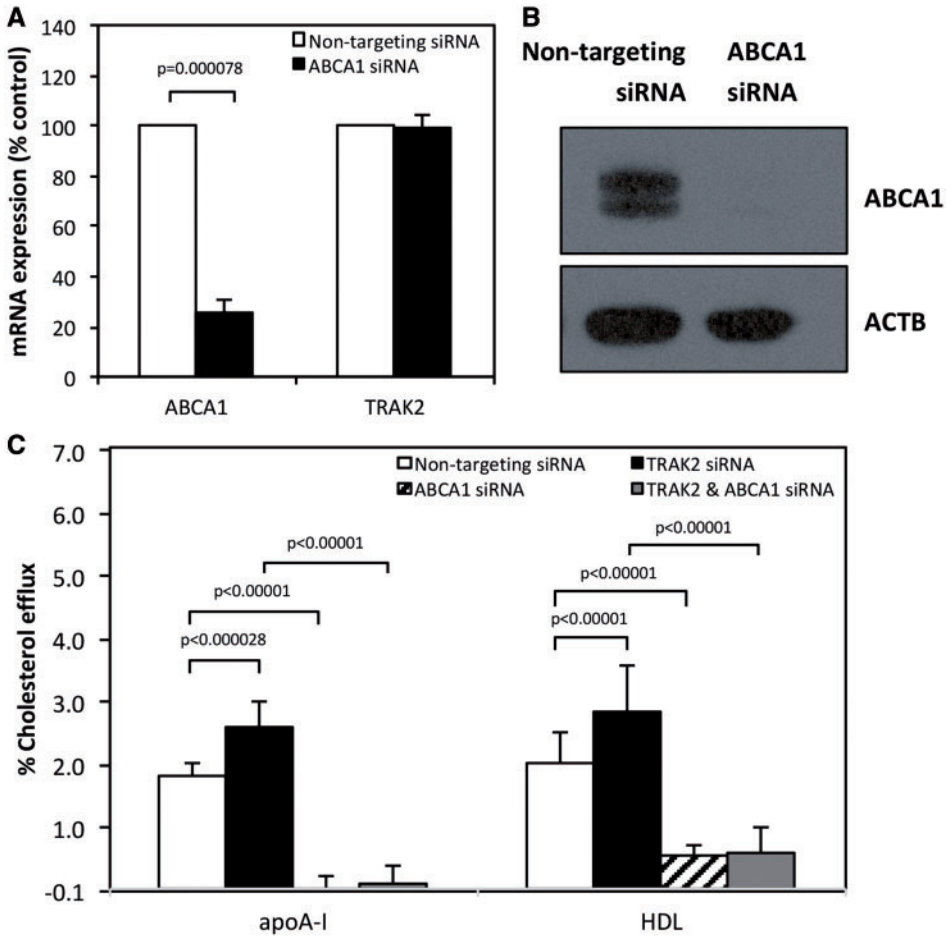

Figure 3 TRAK2's effect on cholesterol efflux is dependent on ABCA1. (*A*) siRNA-mediated gene knockdown of ABCA1 significantly decreased *ABCA1* mRNA expression relative to non-targeting control (NTGC) siRNA in HepG2 cells. *TRAK2* mRNA expression was not affected by knockdown of ABCA1; $n = 3$. (*B*) siRNA-mediated knockdown of ABCA1 ablated protein expression in HepG2 cells. (*C*) TRAK2 siRNA treatment significantly increased cholesterol efflux while transfection with ABCA1 siRNA, or with both TRAK2 and ABCA1 siRNA, ablated cholesterol efflux to apoA-I and to HDL relative to NTGC siRNA in HepG2 cells; $n = 3$. Data are represented as the mean ± standard deviation of n independent experiments.

organization was observed (see Supplementary material online, *Figure S7*).

In summary, these data support that TRAK2 mediates its effect on cholesterol efflux through regulating the expression of *ABCA1*.

TRAK2 knockdown increases LXR binding to LXR response element regions within the *ABCA1* promoter

LXRs regulate *ABCA1* expression by binding to LXR response elements (LXR-RE) within the promoter region where they mediate activation and repression of transcription.[20] LXR agonists promote transcription by increasing LXR binding to the promoter and causing the release of co-repressors.[20,21] To determine if TRAK2 was regulating ABCA1 expression by modulating LXR-mediated transcription of *ABCA1*, we utilized a ChIP assay to evaluate LXR-α binding at four LXR-RE regions within the *ABCA1* promoter. Treatment of HepG2 cells with LXR agonist TO901317 significantly increased LXR-α binding at the *ABCA1* promoter within Regions 2 and 3 by 150% (*Figure 5*, $P = 0.032$ and $P = 0.00025$, respectively), consistent with their location near the *ABCA1* transcription start site. No LXR-α binding was detectable at Region 1 within the 5' region of intron 1, while a trend to increase LXR-α binding was observed at Region 4 which was the most 5' distal sequence (see Supplementary material online, *Figure S8*, $P = 0.145$). TRAK2 siRNA-mediated knockdown had a larger effect on LXR-α binding than TO901317 alone, significantly increasing LXR-α binding at the *ABCA1* promoter within Regions 2 and 3 by ~450% (*Figure 5*, $P = 0.0496$ and $P = 0.038$, respectively), while a non-significant trend to increase LXR-α binding was observed at Region 4 (see Supplementary material online, *Figure S8*, $P = 0.38$). Interestingly, treatment of cells transfected with TRAK2 siRNA with TO901317 abrogated the effect of TRAK2 knockdown on LXR-α binding, where

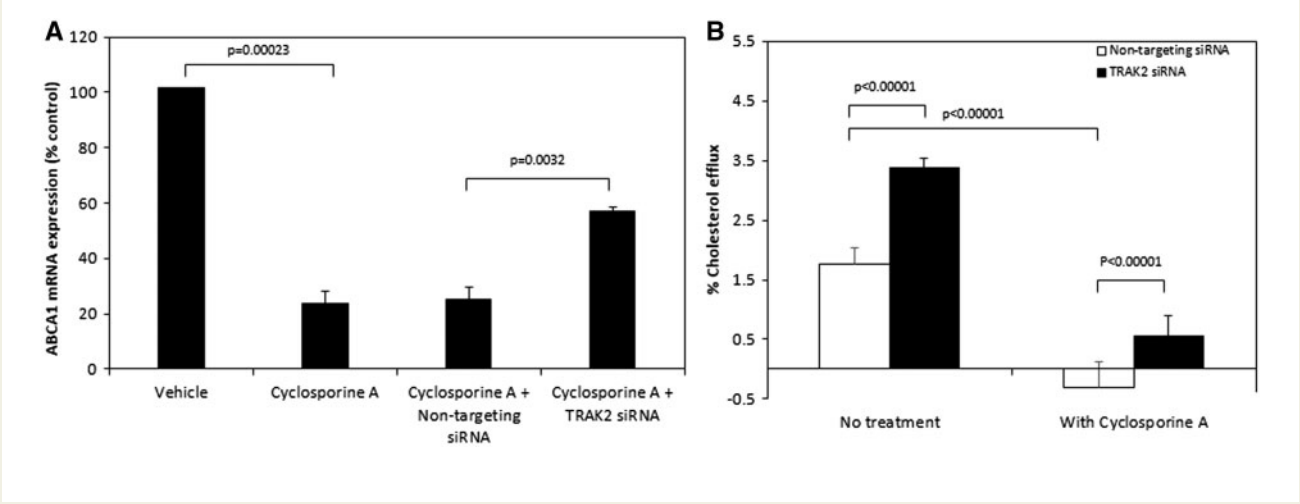

Figure 4 The effect of Cyclosporine A (CsA) treatment on cholesterol efflux and ABCA1 gene expression. (*A*) *ABCA1* mRNA expression was significantly reduced in HepG2 cells treated with CsA relative to vehicle control. TRAK2 knockdown significantly increased the expression of *ABCA1* in cells treated with CsA relative to non-targeting control (NTGC) siRNA; $n = 2$. (*B*) CsA treatment suppressed cholesterol efflux to apoA-I in HepG2 cells transfected with NTGC siRNA. TRAK2 siRNA treatment significantly increased cholesterol efflux to apoA-I relative to NTGC siRNA; $n = 3$. Data are represented as the mean ± standard deviation of *n* independent experiments.

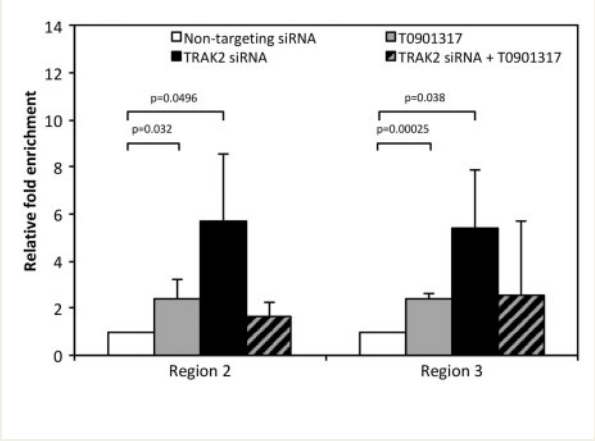

Figure 5 TRAK2 siRNA-mediated knockdown increases LXR-α binding to the *ABCA1* promoter in HepG2 cells. TO901317 treatment and siRNA-mediated knockdown of TRAK2 treatment significantly increased LXR-α binding to LXR-α response element regions within the *ABCA1* promoter relative to control. Treatment with TO901317 diminished the effect of TRAK2 knockdown. Data are represented as the mean ± standard deviation of three independent experiments.

an increase comparable to cells treated with TO901317 alone was observed (*Figure 5*). The mRNA expression of LXRα and several other transcription factors involved in activating or repressing *ABCA1* transcription (RXRα, PPARα, SREBP2, and ZNF202)[20] was examined in cells transfected with siRNA, however TRAK2 knockdown had no significant effect on the expression of these genes (see Supplementary material online, *Figure S9*). Overall, these data indicate that TRAK2 regulates ABCA1 expression and cholesterol efflux through an effect on LXR-mediated transcription of *ABCA1*.

Discussion

Given the disappointing clinical trial outcomes for HDL-C raising therapies,[8] it is evident that our understanding of HDL metabolism is incomplete. To address this, we utilized an innovative joint analysis of human metabolic, GWA and transcriptomic data to identify novel regulators of *in vivo* plasma HDL-C concentration, and reported *TRAK2* as a highly ranking candidate gene for further investigation.[10] TRAK2 belongs to the TRAK protein family which are best characterized as motor-adaptor proteins that link mitochondria to microtubule-based motors kinesin and dynein.[11,12] Since TRAK2 had not been previously implicated in HDL or cholesterol metabolism, the molecular basis of this association with HDL-C was unknown. Here, for the first time, we demonstrate that TRAK2 is an important contributor to the maintenance of HDL-C levels, HDL particle diameter, and HDL subpopulation levels. We further characterize a biological role for TRAK2 as a regulator of macrophage cholesterol efflux and hepatic HDL biogenesis, demonstrating that TRAK2 siRNA-mediated knockdown significantly increases LXR-α binding at the *ABCA1* promoter, *ABCA1* expression, and cholesterol efflux (*Figure 6*). Our data thereby validate our initial *in silico* approach for identifying novel regulators of *in vivo* HDL metabolism, and furthermore demonstrates how GWA data can be effectively leveraged to identify novel regulators of multifactorial traits and disorders.

Expanding on our earlier study,[10] we have shown that TRAK2 is significantly and negatively correlated with *in vivo* HDL-C and HDL particle diameter, and provided additional evidence supporting a

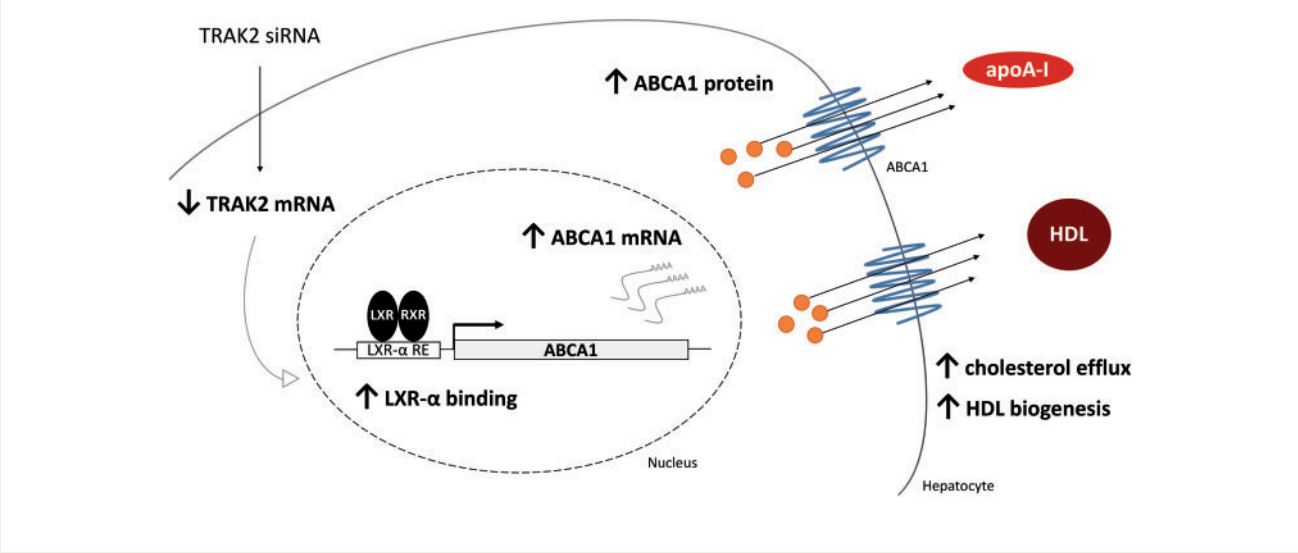

Figure 6 Summary of key findings. TRAK2 siRNA-mediated knockdown significantly increases LXR-α binding at LXR response elements (LXR RE) in the *ABCA1* promoter, which increases ABCA1 mRNA and protein expression. This leads to an increase in cholesterol efflux to both apoA-I and isolated HDL particles. Therefore low-TRAK2 levels can promote both HDL biogenesis and RCT.

genetic association between *TRAK2* and plasma HDL-C concentration. Fractionation of the major HDL subpopulations further revealed a significant negative correlation between *TRAK2* expression and the abundance of larger HDL2b particles, while a significant positive correlation with smaller HDL3a and HDL3b species was observed. The basis of the shift towards increased HDL2b and decreased HDL3a and HDL3b particle abundance in conditions of low-TRAK2 expression is unclear, though it is consistent with recent observations of a slower clearance rate of large HDL2b particles and a faster clearance of small HDL3a and HDL3b particles in a setting of high HDL-C levels.[22] TRAK2's role in *in vivo* HDL homeostasis is therefore broader than an association with HDL-C concentration.

We have demonstrated that TRAK2 siRNA knockdown significantly promotes cholesterol efflux from human macrophage and liver cells. The capacity of TRAK2 knockdown to significantly increase cholesterol efflux and HDL production *in vitro* is consistent with the observed negative *in vivo* correlation between *TRAK2* mRNA and human plasma HDL-C concentration and HDL particle diameter. The absence of an effect of TRAK2 knockdown on cholesterol efflux in human intestinal FHs74Int cells does not contradict the *in vivo* association between TRAK2 and HDL-C abundance, but rather indicates that TRAK2's role in HDL biogenesis is specifically hepatic. Our data therefore signify the role of TRAK2 in HDL biogenesis, given the liver produces the majority of plasma HDL particles.[23] Evidence of increased apoA-I expression in HepG2 cells transfected with TRAK2 siRNA suggests that TRAK2's role in hepatic HDL biogenesis may be multifaceted.

While cholesterol efflux from macrophages may not significantly contribute to total HDL-C levels,[24] it is an important atheroprotective function of HDL.[2] We demonstrated that siRNA-mediated knockdown of TRAK2 significantly increases cholesterol efflux from human macrophage THP-1 cells, highlighting TRAK2 as a potential pharmacological target which could promote the atheroprotective pathway of RCT both by upregulating cholesterol removal from macrophages and by increasing HDL biogenesis. Indeed the ability to promote ABCA1-mediated efflux may be a key determinant of macrophage cholesterol efflux.[25]

We have demonstrated that TRAK2 knockdown promotes cholesterol efflux by increasing ABCA1 mRNA and protein levels, which is supported by several lines of evidence confirming that the effect of TRAK2 knockdown on efflux is suppressed when ABCA1 expression is diminished. Although a modest but significant reduction in *SCARB1* mRNA was observed, it did not appear to affect the protein abundance of SR-BI and therefore it is unlikely to be relevant. Our data indicate that TRAK2 knockdown significantly increases LXR-α binding at the *ABCA1* promoter and establishes that TRAK2 regulates ABCA1 expression by modulating the LXR-mediated transcription of this gene. LXR agonists increase LXR binding to target promoters by causing the release of co-repressors.[20,21] Therefore, the suppression of the effect of TRAK2 knockdown on LXR-α binding by LXR agonist TO901317 suggests that TRAK2 knockdown, like TO901317, may decrease LXR co-repressor levels at the *ABCA1* promoter. The lack of a decrease in ABCA1 expression when TRAK2 is overexpressed suggests that TRAK2 may repress LXR by an unknown mechanism. The role of TRAK2 in LXR co-repressor binding therefore requires further investigation. LXRs are involved in regulating the expression of several genes associated with HDL-C metabolism, including *ABCG1*.[4,5] Further studies are needed to determine the effect of TRAK2 knockdown on other LXR target genes, towards clarifying whether the results reflect a general increase in LXR activity or a ABCA1-specific mechanism. The absence of an effect of TRAK2 knockdown on the expression of LXR-target gene *ABCG1*, and on LXRα mRNA levels, suggests that there may be specificity in the activation of LXR-mediated transcription of *ABCA1* by TRAK2.

Cholesterol accumulation in the late endosomal network enhanced the effect of TRAK2 on *ABCA1* expression, with U18666A treatment doubling the increase in *ABCA1* expression elicited by TRAK2 knockdown. This indicates that the regulation of *ABCA1* expression by TRAK2 is sensitive to conditions of intracellular cholesterol accumulation and perturbed efflux, which is consistent with an effect of TRAK2 knockdown on LXR-mediated ABCA1 transcription given the role of LXRs in responding to intracellular cholesterol content.[4] TRAK2 has previously been described as a regulator of endosomal trafficking,[19] however we could not identify any striking differences in early or late endosomal organization in HepG2 cells transfected with TRAK2 siRNA relative to controls. Whether TRAK2's involvement in LXR-mediated ABCA1 expression and cholesterol efflux is related to its trafficking function is currently unknown and will be of keen interest to determine.

Here we have not only validated our genetic findings, but we have also characterized TRAK2 as a novel regulator of LXR-mediated *ABCA1* expression, macrophage cholesterol efflux and hepatic HDL biogenesis. This study therefore contributes new knowledge about HDL metabolism and functionality. Current approaches to improve CVD have focused on the activation of LXRs to increase ABCA1 expression and cholesterol transport, which highlights the potential for TRAK2 to form part of a therapeutic strategy. Indeed, the negative correlation between *TRAK2* mRNA levels and *ABCA1* expression, cholesterol efflux, and HDL-C concentration makes TRAK2 an attractive therapeutic target for the treatment of CVD and associated disorders.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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References

1. Kingwell BA, Chapman MJ, Kontush A, Miller NE. HDL-targeted therapies: progress, failures and future. *Nat Rev Drug Discov* 2014;**13**:445–464.
2. Khera AV, Cuchel M, de la Llera-Moya M, Rodrigues A, Burke MF, Jafri K, French BC, Phillips JA, Mucksavage ML, Wilensky RL, Mohler ER, Rothblat GH, Rader DJ. Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. *N Engl J Med* 2011;**364**:127–135.
3. Cuchel M, Rader DJ. Macrophage reverse cholesterol transport: key to the regression of atherosclerosis? *Circulation* 2006;**113**:2548–2555.
4. Zhao C, Dahlman-Wright K. Liver X receptor in cholesterol metabolism. *J Endocrinol* 2010;**204**:233–240.
5. Sabol SL, Brewer HB Jr, Santamarina-Fojo S. The human ABCG1 gene: identification of LXR response elements that modulate expression in macrophages and liver. *J Lipid Res* 2005;**46**:2151–2167.
6. Costet P, Luo Y, Wang N, Tall AR. Sterol-dependent transactivation of the ABC1 promoter by the liver X receptor/retinoid X receptor. *J Biol Chem* 2000;**275**:28240–28245.
7. Miller GJ, Miller NE. Plasma-high-density-lipoprotein concentration and development of ischaemic heart-disease. *Lancet* 1975;**1**:16–19.
8. Toth PP, Barter PJ, Rosenson RS, Boden WE, Chapman MJ, Cuchel M, D'agostino RB Sr, Davidson MH, Davidson WS, Heinecke JW, Karas RH, Kontush A, Krauss RM, Miller M, Rader DJ. High-density lipoproteins: a consensus statement from the national lipid association. *J Clin Lipidol* 2013;**7**:484–525.
9. Tuteja S, Rader DJ. High-density lipoproteins in the prevention of cardiovascular disease: changing the paradigm. *Clin Pharmacol Ther* 2014;**96**:48–56.
10. Charlesworth JC, Peralta JM, Drigalenko E, Goring HH, Almasy L, Dyer TD, Blangero J. Toward the identification of causal genes in complex diseases: a gene-centric joint test of significance combining genomic and transcriptomic data. *BMC Proc* 2009;**3(Suppl 7)**:S92.
11. van Spronsen M, Mikhaylova M, Lipka J, Schlager MA, van den Heuvel DJ, Kuijpers M, Wulf PS, Keijzer N, Demmers J, Kapitein LC, Jaarsma D, Gerritsen HC, Akhmanova A, Hoogenraad CC. Trak/Milton motor-adaptor proteins steer mitochondrial trafficking to axons and dendrites. *Neuron* 2013;**77**:485–502.
12. Brickley K, Stephenson FA. Trafficking kinesin protein (trak)-mediated transport of mitochondria in axons of hippocampal neurons. *J Biol Chem* 2011;**286**:18079–18092.
13. Goring HH, Curran JE, Johnson MP, Dyer TD, Charlesworth J, Cole SA, Jowett JB, Abraham LJ, Rainwater DL, Comuzzie AG, Mahaney MC, Almasy L, MacCluer JW, Kissebah AH, Collier GR, Moses EK, Blangero J. Discovery of expression QTLs using large-scale transcriptional profiling in human lymphocytes. *Nat Genet* 2007;**39**:1208–1216.
14. Consortium GT. The genotype-tissue expression (GTEx) project. *Nat Genet* 2013;**45**:580–585.
15. Rosenson RS, Brewer HB Jr, Chapman MJ, Fazio S, Hussain MM, Kontush A, Krauss RM, Otvos JD, Remaley AT, Schaefer EJ. HDL measures, particle heterogeneity, proposed nomenclature, and relation to atherosclerotic cardiovascular events. *Clin Chem* 2011;**57**:392–410.
16. Sviridov D, Fidge N. Pathway of cholesterol efflux from human hepatoma cells. *Biochim Biophys Acta* 1995;**1256**:210–220.
17. Le Goff W, Peng DQ, Settle M, Brubaker G, Morton RE, Smith JD. Cyclosporin A traps ABCA1 at the plasma membrane and inhibits ABCA1-mediated lipid efflux to apolipoprotein A-I. *Arterioscler Thromb Vasc Biol* 2004;**24**:2155–2161.
18. Neufeld EB, Stonik JA, Demosky SJ Jr, Knapper CL, Combs CA, Cooney A, Comly M, Dwyer N, Blanchette-Mackie J, Remaley AT, Santamarina-Fojo S, Brewer HB Jr. The ABCA1 transporter modulates late endocytic trafficking: insights from the correction of the genetic defect in Tangier disease. *J Biol Chem* 2004;**279**:15571–15578.
19. Kirk E, Chin LS, Li L. GRIF1 binds Hrs and is a new regulator of endosomal trafficking. *J Cell Sci* 2006;**119**:4689–4701.
20. Schmitz G, Langmann T. Transcriptional regulatory networks in lipid metabolism control abca1 expression. *Biochim Biophys Acta* 2005;**1735**:1–19.
21. Wagner BL, Valledor AF, Shao G, Daige CL, Bischoff ED, Petrowski M, Jepsen K, Baek SH, Heyman RA, Rosenfeld MG, Schulman IG, Glass CK. Promoter-specific roles for liver X receptor/corepressor complexes in the regulation of ABCA1 and SREBP1 gene expression. *Mol Cell Biol* 2003;**23**:5780–5789.
22. Mendivil CO, Furtado J, Morton AM, Wang L, Sacks FM. Novel pathways of apolipoprotein A-I metabolism in high-density lipoprotein of different sizes in humans. *Arterioscler Thromb Vasc Biol* 2016;**36**:156–165.
23. Timmins JM, Lee JY, Boudyguina E, Kluckman KD, Brunham LR, Mulya A, Gebre AK, Coutinho JM, Colvin PL, Smith TL, Hayden MR, Maeda N, Parks JS. Targeted inactivation of hepatic ABCA1 causes profound hypoalphalipoproteinemia and kidney hypercatabolism of apoA-I. *J Clin Invest* 2005;**115**:1333–1342.
24. Zhu X, Lee JY, Timmins JM, Brown JM, Boudyguina E, Mulya A, Gebre AK, Willingham MC, Hiltbold EM, Mishra N, Maeda N, Parks JS. Increased cellular free cholesterol in macrophage-specific ABCA1 knock-out mice enhances pro-inflammatory response of macrophages. *J Biol Chem* 2008;**283**:22930–22941.
25. de la Llera-Moya M, Drazul-Schrader D, Asztalos BF, Cuchel M, Rader DJ, Rothblat GH. The ability to promote efflux via abca1 determines the capacity of serum specimens with similar high-density lipoprotein cholesterol to remove cholesterol from macrophages. *Arterioscler Thromb Vasc Biol* 2010;**30**:796–801.