Genome-wide RNAi screen reveals ALK1 mediates LDL uptake and transcytosis in endothelial cells

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In humans and animals lacking functional LDL receptor (LDLR), LDL from plasma still readily traverses the endothelium. To identify the pathways of LDL uptake, a genome-wide RNAi screen was performed in endothelial cells and cross-referenced with GWAS-data sets. Here we show that the activin-like kinase 1 (ALK1) mediates LDL uptake into endothelial cells. ALK1 binds LDL with lower affinity than LDLR and saturates only at hypercholesterolemic concentrations. ALK1 mediates uptake of LDL into endothelial cells via an unusual endocytic pathway that diverts the ligand from lysosomal degradation and promotes LDL transcytosis. The endothelium-specific genetic ablation of Alk1 in Ldlr-KO animals leads to less LDL uptake into the aortic endothelium, showing its physiological role in endothelial lipoprotein metabolism. In summary, identification of pathways mediating LDLR-independent uptake of LDL may provide unique opportunities to block the initiation of LDL accumulation in the vessel wall or augment hepatic LDLR-dependent clearance of LDL.

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**Atherosclerotic cardiovascular disease** is the leading cause of death worldwide. The development of atherosclerosis is triggered by the subendothelial retention of plasma-derived apoB-lipoproteins, particularly LDL and apolipoprotein-B (apoB)-containing remnants. The most effective therapy to date that reduces atherosclerotic cardiovascular disease, namely lowering plasma LDL levels, works by decreasing circulating apoB containing lipoprotein levels, thereby reducing the likelihood that these particles will enter and become retained within the arterial wall. Despite this well appreciated series of events leading to disease and effective therapies lowering plasma LDL levels, the molecular mechanisms of how LDL is transported into and across the endothelium have not been elucidated.

LDL in the blood has to enter the endothelium and cross it to reach the subendothelial area, where it is retained and accumulates over time. Electron microscopy studies have demonstrated that physiological levels of LDL particles can be internalized by two pathways; an LDL receptor (LDLR) dependent and LDLR independent pathways. The LDLR-mediated pathway promotes LDL degradation and is downregulated at the higher concentrations of LDL, while the latter pathway is enhanced with hypercholesterolemic concentrations of LDL. Studies have shown that at least 50% of the LDL that is endocytosed by the endothelium traverses the cells to reach the basolateral side via an unknown active transport mechanism and an LDLR independent initial route of LDL permeation into the artery wall has been previously described. A recent paper has described that reducing ApoB containing lipoproteins in an established model of atherosclerosis rapidly reduces LDL permeation into the vessel wall. Interestingly the flux of LDL is separable from the total content of LDL in vessel wall and this improved barrier function precedes plaque regression. Thus, the regulated entry of LDL into the vessel wall is essential for both the formation and regression of atherosomas.

With this background in mind, we sought to elucidate the genes required for native LDL uptake into endothelial cells using a genome-wide RNAi approach to target over 18,000 genes followed by high content confocal imaging of fluorescent DiI-LDL uptake. Primary gene hits were further analysed in secondary screens to assess broad effects on endocytosis and sterol sensing permitting the identification of 34 high confidence hits, three of which are unique to the endothelium. The gene hits were also cross-referenced against publicly available genome-wide association studies (GWAS).

**Results**

**Genome-wide RNAi screen in endothelial cells.** The uptake, transfer and retention of LDL particles across the endothelial layer of blood vessels is considered a primary mechanism to initiate atherogenesis. However, since the LDLR is typically occupied and downregulated when plasma lipids are elevated, we undertook a genome-wide RNAi screen to identify genes involved in native LDL uptake independent of LDLR activity. Considering the importance of genetic stability and reproducibility required for a screen of this calibre, the human endothelial cell line, EA.hy926 (ref. 11) was used and cultured under conditions where endogenous LDLR had been downregulated by excess of the ligand LDL. In the initial screen, run in triplicate over a 3 months period, cells were transfected with a Dharmaco short interfering RNA (siRNA) library containing four pooled siRNAs/gene to silence 18,119 genes in the human genome (Supplementary Data set 1). Transfected cells were then incubated with excess human LDL (25 μg ml⁻¹) overnight to downregulate LDLR overnight, before the uptake of fluorescently labelled LDL (DiI-LDL) was examined after 60 min using a 384 well confocal microscope. The results from the screen were fit to an expected inverse sigmoidal robust z-score distribution (Fig. 1b), indicating that gene knockdown either increased or decreased DiI-LDL uptake and demonstrated a high level of reproducibility between different data sets (Fig. 1c).

As seen in Fig. 1a, silencing of 887 genes showed an effect on DiI-LDL uptake with a robust z-score ≤ −2.5. A manual, computer-assisted data clearance algorithm removed promiscuous genes (that typically show up in various screens), toxic genes, and artefacts by visual inspection of the confocal images from individual hits. The data were mined to include cell surface molecules and novel gene products, but to exclude genes for transcription factors, obvious components of the endocytic machinery and sterol regulated genes. After inspection of individual hits, a final set of 140 genes (Supplementary Data set 2) was re-screened using four individual siRNAs per gene resulting in the confirmation of 55 genes (with ≥ 2 siRNAs/gene showing ≥50% reduction of DiI-LDL uptake) required for DiI-LDL uptake (Fig. 1d,f). To identify pathways specific for LDL and not classical cargo molecules, a secondary screen examining the uptake of transferrin-fluorescein isothiocyanate (FITC), a marker for clathrin-mediated endocytosis was performed. The silencing of 35/55 genes did not affect the uptake of transferrin (Fig. 1e). Finally, the contribution of LDLR, in conjunction with the newly identified genes, was tested using cells stably expressing short hairpin RNAs (shRNAs) against LDLR (Supplementary Fig. 1) for LDLR messenger RNA and protein levels and 34 of these genes reduced DiI-LDL uptake independent of LDLR levels. Furthermore, since the original screen was conducted in an endothelial line, the 34 hits identified were re-tested in primary cultures of human umbilical vein endothelial cells (HUVEC) and all 34 hits were re-confirmed. Analysis of the 34 genes with Ingenuity Pathway Analysis (Fig. 1g, Supplementary Fig. 2 and Supplementary Table 1) showed that 19 hits cluster in metabolic/neurological pathways and 14 belong to lipid/carbohydrate metabolic pathways and only three genes were uniquely expressed in endothelial cells. Analysis of publically available GWAS-data sets revealed an association for 14 gene hits in regard to cardiovascular traits and/or lipids (Supplementary Fig. 3 and Supplementary Data set 3). ACVR1L, ANGPT4 and GPR182 fulfilled all the criteria of the follow-up screen (Fig. 1a and Supplementary Fig. 3). Since ANGPT4 is not well characterized as a ligand and GPR182 is an orphan receptor, the initial follow-up focuses on ALK1 as an LDL-binding protein mediating LDL uptake and transcytosis.

**Specificity of ALK1 deficiency for apoB containing lipoproteins.** ALK1 is a TGF-β-type 1 receptor that binds bone morphogenetic proteins (BMP) −9 and −10 ligands with high affinity. The receptor is highly expressed in primary human endothelial cells compared primary human hepatocytes (Supplementary Fig. 4). To examine how this receptor may regulate LDL uptake, in depth analysis of ALK1 was undertaken in a variety of systems. Knockdown of ALK1 reduced transcript levels in human endothelial cells (Fig. 2a) and mouse lung endothelial cells (MLEC; Supplementary Fig. 5a). All four individual shRNAs against human ALK1 from the genome-wide RNAi screen were analysed for their knockdown efficiency, showing that shRNA 06 led to the strongest inhibition (Supplementary Fig. 5b).
Since several commercially available antibodies do not detect ALK1 protein specifically, we used BMP9 signalling to SMAD1/5 as a surrogate readout for the loss of ALK1 function. Indeed, knockdown of ALK1 impaired BMP9 induction of canonical SMAD 1/5 phosphorylation in HUVECs (Fig. 2b) and MLEC (Supplementary Fig. 5c). Moreover, ALK1 silencing in EA.hy926 cells, primary HUVEC and MLEC resulted in reduced uptake of DiI-LDL (Fig. 2c). To test the sufficiency of ALK1 for LDL uptake, MLEC were isolated from Acvrl1fl/fl, Ldlr−/− double knockout (KO) mice, immortalized with middle T antigen13 cultured in lipoprotein-deficient serum (LPDS) overnight to maximize LDL uptake and infected with adenovirus-expressing green fluorescent protein (AdGFP) as a control or adenviral Cre-recombinase (AdCre) to excise the Acvrl1 allele. The loss of LDLR markedly reduced 125I-LDL uptake into MLEC infected with AdGFP consistent with its known role of LDLR (Fig. 2d), however, AdCre-mediated excision of Acvrl1 (e) did not affect the uptake of FITC transferrin (scale bar, 50 μm). (f) Pie chart of 140 hits tested for DiI-LDL uptake inhibition in the follow-up screen, showing the number (and percentage) of active siRNAs for each hit. (g) Pathway clustering of the final 34 hits.

Figure 1 | Screen to identify pathways regulation LDL uptake. (a) Summary of the results from the RNAi screen. Results from genome-wide RNAi screen are red while follow-up screens are in blue. (b) Robust z-score-scores from the original screen. The inverse sigmoidal robust z-score-score distribution of genes indicates that genes can either increase or decrease Dil-LDL uptake and (c) shows the reproducibility between two individual sets of plates from the screen. (d) Representative image (scale bar, 50 μm.) of a gene knockdown of ALK1 resulting in less Dil-LDL uptake, whereas the loss of ALK1 (e) did not affect the uptake of FITC transferrin (scale bar, 50 μm). (f) Pie chart of 140 hits tested for DiI-LDL uptake inhibition in the follow-up screen, showing the number (and percentage) of active siRNAs for each hit. (g) Pathway clustering of the final 34 hits.
Chylomicrons or its remnants were not tested. It has been hypothesized that oxidized LDL (OxLDL) contributes to the development of atherosclerosis, and it is known that the biological behaviour and receptor recognition of OxLDL is significantly different from native LDL. The uptake of Dil-OxLDL is not affected by the knockdown of ALK1 (Fig. 2g). ALK1 is one of seven ALK receptors (ALK1-ALK7) and sequence alignment of this family reveals low homology in the amino-terminal/extracellular domains, but a high homology of the carboxy-terminal/intracellular domain. As the ligands bind to the amino-terminal domain of the ALK family we compared ALK1 and ALK2, which share 59% homology among the entire protein and just 23% for the extracellular domain. Whereas LDLR and ALK1 overexpression increases Dil-LDL uptake we could not detect an effect of ALK2 overexpression on Dil-LDL uptake (Fig. 2h). As the knockdown of ALK1 in endothelial cells results in less uptake of LDL, the

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ALK1 knockdown does not affect sterol sensing. As cholesterol homeostasis is crucial for cell growth and maintenance, cholesterol uptake, synthesis and metabolism, is largely regulated through sterol regulatory element-binding protein 2 (SREBP2) of sterol responsive genes. As seen in Fig. 3a, knockdown of dynamin 2 (DNM2) in EA.hy926 cells grown in complete media and serum, did not influence ACVR1L1 gene expression but upregulated the transcript levels of several SREBP2-dependent genes (LDLR, HMGCR, INSIG1 and PCSK9), whereas the loss of ACVR1L1 did not affect SREBP2-dependent gene expression. A further analysis was performed in EA.hy926 cells cultured in LPDS or LPDS containing LDL (25 µg ml⁻¹, Supplementary Fig. 6). As expected, the addition of LDL to LPDS resulted in downregulation of LDLR, HMGCR and INSIG1—all SREBP2-dependent genes. Importantly, the loss of DNM2, but not ACVR1L1, blunted the ability of LDL to reduce gene expression showing that ALK1 does not influence sterol sensing. Moreover, silencing of ACVR1L1 had no effect on the total levels of LDLR in cell lysates by western blotting (Fig. 3b) or on cell surface LDLR quantified by fluorescence-activated cell sorting (FACS) (Fig. 3c). Because the uptake of LDL by the LDLR results in its lysosomal degradation, we assessed the uptake and degradation of 125I-LDL in cells deficient in LDLR or ACVRL1. EA.hy926 were transfected with control, LDLR or ACVRL1 siRNAs, and then exposed to excess LDL (25 µg ml⁻¹) overnight. The next morning, cells were incubated with 125I-LDL and the uptake and degradation (after 4 h) was assessed. The net uptake of 125I-LDL in LDLR-transfected EA.hy926 cells (Fig. 3d) was lower than that in MLEC cultured in LPDS, Fig. 2d). Knockdown of ALK1 in LDLR-transfected EA.hy926 cells reduced 125I-LDL internalization, whereas knockdown of LDLR did not. However, the degradation of 125I-LDL, assessed by free 125I-tyrosine in the medium, was reduced by the loss of LDLR, but not the loss of ALK1. The data suggests that the suppression of LDLR by pretreatment with LDL is incomplete, and so the subsequent addition of siRNA against LDLR further suppressed LDL internalization and degradation via the LDLR. As ALK1-mediated LDL uptake did not affect sterol sensing or LDL degradation, the pool of non-esterified, free cholesterol was analysed using Filipin-III staining (Fig. 3e). The knockdown of ACVRL1, LDLR or DNM2 did not increase the pool of free cholesterol, whereas both positive controls (U186866 (ref. 16) and NPC2 siRNA) did. These data demonstrate that ALK1 facilitates LDL uptake but does not target LDLR for degradation.

ALK1 increases LDL uptake independent of its kinase activity. To examine the sufficiency of ALK1 for LDL uptake, rescue and gain-of-function experiments were performed. As shown in Fig. 4a, DiI-LDL uptake is significantly decreased in endothelial cells after knockdown of ALK1, an effect rescued, in a dose-dependent manner (0–100 MOI of adenovirus), by the expression of a non-adenovirus encoding ALK1-GFP (AdALK1). Moreover, infection of AdALK1-GFP into Ldlr-KO mouse lung fibroblasts (MEF) also dose-dependently increased DiI-LDL uptake (Fig. 4b), showing again that ALK1 mediates LDL uptake independently of the LDLR.

To examine if the kinase activity of ALK1 contributed to its ability to promote LDL uptake, GFP (negative control), wild-type (ALK1wt), a constitutively active mutant (Q201D; ALK1ca) or an inactive variant (R374Q; ALK1so) of ALK1 were expressed in HeLa cells, which lack ALK1. In ALK1wt transfected cells, BMP9 (10 ng ml⁻¹) stimulated the phosphorylation of SMAD 1/5 (Fig. 4c) an effect augmented in ALK1ca expressing cells and diminished in ALK1so cells. Expression of all three ALK1 constructs increased DiI-LDL uptake compared with GFP in HeLa cells, but no difference among these three variants could be detected (Fig. 4d), showing that the uptake of LDL is independent of ALK1 kinase activity. As a positive control, LDLR-GFP also increased DiI-LDL uptake. Moreover, in HUVEC, activation of ALK1 with BMP9, neutralization of BMP9 with soluble ALK1 ectodomain (400 ng ml⁻¹, tenfold molar excess over BMP9) or the pharmacological ALK1-inhibitor (LDN193189, 50 nM) resulted in the expected effect on p-SMAD 1/5 levels (Fig. 4e), but all three conditions did not affect the uptake of DiI-LDL (Fig. 4f).

LDL binds to ALK1 directly. To examine novel functions of ALK1 that could mediate LDL uptake, we used several approaches. First, LDLR-KO MEF were infected with adenoviral constructs expressing GFP (AdGFP) or ALK1-GFP (AdALK1-GFP) and the cell surface binding of 125I-LDL was examined. In ALK1-GFP transfected LDLR-KO MEF, the cell surface binding of 125I-LDL was enhanced in these cells by 30% to 67 ± 3 ng LDL mg⁻¹−1 with a K_d of 26 ± 3 µg ml⁻¹ (Fig. 5a). Next to directly examine protein–protein interactions between LDL and ALK1, surface plasmon resonance (SPR) was used with LDL immobilized on the chip and purified fragments of the ectodomains of LDLR and ALK1 were used as analytes of 125I-LDL. As seen in Fig. 5b, the specific binding of LDLRecto and ALK1ecto was clear, with an apparent K_d of 7 and 200 nM for binding to LDLR and ALK1, respectively. To test if LDLR and ALK1 compete for binding to immobilized LDL, LDLR-KO pre-bound with ALK1 or LDLR ectodomains followed by the addition of the other ectodomain. But neither the binding of LDLRecto was altered when the LDL surface was pretreated with

**Figure 2 | Validation of ALK1 in mediating LDL uptake into endothelium.** (a) Quantitative PCR analysis for the knockdown efficiency of the ALK1 siRNA in human endothelial cells (HUVEC). Data represent the mean ± s.e.m. and are representative of three experiments in duplicate. *P* < 0.05, Student’s *t*-test. (b) Western blot analysis showing the knockdown efficiency of the ACVR1L1 siRNA in HUVEC based on the BMP9 (10 ng ml⁻¹) induced phosphorylation of canonical SMAD 1/5 phosphorylation. A non-cropped western blot for this experiment can be found in Supplementary Fig. 9a. (c) DiI-LDL uptake was reduced in various human (EA.hy926 and HUVEC) and mouse (MLEC) endothelial cells treated with ACVR1L1/ACVR1l siRNA. Scale bar, 50 µm. (d) 125I-LDL uptake into WT and Acvr1l/Fl/Fl/ldlr⁻⁻/⁻ MLEC. Acvr1l/Fl/Fl/ldlr⁻⁻/⁻ MLEC were infected with AdGFP (control) or AdCre/GFP, then the uptake (includes bound LDL) of 125I-LDL was examined. Cells were kept in LPDS overnight before uptake studies. Data represent the mean ± s.e.m. and are representative of three experiments. *P* < 0.05, Student’s *t*-test. (e) Cells were treated with control siRNA, LDLR siRNA or ACVRL1 siRNA and placed into regular media, washed and exposed to increased concentrations of DiI-LDL. Data represent the mean ± s.e.m. and are representative of three experiments. *P* < 0.05, Student’s *t*-test. (f) Uptake analysis of DiI-HDL, -LDL and -VLDL (2.5 µg ml⁻¹) into endothelial cells treated with control siRNA and ACVR1L1 siRNA. Data represent the mean ± s.e.m. and are representative of three experiments. *P* < 0.05, Student’s *t*-test. (g) Uptake of oxidized LDL (2.5 µg ml⁻¹) into EA.hy926 cells treated with control siRNA and ACVR1L1 siRNA cultured overnight in LPDS media supplemented with 25 µg ml⁻¹ LDL. Data represent the mean ± s.e.m. and are representative of three experiments. *P* < 0.05, Student’s *t*-test. (i) Uptake of DiI-LDL in EA.hy926 cells in the presence of increasing concentrations of ALK1-Fc was measured. Addition of 10⁻⁴ M ALK1-Fc is equimolar to DiI-LDL (500 ng ml⁻¹). Data represent the mean ± s.e.m. and are representative of three experiments. *P* < 0.05, Student’s *t*-test.
Figure 3 | ALK1 deficiency does not affect sterol sensing in the endothelium. (a) Quantitative PCR analysis of SREBP2-dependent genes after knockdown of DNM2 or ACVRL1. The loss of DNM2 increases SREBP2-dependent gene expression, whereas the loss of ALK1 does not. Data represent the mean ± s.e.m. and are representative of three experiments in duplicates. *P < 0.05, Student’s t-test. (b) Western blot analysis of the BMP9 (10 ng ml⁻¹) induced phosphorylation of SMAD 1/5. HUVEC were incubated in LPDS and exposed to BMP9 for 60 min. In lane 2, cells were pretreated with LDL (25 mg ml⁻¹) to downregulate LDLR. In lanes 3 and 4, ALK1 or LDLR was silenced with siRNA, respectively. A non-cropped western blot for this experiment can be found in Supplementary Fig. 9b. (c) The loss of ALK1 does not influence LDLR on the cell surface. Flow cytometric analysis of cell surface LDLR levels in endothelial cells treated with control, ACVRL1 or LDLR siRNAs. The Ab C7 was used for LDLR and IgG is an isotype control and data quantified in right panel. Data represent the mean ± s.e.m. and are representative of three experiments in triplicates. *P < 0.05, Student’s t-test. (d) 125I-LDL internalization and degradation in cells treated with control, ACVRL1 or LDLR siRNAs. EA.hy926 cells were pre-incubated with LDL (25 mg ml⁻¹) overnight and the internalization and degradation of 125I-LDL was after 4 h of incubation. ACVRL1 siRNA reduced internalization and had no effect on LDL degradation, whereas the LDLR siRNA (as a positive control) reduced LDL internalization and led to less degradation of LDL. Data represent the mean ± s.e.m. and are representative of three experiments in duplicates. *P < 0.05, Student’s t-test. (e) Loss of ALK1 does not increase cellular free cholesterol. Filipin-III staining was examined in endothelial cells treated with siRNAs for ACVRL1, LDLR, DNM2 and NPC2 siRNA or treated with U18666 to enhance free cholesterol. Scale bar, 10 μm. Data are representative of at least four experiments. ns, not significant.
ALK1ecto (Fig. 5c), nor was the binding of ALK1ecto altered when the LDL surface was pretreated with LDLRecto (Fig. 5d). These results indicate that LDLR and ALK1 bind to different sites on LDL, presumably on ApoB. Finally, ALK1ecto binding to LDL was analysed in the presence of equimolar concentrations of its cognate ligand BMP9. ALK1ecto binding was not perturbed by the presence of BMP9 (Fig. 5e) suggesting at least two distinct sites on the extracellular domain of ALK1 for binding each protein.

ALK1-LDL complex internalizes into perinuclear compartment.

To examine if the binding of LDL to ALK1 influences its internalization, imaging experiments were performed in LDLR-KO MEFs infected with AdALK1-GFP and incubated with Dil-LDL from 0–60 min. As seen in Fig. 6a, ALK1-GFP co-localizes in a time-dependent manner with Dil-LDL in the cell with a perinuclear accumulation at 60 min. At 60 min, ALK1-GFP localized in an early endosomal compartment marked by EEA1 (Fig. 6b). Analysis of the images using Pearson correlation (Fig. 6c) showed a significant increase in co-localization of ALK1-GFP and EEA1 after stimulation with either LDL or BMP9.

ALK1 mediates LDL transcytosis.

Collectively, the data suggests that LDL uptake can be mediated by the direct binding of LDL to ALK1 followed by its internalization via a non-degradative pathway raising the question if ALK1 can mediate LDL transcytosis across the endothelium. Recently, a novel method to assess LDL transcytosis using total internal reflectance microscopy (TIRF) was developed where the docking, fusion...
and transcytosis of LDL could be easily quantified in human coronary arterial endothelial cells (HCAEC) treated with PCSK9 to remove LDLR from the surface (see Supplementary Fig. 7). Using this assay, knockdown of ALK1 leads to significantly reduced transport of DiI-LDL from the apical to the basolateral membrane (Fig. 7b and Supplementary Movies 1 and 2), indicating that ALK1 mediates LDL transcytosis. Moreover, over-expression of ALK1, but not ALK2, increases LDL transcytosis (Fig. 7b). To complement the TIRF analysis, the transport of 125I-LDL through a confluent layer of HCAEC cultured on transwell chambers (0.4 µm) confirmed the effect of ALK1 on LDL transcytosis (Fig. 7c). Finally, 3D reconstruction of Z stacks of EA.hy926 cells transfected with control siRNA or ACVRL1 siRNA (Fig. 7d, top panel) demonstrate less DiI-LDL particles near the basolateral membrane (indicated by white arrows) in cells transfected with ALK1 siRNA and overexpression of ALK1 using adenoaviral transduction resulted in the opposite finding. EA.hy926 cells infected with AdALK1-GFP show more DiI-LDL particles near the basolateral side (Fig. 7d, bottom panel). These data firmly show that ALK1 mediates LDL transcytosis.

To test if the loss of ALK1 could influence LDL uptake into the vessel wall in vivo we moved to genetic mouse models. Previous work has shown that genetic ablation of Acvrl1fl/fl using an inducible global ROSA-Cre recombinase in mice leads to a rapid lethality (within 10 days) even when excised in adulthood 19. Similarly, the breeding of Acvrl1fl/fl mice to endothelial specific, tamoxifen inducible Cdh5-CreERt mice (Supplementary Fig. 8a–d) results in lethality after 10 days, as previously described20 precluding the ability to examine LDL uptake and
Figure 6 | Time-dependent internalization and co-localization of DiI-LDL and ALK1. (a) Ldr-KO MEFs were infected with ALK1-GFP, incubated in LPDS overnight and the time-dependent internalization of DiI-LDL examined at 37 °C. Cells were imaged for ALK1-GFP (green), DiI-LDL (red) and white reflecting co-localization of ALK1-GFP/DiI-LDL (Menders correlation). The internalization of DiI-LDL and its co-localization with DiI-LDL shows at 10–20 min and accumulates in a perinuclear compartment after 60 min. Scale bar, 10 μm. The data is representative for three independent experiments. (b) Analysis of ALK1-GFP localization in control (untreated), LDL (25 μg ml⁻¹) or BMP9 (10 ng ml⁻¹) treated EA.hy926 cells. Cells were infected for 48 h and transferred to LPDS for the remaining 24 h. Cells were treated as described for 1 h at 37 °C, fixed and imaged. Upper panels show original confocal laser scanning microscopy images (green, ALK1-GFP; red, EEA1; blue, nuclei). Lower panels show Menders correlation (green, ALK1-GFP alone; red, EEA1 alone; white, ALK1-GFP/EEA1 co-localized). Scale bar, 20 μm. The data is representative for three independent experiments. (c) Bar graph shows Pearson correlation of these three conditions. The result indicates a co-localization of ALK1 with the early endosome marker EEA1 upon stimulation with either LDL or BMP9 within 1 h. Data represent the mean ± s.e.m. and are representative of three experiments. *P<0.05, Student’s t-test.
atherosclerosis in adult mice. Therefore, the effects of endothelial ALK1 deletion on DiI-LDL uptake was analysed in adult Ldlr-KO mice after 5 days of consecutive tamoxifen injections. Under these conditions, mice appeared normal without obvious signs of haemorrhage and anaemia and BMP9 signalling was only slightly reduced in isolated EC (Supplementary Fig. 8e). Here the deletion of ALK1 in endothelium leads to significantly reduced DiI-LDL uptake into aortic endothelium as shown by *en face* confocal imaging of isolated blood vessels (Fig. 7e,f) indicating that ALK1 mediates endothelial LDL uptake *in vivo*.

**Discussion**

The central goal of this study was to identify the pathways of LDL uptake by the endothelium using an unbiased, genome-wide screening approach. The rationale for identifying new pathways for LDL transit is predicated on the observations that (1) the uptake, transport and retention of sub-endothelial LDL particles can occur in an LDLR independent manner and contribute to the initiation of atherosclerosis; and (2) hypercholesterolemic levels of LDL will downregulate LDLR by SREBP2-dependent suppression of genes controlling intracellular cholesterol levels and

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**Figure 7 | ALK1 mediates transcytosis and *in vivo* uptake of LDL.** (a) HCAECs were treated with PCSK9 to remove LDLR and transfected with either control siRNA or ACVRL1 siRNA. TIRF-based transcytosis assay was performed to measure the effect of ALK1 on LDL transcytosis. Each data point represents over five individual cells measured in six independent experiments. *P* < 0.05, Student’s *t*-test. (b) PCSK9 treated HCAECs were transfected with GFP, ALK1 or ALK2 to measure transcytosis using TIRF imaging. Each data point represents over 15 individual cells measured in three independent experiments. *P* < 0.05, Student’s *t*-test. (c) Transwell assay for LDL transcytosis in HCAECs incubated with 125I-labelled LDL. Data represent the mean ± s.e.m. and are representative of three independent experiments with two batches of 125I-LDL. *P* < 0.05, Student’s *t*-test. (d) Cross-section imaging of EA.hy926 cells transfected with either control siRNA or ALK1 siRNA or infected with adenovirus encoding GFP or ALK1-GFP (blue, nucleus; green, top panel: lectin/bottom panel: GFP, red, DiI-LDL). The data is representative for three independent experiments. (e) Representative *en face* images of DiI-LDL uptake into the inner curvature of the aortic arch of Ldlr-KO animals with or without endothelial expression of Acvrl1 (blue, nucleus; red, DiI-LDL). Scale bar, 50 µm. (f) Quantification of DiI-LDL uptake into the inner curvature endothelium of the aortic arch Ldlr-KO animals with or without endothelial expression of ALK1. Data represent the mean ± s.e.m. and are from 4 and 3 mice, respectively. *P* < 0.05, Student’s *t*-test. ns, not significant.
Previous work has described a transcytotic route of LDL affecting net LDL uptake in endothelial cells besides preventing the uptake of LDLR (Fig. 2b), indicating that these candidates are involved in the vesicular trafficking pathway (highlighted in red in Supplementary Fig. 3). About 20% of the genes/proteins identified in the screen cluster in the “Sterol-sensing” pathway, implicating ALK1 in atherosclerosis directly and indirectly. ALK1 mediates LDL uptake and transcytosis in an LDLR and sterol-sensing independent manner by the direct binding of LDLR to the ectodomain of ALK1. These results define the genetic pathways that promote LDL uptake into endothelium and offers alternative strategies to modify the uptake and transport of LDL from the blood into the vessel wall.

The initial screen was designed to identify pathways that mediate rapid (60 min) LDL uptake in endothelial cells previously exposed to an excess of LDL. Using this approach, we identified multiple genes implicated in the development of atherosclerosis or in regulating cellular cholesterol levels and/or LDL uptake. Cross-referencing the genes of the follow-up list with GWAS revealed that ARHGAP9, SDC1 and SLCO3A3 are linked to changes in plasma lipids, whereas ATP6V1C1 is associated with variation in the QT-interval.

CX3CR1 is a chemokine receptor and genetic or pharmacological inhibition of this receptor attenuates atherosclerosis in mice by reducing inflammatory responses22-24. Endonuclease G is thought to be involved in DNA fragmentation during apoptosis of aorta and a recent study has shown that carbamylated LDL stimulates endonuclease G (ref. 25). Leukaemia inhibiting factor (LIF) stimulates endonuclease G, which is a type II receptor. Leukemia inhibiting factor (LIF) is thought to be involved in DNA fragmentation during apoptosis of aorta25. Moreover, similar to findings using a genome wide, RNAi screen for genes regulating cholesterol metabolism in HeLa cells27, BHMT2 and CLN5 were identified as genes necessary for LDL uptake in endothelial cells. About 20% (7/34) of the genes/proteins identified in the screen cluster in the vesicular trafficking pathway (highlighted in red in Supplementary Fig. 2b), indicating that these candidates are involved in transcytosis of LDL, which could lead to another route affecting net LDL uptake in endothelial cells besides preventing the initial binding to cell membrane proteins such as LDLR or ALK1. Previous work has described a transcytotic route of LDL permeation across endothelial cells via a DMM2 dependent, caveolin-1 (C1V1)-dependent mechanism28,29. In mice deficient in CAV1, albumin and LDL uptake are reduced in isolated aortas and 125I-LDL and Dil-LDL uptake are reduced in vivo28,29, resulting in less atherosclerosis despite elevated lipids28,30. Since LDL can bind CD36 (ref. 31), the reduction in atherosclerosis in CAV1-deficient mice may be due to the reduced CD36 levels observed in CAV1-KO vessels30. Interestingly, ALK1 has been localized to caveolae and may also contribute to reduced LDL uptake in CAV1-knockout mice32. Most recently, the lipoprotein scavenger receptor for HDL, SR-B1, has been identified as a new candidate mediating the uptake and transcytosis of LDL in endothelial cells.18 However, the loss of SR-B1 enhances atherosclerosis, possibly by reducing hepatic HDL clearance53.

As our study focuses on the endothelium, the expression patterns of the 34 genes identified were analysed by Ingenuity Pathway Analysis and published work in MEDLINE (Supplementary Fig. 3). Only three genes are uniquely expressed in the endothelium: ACVR1I, ANGPT4 and GPR182. ANGPT4 is the least characterized member of the angiopoietin/TIE-receptor pathway and is the human ortholog of the murine ANGPT3. Interestingly, the structure of these orthologs differ more from each other than the human and mouse counterparts of Ang1 and Ang2 (refs 34-36). This explains, why ANGPT4 has not been characterized in depth yet. Recently, GPR182 was found to be upregulated in tumour-specific endothelial cells using a microarray37. This GPCR has no known ligand or function in endothelial cells, but is enriched in the embryonic vasculature38. Future work will address the relative importance of these endothelial cell specific genes to LDL uptake and function.

Cholesterol is crucial for cell growth and maintenance, but is toxic in excess, therefore, intracellular cholesterol homeostasis is tightly regulated by the transcription factor, SREBP2. As ACVR1I knockdown leads to decreased internalization of LDL, the regulatory feedback on sterol sensing through SREBP2 was investigated through the expression of SREBP2 regulated genes. Silencing of DNMT2, effectively blocking endocytosis, leads to increased transcript levels of SREBP2-regulated genes (LDLR, HMGCR, INSIG1 and PCSK9), whereas knockdown of ACVR1I shows no effect on sterol-mediated gene expression. Varying LDLR expression through siRNA or LDL concentration in the media did not affect ALK1 expression or signalling, moreover the loss of ALK1 does not influence LDLR levels in extracts or the amount of LDLR on the cell surface detected by FACS. Interestingly ALK1-dependent uptake of LDL does not result in its lysosomal degradation (measured by 125Iodotyrosine release into the media) implying the route of entry of LDL bound to ALK1 is different from LDL bound to LDLR, thus independently confirm that ALK1 does not affect sterol sensing which requires LDLR degradation and metabolism of cholesterol esters. Early studies in the field suggested an LDLR independent route can account for 40–50% of LDL internalization through a non-lysosomal, non-degradative pathway39. This observation was corroborated by an EM-study39 and has been observed also in capillary endothelial cells of the blood–brain-barrier40. Since ALK1 expression in LDLr-KO MEFs augments LDL uptake, other components of the BMP/ALK1 pathway were examined for their effects on LDL internalization. siRNA silencing of BMPRII, ActRII, GDF2, BMP9/BMP10 or ENG (endoglin) in the initial screen did not affect LDL uptake implying specificity for ALK1. ALK2 was overexpressed in HeLa and HCAECs and analysed for its effect on Dil-LDL uptake and transcytosis, but the results indicate a specific effect for ALK1. In addition, ALK1-mediated LDL uptake is not affected by its kinase activity, as overexpression of constitutively active or inactive mutants and pharmacological modulation of ALK1 kinase activity had no effect on LDL uptake. The role of ALK1 signalling during atherogenesis has been suggested. For example, ALK1 expression is increased in human coronary atherosclerotic lesions41. However, previous studies implicating ALK1 in atherosclerosis mainly focused on signalling of the TGF-β family. Indeed, independent studies have shown that inhibition of BMP signalling attenuates the formation of atherosclerotic plaques42,43. Because ALK1 mediates LDL uptake through direct binding via its extracellular domain and transduces BMP signalling, ALK1 exerts at least two independent functions during the development of atherosclerosis. Although our data clearly demonstrates a role for ALK1 in the uptake of LDL in endothelial cells in vitro, examining if the loss of ALK1 influences LDL clearance and atherogenesis in vivo is challenging. The genetic deletion of ALK1 causes early embryonic vascular defects44, while conditional, post-natal deletion of ALK1 in endothelial cells also induces death within 8–10 days due to its critical role in BMP9/10 signalling45. Based on SPR experiments, LDL and BMP9 bind to discrete sites on LDL only in the presence of BMP9 did not compete for the binding of ALK1 to LDL.

In addition to compelling in vitro data showing that LDL binds ALK1 and mediates LDL uptake and transcytosis, data in endothelial specific Acvrl1-deficient mice on a Ldlr-KO
background demonstrates less LDL uptake into the aortic wall. Due to the lethality of sustained ALK1 inactivation in adult endothelium as seen by others[19,22] and confirmed here, we could not examine if the loss of ALK1 would reduce the extent of atherosclerosis. Future studies will need to focus on separating the LDL-uptake from the BMP-signalling pathway of ALK1, which may be feasible based on the SPR data presented here.

In summary, this work defines new pathways for LDL uptake into endothelial cells and provides a molecular basis to begin unravelling LDL uptake, transcytosis and retention in the vessel wall. Although lipid lowering therapy is the mainstay for the prevention and treatment of atherosclerotic vascular disease[45]; additional therapeutic approaches targeting the early events of atherogenesis in the vessel wall such as LDL transport, retention or endothelial cell dysfunction are interesting and feasible. For example, a putative pharmacological treatment that antagonizes the LDL/ALK1 interaction in endothelial cells without affecting BMP5/BMP10-dependent signalling may afford unique and synergistic benefits with lipid lowering therapies.

**Methods**

RNAi Screen. The siGenome human genome library from Dharmacon (GE Healthcare) was used for the genome-wide RNAi screening. The images were collected with the Opera High Content Screening System (PerkinElmer) and analysed with the according Acapella software. As a primary readout the average centre intensity was determined. The primary screen was analysed based on the robust z-score46 per individual screening plate whereas a robust z-score greater than 2.5 was called a hit. Each library siRNA plate was used in three independent experiments. The quality of each plate was ensured by only accepting plates with a Z’ factor $ > 0.2$, based on the control siRNA (negative control) and DM2 siRNA (positive control, Supplementary Table 2). The validation (with deconvoluted siRNAs) screen and the follow-up screens were analysed based on the percent effect of the positive control (DM2 siRNA). The secondary library siRNA plates with individual siRNAs were assayed twice. To exclude the possibility of loss in signal due to low cell number cell death over 20% was not accepted and only images with at least 100 cells were taken into account.

In brief, cells (EA.hy926: 4,000 cells per well | HUVEC: 2,000 cells per well) were seeded in 384-well plates containing 20 nM siRNA (reverse transfection) with Lipofectamine RNAiMAX (Life Technologies, 1:500). Forty-eight hours after transfection, the media was exchanged to a media enriched with 25 μg ml$^{-1}$ human LDL. The concentration of 25 μg ml$^{-1}$ was identified during the development of the high-throughput screen by using different concentrations of LDL. At 25 μg ml$^{-1}$ the effect of LDL-pretreatment plateaued indicating that the maximum effect was reached. The identified concentration for LDLR saturation falls within the range identified by other groups[47]. Twenty-four hours later the media was removed and a serum-free media with 2.5 μg ml$^{-1}$ Dil-LDL or 12.5 μg ml$^{-1}$ DiI-LDL was added and transffi-FTTC (TF-FTTC) was added for 1 h. Cells were washed for 5 min with PBS at room temperature and resuspended in 1% FCS in PBS at pH 4.0, fixed with 4% paraformaldehyde (PFA) for 10 min and the nuclei were stained with Hoechst 33342 for 5 min, before washed extensively with PBS before imaged. Threshold for Dil-LDL uptake was set to 50% inhibition and for TF-FTTC to 30% inhibition.

**Cells.** EA.hy926 (#CRL-2922), HEK-293T (#CRL-11268) and HeLa cells (#CCL-2) were purchased from American Type Culture Collection. HUVEC were obtained from the Yale University Vascular Biology and Therapeutics (VBT) Core facility. Human coronary artery endothelial cells (HCAEC) were purchased from Lonza (#CC-2585) for $^{[2]}$-LDL transfection and from PromoCell (#CC-12221) for TIRF-based transfection. Mouse embryonic fibroblasts (MEF) from LdrKO animals were a kind gift from Joachim Herz. MLEC were isolated[19]. Three female mice with mixed background were injected with 100 μg $^{[3]}$-Biotin labelled LDL-LDL or $^{[15]}$S-D5-CD3-EGR (glycosylation phenotype at the age of 3 weeks) were euthanized using ketamine/xylazine. The lungs were isolated, minced with acid wash (25 mM Glycine, 3% (m/V) BSA in PBS at pH 4.0), fixed and resuspended with 4% paraformaldehyde. The lungs were incubated for 30 min. Primary antibodies were added and incubated for 1 h at room temperature. After washing, the surface portion of the LDLR was measured using FACS54.

**Animal studies.** The Institutional Animal Care Use Committee of Yale University approved all mouse experiments. Acvr1f0f0/Cdh5-CreERT2 (ref. 51) animals were bred to Ldr-KO animals (JAX, #007068) to generate Acvr1f0f0/LdrKO/Kol/Cdh5-CreERT2 mice. At 8 weeks of age, littermate male mice with mixed background were injected with 100 μg $^{[3]}$-Biotin labelled TMX (TMX) intraperitoneally for 5 consecutive days to induce deletion of the Acvr1 allele. At day 6, mice were injected via femoral vein with 100 μl Dil-LDL (300 μg), which was allowed to circulate for 30 min. Mice were euthanized and aorta were perfused using 10 ml PBS following by 10 ml of 4% PFA fixation. Mouse aortae (athero-prone area; low curvature of aortic arch) were dissected and mounted on glass slides. 4,6-diamidino-2-phenylindole staining. Images were acquired using confocal microscopy (Leica SP5) and quantified with the Image J. The results are expressed in terms of total positive area of each animal per field.

**Cloning and protein expression.** AdGFP and AdCre/GFP were bought from the Viral Vector Core Facility at the University of Iowa, Carver College of Medicine. Human full-length ACVRL1 was obtained by PCR from HVEC complementary DNA (cDNA), C terminal fused to eGFP and cloned into pENTR (Invitrogen). For adenovirus production, HEK293T cells were transfected with Pac1-linearized adenoviral construct using Lipofectamine 2000 (Invitrogen). After 7–10 days, the adenovirus containing supernatant was harvested, amplified by re-infection of HEK293 cells and purified with the Adeno-X Maxi Purification Kit (Clontech). The titre of the virus was determined using Adeno-X Rapid Titer Kit (Clontech) according to the instructions of the manufacturer.

**siRNAs and shRNAs.** All siRNAs pools used in the genome-wide RAWi screen are listed in Supplementary Data set 1. All single siRNAs used in the follow-up study are listed in Supplementary Data set 2. All other siRNAs and shRNAs are listed in Supplementary Table 2.

**Flow cytometry.** The surface portion of the LDLR was measured using FACSC54. Cells were detached from the cell culture dish using Versene (Gibco, #15080066), before the cells were labelled with anti-LDLR antibody or mouse IgG2b as a control (Supplementary Table 3). After incubation for 1 h, the cells were washed to remove unbound antibodies, before the secondary antibody (Supplementary Table 3) was added and incubated for additional 30 min. The cells were washed again, resuspended in PBS and analysed immediately. If Dil-LDL uptake into cells was determined by FACS, cells were washed with PBS, treated with Dil-LDL (25 μg ml$^{-1}$) for 1 h, washed with acid wash (25 mM Glycine, 3% (m/V) BSA in PBS at pH 4.0), washed with PBS, trypsinized, and resuspended in PBS before analysed immediately. The cells were measured by FACS Calibur or LSR II (both BD Bioscience) flow cytometer and analysed using FlowJo software.

**Surface plasmon resonance.** Binding studies were performed at 25 °C using a Biacore T100 optical biosensor (GE Healthcare) equipped with a CM5 research-grade sensor chip coated with LDL. The LDL surface was created using amine coupling and equilibrated with running buffer (25 mM HEPES, pH 7.4, 12 mM NaN$$_3$$).
150 mM NaCl and 0.1 mM CaCl₂ (ref. 17). The binding of LDLRecto or ALK1ecto was monitored during injections of five different concentrations each (FC-0, 0.2, 0.8, 3.5, 14 mM ALK1ecto; 0.15, 0.39, 1.7, 5.1, 15.3 mM LDLRecto, 5.1, 14, 129, 388 mM) using single cycle kinetics (no regeneration steps). The binding responses were double-referenced against the non-specific binding to dextran surface alone and injections of buffer alone. The resulting sensorgrams were fit to a simple 1:1 binding model to determine apparent binding affinity using Biosensor software (GE Healthcare). To establish the non-specific nature of ALK1ecto and LDLRecto binding to LDL, the binding of ALK1ecto was monitored on native LDL or on LDL that was saturated with LDLRecto before ALK1 ecto binding experiments and vice versa. The binding of FC, ALK1ecto, BMP9 and ALK1 ecto incubated with equimolar amounts of BMP9 were tested at 2 μM protein concentration in the presence of 0.48 mM hydrochloric acid (needed to reconstitute the lyophilized BMP9). Due to the complex nature of buffers used in preparation of lyophilized proteins used in this experiment that prevented proper referencing of the association signal, the binding of FC, ALK1ecto and BMP9 were quantitated at the stability reference point (initial phase of the dissociation step) to eliminate the responses due to refractive index change during buffer differences between protein samples and the running buffer; these responses were normalized against the amplitude generated during ALK1 ecto binding.

**Experimental procedure using 125I-LDL**

Native LDL was bought from Kalen Biomedical and labelled with 125-iiodine by PerkinElmer as a customized order. The activity at the time of delivery was about 3 μCi/μl i.-1 protein. Uptake, binding and degradation were measured as described in a detailed protocol published before61. Uptake studies with 125I-LDL were performed with primary human aortic endothelial cells (AAECs) transfected in serum free DMEM with 1 μg/ml PEG-Fc receptor and assessed by TIRF microscopy61. HCAECs were seeded at a density of 20,000 cells per insert onto 0.4 pore polyester transwells (0.33 cm²) and grown until confluency (0.1 ml upper chamber/0.6 ml lower chamber), before 125I-LDL (25 μg/ml) was added to the upper chamber. LDL transcytosis was determined by measuring the radioactivity of 100 μl LDL-protein.

**Degradation studies.** Cells were washed with PBS and treated with plain DMEM containing 0–100 μg ml⁻¹ LDL with a fixed amount of 125I-LDL (‘spiked’) for 3 h at 4°C. After the incubation, cells were washed five times with buffer A (150 mM NaCl, 50 mM Tris-HCl, pH 7.4). Cells were lysed as described before, protein concentration was measured and lysate was counted.

**Transfection**

Cells were either transfected with control siRNA or scrambled control siRNA using HiPerFect. LDL transcytosis was measured by TIRF imaging 72 h after transfection. For overexpression experiments, HCAECs at 80% confluence were transfected in Opti-MEM with 16 nM AAVR11 siRNA or scrambled control siRNA using HiPerFect. LDL transcytosis was measured by TIRF imaging 72 h after transfection. For overexpression experiments, HCAECs at 80% confluence were transfected with 0.5 μg/ml plasmid DNA (GFP, ALK1-GFP, ALK2-GFP) using a transfection reagent. The rest of the lysate was used with Ultima Gold scintillation liquid (PerkinElmer) before measured using a Tri-Carb 2100 liquid scintillation counter (PerkinElmer).

**Quantification of LDL transcytosis**

Samples from every two experiments were pooled. SDS–polyacrylamide gel electrophoresis was performed on an 8% polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% BSA for 1 h at room temperature, followed by overnight incubation at 4°C with anti-human LDL receptor antibody or anti-β-actin antibody (Supplementary Table 3). Membranes were washed with TBST followed by incubation with HRP-conjugated secondary antibodies for 1 h at room temperature. After several washes in TBST, membranes were visualized with enhanced chemiluminescence detection.

**Cross-section imaging**

Cross-section imaging (x/y-axes) was performed following previous published work48 with a few modifications. In brief, EA.hy926 were either transfected with control siRNA or AAVR11 siRNA or infected with adenovirus encoding GFP or ALK1-GFP on coverslips. Cells were starved in serum-free media with serum for 4 h before they were incubated with DiI-LDL (2 μg/ml) at 4°C for 10 min to allow membrane binding. Cells were then treated with serum-free media and placed in the incubator at 37°C for 30 min, then washed three times with PBS, fixed and stained with 20 μg/ml -1 lectin (Vector Labs, UEA 1 (ref. 62)). Cells were stained with counterstained with Hoechst, mounted on slides, and imaged on an SP5 confocal microscope using an 150x objective and 561 nm excitation laser and a penetration depth of ~110 nm. In siRNA experiments, the 46-diamido-2-phenylindole epifluorescent channel was used to randomly select cells for imaging: 15 TfR videos were taken per condition. In overexpression experiments, transfected cells were identified using the green epifluorescent channel; 10-12 TfR videos were taken per condition. Cross-section imaging was performed and has been previously described in detail48. Briefly, a MATLAB algorithm (written by Bryan Heit, Western University, Canada) was used to track individual LDL-containing vesicles as they enter the TIRF field. Vesicles are filtered based on size, circularity and fluorescent intensity above background; vesicles undergoing fusion with the basal membrane are removed. Cross-section imaging was performed and has been previously described in detail48. Briefly, a MATLAB algorithm (written by Bryan Heit, Western University, Canada) was used to track individual LDL-containing vesicles as they enter the TIRF field. Vesicles are filtered based on size, circularity and fluorescent intensity above background; vesicles undergoing fusion with the basal membrane are removed. Cross-section imaging was performed and has been previously described in detail48. Briefly, a MATLAB algorithm (written by Bryan Heit, Western University, Canada) was used to track individual LDL-containing vesicles as they enter the TIRF field. Vesicles are filtered based on size, circularity and fluorescent intensity above background; vesicles undergoing fusion with the basal membrane are removed. Cross-section imaging was performed and has been previously described in detail48. Briefly, a MATLAB algorithm (written by Bryan Heit, Western University, Canada) was used to track individual LDL-containing vesicles as they enter the TIRF field. Vesicles are filtered based on size, circularity and fluorescent intensity above background; vesicles undergoing fusion with the basal membrane are removed. Cross-section imaging was performed and has been previously described in detail48. Briefly, a MATLAB algorithm (written by Bryan Heit, Western University, Canada) was used to track individual LDL-containing vesicles as they enter the TIRF field. Vesicles are filtered based on size, circularity and fluorescent intensity above background; vesicles undergoing fusion with the basal membrane are removed.
**Genotyping.** Acrv1 flxed alleles, Ldlr-KO allele and Cells-CreERT2 were genotyped by PCR using primers and the PCR programs described in Supplementary Table 4.

**Quantitative PCR analyses.** Cells were washed with PBS, messenger RNA was isolated with RNeasy Mini Kit (Qiagen), cDNA was reverse transcribed with iScript cDNA Synthesis Kit (Bio-Rad) before used with iQ SYBR Green Supermix (Bio-Rad) and the quantitative PCR primers listed in Supplementary Table 5 on an iQ5 cycler (Bio-Rad).

**Immunoblotting.** For immunoblotting, tissues or cells were placed in lysis buffer (50 mM Tris-HCl, 1% NP-40, 0.1% SDS, 0.1% Deoxycholate, 0.1 mM EDTA, 0.1 mM EGTA, 200 μM Na3VO4, 20 μM Na-pyrophosphate, 2 mg ml⁻¹ complete protease inhibitor, 0.3 mg ml⁻¹ Pefabloc phosphatase inhibitor, 40 mM β-glycerophosphate, 2 mM NaN3). Tissues were homogenized before sonication. Cell lysates were centrifuged for 15 min at top speed at 4°C. Protein concentrations were determined (Bio-Rad Protein Assay). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis or 4-20% gradient gels (Bio-Rad) and transferred to nitrocellulose membranes. After Ponceau S staining, the membranes were blocked with 1% casein for 1 h at room temperature under continuous rotation before incubation with the primary antibody ON at 4°C. Supplementary Table 5 lists all primary antibodies used in this study. Li-COR compatible secondary antibodies (680 or 800 nm) were used based on species origin of the primary antibody. All antibodies in this study are listed in Supplementary Table 3.

**Immunofluorescence.** Cells were washed with PBS, fixed with PFA (4%) for 10 min, permeabilized with 0.2% Triton X-100 before stained with antibodies listed in Supplementary Table 5 using a regular immunofluorescence protocol and imaged with a confocal laser scanning microscopy (CLSM). Respective secondary antibodies were applied before Hoechst staining and subsequent imaging, as previously described. If quantitative assessments were performed, CellProfiler63,64 was used to determine the intensity of blue (Hoechst), green (GFP), red (DiI) for further analyses. Pearson correlation was also analysed using CellProfiler. Both CellProfiler pipelines can be requested. All antibodies in this study are listed in Supplementary Table 3.

**Filipin-III Staining.** Filipin-III was used to visualize free cholesterol in cells15. A 25 mg ml⁻¹ stock solution of Filipin-III in dimethylsulfoxide was prepared and kept at −20°C. Cells were washed twice with PBS, fixed with PFA (4%) for 10 min, washed twice with PBS, permeabilized with 0.1% Triton-X-100 in PBS for 5 min at room temperature, washed twice with PBS, before stained with 50 μg ml⁻¹ (1:500) Filipin-III for 1 h in the dark at room temperature. Cells were washed twice with PBS, before imaged immediately (staining lades rapidly) with epifluorescence microscope.

**Chemicals.** All chemicals, in the highest grade of purity, were obtained from Sigma-Aldrich unless otherwise noted. The human LDL, DiI-LDL, DiI-OxLDL, and the follow-up screen can be found in the supplementary information. All chemicals, in the highest grade of purity, were obtained from Sigma-Aldrich unless otherwise noted. The human LDL, DiI-LDL, DiI-OxLDL, and the follow-up screen can be found in the supplementary information. All chemicals, in the highest grade of purity, were obtained from Sigma-Aldrich unless otherwise noted. The human LDL, DiI-LDL, DiI-OxLDL, and the follow-up screen can be found in the supplementary information. All chemicals, in the highest grade of purity, were obtained from Sigma-Aldrich unless otherwise noted. The human LDL, DiI-LDL, DiI-OxLDL, and the follow-up screen can be found in the supplementary information. All chemicals, in the highest grade of purity, were obtained from Sigma-Aldrich unless otherwise noted. The human LDL, DiI-LDL, DiI-OxLDL, and the follow-up screen can be found in the supplementary information. All chemicals, in the highest grade of purity, were obtained from Sigma-Aldrich unless otherwise noted. The human LDL, DiI-LDL, DiI-OxLDL, and the follow-up screen can be found in the supplementary information. All chemicals, in the highest grade of purity, were obtained from Sigma-Aldrich unless otherwise noted. The human LDL, DiI-LDL, DiI-OxLDL, and the follow-up screen can be found in the supplementary information. All chemicals, in the highest grade of purity, were obtained from Sigma-Aldrich unless otherwise noted. The human LDL, DiI-LDL, DiI-OxLDL, and the follow-up screen can be found in the supplementary information. All chemicals, in the highest grade of purity, were obtained from Sigma-Aldrich unless otherwise noted. The human LDL, DiI-LDL, DiI-OxLDL, and the follow-up screen can be found in the supplementary information. All chemicals, in the highest grade of purity, were obtained from Sigma-Aldrich unless otherwise noted. The human LDL, DiI-LDL, DiI-OxLDL, and the follow-up screen can be found in the supplementary information. All chemicals, in the highest grade of purity, were obtained from Sigma-Aldrich unless otherwise noted. The human LDL, DiI-LDL, DiI-OxLDL, and the follow-up screen can be found in the supplementary information. All chemicals, in the highest grade of purity, were obtained from Sigma-Aldrich unless otherwise noted. The human LDL, DiI-LDL, DiI-OxLDL, and the follow-up screen can be found in the supplementary information. All chemicals, in the highest grade of purity, were obtained from Sigma-Aldrich unless otherwise noted. The human LDL, DiI-LDL, DiI-OxLDL, and the follow-up screen can be found in the supplementary information. All chemicals, in the highest grade of purity, were obtained from Sigma-Aldrich unless otherwise noted. The human LDL, DiI-LDL, DiI-OxLDL, and the follow-up screen can be found in the supplementary information. All chemicals, in the highest grade of purity, were obtained from Sigma-Aldrich unless otherwise noted. The human LDL, DiI-LDL, DiI-OxLDL, and the follow-up screen can be found in the supplementary information. All chemicals, in the highest grade of purity, were obtained from Sigma-Aldrich unless otherwise noted. The human LDL, DiI-LDL, DiI-OxLDL, and the follow-up screen can be found in the supplementary information. All chemicals, in the highest grade of purity, were obtained from Sigma-Aldrich unless otherwise noted. The human LDL, DiI-LDL, DiI-OxLDL, and the follow-up screen can be found in the supplementary information. All chemicals, in the highest grade of purity, were obtained from Sigma-Aldrich unless otherwise noted. The human LDL, DiI-LDL, DiI-OxLDL, and the follow-up screen can be found in the supplementary information. All chemicals, in the highest grade of purity, were obtained from Sigma-Aldrich unless otherwise noted. The human LDL, DiI-LDL, DiI-OxLDL, and the follow-up screen can be found in the supplementary information. All chemicals, in the highest grade of purity, were obtained from Sigma-Aldrich unless otherwise note...
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Authors contributions

J.H.C and W.C.S designed the initial RNAi screen. J.H.C and L.K performed the genome-wide screen. J.H.C and W.C.S cleared the data of the genome-wide screen and decided on the 140 follow-up gene candidates. J.R.K and W.C.S planned the follow-up screen and J.R.K with help from C.R carried out the experiments. M.Y.L helped developing the follow-up screens. J.R.K and W.C.S designed the ALK1 related study (Fig. 2A) and M.G.S. and W.L.L designed, performed and analysed the TIFE-based transcytosis assay. J.W.F. performed the TIFE/FACS experiments. X.Z. and J.R.K. performed the in vivo analysis of Dil-LDL uptake in the aortic arch. B.T. analysed the ALK1-dependent LDL transcytosis qualitatively. E.J.P. isolated and immortalized M.w.n. and N.R. generated the ALK1-GFP encoding adenovirus and bred the Cdh5- and A.E. generated the ALK1-GFP encoding adenovirus and bred the Acvrl1fl/fl mouse with endothelial cells. C.F.-H helped analysing total and surface localized LDLER levels. K.C. and K.J.W. analysed the degradation and uptake of Acvrl1fl/fl LDL, B.L., R.O. and A.E. generated the ALK1-GFP encoding adenovirus and bred the Acvrl1fl/fl mouse to the Cdh5-CreERt2 line. E.F.-S. performed the surface plasma resonance analyses. W.Z. carried out the initial characterization of ANGPT4. M.W.N. collected the GWAS-data using publically available databases. J.H. provided the LDLR-KO mouse embryonic fibroblasts. J.R.K., J.H., C.F.-H., K.J.W. and W.C.S. wrote the paper.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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