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Chapter 10
EEP1D1 is novel LXR target gene in macrophages that regulates ABCA1 abundance and cholesterol efflux

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**EEPD1 is a novel LXR target gene in macrophages that regulates ABCA1 abundance and cholesterol efflux**

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Abstract

The sterol-responsive nuclear receptors Liver X receptors α (LXRβ, NR1H3) and β (LXRβ, NR1H2) are key determinants of cellular cholesterol homeostasis. LXRs are activated under conditions of high cellular sterol load and induce expression of the cholesterol efflux transporters \textit{ABCA1} and \textit{ABCG1} to promote efflux of excess cellular cholesterol. However, the full set of genes that contribute to LXR-stimulated cholesterol efflux is unknown. To address this we used a systems approach in which we compared the transcriptional response of macrophages to distinct classes of LXR ligands. This allowed us to identify both common and ligand-specific transcriptional responses in macrophages. Amongst these, we identified endonuclease-exonuclease-phosphatase family domain containing 1 (\textit{EEPD1}) as a direct transcriptional target of LXRs in human and murine macrophages. \textit{EEPD1} specifically localizes to the plasma membrane owing to the presence of myristoylation and palmitoylation sites in its N-terminus. Consistent with this, the first 10 amino acids of \textit{EEPD1} are sufficient to confer plasma membrane localization in the context of a chimeric protein with GFP. Functionally, we report that silencing \textit{EEPD1} blunts maximal LXR-stimulated ApoAI-dependent efflux and demonstrate that this is the result of reduced abundance of \textit{ABCA1} in human and murine macrophages. In conclusion, we identify \textit{EEPD1} as a novel LXR regulated genes and propose that it promotes cellular cholesterol efflux by controlling \textit{ABCA1} levels.
Introduction

Disturbed cholesterol homeostasis is intimately linked with human diseases, most notably atherosclerosis and ensuing cardiovascular complications. Despite an increase in our understanding of the basic mechanisms underlying atherogenesis, and the availability of therapeutic modalities to treat dyslipidemia, cardiovascular complications remain the leading cause of death in Western countries. Atherosclerosis is a lipid-driven disease that is also characterized by the presence of low-grade inflammation in the vascular wall. Within the atherosclerotic plaque, macrophages are able to, amongst others, internalize modified low-density lipoprotein, promote removal of excess cholesterol from the developing atherosclerotic plaque, and respond to local and systemic inflammatory cues. Owing to their ability to integrate lipid and inflammatory signaling the central role played by macrophages in atherosclerosis is well recognized. This also emphasizes the need to elucidate the genetic programs and genes governing macrophage function in atherogenesis.

The liver X receptors α (LXRα, NR1H3) and β (LXRβ, NR1H2) are central transcriptional regulators of cholesterol metabolism. LXRα are sterol-responsive nuclear receptors that are activated under conditions of elevated cellular sterol load. In macrophages, their activation leads to induction of a transcriptional program that is aimed towards reducing the cellular cholesterol burden and concomitantly inhibiting inflammatory signaling. This is largely achieved through LXRα’s ability to (1) enhance cholesterol transport through their target genes ABCA1, ABCG1, and APOE (2) limit uptake of lipoprotein-derived cholesterol by inducing expression of the E3 ubiquitin ligase Inducible degrader of the LDLR (IDOL), and (3) trans-repress inflammatory signaling induced by inflammatory cues. Accordingly, Lxrαβ−/− macrophages accumulate cholesterol in vivo and are hyper-responsive to inflammatory stimuli. Reciprocally, pharmacological engagement of LXRα promotes cholesterol efflux and decreases atherosclerotic plaque development in ApoE−/− and Ldlr−/− mice fed an atherogenic diet. Importantly, this protection is largely due to macrophage-
specific LXR activity as it is lost in \( \text{Ldlr}^{-/-} \) mice transplanted with \( \text{Lxra}\beta^{-/-} \) bone-marrow, most likely due to the importance of LXR stimulated cholesterol efflux in these cells \( ^{17,19} \).

LXRs are ligand-dependent transcription factors and their activation, and hence stimulation of cholesterol efflux, requires receptor-ligand binding. Their endogenous ligands are oxysterols, including 22(R), 24(S), and 27-hydroxycholesterol, and intermediates of the cholesterol biosynthetic pathway, most notably desmosterol \( ^{20-22} \). Similarly, endocytosis of (modified) lipoproteins or efferocytosis increases the cellular cholesterol and oxysterol pool and also promotes LXR signaling \( ^{23,24} \). High affinity synthetic agonists have been also developed to therapeutically target LXRs, with several reported to have differential activation of \( e.g. \) LXR\( \alpha \) over LXR\( \beta \) resulting in a differential transcriptional response \( ^{25} \). While these different classes of agonists activate LXRs, natural and synthetic agonist markedly differ with respect to their inhibitor effect on the sterol-regulatory element binding proteins (SREBP) pathway. Oxysterols and intermediates of the cholesterol biosynthetic pathway prevent processing and maturation of SREBPs to their transcriptionally active form, whereas synthetic ligands do not \( ^{26,27} \). This implies that the LXR-induced transcriptional response to these ligands should be distinct. Furthermore, whether the distinct endogenous ligands induce a differential LXR transcriptional response has not been systematically investigated.

To systematically evaluate the LXR response in macrophages we treated cells with a panel of distinct LXR ligands. Transcriptional profiling allowed us to identify both overlapping, as well as ligand-specific LXR-dependent transcriptional responses to these ligands. Amongst LXR-responsive genes, we identified endonuclease-exonuclease-phosphatase family domain containing 1 (EEPD1/KIAA1706) as a previously unrecognized direct transcriptional target of LXRs in macrophages. We report here that EEPD1 promotes LXR-stimulated cholesterol efflux by regulating abundance of ABCA1 at the plasma membrane.
Materials and Methods

Reagents

Lipoprotein deficient serum (LPDS) was prepared as previously reported and confirmed to contain no lipoproteins by FPLC analysis (not shown)\(^2\). 22R-hydroxycholesterol (22R-HC) and Desmosterol were purchased from Steraloids. Simvastatin was purchased from Calbiochem. GW3965, T0901317, Mevalonate, Cycloheximide, Doxycycline (DOX), Phorbol 12-myristate 13-acetate (PMA), Oil red O powder and 2-Hydroxyhexadecanoic acid were purchased from Sigma. Blasticidin was purchased from Invitrogen. rApoA1 was purchased from Calbiochem. Acetylated-LDL (Ac-LDL) was purchased from Alfa Aesar.

Cell culture and transient transfections

THP1, RAW264.7, J774, Hela, COS7 and HepG2 cells were obtained from the ATCC. THP1 were maintained in RPMI, medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 10,000 U/mL Pencillin-Streptomycin (Gibco) at 37 °C and 5% CO\(_2\). All other cells were cultured at 37°C and 5% CO\(_2\) in Dulbecco's modified eagle's medium (DMEM, Gibco) supplemented with 10% FBS. Human A431 cells were a kind gift from Dr. Elina Ikonen (University of Helsinki, Finland). THP1 monocytes were differentiated into macrophages with the addition of 20nM PMA for 48 hours prior to the described experiments. Where indicated in the figures and figure legends, cells were sterol-depleted by culture in sterol-depletion medium (DMEM or RPMI supplemented with 10% LPDS, 5 µg/mL simvastatin, and 100 µM mevalonate). To induce LXR transcriptional activity, cells were treated with either 1µM GW3965, 5µM Desmosterol, 5µM 22R-Hydroxycholesterol (22R-HC), or 50µg/mL Acetylated-LDL (Ac-LDL) for the indicated time. COS7 cells were transfected using the JetPrime transfection reagent (Polyplus) for 48 hours. To generate RAW264.7 cells with stable expression of inducible EEPD1 constructs we infected cells with lentiviral particles encoding EEPD1-GFP or EEPD1-Myc. Subsequently, cells were selected.
with 5µg/mL Blasticidin and surviving cells were used in experiments. Expression of EEPD1 in these cells was induced by addition of 1µg/mL DOX to the culture medium for 48 hours.

**Mice, Bone Marrow isolation and culture**

Bone marrow cells was isolated from femurs and tibiae of four wild-type (WT) mice and four Lxrαβ(-/-) mice as described. In brief, cells were cultured in RPMI (Gibco) with 10000 U/ml penicillin/streptomycin (Gibco), 10% FBS (Gibco) and 15% L929 conditioned-medium for 8 days to generate bone marrow derived macrophages (BMDM) that were seeded 24 hours before the indicated treatments at a density of 1.5 x 10^5 cells/cm^2. Mouse tissues for RNA isolation were collected from 3-6 male C57Bl/6 mice at 12 weeks of age. All handling of mice were according to institutional guidelines and regulations.

**RNA isolation and quantitative PCR**

Total RNA was isolated from cells and tissues using the Direct-zol RNA miniprep kit (Zymo Research). One microgram of total RNA was reverse-transcribed using the iScript reverse transcription reagent (BioRad). FastStart Sybr green Master (Roche) or SensiFAST SYBR (Bioline) was used for real-time quantitative PCR (qPCR). Assays were performed on a LightCycler 480 II system (Roche). Gene expression levels were determined and normalized to the expression level of 36B4. Sequences for qPCR primers are available upon request.

**RNA sequencing-based transcriptional profiling and bioinformatics analysis**

Human THP1 macrophages were cultured and treated as described above. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and converted into strand-specific cDNA libraries using the TruSeq Stranded mRNA sample preparation kit (Illumina, Part # 15031047 Rev. E) according to the manufacturer's instructions. Briefly, polyadenylated RNA was enriched using oligo-dT beads and subsequently fragmented, random primed and reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Second strand synthesis was performed using Polymerase I and RNaseH with
replacement of dTTP for dUTP. The generated cDNA fragments were 3’ end adenylated, ligated to Illumina paired-end sequencing adapters, and subsequently amplified by 12 cycles of PCR. The libraries were analyzed on a 2100 Bioanalyzer using a 7500 chip (Agilent, Santa Clara, CA) and subsequently sequenced with 65 base single reads on a HiSeq2500 using V4 chemistry (Illumina Inc.). Transcripts were aligned to the Human Feb. 2009 (GRCh37/hg19) assembly using TopHat (version 2.1) 29. Gene expression sets were prepared using ICount, which is based on HTSeq-count. Uniquely mapped reads were normalized to 10 million reads followed by \( \log_2 \) transformation. In order to avoid negative normalized values, 1 was added to each gene expression value. For downstream bioinformatics analyses, only protein-coding transcripts with a \( \log_2 \) normalized read count higher than 3.33 and a false discovery rate (FDR)-adjusted p-value lower than 0.05 were included (Supplementary figure 1). Data were analyzed using QIAGEN’s Ingenuity® Pathway Analysis (IPA®, QIAGEN), Gene Set Enrichment Analysis 30, and Metascape Gene Annotation & Analysis Resource 31.

**siRNA interference experiments**

Differentiated THP1 or J774 macrophages were transfected with 30 nM small interfering RNAs (siRNA) using Lipofectamine RNAiMAX (Invitrogen) for 48 hours. In murine lines, ON-TARGETplus SMART pool control (D-001810-10) and Eepd1 (L-055781-01-0005) siRNAs from Dharmacon were used. To silence human EEPD1 Ambion Silencer Select single EEPD1 siRNAs (catalog numbers s37411 and s37212) and controls (AM4611) were purchased from Invitrogen and used as indicated.

**Plasmids and expression constructs**

The human and mouse EEPD1 cDNA was amplified from THP1 and J774 cells, respectively, and cloned into expression plasmids with Gateway-mediated recombination (Invitrogen). A human EEPD1 C-terminally tagged GFP expression plasmid was constructed by cloning EEPD1 cDNA as an EcoRI and BamHI fragment into pEGFP-N1 (Clontech). Mutations in EEPD1 were introduced using the QuickChange site directed mutagenesis kit. To generate the
EEP1D1(1-10)-eGFP construct the first 10 amino acids of hsEEP1D1 were designed as oligos with EcoR1 and BamH1 sites, annealed, and cloned into pEGFP-N1 (Clontech). The pH2B-mRFP plasmid was a kind gift from Dr. Martijn Kedde (Academic Medical Center, the Netherlands). The pInducer20 vector was modified to contain a Blasticidin resistance gene in place of the Neomycin resistance cassette, and referred to as pInducer-BlaR. We generated pInducer-EEP1D1-eGFP and pInducer-EEP1D1-Myc by cloning the corresponding cDNAs into pInducer-BlaR with gateway recombination. All plasmids used in this study were isolated by CsCl2 gradient centrifugation and their correctness verified by sequencing and digestion analysis.

**Antibodies and immunoblot analysis**

Total cell lysates were prepared in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 100 mM Tris-HCl, pH 7.4) supplemented with protease inhibitors (Roche). Lysates were cleared by centrifugation at 4 °C for 10 min at 10,000 x g. Protein concentration was determined using the Bradford assay with BSA as reference. Samples (10–40µg) were separated on NuPAGE Novex 4 to 12% Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes. Membranes were probed with the following antibodies: ABCA1 (Novus biologicals, NB400-105), ABCG1 (Novus biologicals, NB400-132), KIAA1706 (EEP1D1, from Sigma, SAB1408033), Transferrin receptor (Invitrogen, 136800), tubulin (Sigma, clone DM1A), β-Actin (Millipore, clone C4), Tom20 (SantaCruz clone FL-145, SC-11415) and MYC-Alexa 488 (Cell Signaling, clone 9B11). Secondary HRP-conjugated antibodies (Invitrogen) were used and visualized with chemiluminescence on a Fuji LAS4000 (GE Healthcare). All immunoblots are representative of at least three independent experiments.
**Preparation of crude membrane fractions**

Cells were cultured as indicated, scraped and collected in PBS and subsequently centrifuged at 4 °C for 5 minutes at 1,000 x g. Intact cells were re-suspended in 1 mL of hypotonic buffer (0.5mM NaPi, 2mM MgCl₂, pH 7.4) and allowed to swell for 30 minutes on ice. Cells were disrupted by passing 10 times through a 27-guage needle and transferred to an ultra centrifuge microfuge tube (9.5x 38mm, 1.5mL volume, Beckman). Samples were spun down at 4 °C for 1 hour at 100,000 x g in a Beckman TLA-55 router. The supernatant was collected as the cytosolic fraction, and the remaining crude membrane pellet was washed with PBS and resuspended in RIPA lysis buffer supplemented with protease inhibitors. Crude membrane lysates were cleared by centrifugation at 4 °C for 10 min at 10,000g. The protein concentration was determined using the Bradford assay with BSA as reference prior to immunoblot analysis.

**Confocal microscopy**

COS7 and RAW264.7 cells were grown on glass slides and treated as indicated. Subsequently, cells were fixed with 4% paraformaldehyde (Invitrogen) and nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI). Live and fixed cells were imaged on a TCS SP8 confocal microscope equipped with a 405 nm laser (for DAPI), a 488 nm laser (for Alexa 488 or GFP), and a 561 nm laser (for RFP) with a 63× objective (Leica Microsystems, Mannheim, Germany).

**ApoA1-mediated cholesterol efflux assay**

THP1 monocytes were differentiated with PMA and transfected with siRNA as described above. Cholesterol efflux assay were performed as described by Wang et al. 33. Briefly, cells were pre-treated with or without 1µM GW3965 for 4 hours and cultured for 18 hours in RPMI supplemented with 0.2% BSA containing 50 µg/ml Acetylated-LDL and 2 µCi/ml [1,2(N)-³H]Cholesterol (GE healthcare). Subsequently, cells were washed 3 times with PBS to remove excess cholesterol. Cholesterol efflux was performed by incubating the cells with
or without 10µg/mL ApoA1. Radioactivity in supernatant medium was determined by liquid scintillation counting. The cells were lysed in 100% isopropanol and their radioactivity content was determined. Cholesterol efflux is expressed as the percentage of the radioactivity released from cells into the medium relative to the total radioactivity in cells and medium combined. Results were calculated as net efflux (efflux with ApoA1 minus 0.2% BSA alone) and shown as relative efflux to siNT-treated control cells (DMSOvehicle treated).

**Foam cell formation assay**

Cells were cultured on glass coverslips and treated as indicated. Subsequently, cells were incubated for 16 hours in RPMI supplemented with 0.2% BSA and 50 µg/ml Acetylated-LDL in the presence or absence of 1µM GW3965. Cells were washed 3 times with PBS and fixed with 4% paraformaldehyde for 10 minutes. Neutral lipids in the cells were stained with Oil Red O for 45 minutes and then briefly destained with 60% isopropanol and washed in PBS. Cells were visualized on an EVOS Imaging System (ThermosFisher) under 40x magnification.

**Statistical analysis**

Data are presented as mean ± SD. The statistical analyses were performed using the GraphPad 5.0 software package. Results were evaluated by a t-test for comparison of two groups, or by one-way analysis of variance (ANOVA) with Bonferroni correction for grouped analyses. Symbols for statistically significant p-values are defined in the figure legends and a p value <0.05 was considered significant.
**Results**

To map the LXR ligand-induced transcriptional program in human macrophages we performed RNA sequencing on sterol-depleted THP1 cells – a human monocytic leukemia cell line that can be readily differentiated to macrophages with PMA – treated with 4 different classes of LXR ligands: GW3695 (synthetic), 22R-HC (oxysterol), Desmosterol (cholesterol biosynthesis intermediate), and Ac-LDL (modified lipoprotein). Sterol-depleted cells were used as a baseline since a lack of sterols is known to reduce basal LXR-dependent signaling, thus allowing for greater sensitivity in detecting LXR-induced transcriptional changes.

We identified 1171 protein-coding transcripts that were differentially expressed with an adjusted false discovery rate (FDR) p-value of less than 0.05 in response to sterol-depletion. Of these, 555 transcripts were up-regulated and 616 transcripts were down-regulated (Supplementary figure 2A). Subsequent treatment of sterol-depleted THP1 macrophages with either 1µM GW3965, 5µM 22R-HC, 5µM desmosterol, or 50µg/mL Ac-LDL resulted in a significant change in expression of 1267 to 1856 genes, depending on the ligand used, with only a modest number of genes (287) being regulated by all ligands tested (figure 1A). Hierarchical clustering of our RNA-seq datasets revealed that GW3965 treatment resulted in a transcriptional profile that was distinct from the other ligands, whose expression profiles clustered more closely together (figure 1B). Gene ontology (GO) analysis revealed that gene clusters that were similarly regulated by all ligands were enriched for genes involved in the cellular response to cytokines and cell activation by extracellular ligands. On the other hand, gene clusters that showed differential regulation between the ligands (primarily GW3965 versus the other ligands) were enriched for genes involved in the positive regulation of immune system processes, wound healing, and lipid biosynthesis (figure 1B). Pathway analysis using IPA revealed a similar profile, with GW3965 treatment resulting in a transcriptional profile that was less enriched for genes involved in cholesterol biosynthesis as compared to either the other ligands versus sterol depletion or sterol depletion versus cholesterol-rich medium (Supplementary figure 2B). Similarly, principal component analysis (PCA) showed that there was little variance between the biological replicates of each of the
experimen
tal conditions and that each ligand induced a distinct gene expression profile as compared to the sterol-depleted baseline, with GW3965 treatment being the most different from all other conditions (figure 1C). Pathway analysis using Metascape on the top 100 most variable genes in each of the principal components showed that most of the variation between the datasets was caused by genes involved in lipid metabolism (PC1 and PC2) and, to a lesser extent, the inflammatory response (PC3) (Supplementary figure 2C).

To filter out genes that were most strongly induced by all four of the ligands tested in our ligand screen we applied a stricter cut-off in which only protein-coding transcripts that showed a 1.5 fold change in either direction with an FDR-adjusted p-value of less than 0.001 were considered (figure 2A). Using this cutoff, we identified several established LXR-responsive genes, such as ABCA17, ABCG110, LXRα34, SMPDL3A35, and IDOL12. Several additional genes that have not been previously described as LXR targets, including EEPD1, PPFIA2, IQSEC1 and PBX4, were also identified. We confirmed these observations from our RNA-seq screen using qPCR, which demonstrated that the LXR ligands induce expression of both the established and novel LXR-regulated genes (figure 2B). Furthermore, as expected, desmosterol, 22R-HC and Ac-LDL inhibited expression of SREBP-regulated genes (e.g. HMGCR and LDLR), whereas GW3965 did not (figure 2A,B).

The initial screen and validation involved treating the cells with the different ligands for 18 hours. This implies that the increase in expression of these genes may not reflect them being direct transcriptional targets of LXR, but rather be an indirect effect of LXR activation that is mediated by another protein. To test this possibility we treated cells with GW3965 for 6 hours together with cycloheximide, a protein synthesis inhibitor, reasoning that if induction is indirect it should be abolished by this treatment. Of the genes tested, induction of PPFIA2, IQSEC1, and PBX4 expression by LXR activation was abolished by cycloheximide suggesting their induction is indirect (figure 3A). In contrast, induction of the other genes studied was unaffected by cycloheximide, a finding that is in line with several of them reported to be direct LXR transcriptional targets, and also consistent with their rapid maximal response to LXR activation (~ 3 hours, Supplementary figure 3A).
Amongst the novel LXR-responsive genes identified in our screen, *EEPDI* is one of the few that was induced by all the ligands tested. Similar to the canonical LXR targets *Abca1, Abcg1,* and *Idol,* expression of *Eepd1* was increased in response to different LXR ligands in BMDM from wildtype cells (figure 3B). Regulation of *Eepd1* expression by the ligands was strictly dependent on LXRα, as it was blunted in macrophages derived from *Lxraβ(-/-)* mice (double knockout mice; DKO). In human macrophages derived from peripheral blood monocytes (PBMCs) expression of *EEPDI* was induced by two synthetic LXR ligands, and akin to other established LXR-regulated genes was sensitive to sterol-depletion (figure 3C). Having established that *EEPDI/Eepd1* is expressed in macrophages we determined its expression in a panel of mouse tissues (figure 3D). We observed expression of *Eepd1* in all tissues that were examined, with a particularly high expression in metabolically active tissues (e.g. skeletal muscle and white adipose tissue). We therefore anticipated that similar to most other LXR-regulated genes, *EEPDI* would be regulated by LXR activation in all cell types. To test this hypothesis we investigated the regulation of *EEPDI/Eepd1* by LXR in a number of human and murine cell lines that originate from different tissues. In these cells, we found that *EEPDI/Eepd1* was only induced in macrophage-like cells (figure 3E). This was not simply due to aberrant LXR signaling in these cells, as in response to LXR ligand all were able to activate the canonical LXR target *ABCA1/Abca1* (Supplementary figure 2B). Collectively, these results show that *EEPDI* is a direct, macrophage-specific LXR target gene.

The *EEPDI* gene encodes a 569 amino acids protein that contains several distinct functional domains. Its N-terminal region contains two adjacent helix-hairpin-helix (HhH) motifs, which often associate with DNA binding. The C-terminal region contains a large Exonuclease-Endonuclease-Phosphatase (EEP) domain (Figure 4A). The HhH motifs present in EEPD1 could suggest association of the protein with DNA and involvement in DNA-binding related processes (e.g. DNA repair). However, *in silico* analysis of its amino acid sequence revealed that EEPD1 is unique amongst the EEP super-family of proteins in that it contains highly evolutionary-conserved myristoylation and palmitoylation lipid-modification
sites, which are known to serve as membrane anchors (Figure 4A). To evaluate the cellular localization of EEPD1 we generated an EEPD1<sub>wt</sub>-eGFP expression construct and studied its localization in COS7 cells (Figure 4B). Consistent with the presence of the highly conserved N-terminal myristoylation and palmitoylation sites, wildtype EEPD1 localized exclusively to the plasma membrane in both live and fixed cells. Remarkably, abolishing the single myristoylation site (EEPD1<sub>G2A</sub>-eGFP) resulted in drastically altered localization of EEPD1 (Figure 4B). A similar shift in cellular localization was also observed when we treated RAW264.7 macrophages that express an inducible EEPD1<sub>wt</sub>-eGFP construct with 2-hydroxymyristic acid, a potent inhibitor of the myristoyl conjugating enzyme N-myristoyltransferase (Supplementary figure 4A). Mutation of the predicted palmitoylation site (EEPD1<sub>C7A</sub>-eGFP), or of both sites simultaneously (EEPD1<sub>G2A,C7A</sub>-eGFP) also resulted in a loss of association of EEPD1 with the plasma membrane, further emphasizing the importance of these lipid anchors in EEPD1 localization (Figure 4B). It should be noted that under these conditions we were unable to observe EEPD1 localized in the nucleus. However, since these lipid modifications are dynamic, we reasoned that either LXR activation or the cellular sterol-status may influence the localization of EEPD1. We tested this possibility by determining the localization of EEPD1 in cells following sterol-depletion and/or treatment with the LXR ligand GW3965. Under both conditions we observed no shift of EEPD1 from the plasma membrane towards an intracellular compartment or the nucleus (Supplementary figure 3B). Finally, to further substantiate the role of the proposed N-terminal lipid modifications on EEPD1 localization we engineered chimeric constructs consisting of the first 10 amino acids of EEPD1 fused to eGPF (EEPD1<sub>(1-10)</sub>-eGFP), with or without the myristoylation and/or palmitoylation sites. We found that the localization of these constructs was similar to that of the corresponding full-length or mutated EEPD1 protein variants (Figure 4C), thereby demonstrating that these first 10 amino acids of EEPD1 are both necessary and sufficient to confer plasma membrane localization. Unfortunately, the commercial antibodies we tested were unable to detect endogenous EEPD1 by immunostaining, but we could detect endogenous EEPD1 protein in crude-membrane
fractions from THP1 cells by immunoblotting (Figure 4D). Moreover, the level of endogenous EEPD1 protein in these crude membrane fractions was increased by LXR activation, as could be anticipated from it being an LXR transcriptional target. In aggregate, these experiments demonstrate that EEPD1 localizes to the plasma membrane and that this is dependent on lipid modifications of the first 10 amino acids.

Having established that EEPD1 is an LXR target in macrophages we then aimed to elucidate its function. Since a major role of LXR in macrophages is to promote cholesterol efflux we evaluated the role of EEPD1 in this process. We effectively silenced EEPD1 in THP1 macrophages using two independent siRNAs that reduced the basal, as well as the LXR-inducible expression of EEPD1 (Figure 5A). Importantly, effective silencing of EEPD1 did not alter the induction of ABCA1, or of other LXR-regulated genes in response to LXR ligand (Figure 5A and not shown), nor did silencing of EEPD1 affect foam-cell formation in THP1 macrophages (Supplementary figure 5). However, LXR-stimulated, ApoA1-dependent cholesterol efflux was strongly attenuated in EEPD1-silenced cells (Figure 5B). In contrast, efflux towards HDL remained unchanged (data not shown). Our results therefore point towards EEPD1 playing a role in promoting cholesterol efflux from macrophages. Since ABCA1 expression remained unchanged in EEPD1-silenced cells (Figure 5A), we evaluated the level of ABCA1 protein. Consistent with reduced efflux we determined that silencing EEPD1 reduced the cellular ABCA1 content by ~50% (Figure 6A). This reduction seemed specific as the level of ABCG1, a different cholesterol transporter and an LXR target, as well as of the transferrin receptor (TfR) remained unchanged (Figure 6A and Supplementary figure 6A,B). Moreover, the reduction in ABCA1 protein was not limited to human macrophages as we observed an equivalent decrease in Abca1 protein with no change in TfR or Abcg1 in murine J774 macrophages following Eepd1-silencing (Figure 6B and Supplementary figure 6C,D). Taken together, our results point toward EEPD1 being an LXR-target that is important for maintaining ABCA1 protein levels and promoting cholesterol efflux from macrophages.
Discussion

Macrophages are central determinants of atherosclerosis. Therefore, studies aimed at elucidating the genes governing their handling of lipids and inflammation is central to understanding their role in the vascular wall in diseased states. As such, the most important finding of our study is that using a systems approach we have identified a novel LXR-regulated gene, EEPD1, which by post-transcriptionally regulating ABCA1 abundance is a determinant of cholesterol efflux from macrophages.

THP1 macrophages are commonly used as a model for human-derived macrophages, and have been previously used to evaluate the transcriptional response to LXR ligands. Our study is distinct from these in that we simultaneously evaluated the transcriptional LXR program in response to distinct classes of ligands. An important aspect of our approach is that it allowed us to differentiate the response between synthetic and endogenous ligands. One obvious and expected finding was the absence of inhibition of the SREBP pathway by the synthetic ligand GW3965, which also underlies hepatosteatosis and increased lipogenesis in livers of mice treated with this compound. Less obvious was the lack of a large overlap between the transcriptional response of cells to the different classes of ligands, with each ligand eliciting a distinct profile. While in some cases this may represent quantitative differences (e.g. in our experimental setting Ac-LDL elicited a smaller change in LXR-dependent gene expression), others changes may reflect ligand-specific effects. For example, our RNA-seq analysis identified strong induction of glycolysis-associated genes, amongst others of PDK4, which is not observed with the other ligands (data not shown). This observation is in line with the notion that LXR ligands with specific transactivation profiles can be developed for LXR so as to e.g. potentially mitigate a lipogenic gene program.

Our study also identified a set of genes in macrophages that are pan-regulated by all the LXR ligands we tested, amongst them the novel target gene, EEPD1. Our studies support the notion that EEPD1 is a direct LXR target gene as we demonstrate that its regulation requires LXRs, it is rapidly induced by LXR ligands, and this induction also occurs in the
face of protein synthesis inhibition, ruling out a secondary transcriptional response. However, in our study we have been unable to identify a specific LXR-responsive element in the vicinity of the transcriptional start site of EEPD1 (data not shown). This does not impinge on our conclusion regarding regulation of EEPD1 expression by LXRα as it has been demonstrated that the relevant regulatory element can be located at a large distance from the regulated gene. Indeed, a ChIP-seq study of LXRα in THP1 macrophages lists LXR-responsive peaks in the vicinity of the EEPD1 locus. We have not evaluated this genomic region, but instead provide compelling evidence that in both murine and human macrophage cell lines and primary cells LXR robustly regulates expression of EEPD1/Eepd1.

LXRαs are central determinants of macrophage cholesterol metabolism, largely owing to their ability to promote reverse-cholesterol efflux. Enhanced cholesterol efflux is critically dependent on transcriptional regulation of ABCA1 and ABCG1 by LXRαs. Accordingly; loss of LXRαs or of these cholesterol efflux transporters has dramatic consequences on accumulation of cholesterol in macrophages and on atherogenesis. As such, the identification of EEPD1 as a transcriptional target of LXRαs in macrophages and the demonstration that this gene is necessary for maximal LXR-stimulated ApoA1-dependent cholesterol efflux contributes to our understanding of LXRαs function in macrophages. We propose that the underlying cause for reduced efflux from EEPD1-silenced cells is reduced cellular abundance of ABCA1 and decreased ABCA1 density on the plasma membrane. Since silencing of EEPD1 does not impair the level of ABCA1 mRNA or its induction by LXR stimulation the decrease of ABCA1 protein in EEPD1/Eepd1-silenced cells likely involves a post-transcriptional event.

ABCA1 is reported to have a relatively short-half life, estimated in murine macrophages to be ~1 hour. However, despite its inherent instability there is ample evidence demonstrating that LXR activation robustly increases ABCA1 in macrophages in a time- and dose-dependent manner. This suggests that next to transcriptional regulation, LXRαs may also promote stabilization of ABCA1. Our results are consistent with the idea that LXR-dependent regulation of EEPD1 contributes to stabilization of ABCA1. The underlying
mechanism for this is still unclear, but may involve modification of cellular membrane lipids, a function that has been ascribed to LXR. Members of the EEP-domain-containing family of proteins, to which EEPD1 belongs, catalyze cleavage of phosphodiester bonds found in nucleic acids, phospholipids, and perhaps also proteins. Specifically, several members of this family have lipid phosphatase activity, mainly towards inositol phosphates. Since inositol phosphates are important determinants of intracellular trafficking, EEPD1 could control ABCA1 abundance by modulating the level of specific inositol phosphate species to promote retention of ABCA1 in the plasma membrane or prevent its trafficking towards degradation pathways. However, we point out that this would need to be rather specific, as EEPD1-silencing does not change the level of ABCG1 or TfR. Alternatively, EEPD1 may directly regulate the stability of ABCA1. A cytoplasmic Proline/Glutamate/Serine/Threonine (PEST) containing-sequence in ABCA1 has been demonstrated to act as a phosphorylation-dependent switch that controls stability of ABCA1. Accordingly, phosphorylation of the PEST domain promotes Calpain-mediated degradation of ABCA1 and attenuates ApoA1-dependent cholesterol efflux. Therefore, while speculative, EEPD1 may act as a phosphatase to directly stabilize ABCA1 at the plasma membrane by preventing its phosphorylation-dependent degradation. Future studies to address these possibilities are clearly warranted.

Reports on the possible physiological roles of EEPD1 are scarce. Next to our study, which is the first to identify EEPD1 as an LXR target and ascribe it a function in cellular sterol homeostasis, two other studies from the Hromas group recently reported that EEPD1 has a function in DNA repair in the nucleus. Wu et al. compellingly demonstrated that loss of EEPD1 in several cell types facilitates repair of stressed replication forks induced by DNA-damaging chemicals, and that it does so by promoting homologous recombination. In our studies we have not observed localization of EEPD1 in the nucleus under any of the conditions evaluated. Rather, endogenous EEPD1 was enriched in crude membrane fractions, and heterologous EEPD1 specifically localized to the plasma membrane. Furthermore, in line with the predicted lipid-modifications of the N-terminal sequence of EEPD1 we found that
the first 10 amino acids are sufficient to localize GFP exclusively to the plasma membrane. These findings are in odds with the nuclear localization of EEPD1 reported by Wu et al.\textsuperscript{51}

We note, however, that for these studies overexpression of a construct encoding GFP-EEPD1 was used, which would mask the native lipidation sites in the N-terminal sequence of EEPD1. Indeed, when we tested localization of an N-tagged EEPD1 construct we found that it is not localized to the plasma membrane (data not shown). Reconciling the proposed functions of EEPD1 is difficult at present yet the sterol-dependent regulation of EEPD1 by LXR\textsubscript{s} is consistent with it having a function in cholesterol homeostasis, similar to other LXR-regulated genes. However, we cannot rule out the possibility that in different contexts, or cell types, EEPD1 adopts a different function. In that respect, the fact we only identify sterol-dependent regulation of EEPD1 in macrophages, but not in other cell types including those studied by Wu et al.\textsuperscript{51} is consistent with the possibility that EEPD1 has cell-type-specific functions that will need to be addressed in future studies.

In conclusion, our study identifies EEPD1 as a sterol-responsive gene that is regulated by LXR\textsubscript{s} in macrophages. We propose that EEPD1 acts as part of the LXR-regulated program to promote ABCA1-dependent cholesterol efflux from cells and that it does so by maintaining stability of ABCA1. Given the importance of cholesterol efflux in macrophage cholesterol homeostasis the ability of EEPD1 to regulate this process may have implications for atherogenesis.
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Conflict of interest

The authors declare that they have no conflicts of interest with the content of this article.

Abbreviations

Liver X Receptors; LXR, Endonuclease-exonuclease-phosphatase family domain containing 1; EEPD1, Phorbol 12-myristate 13-acetate; PMA, Fetal bovine serum ; FBS, 22R-hydroxycholesterol ; 22R-HC, Acetylated-LDL ; Ac-LDL, Doxycycline ; DOX, Transferrin receptor ; TfR, Inducible degrader of the LDLR ; IDOL, Bone marrow derived macrophages ; BMDM, peripheral blood monocyte cells ; PBMC, ATP-binding cassette transporter A1 ; ABCA1, ATP-binding cassette transporter G1 ; ABCG1, sterol-regulatory element binding proteins; SREBP, false discovery rate ; FDR, Gene ontology ; GO, principal component analysis ; PCA, Ingenuity Pathway Analysis ; IPA
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Figure 1. Transcriptional profiling of LXR activation in human THP1 macrophages. (A) THP1 macrophages were sterol-depleted and treated with 1μM GW3965, 5μM 22R-HC, 5μM Desmosterol, or 50μg/mL Ac-LDL for 18 hours. The Venn diagram details the overlap in differentially regulated genes (B) Hierarchical clustering of differentially expressed genes in response to the different LXR ligands. The top 3 GO biological processes that were enriched in each the major gene clusters are indicated (C) PCA plot of the changes in gene expression in response to LXR ligands and sterol depletion. Axis titles show the percentage of variance explained by each of the principal components.
Figure 2. Transcriptional profiling identifies LXR-regulated genes in THP1 macrophages. (A) Heat map showing the fold-change (log2-transformed) in expression of the 39 most differentially expressed transcripts between the different LXR-ligands with steroldepletion as baseline. Genes in bold were further validated and characterized. (B) THP1 differentiated macrophages were treated for 18 hours in sterol-depleted medium with vehicle, 1μM GW3965, 5μM Desmosterol, 5μM 22R-HC, or 10μg/mL Ac-LDL. Subsequently, expression of the indicated genes was determined by qPCR. Each bar represents the mean ± SD (n=4). *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.
Figure 3. EEPD1 is a direct LXR target gene in human and mouse macrophages. (A) THP1 macrophages were treated for 6 hours with 1μM GW3965 or vehicle control in the presence of 10μg/mL cycloheximide. Expression of the indicated genes was determined by qPCR and displayed as relative mRNA expression compared to vehicle treated controls. Each bar represents the mean ± S.D. (n=4). (B) BMDMs from wildtype (WT) and Lxrαβ (-/-) (DKO) mice were cultured in sterol-depleted medium for 18 hours and subsequently treated with 1μM GW3965, 5μM Desmosterol, 5μM 22R-HC or vehicle control for 6 hours. Expression of the indicated genes was determined and each bar represents the mean ± SD (n=4). (C) Human PBMCs were differentiated to macrophages and treated with vehicle, 1μM GW3965, or 1μM T0901317. Expression of EEPD1 was determined and each bar represents the mean ± SD (n=3). (D) The indicated tissues were isolated from WT mice (n=3) and expression of Eepd1 determined by qPCR. Each bar represents the mean ± SD (E) The indicated human (left) and murine (right) cell lines were treated with 1μM GW3965 or vehicle control for 18 hours. EEPD1/Eepd1 mRNA expression was determined by qPCR and each bar represents the mean ± SD (n=4). *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.
**Figure 4. EEPD1 is anchored to the plasma membrane.** (A) Schematic representation of the structure of EEPD1 (569 amino acids) depicting the two N-terminal helix-hairpin-helix (HhH) and the C-terminal Exonuclease-Endonuclease-Phosphatase (EEP) domains. Box: Evolutionary conservation of the first 20 amino acids of EEPD1 with the predicted myristoylation and palmitoylation sites indicated (B) COS7 cells were transfected with WT or mutated EEPD1-GFP constructs as indicated. Representative images from fixed and live cells were taken 48 hours after transfection. (C) COS7 cells were transfected with WT or mutated EEPD1(1-10)-GFP constructs and representative images are shown. (B,C) Scale bar is 5 μm. (D) THP1 macrophages were grown in either sterol-containing or sterol-depletion medium for 16 hours with or without 1μM GW3965 or vehicle control. Subsequently, crude membrane fractions were prepared and analyzed by immunoblotting as indicated. Endogenous EEPD1 levels were quantified and normalized to the level of TOM20 (an abundant and stable mitochondrial membrane protein) and displayed as the average of 3 independent experiments.
Figure 5. EEPD1 silencing decreases ApoA1-dependent cholesterol efflux. (A) THP1 macrophages were transfected with control (non-targeting, siNT), or two independent EEPD1 siRNAs (siEEPD1) for 48 hours. Subsequently, cells were treated with 1μM GW3965 or vehicle control for 18 hours and expression of the indicated genes determined by qPCR. Each bar represents the mean ± SD (n=4). (B) THP1 macrophages were transfected as above and then treated with or without 1μM GW3965 for 4 hours followed by an additional 18 hours with medium containing 2 μCi/mL [3H] Cholesterol and 50 μg/ml Ac-LDL. Cholesterol efflux was initiated by incubating the cells with or without 10μg/mL ApoA1 for 6 hours. Cholesterol efflux is expressed as the percentage of the radioactivity released from cells into the medium relative to the total radioactivity in cells and medium combined. Results were calculated as net efflux (efflux with ApoA1 minus 0.2% BSA alone) and shown relative to siNT-treated vehicle cells. Each bar represents the mean ± SD of 4 independent experiments done in duplicate. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

Figure 6. Silencing of EEPD1/Eepd1 decreases abundance of ABCA1 protein. (A) THP1, or (B) J774 macrophages were transfected with control (non-targeting, NT) or EEPD1/Eepd1 siRNAs 48 hours. Subsequently, cells were treated with 1μM GW3965 or vehicle control for 16 hours and total cell lysates were immunoblotted as indicated. The level of ABCA1 was determined by densitometry after normalization to actin and the average indicated (A, n=3; B, n=4).
Supplementary figure 1. Quality control of RNAseq analysis. (A) Correlation plots of the log2 read counts for each of the ligands compared to the sterol-depleted baseline. (B) Volcano plots of the –log10 FDR-adjusted p-value versus the log2 fold-change in gene expression for each of the ligands compared to the sterol-depleted baseline. (C) MA plots of the log2 fold-change in gene expression versus the average log2 read count for each of the ligands tested compared to the sterol-depleted baseline. Black dots represent non-significantly changed transcripts, while colored dots represent significantly changed transcripts with an FDR-adjusted p-value < 0.05.
Supplementary figure 2. Pathway analysis of differentially changed genes. (A) Graphical representation of the number of genes differentially expressed in cells cultured in sterol-containing and sterol-depleted culture medium (B) IPA top 10 canonical pathways enriched in cells cultured in sterol-depletion medium versus cells cultured in sterol-containing medium for each of the four different ligands versus sterol-depleted conditions. (C) GO analysis of top 100 genes responsible for the majority of the variance in each of the three principal components plotted in Figure 1C.
Supplementary figure 3. Regulation of LXR target genes by GW3965. (A) THP1 macrophages were cultured in sterol depletion medium for 24 hours. Subsequently, cells were treated with 1μM GW3965 for 3, 6, 12, or 24 hours and expression of the indicated genes determined by qPCR. Each bar represents the mean ± SD (n=3). (B) The indicated cells were cultured and treated as indicated in figure 3D and expression of ABCA1/Abca1 determined by qPCR. Each bar represents the mean ± SD (n=4). **p<0.01, ***p<0.001, and ****p<0.0001.
Supplementary figure 4. EEPD1 localizes to the plasma membrane (A) EEPD1-GFP expression was induced in RAW264.7 by treating the cells with 1μg/mL DOX for 48 hours. Subsequently, cells were treated with 10μM myristic acid for 8 hours, fixed and nuclei counterstained with DAPI. Image is representative of three independent experiments. (B) EEPD1-MYC expression was induced as described above. Subsequently, cells were cultured in sterol-containing or sterol depletion medium with or without 1μM GW3965 for 18 hours prior to fixation. The Myc tag was detected by immunofluorescence and nuclei were counterstained with DAPI. Scale bar 5μm.

Supplementary figure 5. EEPD1 silencing does not influence foam cell formation in THP1 macrophages. Cells were cultured and treated as described in figure 5B. Subsequently, cells were fixed and lipid droplets assessed with Oil Red O staining. Representative images are shown. Scale bar 10 μm.
Regulation of ABCA1 by EEPD1

Supplementary figure 6. EEPD1 silencing does not change basal and LXR inducible levels of ABCG1 in macrophages. THP1 (A,B) or J774 (C,D) macrophages were treated as described in figure 6A,B and analyzed by immunoblotting (A,C) or by qPCR (B,D). Representative immunoblots are presented (n=4). Each bar represents the mean ± SD (n=4). *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.