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EEPD1 Is a Novel LXR Target Gene in Macrophages Which Regulates ABCA1 Abundance and Cholesterol Efflux

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Objective—The sterol-responsive nuclear receptors, liver X receptors α (LXRα, *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 *ABCA1* and *ABCG1* to promote efflux of excess cellular cholesterol. However, the full set of genes that contribute to LXR-stimulated cholesterol efflux is unknown, and their identification is the objective of this study.

Approach and Results—We systematically compared the global transcriptional response of macrophages to distinct classes of LXR ligands. This allowed us to identify both common and ligand-specific transcriptional responses in macrophages. Among these, we identified endonuclease–exonuclease–phosphatase family domain containing 1 (*EEPD1/KIAA1706*) as a direct transcriptional target of LXRs in human and murine macrophages. EEPD1 specifically localizes to the plasma membrane owing to the presence of a myristoylation site in its N terminus. Accordingly, the first 10 amino acids of EEPD1 are sufficient to confer plasma membrane localization in the context of a chimeric protein with GFP. Functionally, we report that silencing expression of *EEPD1* blunts maximal LXR-stimulated Apo AI-dependent efflux and demonstrate that this is the result of reduced abundance of ABCA1 protein in human and murine macrophages.

Conclusions—In this study, we identify *EEPD1* as a novel LXR-regulated gene in macrophages and propose that it promotes cellular cholesterol efflux by controlling cellular levels and activity of ABCA1.

Visual Overview—An online visual overview is available for this article. **(*Arterioscler Thromb Vasc Biol*. 2017;37:423-432. DOI: 10.1161/ATVBAHA.116.308434.)**

Key Words: ABCA1 ■ cholesterol efflux ■ cholesterol metabolism ■ LXR ■ macrophages ■ nuclear receptors

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.[1] Atherosclerosis is a lipid-driven disease that is also characterized by the presence of low-grade inflammation in the vascular wall.[2] Within the atherosclerotic plaque, macrophages are able to, among others, internalize modified low-density lipoprotein, promote removal of excess cholesterol from the developing atherosclerotic plaque, and respond to local and systemic inflammatory cues.[3] 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.[4,5] LXRs 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 toward reducing the cellular cholesterol burden and concomitantly inhibiting inflammatory signaling. This is largely achieved through LXRs' ability to (1) enhance cholesterol transport through their target genes ABCA1 (ATP-binding cassette transporter A1),[6–8] ABCG1 (ATP-binding cassette transporter G1),[9] and APOE,[10] (2) limit uptake of lipoprotein-derived cholesterol by inducing expression of the E3 ubiquitin ligase IDOL (inducible degrader of the low-density lipoprotein [LDL] receptor),[11,12] and (3) transrepress inflammatory signaling induced by inflammatory cues.[13–15] Accordingly, *Lxrαβ*$^{-/-}$ macrophages accumulate cholesterol in vivo and are hyper-responsive to inflammatory stimuli.[13,16] Reciprocally, pharmacological

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Nonstandard Abbreviations and Acronyms	
22R-HC	22R-hydroxycholesterol
ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette transporter G1
Ac-LDL	acetylated-LDL
EEPD1	endonuclease-exonuclease-phosphatase family domain containing 1
IDOL	inducible degrader of the LDL receptor
LDL	low-density lipoprotein
LXR	liver X receptors
LXRE	LXR-responsive element

engagement of LXRs promotes reverse cholesterol transport and decreases atherosclerotic plaque development in *ApoE*$^{-/-}$ and *Ldlr*$^{-/-}$ mice fed an atherogenic diet.[17] LXR activity in macrophages, liver, and the intestine has been reported to contribute to their antiatherosclerotic function.[16,18–22]

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.[23–25] Similarly, endocytosis of (modified) lipoproteins or efferocytosis increases the cellular cholesterol and oxysterol pool and also promotes LXR signaling.[26,27] High-affinity synthetic agonists have been also developed to therapeutically target LXRs, with several reported to have preferential activation of, for example, LXRβ over LXRα resulting in a differential transcriptional response.[28] Although these different classes of agonists activate LXRs, natural and synthetic agonist markedly differ with respect to their inhibitory effect on the sterol-regulatory element-binding proteins pathway. Oxysterols and intermediates of the cholesterol biosynthetic pathway prevent processing and maturation of sterol-regulatory element-binding proteins to their transcriptionally active form, whereas synthetic ligands do not.[29,30] 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 thoroughly 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 and ligand-specific LXR-dependent transcriptional responses to these ligands. Among 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

Materials and Methods are available in the online-only Data Supplement.

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 phorbol 12-myristate 13-acetate—treated with 4 different classes of LXR ligands: GW3695 (synthetic), 22R-hydroxycholesterol (22*R*-HC; oxysterol), desmosterol (cholesterol biosynthesis intermediate), and acetylated-LDL (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 *P* value of <0.05 in response to sterol depletion. Of these, 555 transcripts were upregulated, and 616 transcripts were downregulated (Figure IIA in the online-only Data Supplement). Subsequent treatment of sterol-depleted THP1 macrophages with 1 μmol/L GW3965, 5 μmol/L 22*R*-HC, 5 μmol/L desmosterol, or 50 μg/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 data sets 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 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 ingenuity pathway analysis revealed a similar profile, with GW3965 treatment resulting in a transcriptional profile that was less enriched for genes involved in cholesterol biosynthesis when compared with either the other ligands versus sterol depletion or sterol depletion versus cholesterol-rich medium (Figure IIB in the online-only Data Supplement). Similarly, principal component (PC) analysis showed that there was little variance between the biological replicates of each of the experimental conditions and that each ligand induced a distinct gene expression profile when compared with 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 data sets was caused by genes involved in lipid metabolism (PC1 and PC2) and, to a lesser extent, the inflammatory response (PC3; Figure IIC in the online-only Data Supplement).

To filter out genes that were most strongly induced by all 4 of the ligands tested in our screen, we applied a stricter cutoff in which only protein-coding transcripts that showed a 1.5-fold change in either direction with an false discovery

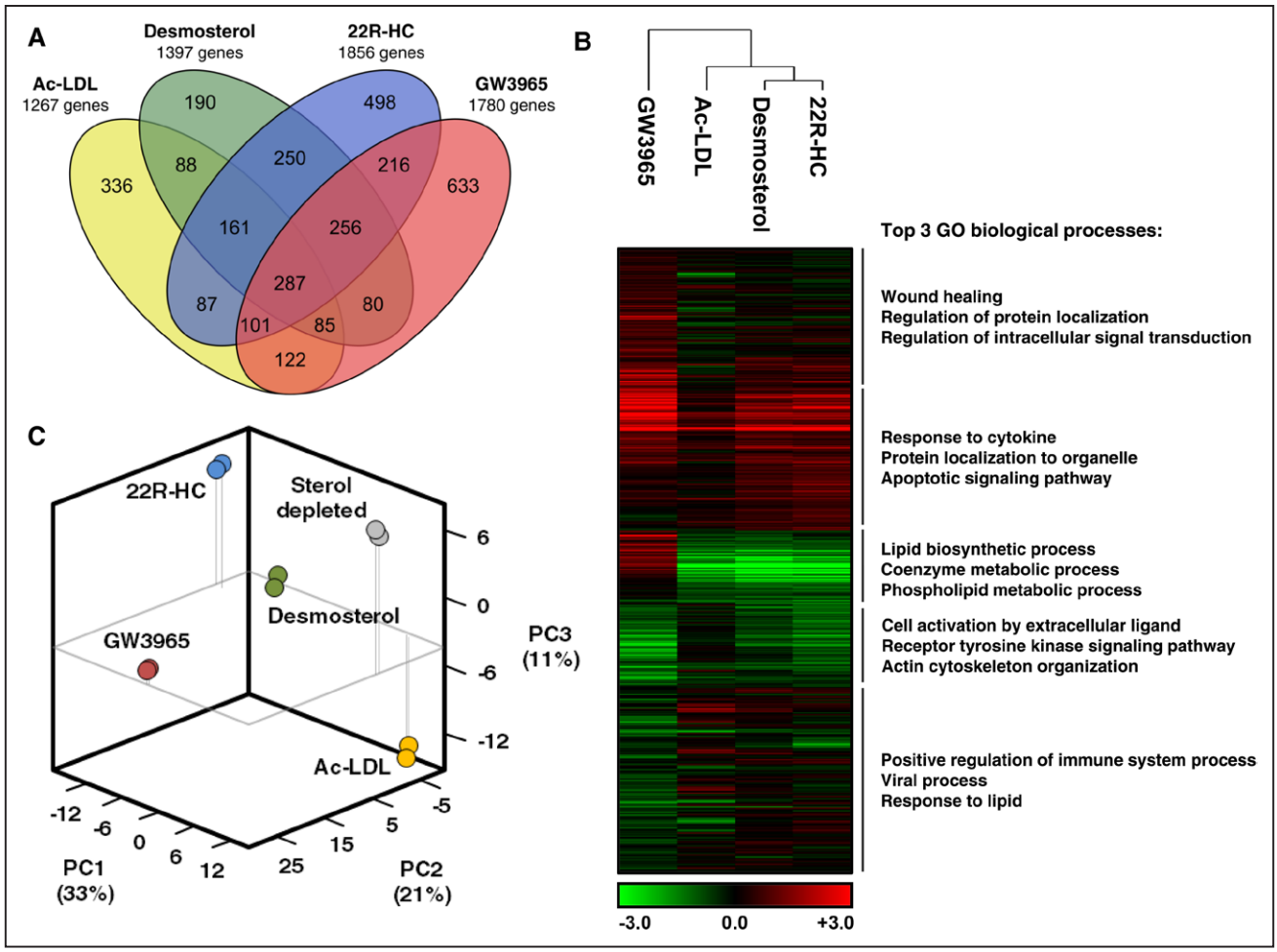

Figure 1. Transcriptional profiling of liver X receptors (LXR) activation in human THP1 macrophages. **A**, THP1 macrophages were sterol depleted and treated with 1 µmol/L GW3965, 5 µmol/L 22R-hydroxycholesterol (*22R*-HC), 5 µmol/L desmosterol, or 50 µg/mL acetylated-low-density lipoprotein (Ac-LDL) for 18 h. 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 Gene ontology (GO) biological processes that were enriched in each the major gene clusters are indicated. **C**, Principal component analysis (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.

rate–adjusted *P* value of <0.001 were considered (Figure 2A). Using this cutoff, we identified several established LXR-responsive genes, such as *ABCA1,*[6] *ABCG1,*[9] *LXRα,*[31] *SMPDL3A,*[32] and *IDOL.*[11] *ApoE*, which is an established LXR target in macrophages,[10] did not meet this cutoff but was also included in the subsequent evaluation. Several additional genes that have not been previously described as LXR targets, including *EEPD1*, *PPF1A2*, *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, 22*R*-HC, and Ac-LDL inhibited expression of sterol-regulatory element-binding protein–regulated genes (eg, *HMGCR* and *LDLR*), whereas GW3965 did not (Figure 2A and 2B).

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 *PPF1A2*, *IQSEC1*, and *PBX4* expression by LXR activation was abolished by cycloheximide, suggesting that 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; Figure IIIA in the online-only Data Supplement).

Among the novel LXR-responsive genes identified in our screen, *EEPD1* is one of the few that were 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 bone marrow–derived macrophages from wild-type cells (Figure 3B). Regulation of *Eepd1* expression by the ligands was strictly dependent on LXRs because

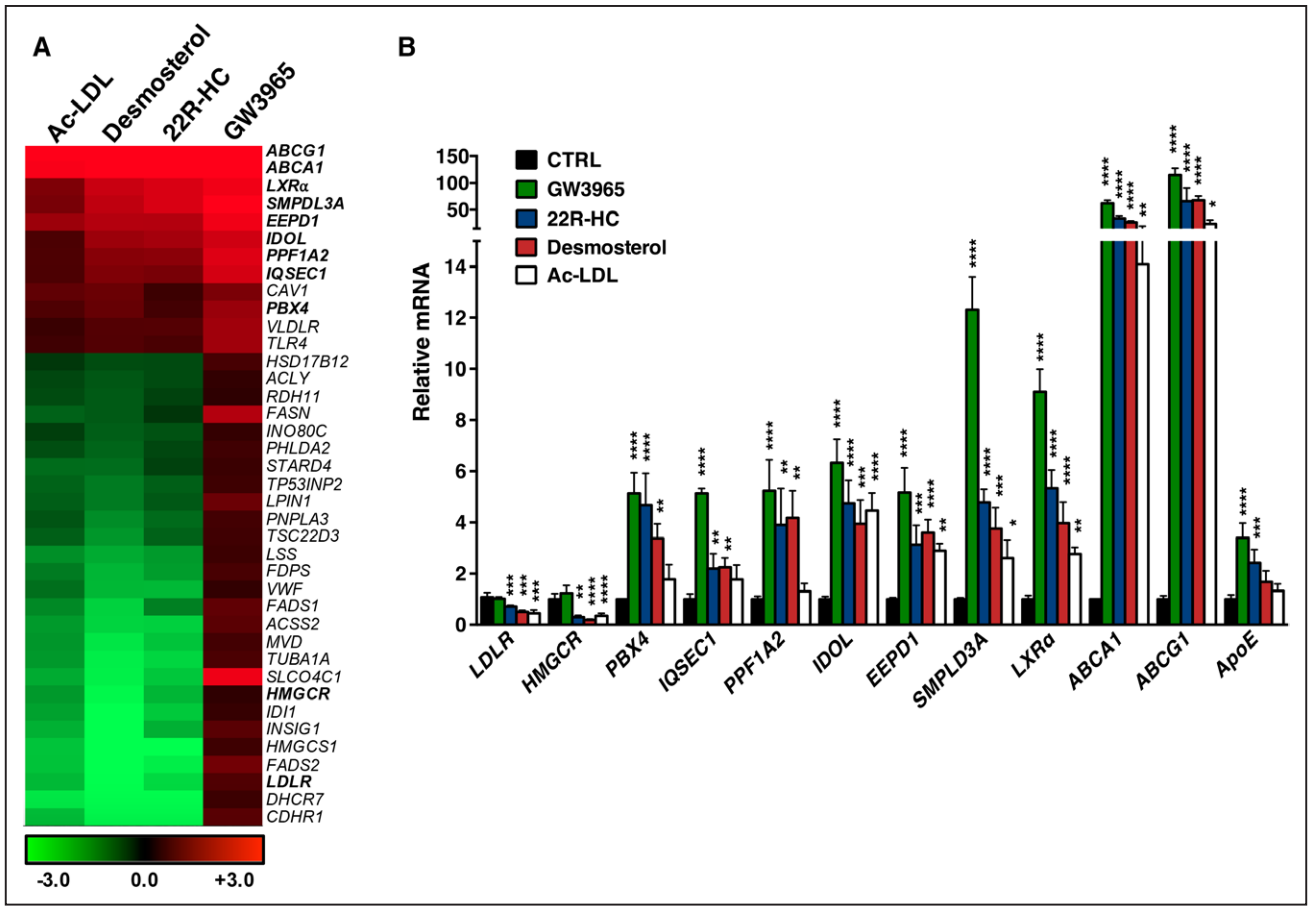

Figure 2. Transcriptional profiling identifies liver X receptors (LXR)–regulated genes in THP1 macrophages. **A**, Heat map showing the fold change (log_2-transformed) in expression of the 39 most differentially expressed transcripts between the different LXR ligands with sterol depletion as baseline. Genes in bold were further validated and characterized. **B**, THP1 differentiated macrophages were treated for 18 h in sterol-depleted medium with vehicle, 1 μmol/L GW3965, 5 μmol/L desmosterol, 5 μmol/L 22R-hydroxycholesterol (22*R*-HC), or 10 μg/mL acetylated-low-density lipoprotein (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, *****P*<0.0001.

it was blunted in macrophages derived from *Lxrαβ*$^{-/-}$ mice (double knockout mice). In human macrophages derived from peripheral blood monocytes, expression of *EEPD1* was induced by 2 synthetic LXR ligands and akin to other established LXR-regulated genes was sensitive to sterol depletion (Figure 3C). Having established that *EEPD1/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 and in macrophage-rich tissues (eg, skeletal muscle, white adipose tissue, and spleen). We therefore anticipated that similar to most other LXR-regulated genes, *EEPD1* would be regulated by LXR activation in multiple cell types. To test this hypothesis, we investigated the regulation of *EEPD1/Eepd1* by LXR in several human and murine cell lines that originate from different tissues. In these cells, we found that *EEPD1/Eepd1* was only induced in macrophage-like cells (Figure 3E). This was not simply because of aberrant LXR signaling in these cells since in response to LXR ligand, all were able to activate the canonical LXR target *ABCA1/Abca1* (Figure IIIB in the online-only Data Supplement). Consistent with LXR-dependent regulation, we identified a potential LXR-responsive element (LXRE) within intron 2 of *EEPD1* by analyzing a previously reported LXRα ChIP-seq study (Figure 4A).[33] In human primary macrophages, this LXRE is adjacent to a macrophage lineage–specifying PU.1 peak. In addition, this genomic region is enriched for H3K27Ac and H3K4me1 histone modifications, all of which were absent in human adipocytes, skeletal muscle, and HepG2 cells (Figure IV in the online-only Data Supplement). These observations suggest that the macrophage-specific regulation of EEPD1 by LXRs is the result of a permissive epigenetic landscape surrounding the LXRE in intron 2 that is not present in other cell types. The corresponding LXRE-containing genomic region could drive expression of a luciferase reporter in response to transfection of LXR/RXR, and furthermore when the cells were cotreated with synthetic LXR/RXR ligands (Figure 4B). Mutating the predicted LXRE in this context ablated the response to both LXR/RXR and the ligands. Collectively, these results show that *EEPD1* is a direct, macrophage-specific LXR target gene.

The *EEPD1* gene encodes a 569 amino acids protein that contains several distinct functional domains. Its N-terminal region contains 2 adjacent helix–hairpin–helix motifs, a motif which is often associated with DNA binding. The C-terminal region contains a large (EEP) domain (Figure 5A). The helix–hairpin–helix motifs present in EEPD1 could suggest

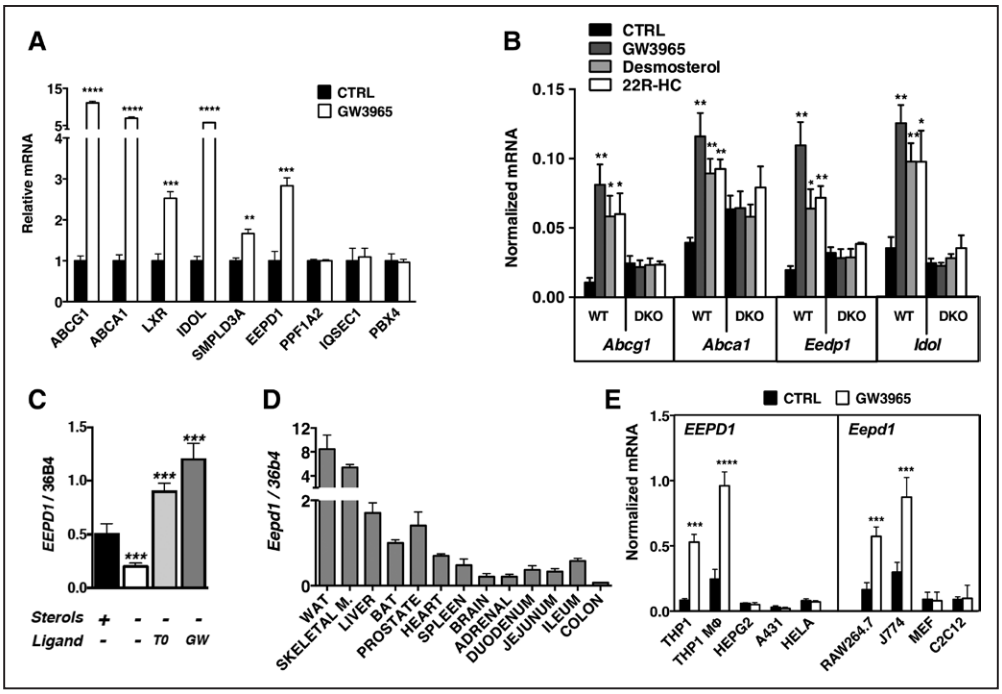

Figure 3. Endonuclease–exonuclease–phosphatase family domain containing 1 (EEPD1) is a direct liver X receptors (LXR) target gene in human and mouse macrophages. **A**, THP1 macrophages were treated for 6 h with 1 μmol/L GW3965 or vehicle control in the presence of 10 μg/mL cycloheximide. Expression of the indicated genes was determined by qPCR and displayed as relative mRNA expression compared with vehicle-treated controls. Each bar represents the mean±SD (n=4). **B**, bone marrow–derived macrophages (BMDMs) from wild-type (WT) and *Lxr*$\alpha\beta^{-/-}$ (double knockout mice [DKO]) mice were cultured in sterol-depleted medium for 18 h and subsequently treated with 1 μmol/L GW3965, 5 μmol/L desmosterol, 5 μmol/L 22R-hydroxycholesterol (22*R*-HC) or vehicle control for 6 h. Expression of the indicated genes was determined, and each bar represents the mean±SD (n=4). **C**, Human peripheral blood monocyte cells (PBMCs) were differentiated to macrophages and treated with vehicle, 1 μmol/L GW3965, or 1 μmol/L 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 1 μmol/L GW3965 or vehicle control for 18 h. *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, *****P*<0.0001

association of the protein with DNA and involvement in DNA-binding related processes (eg, DNA repair). However, in silico analysis of its amino acid sequence revealed that EEPD1 is unique among the EEP superfamily of proteins in that it contains highly evolutionary-conserved myristoylation and palmitoylation lipid modification sites, which are known to serve as membrane anchors (Figure 5A). To evaluate the cellular localization of EEPD1, we generated an $EEPD1_{wt}$-eGFP (enhanced green fluorescent protein) expression construct and studied its localization in COS7 cells (Figure 5B). Consistent with the presence of the highly conserved N-terminal myristoylation and palmitoylation sites, wild-type EEPD1 localized exclusively to the plasma membrane in both live and fixed cells. Remarkably, abolishing the single myristoylation site ($EEPD1_{G2A}$-eGFP) resulted in drastically altered localization of EEPD1 (Figure 5B). A similar shift in cellular localization was also observed when we treated RAW264.7 macrophages that express an inducible $EEPD1_{wt}$-eGFP construct with 2-hydroxymyristic acid, a potent inhibitor of the myristoyl conjugating enzyme *N*-myristoyltransferase (Figure VA in the online-only Data Supplement). Mutation of the predicted palmitoylation site ($EEPD1_{C7A}$-eGFP), or of both sites simultaneously ($EEPD1_{G2A/C7A}$-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 5B). It should be noted that under these conditions, we were unable to observe EEPD1 localized in the nucleus. However, because these lipid modifications are dynamic,[34] 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 after sterol depletion and 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 (Figure VB in the online-only Data Supplement). 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 ($EEPD1_{(1–10)}$-eGFP), with or without the predicted myristoylation and 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 5C), 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 5D). Moreover, the level of endogenous EEPD1 protein in these crude membrane fractions was

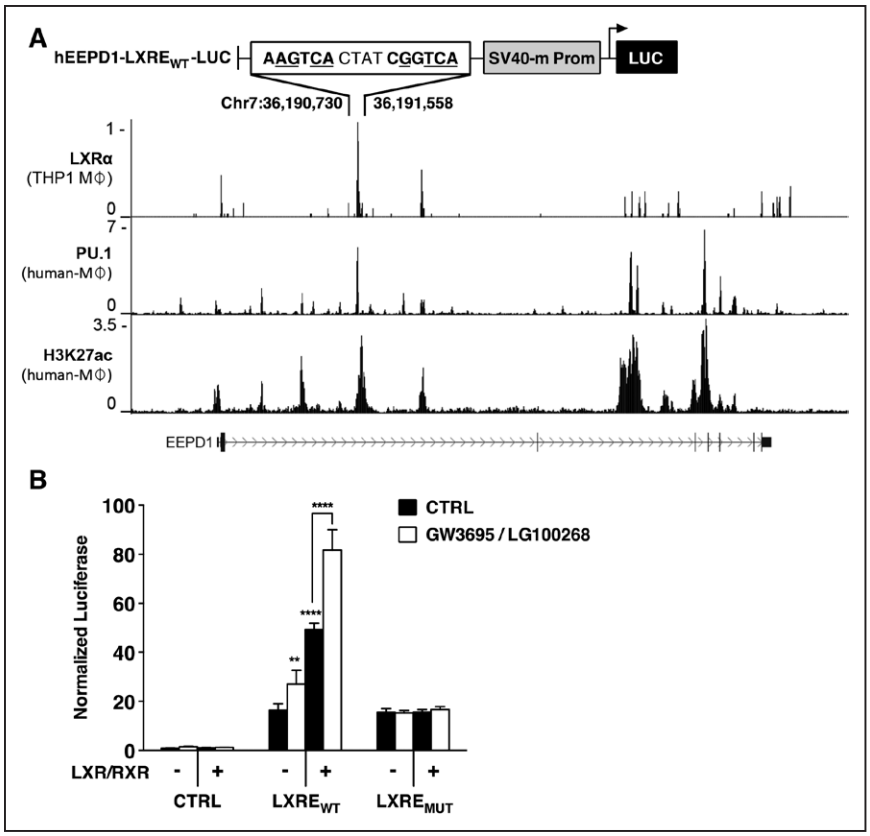

Figure 4. A liver X receptors–responsive element (LXRE) in intron 2 of endonuclease–exonuclease–phosphatase family domain containing 1 (*EEPD1*) drives LXR-dependent expression. **A**, An LXR ChIP-seq experiment was analyzed and used to identify an active LXRE in human THP1 cells (GSM700470). Similarly, PU.1 binding sites (GSM785501) and activate enhancer regions marked by H3K27Ac (GSM785500) were evaluated in human monocyte-derived macrophages (human MΦ). The wild-type LXRE-containing region was cloned into pGL2-SV40 firefly luciferase (LXRE$_{WT}$). The underlined nucleotides were altered to create a mutant LXRE (LXRE$_{MUT}$). **B**, HEK293T cells were transfected with the indicated luciferase reporters with or without LXR and RXR expression plasmids. Subsequently, cells were treated with 1 μmol/L GW3965 and 100 nmol/L LG100268 for 24 h. In all luciferase experiments, the transfection efficiency was normalized using Renilla luciferase, which was cotransfected. Each bar represents the mean±SD relative to vehicle-treated control cells (CTRL; n=6). $^{**}P<0.01$, $^{****}P<0.0001$.

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/Eepd1* in THP1 and J774 macrophages, respectfully, using independent siRNAs that reduced the basal, as well as the LXR-inducible expression of *EEPD1/Eepd1* mRNA and EEPD1 protein levels (Figure VIA through VIC in the online-only Data Supplement). Importantly, effective silencing of *EEPD1/Eepd1* did not alter the induction of *ABCA1/Abca1* or of other LXR-regulated genes in response to LXR ligand (Figure VIA through VIC). However, LXR-stimulated Apo A1-dependent cholesterol efflux was attenuated in *EEPD1/Eepd1*-silenced THP1 and J774 cells (Figure 6A and 6B). In contrast, efflux toward high-density lipoprotein remained unchanged, in line with no changes in ABCG1 protein levels (Figure VII in the online-only Data Supplement). Our results therefore point toward EEPD1 playing a role in promoting cholesterol efflux from macrophages. Because *ABCA1/Abca1* expression remained unchanged in *EEPD1/Eepd1*-silenced cells, we evaluated the level of ABCA1 protein. Consistent with reduced efflux, we determined that silencing *EEPD1* reduced the LXR-stimulated level of cellular ABCA1 content by ≈50% in both macrophage cell types (Figure 6C and 6D). This reduction seemed specific, as the level of 2 established cholesterol transporters, ABCG1/Abcg1 and SR-BI, and of the transferrin receptor remained unchanged (Figure 6C and 6D; Figure VIA in the online-only Data Supplement). Taken together, our results point toward EEPD1 being an LXR-regulated target gene that is important for maintaining ABCA1 protein levels and promoting cholesterol efflux from macrophages.

Discussion

Macrophages are central determinants of atherosclerosis.[3] Therefore, studies aimed at elucidating the genes governing their handling of lipids and inflammation are central to understanding their role in the vascular wall in diseased states. As such, the most important finding of our study is that using global transcription analysis, 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.[30,35] 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 sterol-regulatory element-binding protein pathway by the synthetic ligand GW3965, which also underlies hepatosteatosis and increased lipogenesis in livers of mice treated with this compound.[36] 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. Although in some cases, this may represent

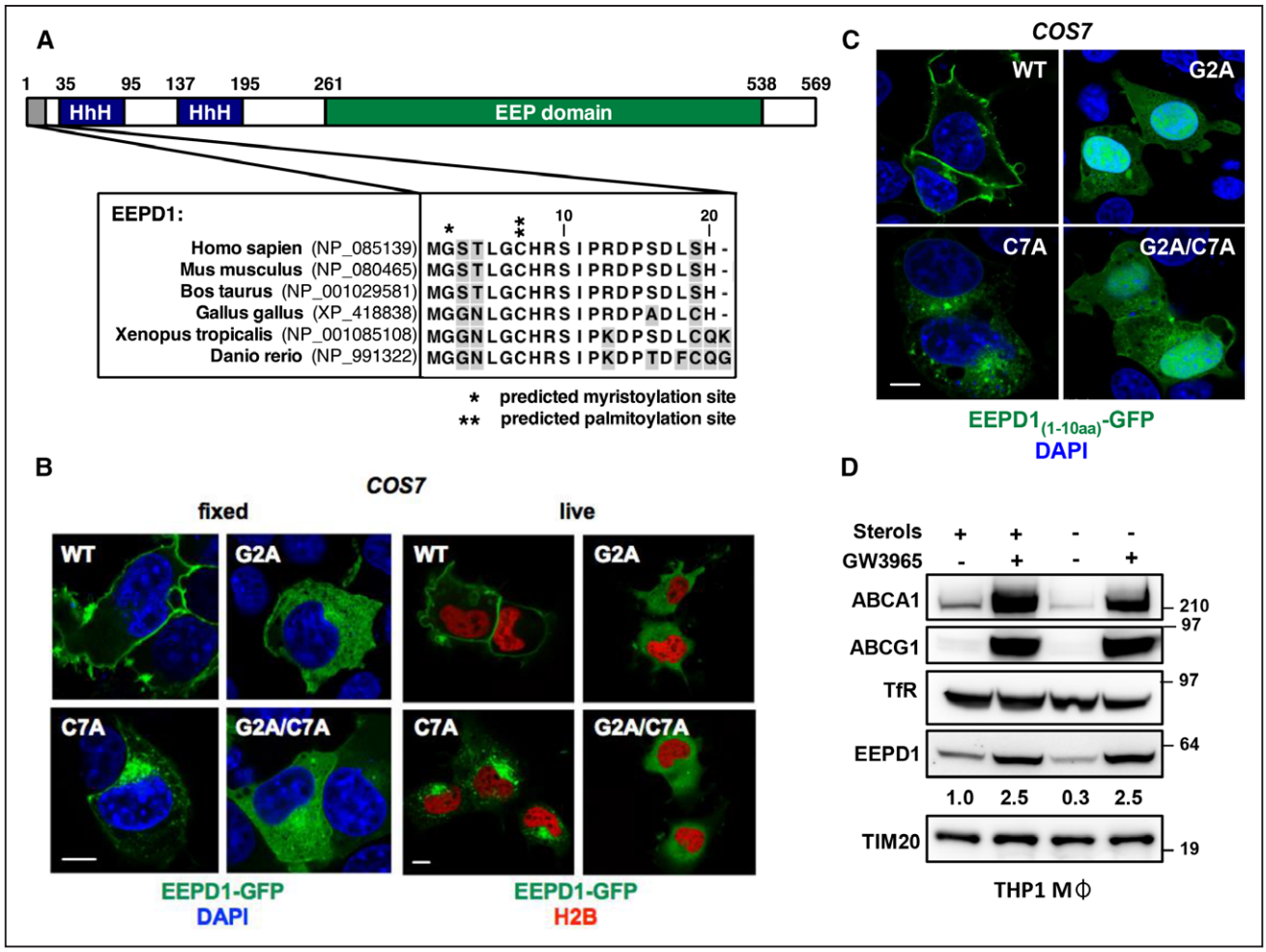

Figure 5. Endonuclease–exonuclease–phosphatase family domain containing 1 (EEPD1) is anchored to the plasma membrane. **A**, Schematic representation of the structure of EEPD1 (569 amino acids) depicting the 2 N-terminal helix–hairpin–helix (HhH) and the C-terminal exonuclease–endonuclease–phosphatase (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 wild-type (WT) or mutated EEPD1-GFP constructs as indicated. Representative images from fixed and live cells were taken 48 h after transfection. **C**, COS7 cells were transfected with WT or mutated $EEPD1_{(1–10)}$-GFP constructs, and representative images are shown. **B** and **C**, Scale bar is 5 μm. **D**, THP1 macrophages were grown in either sterol-containing or sterol-depleted medium for 16 h with or without 1 μmol/L 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.

quantitative differences (eg, 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, among 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, for example, potentially mitigate a lipogenic gene program.[28,36,37]

Our study also identified a set of genes in macrophages that are pan-regulated by all the LXR ligands we tested, among 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. We also provide compelling evidence showing that *EEPD1/Eepd1* is regulated in an LXR-dependent manner in murine and human macrophage cell lines and primary cells. Furthermore, by analyzing a reported ChIP-seq study of LXRα in THP1 macrophages,[35] we identified an LXR-associated peak within intron 2 of *EEPD1,* which is absent in adipocytes, and demonstrate that this genomic region harbors a functional LXRE. In human macrophages, the LXRE coincides with a strong peak of the macrophage lineage–specifying PU.1 transcription factor, as well as enrichment of H3K27Ac and H3K4me1 histone modifications that are absent in other cell types. These findings are consistent with a permissive epigenetic landscape surrounding the LXRE in intron 2 of *EEPD1* in macrophages. In conclusion, we propose that *EEPD1* is a bonefide LXR-responsive target gene in macrophages.

LXRs are central determinants of macrophage cholesterol metabolism, largely owing to their ability to promote reverse cholesterol efflux.[38] Enhanced cholesterol efflux is critically

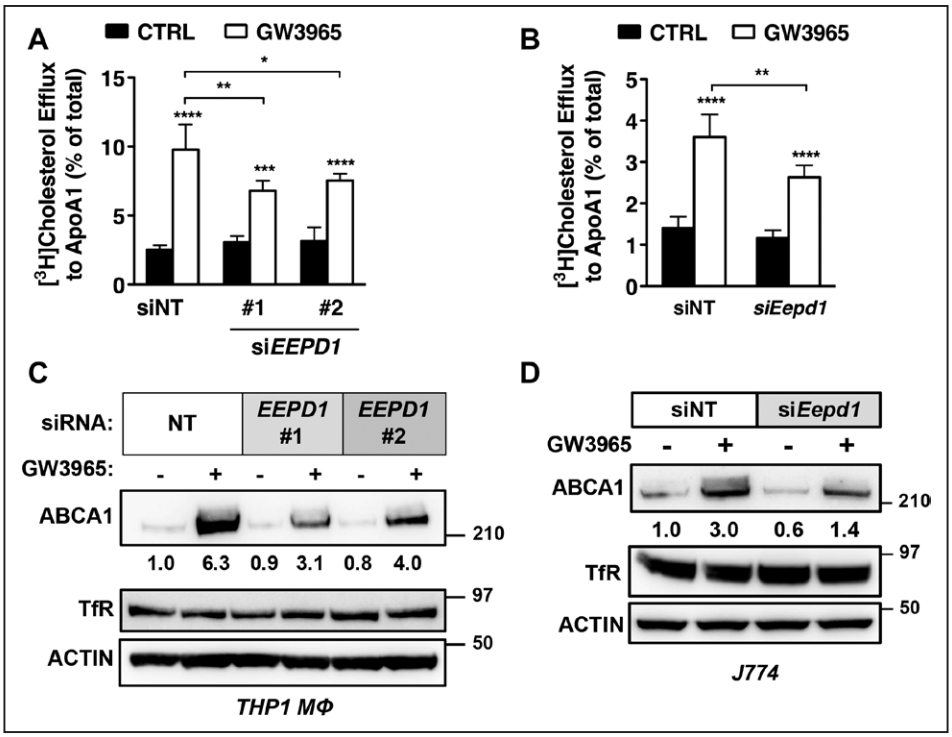

Figure 6. Endonuclease–exonuclease–phosphatase family domain containing 1 (*EEPD1*) silencing decreases Apo A1-dependent cholesterol efflux and ABCA1 abundance. **A**, THP1 or (**B**) J774 macrophages were transfected with control (nontargeting [NT]) or *EEPD1*/*Eepd1* siRNAs 48 h. Subsequently, cells were treated with or without 1 μmol/L GW3965 for 4 h followed by an additional 18 h with medium containing 2 μCi/mL [3H]Cholesterol and 50 μg/mL acetylated-low-density lipoprotein (Ac-LDL). Cholesterol efflux was initiated by incubating the cells with or without 10 μg/mL Apo A1 for 6 h. 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 Apo A1 minus 0.2% bovine serum albumin alone). Each bar represents the mean±SD of 4 independent experiments done in duplicate. **C**, THP1 or (**D**) J774 macrophages were transfected as described above and subsequently treated with 1 μmol/L GW3965 or vehicle control for 18 h. Total cell lysates were immunoblotted as indicated. The level of ABCA1 was determined by densitometry after normalization to actin and the average indicated (**C**, n=3; **D**, n=4). **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001

dependent on transcriptional regulation of *ABCA1* and *ABCG1* by LXRs.[6,9,39] Accordingly, loss of LXRs or of these cholesterol efflux transporters has dramatic consequences on the accumulation of cholesterol in macrophages.[6,40–42] However, recent studies emphasize the atheroprotective activity of LXRs in the intestine and liver and question the importance of ABCA1- and ABCG1-dependent cholesterol efflux from macrophages in this setting.[19–22] Nevertheless, the identification of *EEPD1* as a transcriptional target of LXRs in macrophages and the demonstration that this gene is necessary for maximal LXR-stimulated Apo A1-dependent cholesterol efflux contribute to our understanding of LXRs function in these cells. We propose that the underlying cause for decreased efflux from *EEPD1*-silenced cells is reduced cellular abundance of ABCA1 and decreased ABCA1 density on the plasma membrane. Because 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 macrophages likely involves a post-transcriptional event.

ABCA1 is reported to have a relatively short half-life, estimated in murine macrophages to be ≈1 hour.[43] 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.[6,9,11] This suggests that next to transcriptional regulation, LXRs 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 is emerging as an important determinant of LXR function in cells.[44,45] Members of the EEP-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 toward inositol phosphates. As inositol phosphates are important regulators of intracellular trafficking,[46] EEPD1 could control ABCA1 abundance by modulating the level of specific inositol phosphate species to promote residence of ABCA1 in the plasma membrane or prevent its trafficking toward degradation pathways. However, we point out that this would need to be rather specific because *EEPD1* silencing does not change the level of ABCG1, SR-BI, or transferrin receptor. An alternative possibility is that EEPD1 may directly regulate the stability of ABCA1. A cytoplasmic proline/glutamate/serine/threonine–containing sequence in ABCA1 has been demonstrated to act as a phosphorylation-dependent switch that controls stability of ABCA1.[47] Accordingly, phosphorylation

of the proline/glutamate/serine/threonine domain promotes calpain-mediated degradation of ABCA1 and attenuates Apo A1-dependent cholesterol efflux.[48] Although speculative, if EEPD1 acts as a phosphatase, it could directly stabilize ABCA1 at the plasma membrane by preventing its phosphorylation-dependent degradation. We have been thus far unable to demonstrate binding between EEPD1 and ABCA1 (data not shown), yet this does not preclude the possibility that such an interaction could be weak or transient. Future studies to address the functional interaction between EEPD1 and ABCA1 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, 2 other studies from the Hromas group recently reported that EEPD1 has a function in DNA repair in the nucleus.[49,50] Wu et al[49] 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 at odds with the nuclear localization of EEPD1 reported by Wu et al.[49] We note, however, that for some of these studies, overexpression of a construct encoding N-terminally tagged EEPD1 was used, a modification that would mask the native lipidation sites in the N-terminal sequence of EEPD1. Accordingly, similar to the EEPD1 lipidation mutations, an N-terminally tagged EEPD1 does not localize exclusively to the plasma membrane (Figure VIII in the online-only Data Supplement). Reconciling the proposed functions of EEPD1 is difficult at present, yet the sterol-dependent regulation of EEPD1 by LXRs 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, cell types, or tissues, EEPD1 adopts a different function. In that respect, the fact that we only identify sterol-dependent regulation of EEPD1 in macrophages, but not in other cell types, including those studied by Wu et al,[49] 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 LXRs in macrophages. We propose that EEPD1 acts as part of the LXR-regulated program to promote ABCA1-dependent cholesterol efflux from macrophages and that it does so by maintaining stability of ABCA1. Whether EEPD1 plays a role in the development of atherosclerosis remains to be investigated.

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Disclosures

None.

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Highlights

- Transcriptional profiling of macrophages defines a distinct liver X receptor (LXR) response to different classes of LXR ligands.
- Endonuclease–exonuclease–phosphatase family domain containing 1 (*EEPD1*) is a previously unrecognized transcriptional target of LXR in human and murine macrophages.
- EEPD1 governs post-transcriptional abundance of ABCA1 and is required for maximal LXR-stimulated cholesterol efflux to Apo A1.