**Prenylcysteine oxidase 1, an emerging player in atherosclerosis**

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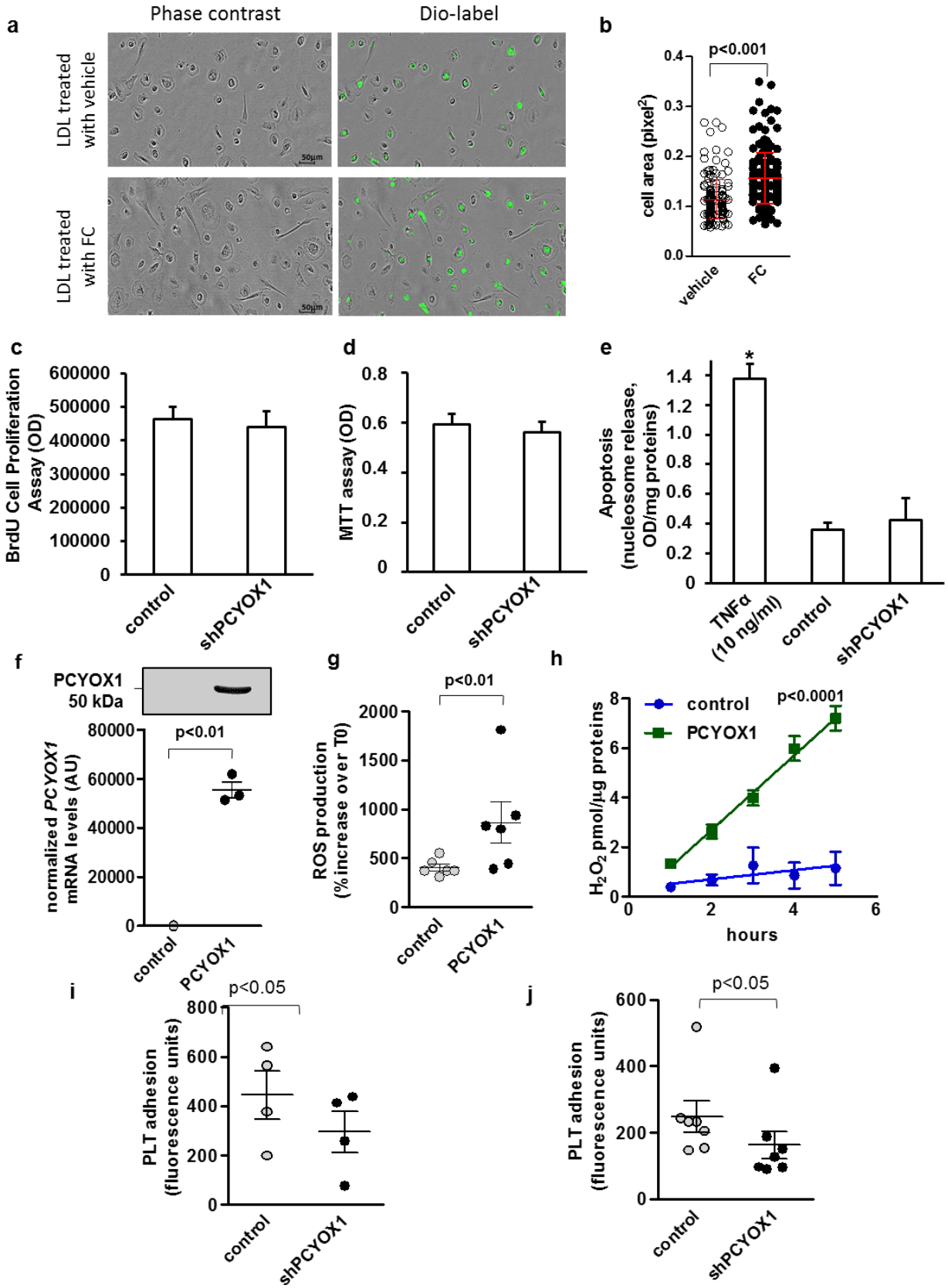
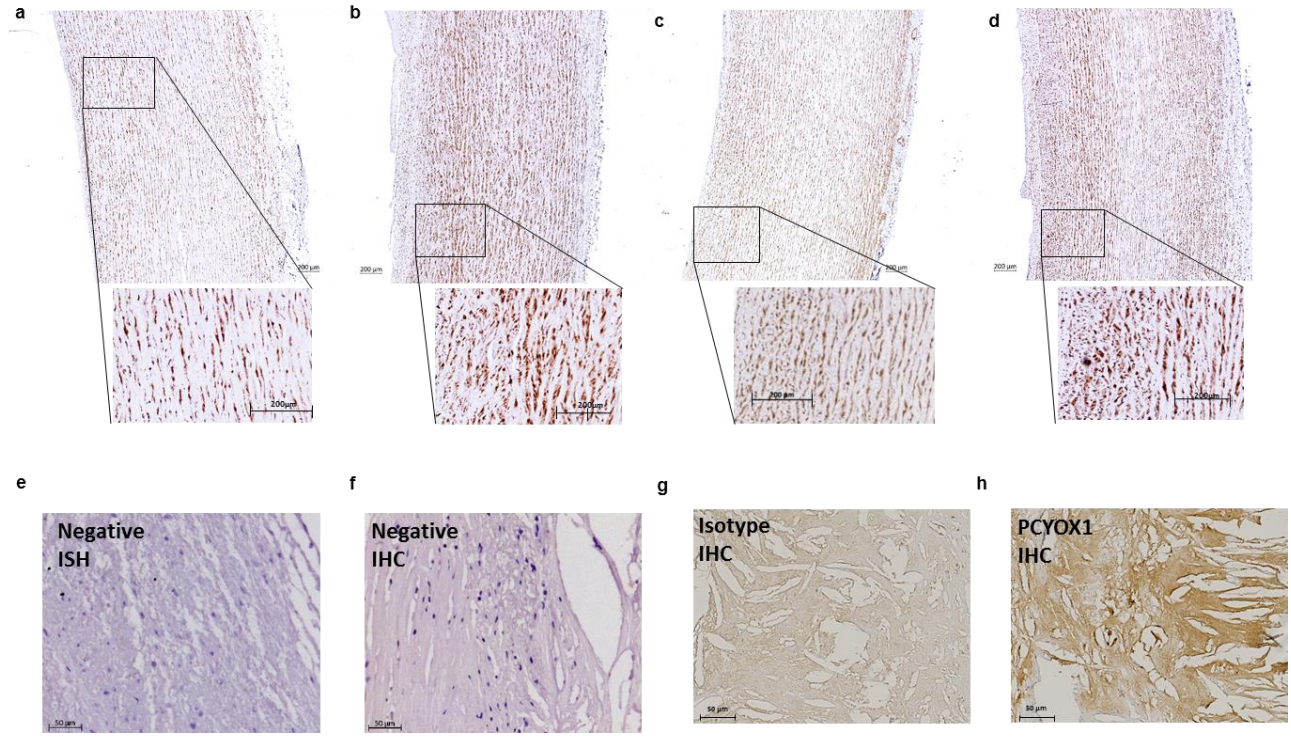
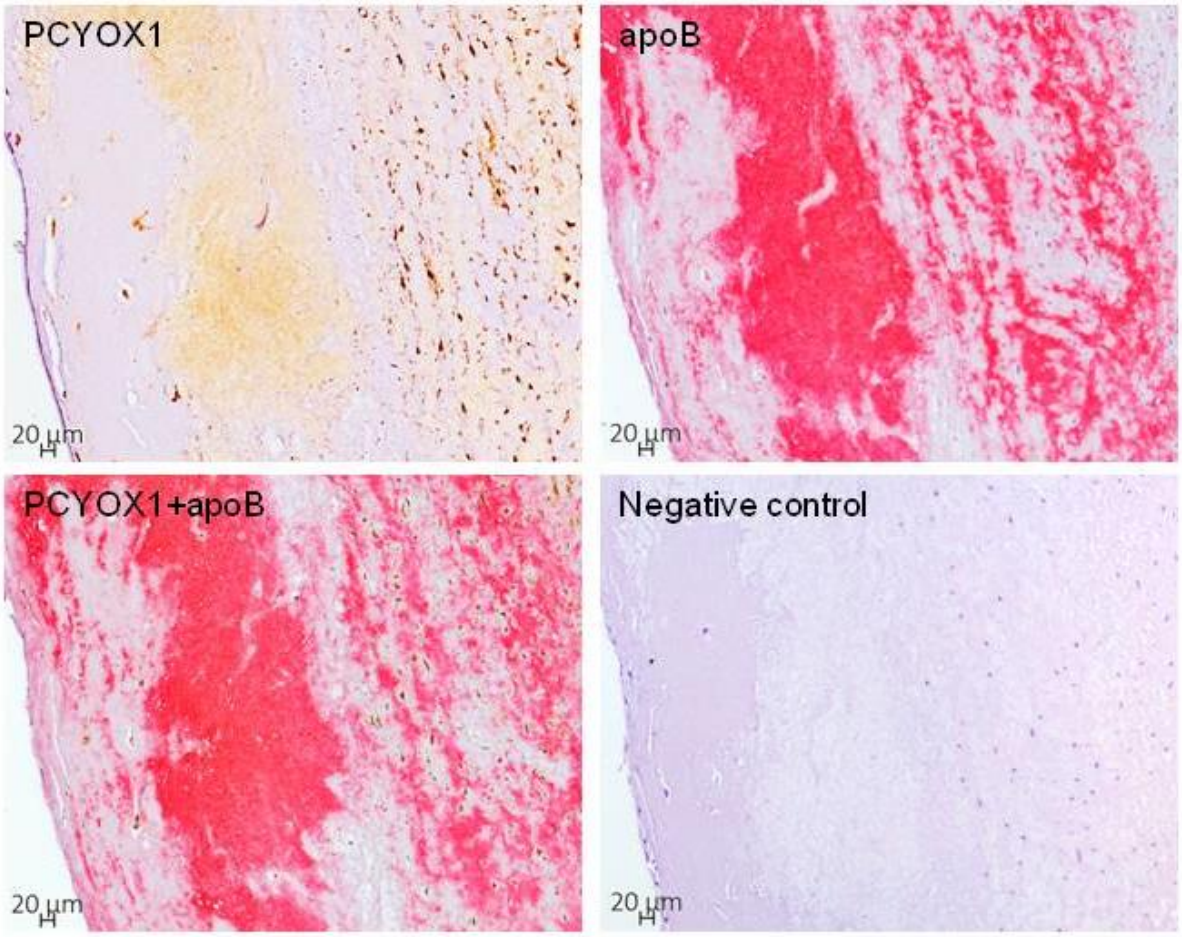
**SUPPLEMENTARY MATERIAL**



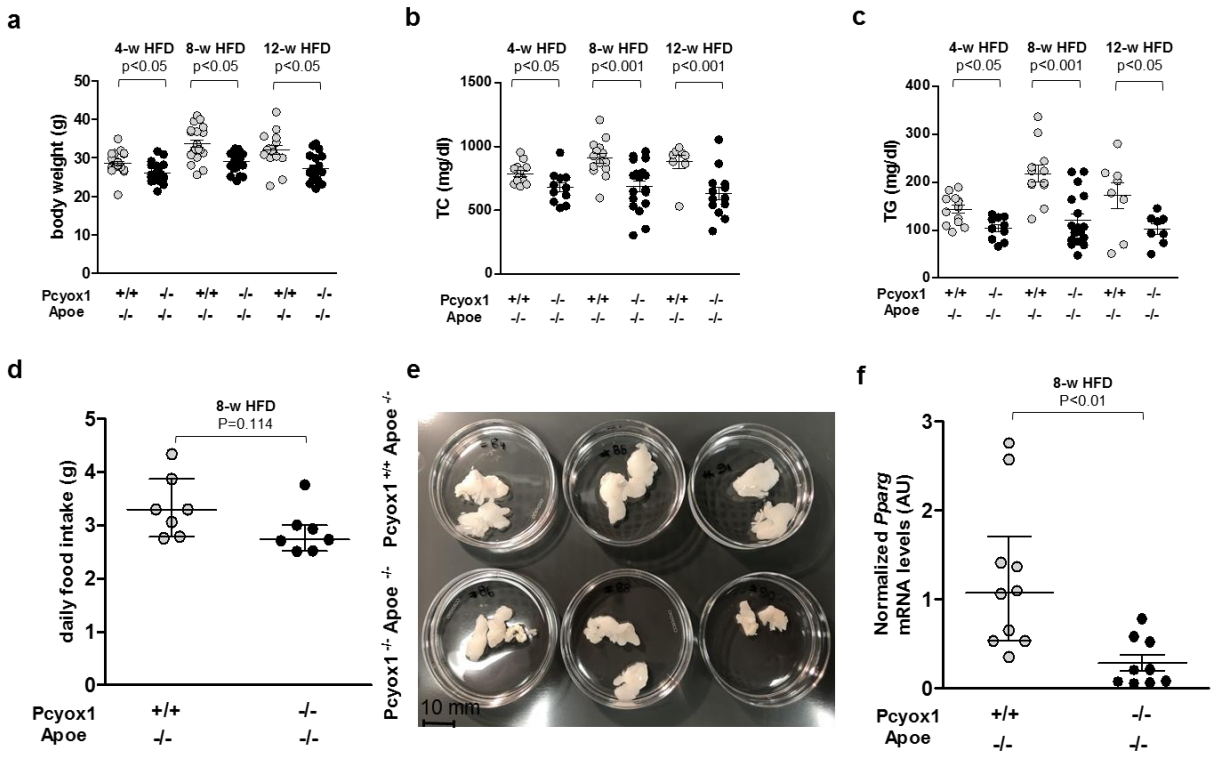
**Supplementary Figure 1.** Human macrophages were incubated with 50 μg/ml of vehicle-treated LDL or FC-treated LDL, both labelled with 3,3′-Dioctadecyloxacarbocyanine perchlorate (Dio). Representative images acquired with Incucyte in phase contrast or for green fluorescence (a) and measurement of cell area obtained with ImageJ software (b). Data are presented as circle-plot, with each circle representing an individual sample and bars showing the mean value±SEM, $p<0.001$ by Student's t test. c-e) Effects of PCYOX1 silencing on HepG2. c) Cell proliferation assayed by BrdU incorporation. Values are the means±SEM of 4 individual experiments. d) MTT assay. Values are the means±SEM of 4 individual experiments. e) Cell apoptosis assay. TNFα (10 ng/ml) was used as positive control. The data are expressed as absorbance/mg of cell proteins as determined by the Bradford protein assay. Values are the means±SD of 4 individual experiments. *$p<0.01$ vs control cells by Student's t test. f) *PCYOX1* mRNA normalized to the housekeeping gene 18S rRNA (n=3), protein (n=3) and intracellular ROS production (n=6) (g) in control or in PCYOX1-overexpressing CHO cells. $p<0.01$ vs control cells by Student's t test. Data are presented as circle-plot, with each circle representing an individual sample and bars showing the mean value±SEM. h) $H_2O_2$ produced by CHO cell lysates in the presence of FC, means±SEM, n=3. Statistical significance was calculated by linear regression, $p<0.0001$. i-j) PCYOX1 silencing reduces the adhesion of human platelets to fibrinogen and endothelial cells. i) Washed human platelets, stained with calcein, were allowed to adhere to immobilized fibrinogen for 30 min in the presence of the secretome from control and PCYOX1-silenced cells. Data are expressed as fluorescence intensity after cell lysis from 4 independent experiments, means±SEM. j) Platelet adhesion to endothelial cells (HUVEC) was quantified based on the fluorescence detection of labelled platelets. $p<0.05$ vs control cells by Student's t test. Data are presented as circle-plot, with each circle representing an individual sample and bars showing the mean value±SEM. Related to Figure 2.



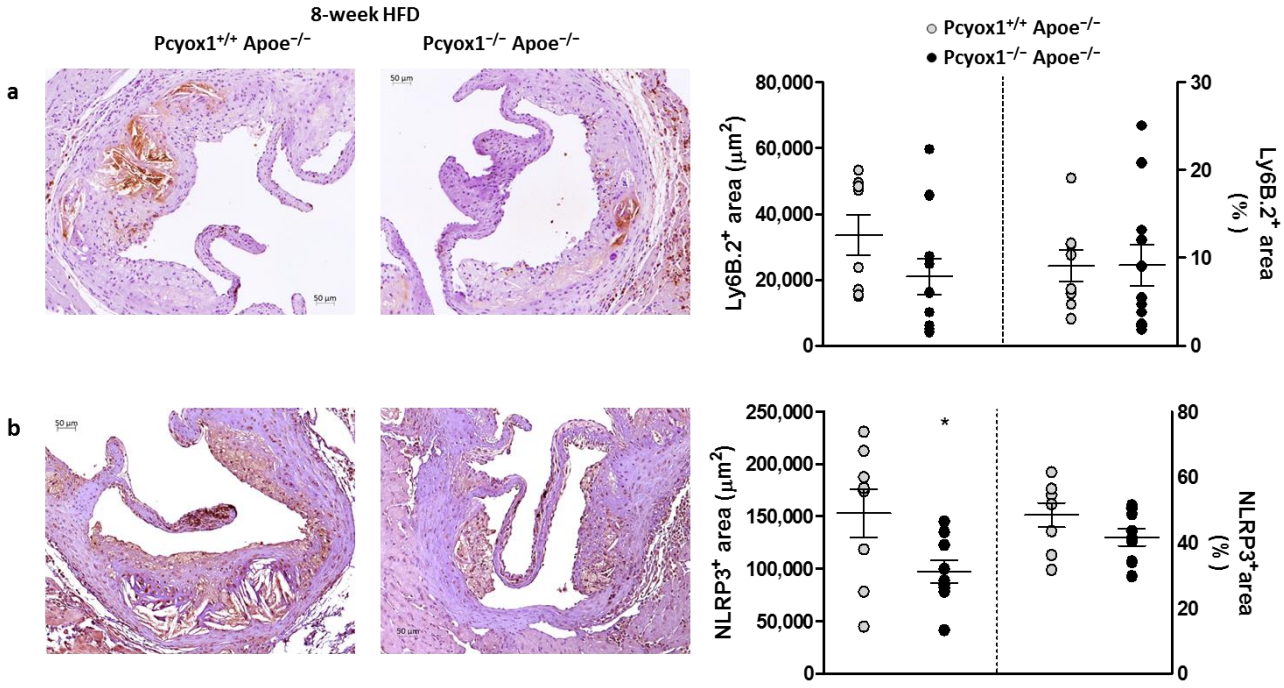

**Supplementary Figure 2.** a-d) Normal human arterial tissue exhibits elevated expression of PCYOX1 in medial SMCs. Formalin-fixed paraffin-embedded sections from normal human arteries were subjected to IHC for the detection of PCYOX1 protein. Primary antibody (sc-136391) was detected as DAB reaction product (brown staining) with a MACH 2 Universal HRP-Polymer (Biocare Medical). Tissue sections were counterstained with hematoxylin. Shown are tissue sections obtained from 2 female subjects (a and b; age: 46 and 52 years, respectively) and 2 male subjects (c and d; age: 25 and 53 years, respectively). e-g) Representative images of negative controls for PCYOX1 staining in the human atherosclerotic lesion. e) Negative control for PCYOX1 in situ hybridization (DapB probe). f) Negative control for PCYOX1 immunostaining obtained without the primary antibody. g) Immunostaining with isotype antibody. h) Immunostaining with PCYOX1 antibody. All images refer to tissue sections from the same donor (Objective 20x). Related to Figure 3.



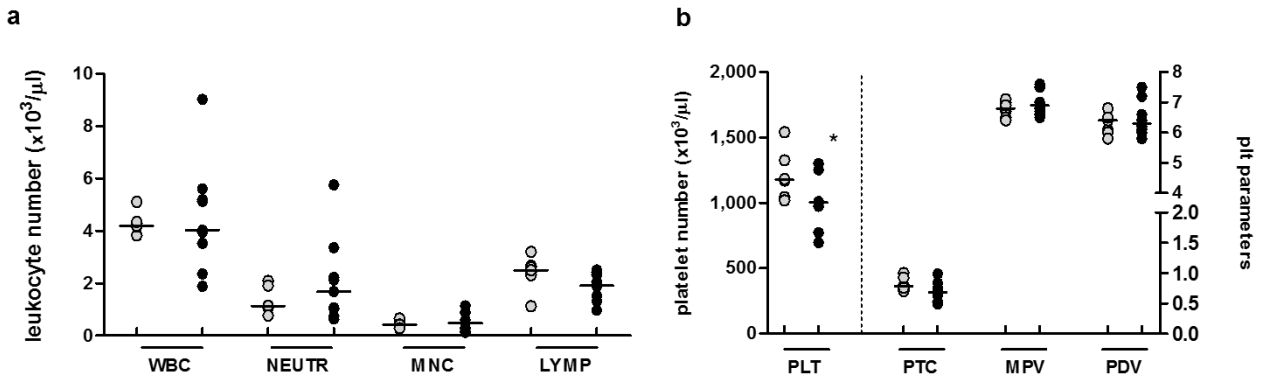

**Supplementary Figure 3.** Extracellular staining for PCYOX1 in human atherosclerotic lesions displays substantial overlapping with apolipoprotein B deposits. Formalin-fixed paraffin-embedded sections of human atherosclerotic lesions were subjected to dual IHC for simultaneous detection of PCYOX1 and apolipoprotein B using a MACH 2 Double Stain 1 micro-polymer detection system (Biocare Medical). Rabbit polyclonal anti-PCYOX1 HPA035193 antibody was detected as DAB reaction product (brown staining) and mouse monoclonal anti-apoB-100 antibody was detected as Biocare's Warp Red reaction product (bright fuchsin-red staining). Shown are representative images from serial sections immunostained for PCYOX1 and apoB, alone or in combination, or incubated in the absence of primary antibodies (negative control). Related to Figure 3.



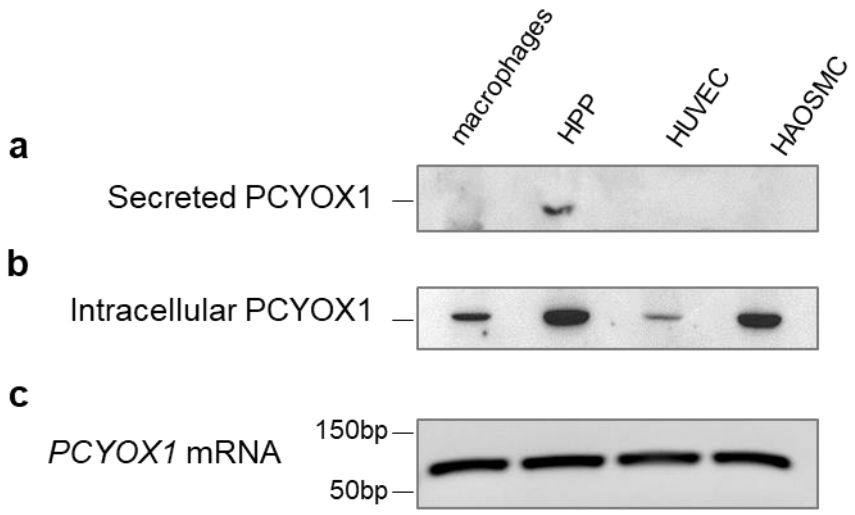

**Supplementary Figure 4.** PCYOX1 deficiency effects on plasma lipids, food intake, adipose tissue depots and liver metabolism gene expression. a-c) Body weight and plasma lipids at different time points. Daily food intake (d) and representative images of adipose tissues (e) from Pcyox1$^{-/-}$/Apoe $^{-/-}$ and Pcyox1 $^{-/-}$ /Apoe $^{-/-}$ fed for 8 weeks with a high-fat diet (HFD). f) *Pparg* mRNA levels normalized to the housekeeping gene *Gapdh* evaluated in the liver of Pcyox1$^{-/-}$ /Apoe $^{-/-}$ and Pcyox1 $^{-/-}$/Apoe $^{-/-}$ fed for 8 weeks with a HFD. p values have been calculated by Student's t test. Data are presented as circle-plot, with each circle representing an individual sample and bars showing the mean value±SEM. Related to Figure 4.



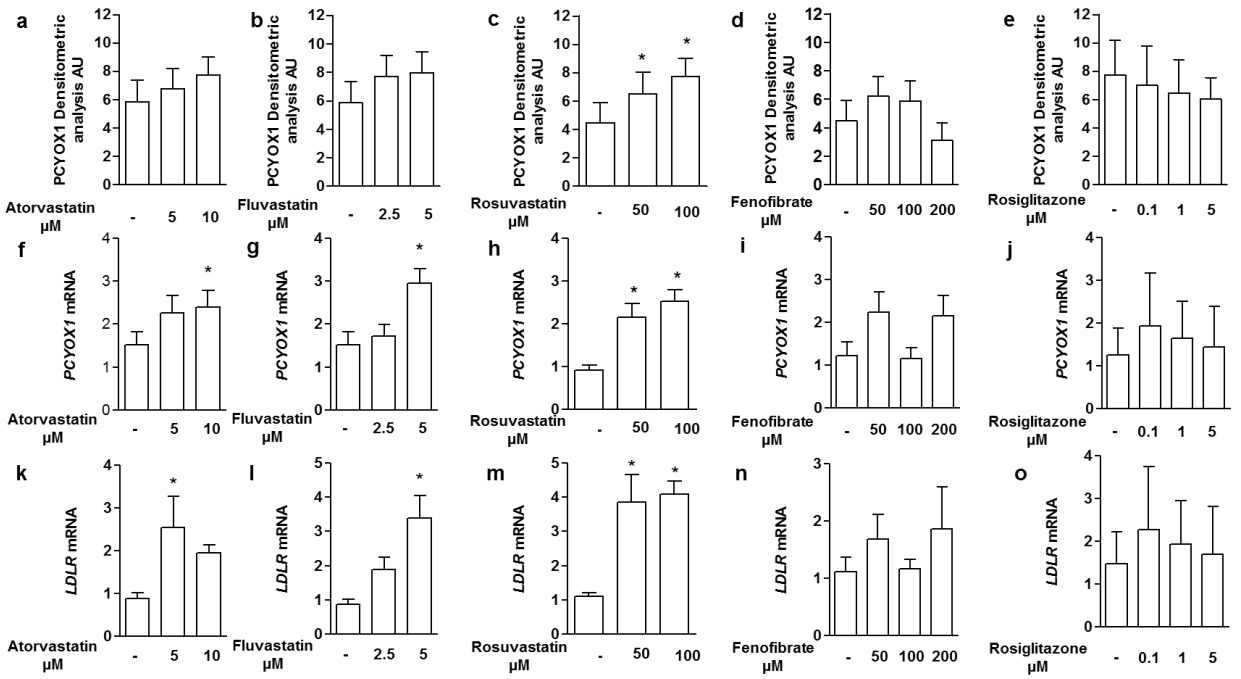

**Supplementary Figure 5.** Pcyox1 deficiency is associated with a minor content of neutrophils and a lower expression of NLRP3 protein. Morphometric analysis of atherosclerotic lesions from aortic roots of Pcyox1$^{+/+}$/Apoe$^{-/-}$ (n=8, panel a; n=8 panel b) and Pcyox1$^{-/-}$ /Apoe$^{-/-}$ (n=11, panel a; n=9 panel b) mice fed a HFD for 8 weeks to assess the effect of Pcyox1 deletion on neutrophil content (a) and NLRP3 expression (b). Representative images and quantification of: a, neutrophil content (Ly-6B.2 staining); b, inflammasome (NLRP3 staining). Shown on the right are the results of the morphometric analysis for the stainings, expressed either as absolute positive areas or as percentage of total plaque area; data are presented as circle-plot, with each circle representing an individual sample and bars showing the mean value±SEM. * $p<0.05$ by Student's t test. Related to Figure 6.




**Supplementary Figure 6.** Pcyox1 deficiency does not affect blood leukocyte counts but is associated with a decreased number of circulating platelets. EDTA-anticoagulated blood samples from Pcyox1$^{+/+}$ /Apoe$^{-/-}$ (grey circles) and Pcyox1$^{-/-}$ /Apoe$^{-/-}$ (black circles) mice fed an HFD for 8 weeks were subjected to hematologic analysis to assess the effect of Pcyox1 deletion on leukocyte and platelet count. The analysis showed that the total leukocyte count (WBC), and the cell count of the main leukocyte subsets (polymorphonuclear neutrophils, NEUTR; monocytes MNC; and lymphocytes, LYMP) were comparable in the two genotypes (a). In contrast, platelet number (PLT) in Pcyox1$^{-/-}$/Apoe$^{-/-}$ mice was significantly lower in comparison to Pcyox1$^{+/+}$/Apoe$^{-/-}$ control mice, with no changes in plateletcrit (PTC), mean platelet volume (MPV) and platelet distribution width (PDV) (b).* $p<0.05$ by Student's t test, Bars indicate the median.




**Supplementary Figure 7.** *PCYOX1* mRNA, intracellular protein level, and PCYOX1 secretion in human macrophages, human primary hepatocytes (HPP), human endothelial cells (HUVEC), and smooth muscle cells (HAOSMC). a and b, an equal amount of proteins were separated by SDS-PAGE and immunoblotted with an antibody against human PCYOX1. c, mRNA levels of *PCYOX1* analysed by RT-PCR.




**Supplementary Figure 8**. Pharmacological modulation of PCYOX1 expression in HepG2 cells by HMG-CoA inhibitors, fenofibrate and rosiglitazone. HepG2 cells were treated for 48 hours in complete medium containing drugs or vehicle, and PCYOX1 levels were evaluated by immunoblotting at protein level (Atorvastatin, a; Fluvastatin, b; Rosuvastatin, c; Fenofibrate, d; Rosiglitazone, e) and real-time PCR at mRNA level (Atorvastatin, f; Fluvastatin, g; Rosuvastatin, h; Fenofibrate, i; Rosiglitazone, j). 18s mRNA has been used as the housekeeping gene for real-time PCR. Data are representative of 3 independent experiments. * $p<0.05$ vs vehicle–treated cells by Student's t test. Pharmacological modulation of LDL receptor (LDLR) expression in HepG2 cells by HMG-CoA inhibitors, fenofibrate and rosiglitazone. HepG2 cells were treated for 48 hours in complete medium containing drugs or vehicle, and *LDLR* levels were evaluated by real-time PCR (Atorvastatin, k; Fluvastatin, l; Rosuvastatin, m; Fenofibrate, n; Rosiglitazone, o). 18s mRNA has been used as housekeeping gene for real-time PCR. Data are representative of 3 independent experiments with bars showing the mean value±SEM. * $p<0.05$ vs vehicle-treated cells by Student's t test.

**Supplementary Table 1.** List of the secreted proteins modulated by PCYOX1 silencing in HepG2 cells identified by means of a label-free mass spectrometry-based proteomic approach, LC-MS$^E$. Data have been generated from four independent experiments performed in triplicate. Related to Figure 2.

| Accession | Description | Highest mean condition | # Peptides[1] | Score | p value | Max fold change |
|---|---|---|---|---|---|---|
| *Proteins less abundant after PCYOX1 silencing* | | | | | | |
| P07996 | Thrombospondin-1, THBS1 | control | 24/20 | 154.2 | 6.69E-11 | 1.64 |
| P02768 | Serum albumin, ALB | control | 68/68 | 702.1 | 0 | 1.73 |
| Q08830 | Fibrinogen-like protein 1, FGL-1 | control | 8/8 | 47.0 | 4.61E-07 | 1.41 |
| P01033 | Metalloproteinase inhibitor 1, TIMP1 | control | 2/2 | 13.4 | 0.058383 | 1.44 |
| P08833 | Insulin-like growth factor-binding protein 1, IGFBP1 | control | 14/14 | 125.6 | 3.36E-07 | 1.49 |
| P01011 | Alpha-1-antichymotrypsin, AACT | control | 17/15 | 148.2 | 0.000252 | 1.35 |
| Q99988 | Growth/differentiation factor 15, GDF15 | control | 5/5 | 35.8 | 2.29E-07 | 1.35 |
| P10646 | Tissue factor pathway inhibitor, TFPI | control | 10/10 | 81.8 | 2.46E-07 | 1.39 |
| P02766 | Transthyretin, TTR | control | 8/8 | 75.0 | 0.073035 | 1.31 |
| P61626 | Lysozyme C, LYZ | control | 11/9 | 91.0 | 0.000168 | 1.30 |
| P05121 | Plasminogen activator inhibitor 1, SERPINE1 | control | 11/11 | 78.2 | 0.045464 | 1.23 |
| P01023 | Alpha-2-macroglobulin, A2M | control | 83/70 | 836.1 | 3.14E-05 | 1.25 |
| P01024 | Complement C3, C3 | control | 102/98 | 983.0 | 2.06E-06 | 1.19 |
| P29279 | Connective tissue growth factor, CTGF | control | 14/12 | 112.8 | 2.75E-05 | 1.27 |
| P19883 | Follistatin, FST | control | 9/9 | 56.2 | 0.000876 | 1.25 |
| *Proteins more abundant after PCYOX1 silencing* | | | | | | |
| P08238 | Heat shock protein HSP 90-beta, HSP90AB | shPCYOX1 | 7/3 | 43.4 | 0.002578 | 1.74 |
| P07437 | Tubulin beta chain, TUBB | shPCYOX1 | 5/4 | 30.0 | 0.000143 | 1.60 |
| P68104 | Elongation factor 1-alpha 1, EEF1A1 | shPCYOX1 | 4/4 | 26.8 | 4.81E-07 | 1.54 |
| P07900 | Heat shock protein HSP 90-alpha, HSP90AA1 | shPCYOX1 | 7/3 | 42.6 | 0.000705 | 1.61 |
| P04406 | Glyceraldehyde-3-phosphate dehydrogenase, GAPDH | shPCYOX1 | 4/3 | 27.3 | 2.53E-05 | 1.50 |
| P11142 | Heat shock cognate 71 kDa protein, HSP8A | shPCYOX1 | 7/2 | 47.8 | 8.97E-07 | 1.38 |
| P60709 | Actin, cytoplasmic 1, ACTB | shPCYOX1 | 13/2 | 116.2 | 0.000819 | 1.29 |
| P14618 | Pyruvate kinase, PKM | shPCYOX1 | 5/3 | 26.7 | 0.000166 | 1.26 |
| P07148 | Fatty acid-binding protein, liver , FABP1 | shPCYOX1 | 4/3 | 26.6 | 0.000125 | 1.26 |
| P00338 | L-lactate dehydrogenase A chain, LDHA | shPCYOX1 | 6/5 | 40.5 | 0.000454 | 1.24 |
| P60174 | Triosephosphate isomerase, TPI1 | shPCYOX1 | 10/8 | 62.7 | 0.008772 | 1.20 |
| P01034 | Cystatin-C, CST3 | shPCYOX1 | 8/8 | 53.6 | 6.97E-06 | 1.25 |
| P62987 | Ubiquitin-60S ribosomal protein L40, UBA52 | shPCYOX1 | 3/3 | 22.4 | 0.026215 | 1.23 |

| | | | | | | |
|---|---|---|---|---|---|---|
| P00558 | Phosphoglycerate kinase 1, PGK1 | shPCYOX1 | 2/2 | 11.4 | 0.006522 | 1.27 |

[1] Peptide count / Peptides used for quantitation

**Supplementary Table 2.** List of the secreted proteins modulated by PCYOX1 silencing in HepG2 cells identified by means of multiplex immunoassay. Related to figure 2.

| Uniprot accession | Protein description | Fold shPCYOX1/control |
|---|---|---|
| *Proteins more abundant after PCYOX1 silencing* | | |
| P13726 | Tissue factor (TF) | 2.05 |
| P04080 | Cystatin-B (CSTB) | 2.02 |
| P12931 | Proto-oncogene tyrosine-protein kinase Src (SRC) | 1.99 |
| Q14790 | Caspase-8 (CASP-8) | 1.88 |
| Q9Y6K9 | NF-kappa-B essential modulator (NEMO) | 1.75 |
| P35318 | Adrenomedullin (AM) | 1.60 |
| P25116 | Proteinase-activated receptor 1 (PAR-1) | 1.50 |
| P09341 | C-X-C motif chemokine 1 (CXCL1) | 1.46 |
| P07711 | Cathepsin L1 (CTSL1) | 1.43 |
| O14763 | TNF-related apoptosis-inducing ligand receptor 2 (TRAIL-R2) | 1.39 |
| P09603 | Macrophage colony-stimulating factor 1 (CSF-1) | 1.35 |
| P25445 | Tumor necrosis factor receptor superfamily member 6 (FAS) | 1.31 |
| O00253 | Agouti-related protein (AGRP) | 1.27 |
| *Proteins less abundant after PCYOX1 silencing* | | |
| P10145 | Interleukin-8 (CXCL8) | 0.83 |
| P19883 | Follistatin (FST) | 0.73 |
| Q9H2A7 | C-X-C motif chemokine 16 (CXCL16) | 0.72 |
| Q99988 | Growth/differentiation factor 15 (GDF-15) | 0.69 |
| P09237 | Matrix metalloproteinase-7 (MMP-7) | 0.58 |
| Q03405 | Urokinase plasminogen activator surface receptor (PLAUR) | 0.52 |
| O94907 | Dickkopf-related protein 1 (DKK-1) | 0.34 |

**Supplementary Table 3.** Body weight, plasma lipids levels and glucose from Pcyox1$^{+/+}$/Apoe$^{-/-}$ and Pcyox1$^{-/-}$/Apoe$^{-/-}$ mice fed a chow diet, and age-matched with mice fed HFD for 8 weeks.

| Measurement | Pcyox1$^{+/+}$/Apoe$^{-/-}$ | Pcyox1$^{-/-}$/Apoe$^{-/-}$ | *P* |
|---|---|---|---|
| Body weight, g | 30.7 ± 2.4 (10) | 27.93 ± 2.2 (6) | <0.05 |
| Total cholesterol, mg/dL | 300 ± 69 (10) | 279 ± 67 (6) | n.s. |
| Free cholesterol, mg/dL | 124 ± 23 (10) | 119 ± 23 (6) | n.s. |
| Cholesterol esters, mg/dL | 176 ± 50 (10) | 161 ± 46 (6) | n.s. |
| Triglycerides, mg/dL | 91 ± 19 (10) | 75 ± 27 (6) | n.s. |
| Phospholipids, mg/dL | 199 ± 31 (10) | 180 ± 40 (6) | n.s. |
| Glucose, mg/dL | 196 ± 50 (10) | 214 ± 86 (6) | n.s. |

Mean ± SD