Identification of the (Pro)renin Receptor as a Novel Regulator of Low-Density Lipoprotein Metabolism

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Running title: (Pro)renin Receptor and LDL Metabolism

Subject Terms:
Lipids and Cholesterol

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In October 2015, the average time from submission to first decision for all original research papers submitted to Circulation Research was 15.18 days.
ABSTRACT

**Rationale:** The (pro)renin receptor [(P)RR] interacts with (pro)renin at concentrations that are >1000 times higher than observed under (patho)physiological conditions. Recent studies have identified renin-angiotensin-system (RAS)-independent functions for (P)RR related to its association with the vacuolar H⁺-ATPase (V-ATPase).

**Objective:** To uncover RAS-independent functions of the (P)RR.

**Methods and Results:** We used a proteomics-based approach to purify and identify (P)RR-interacting proteins. This resulted in identification of sortilin-1 (SORT1) as a high-confidence (P)RR-interacting protein, a finding which was confirmed by co-immunoprecipitation of endogenous (P)RR and SORT1. Functionally, silencing (P)RR expression in hepatocytes decreased SORT1 and low-density-lipoprotein (LDL) receptor (LDLR) protein abundance and, as a consequence, resulted in severely attenuated cellular LDL uptake. In contrast to LDL, endocytosis of EGF or transferrin remained unaffected by silencing of the (P)RR. Importantly, reduction of LDLR and SORT1 protein abundance occurred in the absence of changes in their corresponding transcript level. Consistent with a post-transcriptional event, degradation of the LDLR induced by (P)RR silencing could be reversed by lysosomotropic agents, such as bafilomycin A1.

**Conclusions:** Our study identifies a RAS-independent function for the (P)RR in the regulation of LDL metabolism by controlling the levels of SORT1 and LDLR.

**Keywords:** (P)RR, sortilin, LDLR, V-ATPase, cholesterol metabolism, low-density lipoprotein, cholesterol homeostasis, renin-angiotensin system, receptor.

**Nonstandard Abbreviations and Acronyms:**
- SORT1: sortilin 1
- LDL: low-density lipoprotein
- LDLR: low-density lipoprotein receptor
- RAS: renin-angiotensin system
- (P)RR: (pro)renin receptor
- V-ATPase: vacuolar H⁺-ATPase
- Tf: transferrin
- EGF: epidermal growth factor
- EGFR: epidermal growth factor receptor
- TGF-β: transforming growth factor β
- Erk1/2: extracellular signal-regulated kinase 1 and 2
- PCSK9: Proprotein convertase subtilisin/kexin type 9
- LPDS: lipid-deficient serum
- 3-MA: 3-Methyladenine
- TRAF4: TNF receptor associated factor 4
- IDOL: inducible degrader of the LDLR
- APOB: Apolipoprotein B
INTRODUCTION

The (pro)renin receptor [(P)RR] has been implicated as a receptor for renin/prorenin [denoted as (pro)renin]-stimulated signaling, and plays a role in local renin-angiotensin system activation by non-proteolytically activating bound prorenin. In experimental assays (pro)renin-(P)RR signaling results in extracellular signal-regulated kinase 1/2 (Erk1/2) activation, and as a consequence up-regulation of profibrotic factors, such as transforming growth factor β (TGF-β), collagen and fibronectin. However, the physiological relevance of the (pro)renin-(P)RR interaction is questionable, as the (pro)renin concentrations required are >1000 times higher than observed under (patho)physiological conditions. Recently, (pro)renin-independent functions for (P)RR have been reported, including a function as an accessory protein of the vacuolar H+-ATPase (V-ATPase). V-ATPases are multisubunit complexes, and are expressed virtually in all cell types. They play an important role in protein trafficking, receptor recycling, and lysosomal degradation by acidifying intracellular compartments. Depletion of the (P)RR results in decreased protein levels of V-ATPase subunits, impaired acidification of intracellular compartments, and defects in autophagy. V-ATPases are also found at the plasma membrane in certain cell types, such as intercalated cells of the collecting duct. Accordingly, we previously reported that the (P)RR is required for both prorenin-dependent and -independent regulation of V-ATPase activity in collecting duct cells. The (P)RR has been also recently implicated in canonical Wnt and PCP signaling, emphasizing the notion that our understanding of (P)RR function remains incomplete.

To address this we used an unbiased proteomics approach to discover potential novel functions of the (P)RR. We mapped the (P)RR-interactome and identified Sortilin 1 (SORT1) as a novel (P)RR-interacting partner. We show that the (P)RR post-transcriptionally controls protein abundance of SORT1, and unexpectedly that of the low-density lipoprotein receptor (LDLR). We demonstrate that as a consequence LDL uptake in several cell types is sensitive to (P)RR levels. Collectively, our results indicate that the (P)RR represents a previously unrecognized regulator of LDL metabolism.

METHODS

A brief description of the methods is provided below. For a detailed description of the methods please refer to the online supplemental material.

Cell culture and transfections.
HEK293, A431, Huh7 and HepG2 cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/mL streptomycin at 37°C and 5% CO₂. LDLA7 CHO cells were kindly provided by Dr Monty Krieger (MIT, USA) and maintained in DMEM/F12 supplemented with 5% FBS, 100 U/ml penicillin and 100 μg/mL streptomycin at 37°C and 5% CO₂. For both siRNA and plasmid transfection, HEK293 cells were transfected with lipofectamine 2000 according to the manufacturer’s protocol. Unless indicated otherwise, cells were cultured in sterol-depletion medium [DMEM supplemented with 10% bovine lipid-deficient serum (LPDS), 5 μg/mL simvastatin, and 100 μM mevalonate], to increase LDLR expression, for 18 hours prior to experiment. A431 and LDLA7 CHO cells were transfected with lipofectamine 2000 according to the manufacturer’s protocol. Unless indicated otherwise, cells were cultured in sterol-depletion medium [DMEM supplemented with 10% bovine lipid-deficient serum (LPDS), 5 μg/mL simvastatin, and 100 μM mevalonate], to increase LDLR expression, for 18 hours prior to experiment. A431 and LDLA7 CHO cells were transfected with 40 nmol/L siRNAs by JetPrime, and HepG2 cells were transfected with 40 nmol/L siRNAs by RNAiMax following the manufacturer’s protocols. For plasmid transfection, HepG2 cells were transfected with JetPrime using the manufacturer’s protocol.

RNA isolation and qPCR.
Total RNA was isolated from cells using TRIzol following the manufacturer’s protocol. One milligram of total RNA was reverse transcribed with the iScript reverse transcription kit (Bio-rad). SYBR Green real-time quantitative PCR assays were performed on a Lightcycler 480 II apparatus (Roche) using SYBR Green.
master mix (Roche). Gene expression was normalized to the expression of 36B4, and expressed as mean ± SEM. Primers are listed in Online Table I.

**Immunoprecipitation and immunoblotting.**

For co-immunoprecipitations HEK293 cells were lysed in IP lysis buffer. Cell lysates were cleared by centrifugation at 1000×g for 5 minutes at 4°C, and protein content was measured with the BCA assay. Of each lysate, one mg of protein was precleared with 50 μL prewashed ProtA Dynabeads for 1 hour at 4°C, and then nutated for 1 hour at 4°C with 9 μg anti-h(P)RR antibodies, anti-SORT1 antibodies, or non-specific rabbit IgGs as control, coupled to 50 μL Protein-A Dynabeads. For protein expression studies, A431 and HepG2 cells were lysed in RIPA buffer. Lysates were cleared by centrifugation at 10,000×g at 4°C for 5 minutes and protein contents were measured using the BCA assay. For immunoblotting, immunocomplexes or lysates containing an equal amount of proteins (10-25 μg) were resolved by SDS-PAGE and probed using the primary antibodies listed in Online Table II. Subsequently, HRP-conjugated goat anti-mouse or goat anti-rabbit antibodies were added and detected with ECL.

**LDL uptake assays.**

LDL uptake was measured using DyLight 488-labeled LDL, as described previously. Briefly, HepG2 or A431 cells were incubated in sterol-depleted medium for 16 hours prior to adding LDL. Cells were incubated with 5 μg/mL DyLight488-labeled LDL in DMEM supplemented with 0.5% BSA for 3 hours at 37°C or 4°C. Subsequently cells were washed twice with ice-cold PBS supplemented with 0.5% BSA and lysed in RIPA buffer. LDL uptake was determined by quantification of the fluorescence signal on a Typhoon imager (GE Healthcare) and corrected for the protein content in the lysate as determined with the BCA assay.

**Measurement of cell surface LDLR by FACS.**

Surface LDLR density after knocking down the (P)RR was measured as described before. Briefly, cells were maintained on sterol-depleted medium for 16 hours prior to the experiment, dissociated with TrypLE Express and incubated in FACS blocking buffer on ice for 30 minutes. Next, 100,000 cells were stained in 50 μL FACS buffer containing PE-conjugated anti-LDLR antibody for 1 hour on ice. Following three washes cells were directly analyzed on a FACS Calibur (BD Biosciences).

**Statistical analysis.**

Data are presented as mean±SEM. One-way ANOVA followed by the Bonferroni correction was performed for comparison of more than two groups. Student’s t-test was performed for comparison of two groups. p values <0.05 were considered significant.

**RESULTS**

The (P)RR-interactome reveals potential novel functions of the (P)RR.

The (P)RR has been recently implicated in cellular functions unrelated to its ability to bind (pro)renin. In order to identify such functions we mapped the (P)RR-interactome in HEK293 cells using a TAP-based approach (Online Figure I). We identified 40 proteins that co-purified with N-terminally TAP-tagged (P)RR in two independent purifications (Online Table III), but not with the tag only. In this set of proteins we found several V-ATPase components, including those known to interact with (P)RR, which validates our proteomics approach and suggests that the N-terminally TAP-tagged (P)RR is at least functional for interacting with the V-ATPase. To better interpret the profile, we compared it with CRAPome, a recently published database of contaminants in currently 343 affinity purification-mass spectrometry profiles (www.crapome.org). Many proteins found in our profile, especially transporters, are also present.
at high frequency in other purifications in the CRAPome. Apart from V-ATPase subunits, we found several (P)RR-interacting partners with low frequency in CRAPome that are involved, amongst others, in signal transduction, lipid metabolism, mitochondrial transport, and protein folding.

From the identified proteins SORT1 is the candidate with the highest Mascot score and is not found in the CRAPome. TRAF4, despite having a Mascot score in the lower range was another attractive candidate, as it can mediate activation of Erk1/2, Akt and Wnt/β-catenin signaling, all of which have been recently suggested to be modulated by (P)RR. This screen was performed with a heterologous construct and we therefore first wanted to validate the interactions of these proteins with endogenous (P)RR. Thus, we tested if SORT1 and TRAF4 interact with endogenous (P)RR by co-immunoprecipitation in HEK293 cells. We found that SORT1, but not TRAF4, co-immunoprecipitated with endogenous (P)RR (Figure 1A). Conversely, (P)RR also co-immunoprecipitated with endogenous SORT1 (Figure 1B), establishing the (P)RR-SORT1 interaction. To determine the function of the (P)RR-SORT1 interaction we first tested the consequence of silencing the (P)RR on SORT1. Unexpectedly, this resulted in a 42% decrease in SORT1 protein abundance, reminiscent of that seen with other V-ATPase subunits (Figure 1C,D). Importantly, this occurred in the absence of changes in SORT1 transcript level (Figure 1E). Additionally, in agreement with the absence of an interaction between (P)RR and TRAF4 we observed no effect of silencing (P)RR on TRAF4 levels (not shown). Our proteomic screen therefore resulted in identification of the (P)RR as a post-transcriptional regulator of SORT1.

**(P)RR regulates cellular LDL uptake.**

SORT1 is implicated in several cellular functions, and has recently been identified as an important determinant of LDL metabolism, and of circulating levels of LDL in humans. The identification of a functional interaction between (P)RR and SORT1 led us therefore to test the role of the (P)RR in LDL metabolism. As SORT1 can also directly contribute to LDL uptake by cells, we first tested whether the (P)RR affects LDL uptake in A431 and HepG2 cells, two cell types that display high LDLR abundance following sterol depletion. In A431 cells, reducing (P)RR mRNA levels (by ~90%) resulted in a two-fold decrease in LDL uptake (Figure 2A-C). To rule out the possibility that this is an off-target effect, we silenced (P)RR expression in these cells using two additional siRNAs. Both siRNAs reduced LDL uptake to the same extent (Online Figure II A), confirming that the decrease in LDL uptake is due to reduced (P)RR expression. Similar to A431 cells, silencing (P)RR expression in HepG2 cells decreased uptake of LDL by 40% (Figure 2D). Silencing (P)RR also reduced LDL uptake in A431 and HepG2 cells when these were cultured in complete medium (i.e., medium containing lipoproteins), even though overall uptake was much lower when compared to that in sterol-depleted cells (Online Figure II B,C). Furthermore, the effect of silencing (P)RR seems to be time-dependent, as LDL uptake was further decreased when silencing was extended to 72 hours (Online Figure II D). Silencing (P)RR also reduced LDL uptake in Huh7 cells to a similar extent as in HepG2 cells (Online Figure II E). Taken together, these data show that (P)RR depletion affects LDL metabolism in multiple cell lines, suggesting that the (P)RR is a common regulator of LDL metabolism.

Endocytosis of LDL via the LDLR pathway is a clathrin-dependent process. Therefore, decreased LDL uptake in response to (P)RR depletion may reflect a gross perturbation of clathrin-dependent endocytosis. To test this possibility we studied the uptake of two ligands that are taken up via a clathrin-dependent pathway, namely transferrin (Tf) and epidermal growth factor (EGF) that are ligands for the Tf receptor (TfR) and EGF receptor (EGFR), respectively. Silencing of (P)RR expression in A431 cells did not influence abundance of either the TfR or EGFR (Online Figure III A). Consistent with this, and in contrast to the observed effects on LDL uptake, Tf uptake in A431 and HepG2 cells was not decreased (Figure 2E,F, Online Figure III B). In fact, Tf uptake was slightly increased in A431 cells following (P)RR silencing (Figure 2F). A431 cells contain high levels of the EGFR, and incubating these cells with Alexa488-labeled EGF for 15 minutes at either low (100 ng/mL) or high (500 ng/mL) levels, allowed us...
to evaluate both clathrin-dependent and -independent internalization pathways, respectively. Irrespective of the dose, silencing of (P)RR expression did not affect EGF uptake in A431 cells (Online Figure III C,D). Taken together, these results indicate that (P)RR depletion does not grossly attenuate clathrin-dependent endocytosis and that (P)RR differentially affects endocytosis of cargo, largely attenuating LDL uptake yet sparing EGF and Tf internalization.

The (P)RR controls stability of the LDLR protein and LDL uptake.

Consistent with our initial observation in HEK293 cells, silencing of (P)RR expression in A431 and HepG2 cells also decreased the abundance of endogenous SORT1 protein (Figure 3A,B). Recent studies from the Rader lab have shown that SORT1 can directly bind LDL and mediate LDL internalization in hepatocytes and macrophages. Hence, a simple explanation for the reduction of LDL uptake in (P)RR depleted cells would be reduced SORT1 abundance. To test this we made use of LDLA7 cells, which is a CHO-derived cell line that lacks functional LDLR resulting in strongly diminished uptake of LDL.

Similar to the other cell lines, silencing (P)RR expression reduced SORT1 abundance in LDLA7 cells, and vice versa, implying that a functional (P)RR-SORT1 interaction does not require the presence of the LDLR (Figure 3C). Overall LDL uptake in LDLA7 cells is low, but nevertheless silencing (P)RR or SORT1 significantly reduced LDL uptake in these cells. However, this reduction was minimal and attenuated in comparison to the effect that silencing these genes had in the other cells tested (Figure 3D, and compare with Figure 2C,D), suggesting that the LDLR is necessary for the large effect of (P)RR on LDL uptake.

Having ruled out the possibility that the effect of si(P)RR on LDL uptake is largely due to direct SORT1-mediated LDL uptake we considered involvement of the LDLR pathway in this phenotype. In fact, under the sterol depletion regimen and the LDL concentration (5 µg/mL) we used in these experiments, it is highly likely that the primary entry portal for LDL internalization would be the LDLR, and not SORT1. In line with this, we found that next to reducing SORT1 levels, silencing of (P)RR also reduced total LDLR abundance (Figures 3A,B, Online Figure IV A,B) and LDLR cell-surface density (Figures 3E,F) without affecting SORT1 and LDLR mRNA expression (Online Figure IV C,D).

Unexpectedly, in these experiments we found that silencing SORT1 also effectively reduced cellular LDLR abundance to an extent comparable to that achieved by (P)RR silencing, and as a consequence also resulted in attenuated LDL uptake (Figure 3G, Online Figure II B-E). Despite the existence of strong evidence linking SORT1 to LDL-cholesterol metabolism in humans, to the best of our knowledge there is no report indicating that SORT1 can affect LDLR levels. Therefore, in view of the mutual effect (P)RR and SORT1 have on the LDLR and on each other’s protein level we reasoned that the two may act in concert to control LDLR function. In support of this concept, we found that in both HepG2 and A431 cells combined silencing of (P)RR and SORT1 did not result in an additive reduction in LDL uptake as compared to depletion of the (P)RR or SORT1 alone (Figure 3G, Online Figure II B,C). Furthermore, we found that over-expression of SORT1 in HepG2 cells mildly increased protein abundance of (P)RR and LDLR (Online Figure V). However, this was not sufficient to overcome degradation of the LDLR induced by silencing (P)RR, indicating that this outcome is not solely dependent on reduced SORT1 protein. These results point towards the (P)RR acting as a post-transcriptional regulator of the LDLR and consistent with this notion three different lysosomotropic agents, bafilomycin A1 (BafA1), NH₄Cl and chloroquine rescued LDLR levels following (P)RR silencing (Figure 4). None of these lysosomal blockers reversed the degradation of SORT1 following (P)RR silencing. We therefore tested alternative degradation pathways that may explain the decrease in SORT1 protein. Unexpectedly, blocking the two other major degradation pathways, autophagy and the proteasome (using 3-MA, or MG132, respectively), also failed to rescue SORT1 protein (Online Figure VI). Therefore, the mechanism underlying reduced SORT1 protein following silencing of (P)RR remains currently unknown.

DOI: 10.1161/CIRCRESAHA.115.306799
A plausible explanation for reduced LDLR levels in \((P)\text{RR}\)-depleted cells is that the receptor is subject to accelerated internalization from the plasma membrane and subsequent degradation. We tested this idea by following surface-biotinylated LDLR in control and \((P)\text{RR}\) depleted cells. In these experiments the cells were initially sterol depleted to increase abundance of the LDLR, and disappearance of surface-biotinylated LDLR was followed over time by addition of medium, which either contained or lacked lipoproteins. In the absence of added lipoproteins, disappearance of labeled-LDLR was negligible (not shown). Similarly, when lipoprotein-containing medium was added, even though effective \((P)\text{RR}\) silencing reduced total cellular LDLR levels, disappearance of the LDLR or TfR (as control) from the plasma membrane was unchanged (Online Figure VII). In this, the effect of \((P)\text{RR}\) seems to be distinct from that mediated by the ubiquitin ligase IDOL and PCSK9 which induce specific removal of the LDLR from the membrane and target the receptor for lysosomal degradation.\(^{20,36,37}\) Accordingly, overexpressing \((P)\text{RR}\) did not affect PCSK9-induced LDLR degradation (Online Figure VIII). Therefore, our results rather point towards an event controlled by the \((P)\text{RR}\) which is required for proper trafficking and/or recycling of the LDLR to the plasma membrane.

**DISCUSSION**

In this study, using an unbiased proteomics approach, we identify a novel role for the \((P)\text{RR}\) in LDL metabolism. The main finding of the current study is the identification of \((P)\text{RR}\) as a post-transcriptional regulator of LDLR abundance of LDL uptake into cells, plausibly by regulating trafficking of the receptor to the plasma membrane.

Our proteomic screen identified several potential \((P)\text{RR}\)-interacting proteins. Of these, SORT1, had the highest Mascot score and was therefore chosen for study. SORT1 was recently identified as a regulator of LDL metabolism, and its genetic locus is strongly associated with plasma LDL levels and the risk for cardiovascular disease.\(^{29,33,38,39}\) SORT1 controls hepatic VLDL secretion, and can also bind and internalize LDL directly, thereby serving as a major regulator in determining plasma LDL levels.\(^{28,29,34}\) In the current study, we found that \((P)\text{RR}\) depletion reduces SORT1 protein abundance without affecting its transcript levels, and attenuates cellular accretion of LDL. Similarly, SORT1 depletion led to a comparable reduction in cellular LDL uptake and decreased \((P)\text{RR}\) abundance reciprocally. Since combined silencing of \textit{SORT1} and \((P)\text{RR}\) did not additively reduce LDL uptake we speculate the two act through a common pathway. Our current study also reveals a previously unrecognized function of SORT1 in regulating LDLR protein levels in hepatocytes. This finding is consistent with recent reports demonstrating that SORT1 deficiency leads to increased plasma LDL cholesterol,\(^{29,40}\) and provides an additional mechanism that may contribute to this outcome. In addition, our results may also provide an explanation why genetic ablation or silencing of SORT1 resulted in a less pronounced increase in plasma LDL-cholesterol on a \textit{Ldlr}^\text{+/−} background.\(^{29,41}\) The ability of SORT1 to control LDLR activity hints that it may influence efficacy of statins by regulating LDLR activity. Interestingly, a recent meta-analysis study found that genetic variances in the \textit{SORT1} locus are associated with the LDL-cholesterol response to statin therapy.\(^{42}\)

Our study demonstrates that regulation of the LDLR by the \((P)\text{RR}\) is a post-transcriptional event culminating in lysosomal degradation of the LDLR. Grossly, two cellular scenarios may fit this pattern of regulation. The first involving accelerated removal and degradation of plasma membrane LDLR, and the second resulting from increased lysosomal targeting of newly synthesized LDLR. Our biotinylation experiments of cell-surface LDLR support the second scenario as we found that silencing \((P)\text{RR}\) expression did not accelerate the degradation of the plasma membrane LDLR pool or affected lysosomal targeting of the LDLR by exogenous PCSK9. Given the established role of SORT1 in intracellular trafficking of APOB, PCSK9, Trk, and Glut4,\(^{28,43,45}\) we speculate that SORT1 is essential for proper trafficking of the nascent LDLR protein towards the plasma membrane, and that this is dependent on \((P)\text{RR}\) function.
This raises the question of how (P)RR influences LDLR trafficking? An attractive possibility may be that this could be due to (P)RR being an accessory component of the V-ATPase complex. This complex is implicated in protein sorting and membrane targeting,\textsuperscript{11,46} and previous studies demonstrated that depleting the (P)RR in podocytes decreases the level of V-ATPase subunits leading to autophagosomal defects.\textsuperscript{13,14} However, we found that lysosomotropic agents reversed (P)RR-induced reduction in LDLR protein abundance, indicating the existence of an intact endolysosomal pH gradient and functional lysosomes. A caveat of these experiments is that we used siRNA to suppress (P)RR expression while the above-mentioned studies used a Cre-Lox approach to genetically ablate (P)RR. Therefore, in our experiments, despite effective silencing, minimal levels of (P)RR may still exist. Nevertheless, several additional lines of evidence suggest that loss of PRR in the studied cells did not grossly affect V-ATPase activity. Endocytosis of EGFR and TfR was not affected by (P)RR silencing. As pH and V-ATPase activity are critical for overall endocytosis and receptor recycling, our findings suggest that a general acidification defect of the endolysosomal compartment does not occur following (P)RR silencing in the cells we studied, consistent with our earlier study.\textsuperscript{15} We find it also unlikely that (P)RR depletion affects the V-ATPase only in a subset of LDLR-enriched endocytic vesicles as our kinetic analysis of plasma membrane removal of the LDLR revealed no major alterations following silencing of (P)RR. In agreement with our conclusion, Kissing et al. recently reported that macrophages lacking PRR do not display altered acidification and phagolysosomal defects.\textsuperscript{47} Therefore, an alternative explanation for our observations may be that the functional interaction of the (P)RR and SORT1 serves to ensure proper trafficking of nascent LDLR to the plasma membrane. This may be reminiscent of the role SORT1 plays in APOB metabolism, where SORT1 has been demonstrated to promote both APOB secretion and degradation in hepatocytes.\textsuperscript{28,48}

Summary

In conclusion, we report that the (P)RR is a previously unrecognized regulator of LDL metabolism, which specifically regulates cellular LDL uptake by modulating LDLR protein abundance. As such, our report warrants future studies to assess the full spectrum of proteins whose trafficking/secretion is subject to regulation by the (P)RR-SORT1 functional interaction, and to elucidate the role of (P)RR in lipoprotein metabolism \textit{in vivo}. 

ACKNOWLEDGEMENTS

We thank members of the Zelcer and Danser labs for their help, and Xiao Chun Yang, Fengting Su, and Hui Lin for their technical assistance. We thank Irith Koster for her comments and suggestions.

SOURCES OF FUNDING

XL is supported by a National Natural Science Foundation of China (grant no. 81500667). NZ is supported by a European Research Council Consolidator grant (617376), by a VIDI grant (17.106.355) from The Netherlands Organization of Science (NWO), and is an Established Investigator of the Dutch Heart Foundation. AHJD is supported by the Top Institute Pharma (T2-301).

DISCLOSURES

None.

DOI: 10.1161/CIRCRESAHA.115.306799
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FIGURE LEGENDS

**Figure 1.** (P)RR interacts with SORT1 and regulates its protein abundance. *(A&B)* Representative blot of two independent experiments showing co-immunoprecipitation of (P)RR with SORT1, but not with TRAF4. Total lysates from HEK293 cells were immunoprecipitated with antibodies against *(A)* the (P)RR or *(B)*, SORT1 or with rabbit IgGs (rIgG) as negative control and immunoblotted as indicated. *(C)* HEK293 cells were transfected with control or (P)RR siRNA for 48 hours. Total lysates were immunoblotted and a representative of two independent experiments in duplicate is shown. *(D)* (P)RR and SORT1 protein abundance was quantified and normalized to the level of β-actin in the same lysate. Each bar and error represents the (P)RR and SORT1 protein abundance relative to that in control siRNA transfected ± SEM (n=8). ***, p<0.001. *(E)* (P)RR silencing in HEK293 cells successfully suppresses (P)RR mRNA levels, and does not affect SORT1 mRNA levels. N=6, ***, p<0.001.

**Figure 2.** (P)RR silencing reduces LDL uptake without grossly affecting endocytosis. *(A)* A431 cells transfected with (P)RR or control siRNAs for 48 hours. Expression of (P)RR was determined by quantitative PCR. *(B)* A431 cells were treated as in A. Subsequently, cells were cultured with Dylight488-labeled LDL for an additional 3 hours. Representative fluorescence images of cells are shown. Nuclei are counterstained with DAPI (blue). Scale bar 10 μm. *(C&D)* Quantitative measurement of LDL uptake in *(C)* A431 cells, and *(D)* HepG2 cells following (P)RR silencing. *(A,C,D,F)* Open and closed bars represent control siRNA and (P)RR siRNA, respectively. Each bar and error are the mean ± SEM (n=6). ***, p<0.001. *(E)* A431 cells were treated as in A and incubated with fluorescent-labeled Tf. Representative fluorescence images are shown. *(F)* Quantitative measurement of fluorescence-labeled Tf uptake in A431 cells. N=6, ***, p<0.001.

**Figure 3.** The (P)RR-SORT1 interaction controls LDLR protein stability and LDL uptake. *(A&B)* A431 *(A)* or HepG2 *(B)* cells were transfected with control, (P)RR, or SORT1 siRNAs for 48 or 72 hours, respectively. Total cell lysates were immunoblotted as indicated and a representative blot of at least three independent experiments is shown. *(C&D)* LDLA7 CHO cells were transfected with control, (P)RR, or SORT1 siRNA for 48 hours. Total cell lysates were immunoblotted as indicated and a representative blot of three independent experiments is shown. *(D)* LDLA7 CHO cells were treated as indicated above, and subsequently cultured with Dylight488-labeled LDL for an additional 3 hours. Quantitative measurement of LDL uptake is shown. N=9. *: p<0.05, **: p<0.01. *(E&F)* LDLR at the cell-surface was measured by FACS in *(E)* A431 and *(F)* HepG2 cells. Open and closed bars represent control and (P)RR siRNA, respectively (n=6). *: p<0.05; **: p<0.001. *(G)* A431 cells were transfected with control, (P)RR, SORT1 siRNA or both [(P)RR+SORT1 siRNA; DK] for 48 hours. Subsequently, cells were cultured with Dylight488-labeled LDL for an additional 3 hours. Quantitative measurement of LDL uptake is shown. N=9, ***, p<0.001.

**Figure 4.** (P)RR-induced reduction in LDLR abundance is rescued by lysosomotropic agents. HepG2 cells were treated with control, or (P)RR siRNA for 72 hours. Cells were incubated with vehicle control, 100 nmol/L bafilomycin A1 (BafA1), 10 mmol/L NH4Cl, or 25 μmol/L chloroquine for 6 hours. Total cell lysates were immunoblotted as indicated, and a representative blot of three independent experiments is shown.
Novelty and Significance

What Is Known?

- The low affinity of the (pro)renin receptor for both renin and prorenin raises doubt about its in-vivo significance as a renin-angiotensin system (RAS) component.
- Recent studies show that the (pro)renin receptor has functions beyond the RAS, including participation in Wnt signaling and modulation of vacuolar H⁺-ATPase activity.

What New Information Does This Article Contribute?

- The (pro)renin receptor, by controlling the protein abundance of sortilin-1 (SORT1) and the low-density lipoprotein receptor (LDLR), is a novel regulator of low-density lipoprotein (LDL).
- SORT1 not only acts as a receptor for low-density lipoprotein, but also controls LDLR protein abundance.

The (pro)renin receptor is now believed to largely have renin-angiotensin system-independent functions. Using a proteomics approach to identify potential novel functions of the (pro)renin receptor, we found that it interacts with sortilin-1, a recently identified receptor for low-density lipoprotein. Silencing the (pro)renin receptor led to decreased SORT1 and LDLR protein abundance, thereby reducing the cellular uptake of low-density lipoprotein. As such, our study identifies the (pro)renin receptor as a new regulator of LDL metabolism, and suggests that mutations in the (pro)renin receptor gene might associate with circulating LDL levels.
Figure 4

<table>
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<tr>
<th>Treatment</th>
<th>Control siRNA</th>
<th>(P)RR siRNA</th>
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<td>Vehicle</td>
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<td>+</td>
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<tr>
<td>BafA1</td>
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<td>+</td>
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<tr>
<td>NH₄Cl</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Chloroquine</td>
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<td>+</td>
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</table>

- **SORT1**
- **LDLR**
- **(P)RR**
- **β-actin**
Identification of the (Pro)renin Receptor as a Novel Regulator of Low-Density Lipoprotein Metabolism

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_Circ Res._ published online November 18, 2015;

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Identification of the (Pro)renin Receptor as a Novel Regulator of Low-density Lipoprotein Metabolism

Xifeng Lu¹²³, Marcel E. Meima¹, Jessica K. Nelson², Vincenzo Sorrentino², Anke Loregger², Saskia Scheij², Dick H. W. Dekkers⁵, Monique T. Mulder¹, Jeroen A. A. Demmers⁵, Geesje M-Dallinga-Thie⁴, Noam Zelcer²,*, and A.H. Jan Danser¹,*

Supplemental Material
Methods

Chemicals and Reagents
DMEM, penicillin, streptomycin, and FBS were purchased from Lonza (Basel, Switzerland). Lipofectamine 2000, RNAiMax, negative control siRNA (Silencer® Select Negative Control No. 1 siRNA), SORT1 siRNA (Ambion® Select, S224557), Stealth RNAi® (P)RR siRNA 1 [GACAGUGUUGCAAAUUCAUUCACU (sense)] and AGUGAAUGGAAUUGCAACACUGUC (anti-sense), Stealth RNAi® (P)RR siRNA 2 [AGCCAAGGAUAUCCUGAUUUA (sense)] and UAAAUCAGGAGAAUCCUGGU (anti-sense), Novex Bis-tris gel, 4×LDS sample buffer, MOPS buffer, colloidal Coomassie blue, streptavidin-agarose beads, ProtA Dynabeads, non-specific rabbit IgG, Alexa 488-conjugated human transferrin (Tf), Alexa 488 EGF complex, TEV protease, TRIzol, TrypLE, Lipofectamine RNAiMax, Lipofectamine 2000, colloidal blue staining kit, and recombinant human PCSK9 were purchased from Life Technologies (Bleiswijk, The Netherlands). Chloroquine, ammonium chloride, mevalonate, simvastatin, and Complete protease inhibitors cocktail™ was purchased from Roche (Woerden, The Netherlands). NeutArvidin agarose beads, enhanced chemiluminescence (ECL) kit, EZ-Link Sulfo-NHS-Biotin, and bicinechoninic acid (BCA) assay were purchased from Pierce (Etten-Leur, The Netherlands). IgG sepharose beads were purchased from GE Healthcare (Luxemburg). The JetPrime transfection reagent was purchased from Polyplus Transfection SA (Illkirch, France). Bafilomycin A1 was purchased from Millipore (Amsterdam, The Netherlands). 3-Methyladenine (3-MA) and MG132 were from Sigma Aldrich (Zwijndrecht, The Netherlands).

Cell Culture and Transfections
HEK293, A431, Huh7 and HepG2 cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/mL streptomycin at 37°C and 5% CO2. LDLA7 CHO cells were kindly provided by Dr Monty Krieger (MIT, USA)¹ and maintained in DMEM/F12 supplemented with 5% FBS, 100 U/ml penicillin and 100 mg/mL streptomycin at 37°C and 5% CO2. For both siRNA and plasmid transfection, HEK293 cells were transfected with lipofectamine 2000 according to the manufacturer’s protocol. Unless indicated otherwise, cells were cultured in sterol-depletion medium [DMEM supplemented with 10% bovine lipid-deficient serum (LPDS), 5 µg/mL simvastatin, and 100 µM mevalonate], to increase LDLR expression, for 18 hours prior to experiment. A431 and LDLA7 CHO cells were transfected with 40 nmol/L siRNAs by JetPrime, and HepG2 cells were transfected with 40 nmol/L siRNAs by RNAiMax following the
manufacturer’s protocols. For plasmid transfection, HepG2 cells were transfected with JetPrime using the manufacturer’s protocol.

**Plasmid Generation**

The pCeMM-NTAP vector was used to express TAP-tagged human (P)RR. The signal peptide of human (P)RR, as determined by the SignalP server, was inserted as a primer duplex in the EcoRI-site immediately upstream of the TAP-tag. The remainder of the human (P)RR sequence was amplified by PCR and inserted into the XhoI and NotI site. Correctness of the construct was confirmed by sequencing. The SORT1 expression plasmid was a kind gift from Dr. Trond P. Leren (University of Oslo). Primers are listed in Online Table I.

**TAP Purification**

Cells were washed twice with ice cold PBS, and lysed on ice with lysis buffer (20 mmol/L Tris-HCl, 3 mmol/L KCl, 1 mmol/L EDTA, 1% NP-40, 150 mmol/L NaCl, 50 mmol/L NaF, 1 mmol/L Na3VO4, complete protease inhibitors cocktail™, pH 7.4). For affinity purification, 10 T-75 flasks were used for each condition. Cell lysates were collected and cleared by centrifugation twice at 1500×g for 10 minutes at 4°C, mixed with 1 mL prewashed IgG Sepharose beads (GE Healthcare), and incubated on a rotating platform for 2 hours at 4°C. Beads were collected by centrifugation at 1000×g for 3 minutes at 4°C, and washed three times with lysis buffer and three times with TEV buffer (50 mmol/L Tris-HCl, 0.5 mmol/L EDTA, complete protease inhibitors cocktail™, pH 8.0). TEV protease (700 U) was added to beads resuspended in 1 mL TEV buffer supplemented with 1 mmol/L DTT. Beads were incubated overnight on rotating platform at 4°C. The supernatants were collected by centrifugation at 1000×g for 3 minutes at 4°C, 100 µL prewashed streptavidin-agarose beads were added, and the supernatants were incubated on a rotating platform for 1 hour at 4°C. After incubation, streptavidin-agarose beads were collected by centrifugation at 1000×g for 3 minutes at 4°C, and washed five times with lysis buffer. Protein complexes were eluted from the beads by adding 80 uL of NuPage® LDS sample loading buffer, and incubating at 95°C for 5 minutes. Proteins were separated by SDS-PAGE on 4-20% Novex® Bis-Tris gradient gels using MOPS buffer and visualized by colloidal coomassie blue staining according to the manufacturer’s protocol.

**Liquid Chromatography/Tandem Mass Spectrometry Analysis**

Complete SDS-PAGE gel lanes were cut into ~1 mm slices using an automatic gel slicer and subjected to in-gel reduction with dithiothreitol, alkylation with chloroacetamide and digestion
with sequencing graded porcine trypsin (Promega) as described. Nanoflow LC-MS/MS was performed on a 1100 series capillary LC system (Agilent Technologies) coupled to an LTQ Orbitrap XL mass spectrometer (Thermo Scientific), operating in positive mode and equipped with a nanospray source. Peptide mixtures were trapped on a ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 1.5 cm × 100 µm, packed in-house) at a flow rate of 8 µL/minute. Peptide separation was performed on ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 15 cm × 50 µm, packed in-house) using a linear gradient from 0 to 80% B (A = 0.1 % formic acid; B = 80% (v/v) acetonitrile, 0.1 % formic acid) in 70 minutes and at a constant flow rate of 200 nL/minute using a splitter. The column eluent was directly sprayed into the ESI source of the mass spectrometer. Mass spectra were acquired in continuum mode; fragmentation of the peptides was performed in data-dependent mode by CID. Peak lists were automatically created from raw data files using the Proteome Discoverer (version 1.3; Thermo). The Mascot search algorithm (version 2.2, MatrixScience) was used for searching against the Uniprot database (release unihuman_2012_02_cont.fasta, taxonomy: all entries). The peptide tolerance was set to 10 ppm and the fragment ion tolerance was set to 0.8 Da. A maximum number of 2 missed cleavages by trypsin were allowed and carbamidomethylated cysteine and oxidized methionine were set as fixed and variable modifications, respectively. The Mascot score cut-off value for a positive protein hit was set to 40.

**Immunoprecipitation and Immunoblotting**

For co-immunoprecipitations (CoIPs), HEK293 cells were lysed in IP lysis buffer (lysis buffer as described above, but with 5 mmol/L instead of 1 mmol/L EDTA) on ice for 15 minutes. Cell lysates were cleared by centrifugation at 1000×g for 5 minutes at 4°C, and protein content was measured with the BCA assay. Of each lysate, one mg of protein was precleared with 50 µL prewashed ProtA Dynabeads for 1 hour at 4°C, and then incubated for 1 hour at 4°C with 9 µg anti-h(P)RR antibodies, anti-SORT1 antibodies, or non-specific rabbit IgGs as control, coupled to 50 µL Protein-A Dynabeads. After washing three times with lysis buffer, the immunocomplexes were eluted with 50 µL 1×LDS sample buffer at 95°C for 5 minutes. The antibodies used to IP endogenous (P)RR and SORT1 have been described before. For protein expression studies, A431 and HepG2 cells were lysed in RIPA buffer (150 mmol/L NaCl, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 100 mmol/L Tris-HCl, complete protease inhibitors cocktailTM, pH 7.4). Lysates were cleared by centrifugation at 10,000×g at 4°C for 5 minutes and protein contents were measured using the BCA assay. For immunoblotting, immunocomplexes or lysates containing equal amount of proteins (10-25 µg) were resolved by SDS-PAGE and transferred to
PVDF membranes. Blots were probed using the primary antibodies listed in Online Table II and HRP-conjugated goat anti-mouse or goat anti-rabbit antibodies and detected by ECL.

**LDL, Transferrin, and EGF Uptake Assays**

LDL uptake was measured using DyLight 488-labeled LDL, as described previously. Briefly, HepG2 or A431 cells were incubated in sterol-depleted medium for 16 hours prior to adding LDL. Cells were incubated with 5 µg/mL DyLight488-labeled LDL in DMEM supplemented with 0.5% BSA for 3 hours at 37°C or 4°C. In these experiments, 100 µg/mL non-labeled LDL was used to correct for non-specific LDL association/binding. Cells were subsequently washed twice with ice-cold PBS supplemented with 0.5% BSA and lysed in RIPA buffer. Specific LDL uptake was calculated as the fluorescent intensity difference between 37°C and 4°C, after subtracting non-specific association/binding. For Tf uptake, cells were serum-starved for 3 hours in binding buffer (DMEM, 0.1% BSA, 20 mmol/L HEPES), and then incubated with 50 µg/mL Alexa 488-conjugated human Tf for 30 minutes. For EGF uptake, cells were serum-starved overnight, and then incubated with Alexa 488 EGF complex in binding buffer (DMEM, 1% BSA, 20 mmol/L HEPES) for 15 minutes. After incubation, cells were washed twice with ice-cold PBS containing 0.1% BSA for Tf uptake or 0.5% BSA for EGF uptake, and lysed in RIPA buffer. LDL, Tf, and EGF uptake were determined by quantification of the fluorescence signal on a Typhoon imager (GE Healthcare) and corrected for the protein content in the lysate as determined with the BCA assay. To visualize LDL, Tf, and EGF uptake, cells were cultured on coverslips. Cells were incubated and washed as described above, then fixed with 4% paraformaldehyde, and mounted with Vectorshield (Vector Laboratories) containing DAPI. Prepared slides were visualized using confocal microscopy (Leica SP5) with a 63x oil lens.

**Measurement of Cell Surface LDLR by FACS**

Surface LDLR density after knocking down the (P)RR was measured as described before. Briefly, cells were maintained on sterol-depleted medium for 16 hours prior to the experiment, dissociated with TrypLE Express and incubated in FACS blocking buffer [PBS supplemented with 2 mmol/L EDTA (or 10 mmol/L EDTA for HepG2), 0.5% BSA, 2% goat serum] on ice for 30 minutes. Next, 100,000 cells were stained in 50 µL FACS buffer containing PE-conjugated anti-LDLR antibody (R&D Systems, FAB2148P, 1:400) for 1 hour on ice. Following three washes with FACS blocking buffer, cells were directly analyzed on a FACS Calibur (BD Biosciences). Viable cells were gated and 10,000 events per condition acquired. Data were analyzed using the CellQuest software package (BD Biosciences).
**RNA Isolation and qPCR**

Total RNA was isolated from cells using TRIzol following the manufacturer’s protocol. One milligram of total RNA was reverse transcribed with the iScript reverse transcription kit (Bio-rad). SYBR Green real-time quantitative PCR assays were performed on a Lightcycler 480 II apparatus (Roche) using SYBR Green master mix (Roche). Gene expression was normalized for the expression of 36B4, and expressed as mean ± SEM. Primers are listed in Table S1.

**Cell Surface Protein Biotinylation**

Degradation of LDLR by PCSK9, and cell surface protein biotinylation were performed as previously described.7-9 Briefly, HepG2 cells were transfected with either control or NTAP-(P)RR plasmid for 48 hours, and sterol-depleted for 18 hours prior to the experiment. Subsequently, cells were incubated with 2.5 µg/mL PCSK9 in DMEM supplemented with 0.5% BSA for 4 hours at 37°C, followed by three washes with ice-cold PBS-CM (PBS, 1 mmol/L MgCl₂, 0.1 mmol/L CaCl₂). Surface proteins were then labelled with 1 mg/mL EZ-Link Sulfo-NHS-Biotin in PBS-CM for 30 minutes with light agitation at 4°C. Free biotin was quenched by washing cells once with quenching buffer (PBS-CM, 100 mmol/L glycine, pH 7.4), and an additional incubation with quenching buffer for 15 minutes at 4°C. When membrane LDLR degradation kinetics were determined, cells were returned to the incubator in DMEM containing 10% FBS for the specified time points. Cells were subsequently washed twice with ice-cold PBS-CM, and lysed with RIPA buffer supplemented with protease inhibitors. Lysates were cleared by centrifugation at 10,000×g at 4°C for 5 minutes. Biotin-labelled surface proteins were isolated by nutating lysates overnight with 50 µL pre-washed NeutrAvidin agarose beads at 4°C. Subsequently, beads were pelleted, washed three times with RIPA containing protease inhibitors and bound proteins eluted by boiling beads with 50 µL 2× LDS sample buffer at 95°C for 5 minutes.

**Statistical Analysis**

Data are presented as mean±SEM. One-way ANOVA followed by the Bonferroni correction was performed for comparison of more than two groups. Student’s t-test was performed for comparison of two groups. P values of <0.05 were considered significant.
References


### Online Table I. List of primers used.

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### Online Table II. List of antibodies used.

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Online Figure I. Scheme of the NTAP-h(P)RR construct and tandem-affinity purification (TAP). Human (P)RR was tagged with the TAP-epitope at the N-terminal side. The signal peptide (SP) of the human (P)RR was inserted at N-terminus before the TAP-tag.
**(Pro)renin receptor and LDL metabolism**

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**Online Figure II. The (P)RR regulates LDL metabolism.** (A) A431 cells were transfected with control siRNA, (P)RR siRNA 1, or (P)RR siRNA 2 for 48 hours, and LDL uptake was determined as indicated before. Quantitative measurement of LDL uptake is shown. N=6, **, p<0.01, ***, p<0.001. (B&C) A431 (B) or HepG2 (C) cells were transfected with control, (P)RR, SORT1 siRNA, or DK [(P)RR siRNA+SORT1 siRNA] for 48 hours, and cultured with normal medium for 18 hours prior to the experiment. Cells were subsequently cultured for an additional 3 hours with Dylight488-labeled LDL. Quantitative measurement of LDL uptake is shown. N=6, *, p<0.05, **, p<0.01, ***, p<0.001. (D) HepG2 cells were treated with control, (P)RR, or SORT1 siRNA for 48 or 72 hours. Quantitative LDL uptake is determined as indicated and shown. N=6, **, p<0.01, ***, p<0.001. (E) Huh7 cells were transfected with control, (P)RR, or SORT1 siRNA for 48 hours, and cultured under sterol-depleted medium for 18 hours prior to the experiment. LDL uptake was measured as in (A). N=6, **, p<0.01, ***, p<0.001.
Online Figure III. (P)RR silencing differentially effects endocytic pathways. (A) A431 cells were transfected with control or (P)RR siRNA for 48 hours. Total lysates were prepared and immunoblotted as indicated. The blot is representative of at least three independent experiments. (B) HepG2 cells were treated as described above and incubated with fluorescent-labeled Tf. Quantitative measurement of fluorescence-labeled Tf uptake is shown. N=6. (C) Representative fluorescent images of fluorescently-labeled EGF uptake (0.5 µg/mL) in A431 cells treated with control siRNA or (P)RR siRNA. (D) Quantitative measurement of EGF uptake (0.1 µg/mL or 0.5 µg/mL). (B&D) open and close bars represent control and (P)RR siRNA treated cells, respectively (n=6).
Online Figure IV. Silencing (P)RR reduces LDLR protein abundance without affecting its transcriptional levels. (A) A431 cells were transfected with control, (P)RR, or SORT1 siRNA for 48 hours, and cultured under sterol-depleted medium for 18 hours prior to the experiment. Total lysates were immunoblotted and protein abundance was quantified and normalized to the level of β-actin in the same lysate. Each bar and error represents the (P)RR, LDLR, and SORT1 protein abundance relative to that in control siRNA transfected ± SEM (n=6). *** p<0.001. (B) HepG2 cells were transfected as indicated in A for 72 hours. (P)RR, LDLR, and SORT1 protein abundance were quantified and normalized as described above. N=6, *** p<0.001. (C&D) The mRNA levels of LDLR, SORT1, and (P)RR were determined by quantitative PCR in (C) A431 or (D) HepG2 cells treated as described above. N=5. ** p<0.01; *** p<0.001.
Online Figure V. Overexpressing SORT1 does not rescue (P)RR-silencing-induced LDLR degradation. (A): HepG2 cells were first transfected with siPRR for 24 hours, and then transfected with either GFP control plasmid or SORT1 plasmid for an additional 48 hours. Total cell lysates were immunoblotted as indicated, and a representative immunoblot of four independent experiments in duplicate is shown. (B&C) LDLR (B) and (P)RR (C) protein abundance were quantified and normalized to the level of β-actin in the same lysate. Each bar and error represents the LDLR and (P)RR protein abundance relative to that in control siRNA and GFP control plasmid transfected ± SEM (n=8). *, p<0.05.
Online Figure VI. Inhibiting autophagy or the proteasome does not prevent si(P)RR induced SORT1 and LDLR degradation. (A&B) HepG2 cells were transfected with siSORT1 (A) or si(P)RR (B) for 72 and 48 hours respectively, and then incubated in the presence of 7.5 mM 3-MA or 10 µM MG132 for 6 hours. Total lysates were immunoblotted and a representative immunoblot of three independent experiments done in triplicate is shown. (B&C) LDLR, SORT1 and (P)RR protein abundance following transfection with si(P)RR (C) or siSORT1 (D) and the indicated treatments were quantified and normalized to the level of β-actin in the same lysate. Each bar and error represents the LDLR, SORT1 and (P)RR and protein abundance relative to that in control siRNA ± SEM (n=3). ***, p<0.001.
**Online Figure VII. Silencing (P)RR does not accelerate membrane LDLR degradation.** HepG2 cells were transfected with control or (P)RR siRNA for 72 hours. Membrane proteins were labeled with biotin as indicated, and subsequently cells were cultured back at 37°C for indicated period. Biotinylated proteins were isolated and purified as indicated. LDLR abundance in total lysate, supernatant (non-biotinylated cytosolic proteins), and membrane (biotinylated membrane proteins) was determined by immunoblotting. TfR is used as a loading control. A representative blot of two independent experiments is shown.
Online Figure VIII. Overexpressing (P)RR in HepG2 cells does not affect PCSK9-induced LDLR degradation. HepG2 cells were treated with control or NTAP-(P)RR plasmid for 48 hours, and subsequently incubated with 2.5 µg/mL PCSK9 for 4 hours. Afterwards, membrane proteins were labeled with biotin and purified as indicated. LDLR abundance in total lysate, supernatant (non-biotinylated cytosolic proteins), and membrane (biotinylated membrane proteins) was determined by immunoblotting. TfR is used as a loading control.