The Deubiquitylase USP2 Regulates the LDLR Pathway by Counteracting the E3-Ubiquitin Ligase IDOL

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Rationale: The low-density lipoprotein (LDL) receptor (LDLR) is a central determinant of circulating LDL-cholesterol and as such subject to tight regulation. Recent studies and genetic evidence implicate the inducible degrader of the LDLR (IDOL) as a regulator of LDLR abundance and of circulating levels of LDL-cholesterol in humans. Acting as an E3-ubiquitin ligase, IDOL promotes ubiquitylation and subsequent lysosomal degradation of the LDLR. Consequently, inhibition of IDOL-mediated degradation of the LDLR represents a potential strategy to increase hepatic LDL-cholesterol clearance.

Objective: To establish whether deubiquitylases counteract IDOL-mediated ubiquitylation and degradation of the LDLR.

Methods and Results: Using a genetic screening approach, we identify the ubiquitin-specific protease 2 (USP2) as a post-transcriptional regulator of IDOL-mediated LDLR degradation. We demonstrate that both USP2 isoforms, USP2-69 and USP2-45, interact with IDOL and promote its deubiquitylation. IDOL deubiquitylation requires USP2 enzymatic activity and leads to a marked stabilization of IDOL protein. Paradoxically, this also markedly attenuates IDOL-mediated degradation of the LDLR and the ability of IDOL to limit LDL uptake into cells. Conversely, loss of USP2 reduces LDLR protein in an IDOL-dependent manner and limits LDL uptake. We identify a tripartite complex encompassing IDOL, USP2, and LDLR and demonstrate that in this context USP2 promotes deubiquitylation of the LDLR and prevents its degradation.

Conclusions: Our findings identify USP2 as a novel regulator of lipoprotein clearance owing to its ability to control ubiquitylation-dependent degradation of the LDLR by IDOL. (Circ Res. 2016;118:410-419. DOI: 10.1161/CIRCRESAHA.115.307298.)

Key Words: E3 ubiquitin ligase ■ LDL cholesterol ■ LDL receptors ■ MYLIP/IDOL ■ ubiquitin ■ USP2

The low-density lipoprotein (LDL) receptor (LDLR) is a major determinant of circulating levels of LDL. Accordingly, mutations in this receptor are the leading cause for development of autosomal-dominant hypercholesterolemia, a disease characterized by reduced hepatic LDL clearance, elevated plasma cholesterol levels, and accelerated atherosclerosis. Owing to its central role in lipoprotein metabolism, the level of the LDLR is subject to tight homeostatic regulation. Transcription of the LDLR is controlled by the sterol regulatory element-binding proteins, and it is increased when cellular cholesterol levels decline to allow increased endocytosis of cholesterol-rich LDL particles.

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Next to transcriptional regulation, post-transcriptional mechanisms are also emerging as important determinants of LDLR abundance. Among these, ubiquitylation of the LDLR plays an important role in controlling the intracellular trafficking of the receptor, similar to the role this process plays for other membrane receptors. We have recently identified inducible degrader of the LDLR (IDOL) as an E3-ubiquitin ligase (E3) that specifically promotes ubiquitylation of the LDLR. IDOL itself is subject to direct transcriptional regulation by the sterol-sensing transcription factors liver X receptors (LXR). Activation of LXR when cellular sterol levels increase leads to induction of IDOL expression and activity, and subsequent ubiquitylation of conserved residues in the short intracellular tail of the LDLR. In turn, ubiquitylation of the LDLR serves to mark the receptor for internalization and subsequent lysosomal degradation. In particular, the plasma membrane pool of LDLR is highly sensitive to IDOL activity, and ubiquitylation leads to rapid removal of this receptor pool via an epsin-dependent but clathrin-independent endocytic
route. The role of IDOL in human lipoprotein metabolism is supported by genome-wide association studies that identify an association between genetic variation in the IDOL locus and circulating levels of LDL-cholesterol. Furthermore, we recently described a rare loss-of-function IDOL variant in individuals with LDL-cholesterol. Consistent with this, silencing of IDOL in nonhuman primates using an RNAi-based approach resulted in a reduction of circulating levels of LDL-cholesterol, which was associated with increased levels of hepatic LDLR protein. Thus, the LXR–IDOL–LDLR axis is a potent ubiquitination-dependent mechanism to acutely limit lipoprotein-derived cholesterol uptake.

Similar to other post-translational modifications, ubiquitylation is reversible; a process dependent on the activity of deubiquitylases. The human genome encodes ≈100 deubiquitylases, 79 of which are predicted to be active. These deubiquitylases are classified into 5 distinct families, the largest being the ubiquitin-specific protease (USP) family. A major challenge in elucidating the cellular function and physiological roles of deubiquitylases is identification of their substrate specificity. Adding to the complexity, recent studies indicate that in addition to removing or remodeling ubiquitin (chains) from ubiquitylated substrates, deubiquitylases may also act directly on E3s, thereby modifying the stability and activity of the latter. Characterizing the deubiquitylase-E3 interactome thus represents an important step in understanding how E3s are themselves regulated by ubiquitylation.

Whether deubiquitylases play a role in cholesterol metabolism is largely unknown. Our work and that of Scotti et al have recently shown that activity of the endosomal-sorting complex required for transport–associated USP8 is required for IDOL-dependent degradation of ubiquitylated LDLR. However, this most likely represents a nonselective deubiquitylation step used to salvage ubiquitin from ubiquitylated cargo before it entering multivesicular bodies. Given its potent LDLR-lowering activity, we hypothesized that the IDOL pathway may be itself subject to post-transcriptional regulation, in addition to transcriptional control by LXRs. Herein, we identify USP2 as a negative regulator of IDOL-dependent degradation of the LDLR, thereby implicating this deubiquitylase as a regulator of lipoprotein metabolism.

## Methods

A brief description of the methods is provided below. Detailed description of the methods are available at the Online Data Supplement.

### Cell Culture and Transfections

HEK293T, HeLa, HuH7, and HepG2 cells were obtained from the ATCC and maintained in DMEM supplemented with 10% fetal bovine serum at 37°C and 5% CO₂. A431 cells were a kind gift from Drs Vilja Pietiainen and Elina Ikonen (University of Helsinki). Wild-type and Idol⁻/⁻ mouse embryonic fibroblasts (MEFs) were a kind gifts from Dr Peter Tontonoz (University of California at Los Angeles). DU145 were from Dr Koen van de Wetering (NKI, Amsterdam). Where indicated, cells were sterol depleted by culture in sterol-depletion medium (DMEM supplemented by 10% lipoprotein-deficient serum, 5 μg/mL simvastatin, and 100 μmol/L mevalonate) or treated with 1 μmol/L GW3935 to induce LXR signaling. HEK293T and HepG2 cells were transfected with the indicated amounts of plasmids using the JetPrime reagent, and transfection efficiency was monitored by cotransfection of a GFP expression plasmid.

### Plasmids and Expression Constructs

Expression plasmids encoding IDOL, LDLR, USP8, very low-density lipoprotein receptor, GFP, Ubiquitin, and Epsin1 were previously reported. Expression plasmids encoding USP2-69 and USP2-43 cDNAs were generated with gateway-mediated recombination (Invitrogen). Unless otherwise indicated, USP2-69 is referred to as USP2.

### Antibodies and Immunoblot Analysis

Total cell lysates were prepared in radioimmunoprecipitation assay buffer (RIPA; 130 mmol/L NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 100 mmol/L Tris-HCl, and pH 7.4) supplemented with protease inhibitors. Lysates were cleared by centrifugation at 4°C for 10 minutes at 10000 g. Samples were separated on NuPAGE Bis-Tris gels and transferred to nitrocellulose. The primary antibodies used are listed in the extended Methods section in Online Data Supplement. Secondary HRP (horseradish peroxidase)-conjugated antibodies were used and visualized with enhanced chemiluminescence. All immunoblots are representative of at least 3 independent experiments.

### LDL Uptake Assay

DyLight 488-labeled LDL was produced as previously described. Briefly, HepG2, HeLa, or A431 cells were incubated in sterol-depletion medium for 16 hours before addition of LDL to increase LDLR abundance. To initiate LDL uptake, cells were incubated with 5 μg/mL DyLight488-labeled LDL in DMEM supplemented with 0.5% BSA at 37°C. Subsequently, cells were washed twice with PBS supplemented with excess sterols (PBS with 10% FCS), followed by an additional wash with PBS. Endocytosis of LDL was determined by lysing cells in RIPA buffer and quantification of the fluorescence signal on a Typhoon imager. Relative LDL uptake was calculated from the measure of fluorescence values corrected for specific uptake (cells treated with labeled LDL in the presence of an excess of nonlabeled LDL) and presented as mean±SD.

### Measurement of Cell Surface LDLR

Surface LDLR density in the different cells was determined as previously reported. Briefly, cells were treated as indicated, dissociated, and incubated in fluorescence-activated cell sorter blocking buffer for 30 minutes on ice. Subsequently, 100000 cells were stained with a R-Phycocerythrin-conjugated mouse antihuman LDLR antibody for 1 hour on ice. Cells were subsequently washed 3× with fluorescence-activated cell sorter buffer and directly analyzed on a fluorescence-activated cell sorter Calibur Flow Cytometer (BD Biosciences). Relative surface LDLR was calculated from geometric values after correction for background using FlowJo, and presented as mean±SD.

### siRNA Transfection

To knockdown USP2 expression, we used ON-TARGETplus Human USP2 SMARTpool (Dharmacon: cat#L-006069-00), or individual ON-TARGETplus Human USP2 siRNAs (Dharmacon: cat#J-006069-11-13). As control, we used the ON-TARGETplus Non-targeting Pool (Dharmacon: cat#D001810-11). A431 and HeLa cells were transfected with siRNAs (30 nmol/L) using the JetPrime reagent. HepG2 and DU145 cells were transfected similarly, but with the RNAiMAX reagent. The efficiency of USP2 silencing was assessed by quantitative polymerase chain reaction. Unless otherwise indicated, siRNA transfection experiments were conducted for 48 hours.
RNA Isolation and Quantitative Polymerase Chain Reaction

Total RNA was isolated from cells using TRIzol and 1 μg of total RNA was reverse transcribed using the iScript reverse transcription reagent. SYBR Green real-time quantitative polymerase chain reaction assays were performed on a Lightcycler 480 II apparatus. Oligonucleotide sequences are available on request.

Yeast-2-Hybrid Screen

Saccharomyces cerevisiae strains used in this study are PJ69-4a (trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ lys2-Δ1 GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ) and PJ69-4a (trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ lys2-Δ1 GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ). Cells were grown at 28°C in YPAD or in minimal glucose medium. PJ69-4α transformed with human IDOL constructs or LDLR intracellular tail (residues 780–860) cloned into pACT2-DEST, and PJ69-4α transformed with the bait constructs cloned into pGBK-T7/DEST were selected on minimal glucose plates lacking both leucine and uracil, with or without histidine, 10 to 20 mmol/L 3-aminotriazol, and 10 μg/mL X-α-gal. Diploids were selected on minimal glucose plates lacking leucine and uracil, with or without histidine, 10 to 20 mmol/L 3-aminotriazol, and adenine. Plates were incubated for 3 days before analysis.

Statistical Analysis

Data are presented as mean±SD. One-way ANOVA followed by the Bonferroni correction was performed for comparison of >2 groups. Student’s t test was performed for comparison of 2 groups. P values of <0.05 were considered significant.

Results

USP2 Is a Novel-Binding Partner of IDOL

The LDLR is a constitutive recycling receptor that rapidly shuttles between the plasma membrane and endosomal compartments. Internalization of the receptor is a clathrin-dependent process that requires adaptor proteins yet does not involve receptor ubiquitylation.1 Next to the classical LDLR pathway, IDOL-stimulated ubiquitylation of the receptor promotes internalization of the receptor via a distinct, clathrin-independent endocytic route that requires activity of the endosomal-sorting complex required for transport–associated deubiquitylase USP8.6,7 As strategies to increase abundance of the LDLR form the cornerstone of current therapies of hypercholesterolemia, we hypothesized that other deubiquitylases (Online Figure IA). However, the assay identified the deubiquitylase USP2-69 isoform (referred to further as USP2), as an interacting partner of the full-length IDOL protein (Online Table I). IDOL contains 2 functional regions, a FERM and a RING domain, which are joined by a short linker (Figure 1A). Using the yeast-2-hybrid assay, we mapped IDOL’s F3 and linker subdomains as the minimal regions required for interacting with USP2 (Figure 1A and 1B; Online Figure IB). Importantly, the interaction with USP2 was also maintained with a severe IDOL RING mutant, IDOL(C387A). This mutation disrupts the conserved RING structure and as a result this mutant is unable to promote LDLR degradation and to support IDOL autoubiquitylation,4 suggesting that both functions are not essential for the physical interaction of USP2 and IDOL. Furthermore, this result also indicates that USP2 does not compete with binding of the E2-ligase to IDOL, an interaction that depends on an intact RING domain.22,23 We next confirmed that this interaction also takes place in mammalian cells (HEK293T). Immunoprecipitation of transfected USP2 or IDOL (1:1 ratio) resulted in copurification of the corresponding binding partner (Figure 1C; Online Figure IIA). Furthermore, the interaction with IDOL was independent of USP2’s catalytic activity because it was maintained in the USP2(C236A) catalytic mutant (Online Figure IIB). In addition to USP2-69 identified in our yeast-2-hybrid screen, a shorter isoform of USP2, USP2-45, has been also described.37 These 2 USP2 isoforms differ in their N-terminal region and tissue distribution, but share the common C-terminal catalytic domain (Online Figure IIC). Like the USP2-69 isoform, the lower-molecular weight isoform, USP2-45 interacted with IDOL as well (Figure 1D). This points to the catalytic C-terminal region of USP2 as the IDOL interacting region, which is the same region implicated in the interaction of USP2 with the E3 MDM2.28 Taken together, these results identify USP2 as a novel-binding partner of IDOL and as a potential regulatory component of the IDOL–LDLR axis.

USP2 Deubiquitylates and Stabilizes IDOL

To study the functional consequence of the USP2–IDOL interaction, we initially used a gain-of-function approach. Remarkably, we found that overexpression of USP2 markedly stabilized IDOL protein (Figure 2A). USP8 however failed to do so, supporting the notion that the antagonizing effects of USP8 on the LXR–IDOL–LDLR axis occur primarily at the level of the ubiquitylated receptor. The stabilization of wild-type IDOL was equivalent to that observed with the proteasome inhibitor MG132, and required USP2 catalytic activity, as it was not seen with USP2(C236A) (Figure 2B). Thus, in spite of the preserved interaction of USP2 with the wild-type IDOL and USP2(C236A), only the active form of USP2 could increase IDOL levels. This finding implies that the deubiquitylase activity of USP2, rather than steric hindrance is required for IDOL stabilization. We have previously reported that IDOL is a highly unstable protein, likely because of its high autoubiquitylation activity.4 Accordingly, using cycloheximide to prevent protein synthesis we could determine that stabilization of IDOL by USP2 was a result of the extension of IDOL’s half-life (Figure 2C; Online Figure III). The most parsimonious explanation for this observation is that IDOL is subject to USP2-mediated deubiquitylation, which

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prevents its degradation. To test this, we conducted experiments in conjunction with pharmacological blocking of proteasomal degradation. This treatment equalizes IDOL levels and promotes accumulation of ubiquitylated IDOL species. Under this condition, both USP2 isoforms reduced the degree of ubiquitylated IDOL (Figure 2D and 2E). Furthermore, consistent with the lack of IDOL stabilization by the catalytic mutant USP2C276A or by USP8, we observed no changes in the degree of IDOL ubiquitylation by these deubiquitylases. If USP2 removes ubiquitin from IDOL and promotes its stability, the opposite can be expected if USP2 is silenced. Unfortunately, we cannot determine the endogenous level of IDOL in cells, as no commercial or homemade antibodies are able to detect it (data not shown). For that reason, we transfected IDOL into hepatocyte-like human HepG2 and Huh7 cells, and determined the level of heterologous IDOL protein after silencing USP2 expression. We observed that effective silencing of USP2 in these cells (55±6% reduction) reduced immunodetectable IDOL protein (Online Figure IV A and IVB), consistent with the notion that USP2 removes ubiquitin from IDOL to prevent its degradation. Thus, our experiments demonstrate that both gain and loss of USP2 function modulate the level of IDOL protein.

**USP2 Counteracts IDOL-Mediated Degradation of the LDLR**

In view of these findings, we predicted that USP2 would promote degradation of the LDLR because of increased IDOL abundance. However, unexpectedly we observed the opposite outcome. Despite massive stabilization of IDOL,
USP2 (−69 and −45) prevented degradation of the LDLR, as well as of a second IDOL target, the very low-density lipoprotein receptor, in a dose- and activity-dependent fashion (Figure 3A–3C; Online Figure VA). Because IDOL is a transcriptional target of LXR, and LXR-induced degradation of the LDLR is IDOL-dependent, we also evaluated whether USP2 can counteract the effect of LXR on the LDLR. In agreement with our findings, USP2 was also able to counteract LXR-induced LDLR degradation, even though expression of IDOL was induced (Online Figure VB and VC). We also considered the possibility that expression of USP2 is sensitive to the cellular sterol status. However, we found that neither LXR activation nor variation of the cellular sterol content altered USP2 expression (Online Figure VD). Functionally, antagonism of IDOL activity by USP2 counteracted the decrease in cellular LDL uptake imposed by IDOL (Figure 3D), positioning this deubiquitylase as a potential regulator of lipoprotein uptake.

Silencing USP2 Promotes LDLR Degradation in an IDOL-Dependent Manner

To understand how USP2 counters the IDOL–LDLR nexus, we tested the consequence of silencing USP2 on the LDLR pathway in hepatic and nonhepatic cell lines. These cells express IDOL and are, therefore, suitable for studying the functional interaction between IDOL and USP2 (Online Figure VIA). Silencing of USP2 in these cells led to a marked reduction in LDLR protein without changing the level of LDLR mRNA (Figure 4A and 4B; Online Figure VIB–VID). The secreted protein PCSK9 promotes degradation of the LDLR, and it is expressed by HepG2 cells. It was therefore conceivable that silencing USP2 increases PCSK9 expression, and that this, in turn, leads to LDLR degradation. However, this does not seem to be the case because in HepG2 cells, PCSK9 expression was not sensitive to the level of USP2 (Figure 4B). In contrast to the effects of silencing USP2 on the LDLR, another membrane receptor, the epidermal growth factor receptor (EGFR), remained either...
unchanged or even slightly increased after knockdown of USP2 in these cells (Figure 4A; Online Figure VIB). Unfortunately, we were unable to detect endogenous USP2 with commercially available antibodies in our experimental assays (not shown). Therefore, to establish the specificity of our USP2 siRNA and to rule out off target effects, we tested 3 individual USP2 siRNAs. All the 3 effectively knocked down expression of USP2 in HepG2 cells and decreased the protein levels of the LDLR (Online Figure VIIA and VIIB). Consistent with IDOL targeting an LDLR pool present at the plasma membrane,

we found that silencing USP2 resulted in prominent removal of LDLR from the cell surface (100±2.6 in control versus 30.4±4.9 in siUSP2, n=8, P<0.0001; Figure 4C), and as a consequence attenuated LDL uptake in different cell types (Figure 4D). Having established that USP2 influences the level of LDLR protein, we now asked if this mechanism requires IDOL activity. To test this, we silenced Usp2 in wild-type or Idol(−/−) MEFs (Figure 4E; Online Figure VIE). Effective silencing of Usp2 in wild-type cells decreased the LDLR. In Idol(−/−) cells LDLR protein was elevated, as previously reported, yet was insensitive to loss of Usp2. Collectively, these results demonstrate the functional importance of the USP2–IDOL nexus in physiological control of LDLR levels.

Figure 3. Ubiquitin-specific protease 2 (USP2) antagonizes the inducible degrader of the low-density lipoprotein (LDL) receptor (IDOL)–mediated degradation of lipoprotein receptors. A and B, HEK293T cells were transfected with expression constructs encoding FLAG-IDOL, LDL receptor (LDLR) (A) or very low-density lipoprotein receptor (VLDLR)-HA; B), and MYC-USP2 wild-type (WT) or catalytic mutant as indicated. Forty-eight hours after transfection cells were collected and total cell lysates immunoblotted as indicated. C, HEK293T cells were transfected with the indicated LDLR, IDOL, and USP2 expression vectors, with increasing amounts of WT or mutant USP2. Forty-eight hours after transfection, cells were harvested for immunoblots analysis as shown (D). HEK293T cells were transfected with expression constructs encoding FLAG-IDOL, LDLR, and MYC-USP2 WT. Twenty-four hours after transfection, cells were incubated for 30 minutes with 5 μg/mL Dylight 488-labeled LDL. After extensive washing, internalized LDL was quantified by measuring fluorescence in cell lysates. LDL uptake in cells not expressing IDOL and USP2 was set to 100%. Each bar and error represent the average±SD (n=8, ***P<0.0001). All immunoblots are representative of at least 3 independent experiments.

IDOL, USP2, and the LDLR Form a Functional Tripartite Complex to Regulate LDLR Ubiquitylation and Degradation

Our results demonstrate that USP2 can control LDLR protein abundance in an IDOL-dependent manner. Yet this raises a paradox; whereas USP2 dramatically stabilizes IDOL by promoting its deubiquitylation, it also inhibits IDOL-stimulated degradation of the LDLR. Conversely, silencing of USP2 decreases IDOL protein yet enhances degradation of the LDLR. To untangle this conundrum, we set out to test the requirements for interaction between IDOL, USP2, and the LDLR, and to determine the consequence of these interactions on ubiquitylation and degradation of the receptor. First, because USP2 can reverse IDOL-mediated degradation of the LDLR, we wanted to test whether elevating the level of USP2 influences ectopic LDLR levels independent of IDOL (Figure 5A). We introduced USP2 into cells at an increasing dose but found that this had no effect on LDLR uptake (Figure 3). We observed in the presence of IDOL (Figure 3). This suggests that for USP2 to modulate LDLR levels, ubiquitylation of the receptor is required. In the yeast-2-hybrid screen, no interaction between USP2 and the intracellular tail of the LDLR was detected (Online Figure IA). Yet, in this assay it is likely that the
tail is not ubiquitylated because yeast do not encode an IDOL homolog, and in this assay the tail is presented out of its normal, membrane-linked context. Therefore, we tested whether USP2 interacts with the LDLR in mammalian cells, and found that USP2 could be pulled down with the LDLR, albeit weakly (Figure 5B, lane 4). We specifically chose to use the FERM domain in these experiments because the FERM domain binds USP2, and it is recruited to, and binds the LDLR at the plasma membrane, but does not stimulate degradation of the receptor because of the lack of the RING domain. As expected, the FERM domain interacted with the LDLR (Figure 5B, lane 7). Yet when all the 3 were introduced into cells, we found that both USP2 and IDOL were readily coimmunoprecipitated with the LDLR (Figure 5B, lane 8). This result supports the idea that a tri-partite complex, containing IDOL, USP2, and the LDLR is formed at the plasma membrane. To capture this complex, we used overexpression of the endocytic adaptor molecule Epsin1, which acts in a dominant negative manner to block Epsin1-dependent endocytosis. We have previously used this approach to demonstrate that it effectively blocks IDOL-stimulated degradation of the LDLR. This is because of Epsin1 blocking internalization of the LDLR following IDOL-mediated ubiquitylation, thus resulting in accumulation of ubiquitylated LDLR at the plasma membrane.
shown, both Epsin1 and USP2 could block IDOL-dependent degradation of the receptor (Figure 5C, lanes 1–4). The use of dominant negative Epsin1 also increased ubiquitylated LDLR species, as expected. Yet when the deubiquitylase USP2 was also present, the level of ubiquitylated LDLR was markedly reduced (Figure 5C, lane 5). Collectively, these experiments support the notion that USP2 can counteract IDOL-dependent ubiquitylation of the LDLR, and prevent the receptor from being directed toward lysosomal degradation.

**Discussion**

The LDLR provides the main entry portal for LDL into the cell, and the mechanisms characterizing its transcriptional regulation and endocytic network have been largely described. Recently, the ubiquitin-proteasome system (UPS) was recognized as a novel regulatory layer in the LDLR pathway through the discovery of ubiquitylation-dependent degradation of the LDLR, mediated by the E3-ubiquitin ligase IDOL. IDOL stimulates clathrin-independent internalization and lysosomal degradation of the LDLR, a process requiring sorting of the ubiquitylated receptor by the endosomal-sorting complex required for transport system. The main finding of this study is the identification of USP2 as a deubiquitylase counteracting IDOL activity, and as a consequence abundance of the LDLR.

In view of the reversible nature of ubiquitylation, we reasoned that deubiquitylases counteracting IDOL activity might exist. To investigate this, we screened IDOL and the intracellular tail of the LDLR for potential interactions with a representative collection of human deubiquitylases. The screen with the LDLR intracellular tail failed to identify any LDLR-interacting deubiquitylases. This was somewhat surprising as previous studies, ours included, reported that USP8 is essential for IDOL-mediated degradation of the LDLR, and USP8 was included in our deubiquitylase screen. This apparent incongruence is possibly because of the fact that the cytosolic LDLR tail is only recognized by USP8 when ubiquitylated and in the context of a biological membrane, and not in the native form that is likely the one prevalent in the yeast-2-hybrid assay. However, by screening the deubiquitylase library against IDOL, we identified USP2 as a novel IDOL-binding protein. Our biochemical and molecular-based findings collectively indicate that USP2 interacts with IDOL and significantly increases its lifespan. The increased stabilization of IDOL is a result of its USP2-dependent deubiquitylation, similar to what has been described for the USP2–MDM2 and USP2–MDMX complexes. Regulation of E3 ligases by deubiquitylase-mediated deubiquitylation has been previously reported and represents an emerging concept in E3 control. Previous reports indicated that deubiquitylases stabilize E3s by limiting their autoubiquitylation. Consequently, deubiquitylation of E3s acts to enhance substrate ubiquitylation. In line with this, USP2 deubiquitylates and stabilizes MDM2 and ITCH, which results in increased ubiquitylation and degradation of their cognate substrates. In contrast to this model, deubiquitylation of IDOL by USP2 leads to a paradoxical outcome in which increased levels of the E3 ligase are mirrored by decreased degradation of its targets (ie, the LDLR and very low-density lipoprotein receptor) and functionally leads to enhanced cellular LDL uptake. In line with this, silencing of USP2 accelerates LDLR degradation and decreases LDL uptake in an IDOL-dependent manner in a variety of cell types.

Our results suggest that IDOL, USP2, and the LDLR form a tri-partite complex at the plasma membrane, and in that context ubiquitylation of the receptor is decreased. The most plausible scenario to explain this outcome is that IDOL-mediated ubiquitylation of the LDLR is counteracted by subsequent removal of ubiquitin chains from the LDLR by USP2. In this sequential scenario, USP2 is recruited by IDOL and acts directly on the receptor, reminiscent of the reported effect of USP2 on EGFR after ligand binding. This would be consistent with the fact that the phenotype is IDOL dependent, and that USP2 is able to process diverse ubiquitin linkages. Although we think this is the probable scenario, our experiments cannot formally rule out a more provocative possibility, namely that ubiquitylation of IDOL itself serves to both regulate its stability (ie, autoubiquitylation) and activity (ie, LDLR ubiquitylation). As such, by removing ubiquitin from IDOL, USP2 could counteract both processes. Although speculative, this form of regulation is not without precedent. Both cis and trans ubiquitylation of E3 ligases has been reported, and we have observed that USP2 can remove ubiquitin chains from an IDOL mutant lacking autoubiquitylation activity (data not shown). The significance of this observation requires further investigation, yet it is possible that these modifications may allow a hierarchical E3-based network for fine tuning of cellular proteostasis.

The role of USP2 in regulating the IDOL–LDLR pathway seems to be exclusively dependent on post-transcriptional events because USP2 expression is unaffected by perturbing the cellular sterol content or by pharmacologically activating LXR in different cell types. Therefore, identifying the signals, or post-transcriptional modifications on USP2 or IDOL that regulate their functional interaction will be an important goal in future studies. The USP2 gene is alternatively spliced into 3 isoforms, all sharing an identical C-terminal catalytic core. The 2 major USP2 isoforms, USP2-69 and USP2-45, promote IDOL deubiquitylation and rescue LDLR degradation. The specific functions and regulation of these 2 distinct isoforms are not well understood. The USP2-69 isoform is induced by androgens both in human and in rat prostate cells and by the AKT–mTORC1 axis in hepatocellular carcinoma. The mouse Usp2-45 isoform is regulated by the transcriptional co-activator PGC-1α, which couples the circadian and nutritional control in the liver in response to starvation. The above-mentioned regulation suggests that expression of USP2 is sensitive to nutritional and circadian input. Given that cholesterol metabolism is subject to these cues as well, it will be important to investigate how USP2 is integrated with the IDOL–LDLR axis in vivo. We speculate that the functional interaction between USP2 and IDOL, and the formation of a tri-partite complex with the LDLR, may be sensitive to post-transcriptional modifications, nutritional cues, or to the dynamic levels of these proteins. However, a serious limitation in experimentally testing the physiological role of the USP2–IDOL nexus is the species-dependent differences in hepatic IDOL activity and transcriptional regulation; whereas in human and in nonhuman primates, the IDOL pathway is highly active in...
hepatocytes and subject to strong sterol-dependent transcriptional regulation, in rodent hepatocytes it is not. Accordingly, Idol−/− mice do not display a marked cholesterol phenotype or changes in hepatic LDLR content. We therefore anticipate that a functional USP2–IDOL interaction will be predominantly active in human hepatocytes. In that respect, the growing body of evidence pointing toward genetic variation in IDOL as a modifier of LDL cholesterol in humans,3–11 warrants further studies to investigate the basic mechanisms governing IDOL activity and regulation by USP2.

In summary, this study reports the identification of the debiquitylase USP2 as an endogenous inhibitor of IDOL and a novel modulator of lipoprotein uptake owing to its ability to counteract IDOL-stimulated degradation of the LDLR (graphically summarized in Online Figure VIII). This finding further supports the notion that inhibition of IDOL activity would result in increased LDLR abundance and enhance LDL uptake, an approach that can be exploited as a therapeutic strategy to treat dyslipidemia and coronary artery disease.

Acknowledgments

We acknowledge members of the Zelcer group, Menno de Winter and Irith Koster for their comments and suggestions.

Sources of Funding

B. Distel is supported by a TOP grant (91209046) from the Netherlands Organization of Scientific Research (NWO). N. Zelcer is an Established Investigator of the Dutch Heart Foundation (2013T111), and he is supported by a VIDI grant (17.106.355) from the Netherlands Organization of Scientific Research (NWO), and by a European Research Council (ERC) Consolidator grant (617376) from the European Research Council.

Disclosures

None.

References

USP2, IDOL, and the LDLR form a tri-partite complex in which USP2 can deubiquitylate the LDLR and prevent its degradation.

What Is Known?
- The E3 ubiquitin ligase IDOL promotes ubiquitylation and subsequent degradation of the low-density lipoprotein receptor (LDLR) and thereby modulates LDL metabolism.
- Common and rare genetic variation in IDOL associates with the circulating level of LDL-cholesterol in humans.

What New Information Does This Article Contribute?
- The deubiquitylase ubiquitin-specific protease 2 (USP2) interacts with IDOL.
- USP2 deubiquitylates IDOL leading to its stabilization, which paradoxically attenuates degradation of the LDLR by IDOL.
- USP2, IDOL, and the LDLR form a tri-partite complex in which USP2 can deubiquitylate the LDLR and prevent its degradation.

Novelty and Significance

The E3 ubiquitin ligase IDOL promotes ubiquitylation and subsequent degradation of the LDLR. As such, IDOL activity represents an acute mechanism to shut down uptake of LDL-derived cholesterol into cells. Yet IDOL also promotes its own ubiquitylation and degradation and is, therefore, a highly unstable protein. Using a genetic approach, we identified the deubiquitylase USP2 as an IDOL-interacting partner. We found that USP2 deubiquitylates IDOL resulting in increased IDOL stability. However, despite elevated levels of IDOL degradation of the LDLR is paradoxically attenuated. Conversely, silencing of USP2 expression enhances IDOL-dependent degradation of the LDLR. Underlying these observations, we demonstrate that IDOL, USP2, and the LDLR can form a tri-partite complex in which USP2 can deubiquitylate the LDLR. As such, our study identifies USP2 as a new regulator of LDL metabolism owing to its ability to counteract IDOL-mediated degradation of the LDLR. Furthermore, this finding emphasizes the important role of the ubiquitin-proteasomal system in lipoprotein metabolism and cardiovascular disease.
The Deubiquitylase USP2 Regulates the LDLR Pathway by Counteracting the E3-Ubiquitin Ligase IDOL

Jessica Kristine Nelson, Vincenzo Sorrentino, Rossella Avagliano Trezza, Claire Heride, Sylvie Urbe, Ben Distel and Noam Zelcer

Circ Res. 2016;118:410-419; originally published online December 14, 2015; doi: 10.1161/CIRCRESAHA.115.307298

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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World Wide Web at:
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The deubiquitylase USP2 regulates the LDLR pathway by counteracting the E3-ubiquitin ligase IDOL

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SUPPLEMENTAL MATERIAL
Materials and methods

Reagents
Simvastatin and the proteasome inhibitor MG-132 were from Calbiochem. Lipoprotein deficient serum (LPDS) was prepared following standard procedures and confirmed to contain no lipoproteins (not shown). GW3965, and Bafilomycin A1 were from Sigma.

Cell culture and transfections
HEK293T, HeLa, Huh7, and HepG2 cells were obtained from the ATCC. Cells were maintained in DMEM supplemented with 10% FBS at 37 °C and 5% CO2. A431 cells were a kind gift from Drs. Vilja Pietiainen and Elina Ikonen (University of Helsinki). Wildtype and Idol(-/-) MEFs were a kind gift from Dr. Peter Tontonoz (University of California at Los Angeles). DU145 were from Dr. Koen van de Wetering (NKI, Amsterdam). Where indicated, cells were sterol-depleted by culture in sterol-depletion medium (DMEM supplemented by 10% LPDS, 5 μg/mL simvastatin, and 100 μM mevalonate) or treated with 1 μM GW3965 to induce LXR signaling. HEK293T and HepG2 cells were transfected with the indicated amounts of plasmids using the JetPrime reagent. In transfection experiments the efficiency of transfection was monitored by co-transfection of an expression plasmid for GFP and was consistently >85% in HEK293T and HepG2 cells.

Plasmids and expression constructs
Expression plasmids encoding IDOL, IDOL-FERM, LDLR, USP8, VLDLR, GFP, Ubiquitin, and Epsin1 were previously reported. Expression plasmids encoding USP2-69 and USP2-45 cDNAs were generated with gateway-mediated recombination (Invitrogen). In experiments where only expression constructs for USP2-69 are used, USP2-69 is indicated just as USP2. The activity disabling C276A mutation in USP2 was introduced using the QuickChange site-directed mutagenesis kit. All plasmids used in this study experiments were isolated by CsCl2 gradient centrifugation and their correctness verified by sequencing.

Antibodies and immunoblot Analysis
Total cell lysates were prepared in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 100 mM Tris-HCl, pH 7.4) supplemented with protease inhibitors. Lysates were cleared by centrifugation at 4 °C for 10 min at 10,000 g. Protein concentration was determined using the Bradford assay with BSA as reference. Samples (10–40 μg) were separated on NuPAGE BisTris gels and transferred to nitrocellulose. Membranes were probed with the following antibodies: LDLR (Epitomics, clone EP1553Y 1:4000), tubulin (Sigma, clone DM1A, 1:5000), FLAG-HRP (Sigma, clone M2, 1:1000), GFP (affinity purified rabbit polyclonal anti-GFP was from Dr. Mireille Riedinger, UCLA, 1:5000, or Santa Cruz, clone B-2, 1:3000), MYC (Cell Signaling, clone 9B11, 1:4000), HA (Covance, clone 16B12, 1:10000), Ubiquitin (ENZO, clone FK2, 1:1000), Epsin1 (rabbit polyclonal serum raised against amino acids 249–401 of rat Epsin1, 1:5), His6 (Rockland, 1:1000), EGFR (affinity purified rabbit polyclonal anti-EGFR sera was from Dr. Simona Polo, 1:40,000). Secondary HRP-conjugated antibodies (Zymed Laboratories Inc.) were used and visualized with chemiluminescence on a Fuji LAS4000 (GE Healthcare). All immunoblots are representative of at least three independent experiments.

LDL uptake assay
DyLight 488-labeled LDL was produced as previously described. Briefly, HepG2, HeLa, or A431 cells were incubated in sterol-depletion medium for 16 h prior to addition of LDL to increase LDLR abundance. To initiate LDL uptake, cells were washed twice with PBS and
incubated with 5 μg/mL DyLight488-labeled LDL in DMEM supplemented with 0.5% BSA for the indicated time at 37°C. Subsequently, cells were washed twice with PBS supplemented with excess sterols (PBS with 10% FCS), followed by an additional wash with PBS. Endocytosis of LDL was measured by lysing cells in RIPA buffer and quantification of the fluorescence signal on a Typhoon imager. Relative LDL uptake was calculated from the measure of fluorescence values corrected for specific uptake (cells treated with labeled LDL in the presence of an excess of non-labeled LDL) and presented as mean ± SD.

**Measurement of cell-surface LDLR**

Cells were treated as indicated, dissociated with TrypLE Express and incubated in FACS blocking buffer (FACS buffer containing 2% goat serum) for 30 min on ice. Subsequently, 100,000 cells were stained in 50μL FACS buffer with a R-Phycoerythrin (PE)-conjugated mouse anti-human LDLR antibody (R&D Systems, #FAB2148P, 1:40) for 1 h on ice. Cells were subsequently washed three times with FACS buffer and directly analyzed on a FACSCalibur Flow Cytometer (BD Biosciences). Viable cells were gated and 10,000 events per condition acquired. Data were analyzed using the CellQuest software package. Relative surface LDLR was calculated from geomean values corrected for background (non-stained cells), and presented as mean ± SD.

**siRNA transfection**

To knockdown USP2 expression we used the ON-TARGETplus Human USP2 SMARTpool (Dharmacon: cat#-006069-00), or the individual ON-TARGETplus Human USP2 siRNAs (Dharmacon: cat#J-006069-11, J-006069-12, J-006069-13). As control we used the ON-TARGETplus Non-targeting Pool (Dharmacon: cat#D001810-10). A431 and HeLa cells were transfected with control or USP2 siRNAs (final concentration 30 nM) using the JetPrime reagent. HepG2 and DU145 cells were transfected similarly, but with the RNAiMAX reagent. The efficiency of USP2 silencing was assessed by quantitative PCR. Unless otherwise indicated, siRNA transfection experiments were conducted for 48 hours.

**RNA isolation and quantitative PCR**

Total RNA was isolated from cells using TRIzol and one microgram of total RNA was reverse-transcribed using the iScript reverse transcription reagent. SYBR Green real-time quantitative PCR assays were performed on a Lightcycler 480 II apparatus. Oligonucleotide sequences are available upon request.

**Yeast two-hybrid screen**

*S. cerevisiae* strains used in this study are PJ69-4a (trp1-901 leu2-3,112 ura3-52 his3-200 gal4D gal80D LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ) and PJ69-4α (trpl-901 leu2-3,112 ura3-52 his3-200 gal4D gal80D LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ). Cells were grown at 28°C in YPAD (2% glucose, 2% peptone, 1% yeast extract and 4 mg/ml adenine) or in minimal glucose medium containing 0.67% Yeast Nitrogen Base (YNB) without amino acids, 2% glucose and amino acids as needed (2 mg/ml uracil, 3 mg/ml leucine, 2 mg/ml tryptophane, 2 mg/ml histidine, 4 mg/ml adenine, 2 mg/ml methionine). The yeast-two-hybrid screening was performed as following. PJ69-4α transformed with human IDOL constructs or LDLR intracellular tail (residues 780-860) cloned into pACT2-DEST, and PJ69-4a transformed with the bait constructs cloned into pGDBU-DEST were selected on minimal agar plates lacking leucine or uracil, respectively. For mating, cells were pre-cultured for 16 h in selection medium, and subsequently 2x10^6 of transformed PJ69-4a and PJ69-4α cells (1:1 ratio) were mated in 2xYPAD (4% glucose, 4% peptone, 2% yeast extract and 8 mg/ml adenine) for 24 h with slow shaking (50 rpm). Diploids were selected on minimal glucose plates lacking both leucine and uracil. To reconfirm interactions we used a spot assay. Briefly, diploid cells were incubated for 16 hrs in minimal glucose medium lacking leucine and uracil, diluted to OD_{600} of 0.2 and grown till an OD_{600} of 1. Cells were
then washed with sterile water and serially diluted so as to plate $10^2$-$10^5$ cells/spot on minimal glucose agar lacking leucine and uracil, and with or without histidine, 10-20 mM 3-aminotriazol (3-AT), and adenine. Plates were incubated for 3 days before analysis.

**Statistical Analysis**

Data are presented as mean ± SD. 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 figure I. Summary of IDOL-DUB yeast-two-hybrid screen. Y2H assay for (A) USP2-LDLRc and (B) IDOL-USP2. The corresponding IDOL constructs that were used are illustrated in Fig. 1A. The intracellular tail of the LDLR or the different IDOL fragments and USP2 were cloned into yeast-two-hybrid “prey” and “bait” plasmids, respectively, and transformed into yeast. Haploid cells were mated, and diploid cells were serially diluted and plated on selective plates. The assay was performed as described in the Methods section, and decreasing amounts of cells are spotted and selected as indicated.

Online figure II. The DUB USP2 is a novel binding partner of IDOL. (A,B) HEK293T cells were transfected with 1:1 ratio of the indicated constructs for IDOL and the USP2 wildtype or catalytic mutant. Total cell lysates were immunoblotted or immunoprecipitated and analyzed as indicated. (C) Schematic representation of UPS2 functional domains. USP2, also known as USP2-69, has a N-terminal extension, involved in contacts with other proteins, and a C-terminal catalytic domain. Mutation of the key cysteine C276 residue in this domain impairs USP2’s DUB activity. USP2-45 is a shorter isoform of USP2, with a partial truncation of its N-terminal domain, but intact catalytic domain. This isoform also interacts with IDOL, as shown in Fig. 1D.

Online figure III. USP2 deubiquitylates and stabilizes IDOL. HEK293T cells were transfected with the indicated IDOL and USP2 expression vectors, with a ratio of IDOL:USP2 of 1:5 (left panel) or 1:10 (right panel). 48 hours after transfection, cells were incubated for the indicated times with 10 μg/mL cycloheximide (CHX), and cells harvested for immunoblots and kinetic analysis as shown in Fig. 2C.

Online figure IV. Silencing USP2 reduces the level of ectopic IDOL protein. (A) HepG2 and (B) Huh7 cells were co-transfected with IDOL and GFP expression plasmids, and either Control (Non-targeting, NT) or USP2 siRNA. 48 hours later total cell lysates were collected and immunoblotted as indicated. A representative immunoblot of three independent experiments is shown. (A,B) the intensity of IDOL protein was quantified and normalized to that of GFP. The relative IDOL protein level is plotted. Each bar and error represent the mean ± SD (N=3). * p < 0.05

Online figure V. USP2 attenuates IDOL and LXR-induced degradation of the LDLR. (A) HEK293T cells were transfected with LDLR and with IDOL and USP2-45 (1:7 ratio). 48 hours after transfection, cells were collected and immunoblotted as indicated. (B,C) HeLa cells were transfected with the indicated USP2 expression vector. 48 hours after transfection, cells were treated with 1 μM GW3965 (GW) for 4 hours to activate the LXR pathway and subsequently harvested for (B) immunoblots and (C) qPCR analysis as shown (n=4, *** p< 0.001). Expression of USP2 does not affect LXR activation, as shown by the increased expression of IDOL and ABCA1. (D) HepG2 cells were cultured in complete medium or in sterol-depletion medium for 16 hours, and subsequently treated with vehicle or 1 μM GW3965 (GW) for 4 hours. The mRNA levels of USP2, LDLR and IDOL were analyzed as indicated. Expression of USP2 is not affected by LXR activation or by variations in the cellular sterol content (n=5, *** p< 0.0001).

Online figure VI. USP2 silencing decreases LDLR protein levels in different cell lines. (A) Relative IDOL expression in the indicated cell lines was determined by qPCR. Each bar and error represent the mean ± SD of three independent experiments (B) HeLa and DU145 cells were transfected as indicated with 30 nM of control (Non-targeting, NT) or USP2 siRNA. 48 hours after transfection cells were shifted to sterol-depletion medium for an additional 24 hours after which total cell lysates were collected and analyzed by immunoblotting. (C,D) A431 and DU145 cells were cultured and treated as in (B) and
analyzed by qPCR to assess LDLR, IDOL and USP2 mRNA levels (n=4, *p<0.05). (E) Wild-type and Idol(-/-) MEFs were cultured and treated as in Fig. 4E. 72 hours after transfection, cells were collected and analyzed by qPCR as indicated (n=4, ***p<0.0001). All immunoblots are representative of at least three independent experiments.

**Online figure VII. USP2 silencing decreases LDLR protein levels (A,B)** The specificity of USP2 silencing in HepG2 cells was tested by transfecting cells as indicated with 30 nM of a control (Non-targeting, NT), three individual USP2 siRNAs, or with a pooled set of USP2 siRNAs. 48 hours after transfection cells were shifted to sterol depletion medium for an additional 24 hours after which cells were collected and total cell lysates were analyzed by immunoblotting (B) or by qPCR (A) to assess the efficiency of USP2 mRNA silencing (n=3, ***p<0.001).

**Online figure VIII. Graphical scheme for the IDOL-USP2 nexus in regulation of the LDLR.** (A) IDOL expression is induced by activation of the sterol-sensing nuclear receptors LXR. This leads to enhanced ubiquitylation of the LDLR, which targets the receptor for lysosomal degradation. In parallel, auto-ubiquitylation of IDOL leads to its proteasomal degradation. (B) USP2 is recruited together with IDOL to the intracellular tail of the LDLR. There, USP2 can counteract IDOL-mediated ubiquitylation of the LDLR by removing these chains from the receptor.
**Online table I: Identification of USP2 as a novel IDOL-binding partner by the Y2H assay**

The full length IDOL, or its RING domain, was screened against a library of human DUBs, comprehensive of the listed enzymes. The assay was performed as described in the Methods section. False positives are indicated by the asterisk, and they represent DUBs whose co-expression in the yeast system with the empty pACT2-DEST vector resulted in selective cell growth even in the absence of IDOL or LDLR. Only yeast diploids co-expressing full length IDOL and USP2-69 showed marked growth on both selective media (+++), and were considered for validation analysis. A similar screen was performed using the LDLR intracellular tail, however it did not identify any potential LDLR-interacting DUBs.

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- IDOL
- IDOL<sub>C837A</sub>
- RING
- FERM
- ΔF1
- ΔF2
- F3+linker
- F3

Spotted cells:
Online figure II

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USP2:  
IB: FLAG-USP2
IB: MYC-IDOL  
IgG  
MYC-IDOL
FLAG-USP2  
TUBULIN  
GFP  
Input  
IP: MYC (IDOL)
IP: FLAG (USP2)

B

USP2:  
IDOL:  
IB: MYC-IDOL  
IgG  
MYC-IDOL
FLAG-USP2  
TUBULIN  
GFP  
Input  
IP: FLAG USP2

C

N-terminal extension  Isopeptidase domain

USP2-69

C276A

USP2-45

Online file
Online figure III

IDOL\textsubscript{WT}:USP2 1:5

MYC-USP2

FLAG-IDOL\textsubscript{WT}\textsuperscript{§}

FLAG-IDOL\textsubscript{WT}

TUBULIN

GFP

0 5 10 30

IDOL:USP2 1:10

CHX (min):

0 5 10 30

MYC-USP2

FLAG-IDOL\textsubscript{WT}\textsuperscript{§}

FLAG-IDOL\textsubscript{WT}

TUBULIN

GFP

0 5 10 30
Online figure IV

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+siRNA: NT USP2

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Relative IDOL protein

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_HUH7_

+siRNA: siNT USP2

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Relative IDOL protein
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**C**

Relative mRNA

GW3965

**D**

Relative mRNA

Sterols

GW3965
Online figure VI

A

B

C

D

E

DU145

HeLa

siRNA: Control USP2 Control USP2

EGFR

LDLR

TUBULIN

DU145

HeLa

siNT siUSP2

LDLR IDOL USP2

A431

siNT siUSP2

LdlrIdol Usp2 WT siNT WT siUsp2 IDOL(-/-) siNT IDOL(-/-) siUsp2

***

***

Relative mRNA

Relative mRNA

Relative mRNA

DU145

siNT siUSP2

USP2 LDLR IDOL

WT siNT WT siUsp2 IDOL(-/-) siNT IDOL(-/-) siUsp2

0.0 0.5 1.0 1.5

0.0 0.5 1.0 1.5

0.0 0.5 1.0 1.5
Online figure VII

A

Relative USP2 mRNA

0.0 0.5 1.0 1.5

NT Pool #1 #2 #3

siUSP2

B

USP2

siRNA: NT pool #1 #2 #3

LDLR Tubulin

HepG2

Relative LDLR protein

0.0 0.5 1.0 1.5

NT Pool #1 #2 #3

siUSP2

***