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Identification of a loss-of-function inducible degrader of the low-density lipoprotein receptor variant in individuals with low circulating low-density lipoprotein

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Aims Recent genome-wide association studies suggest that *IDOL* (also known as *MYLIP*) contributes to variation in circulating levels of low-density lipoprotein cholesterol (LDL-C). IDOL, an E3-ubiquitin ligase, is a recently identified post-transcriptional regulator of LDLR abundance. Briefly, IDOL promotes degradation of the LDLR thereby limiting LDL uptake. Yet the exact role of IDOL in human lipoprotein metabolism is unclear. Therefore, this study aimed at identifying and functionally characterizing IDOL variants in the Dutch population and to assess their contribution to circulating levels of LDL-C.

Methods and results We sequenced the *IDOL* coding region in 677 individuals with LDL-C above the 95th percentile adjusted for age and gender (high-LDL-C cohort) in which no mutations in the *LDLR, APOB,* and *PCSK9* could be identified. In addition, *IDOL* was sequenced in 560 individuals with baseline LDL-C levels below the 20th percentile adjusted for age and gender (low-LDL-C cohort). We identified a total of 14 *IDOL* variants (5 synonymous, 8 non-synonymous, and 1 non-sense). Functional characterization of these variants demonstrated that the p.Arg266X variant represents a complete loss of IDOL function unable to promote ubiquitylation and subsequent degradation of the LDLR. Consistent with loss of IDOL function, this variant was identified in individuals with low circulating LDL-C.

Conclusion Our results support the notion that *IDOL* contributes to variation in circulating levels of LDL-C. Strategies to inhibit IDOL activity may therefore provide a novel therapeutic venue to treating dyslipidaemia.

Keywords LDL • LDL receptor • IDOL • E3-ubiquitin ligase • Rare coding variants

Introduction

Elevated levels of plasma low-density lipoprotein cholesterol (LDL-C) represent a major risk factor for development of atherosclerosis and cardiovascular disease (CVD).[1] Owing to its ability to promote LDL uptake into cells, the LDL receptor (LDLR) is an important determinant of plasma LDL levels and is therefore a target for therapy in patients at risk for cardiovascular events.[1–3]

The pivotal role of the LDLR in LDL metabolism is exemplified by the fact that *LDLR* mutations are the leading cause for familial hypercholesterolaemia,[3,4] a disease characterized by reduced hepatic LDL clearance, elevated plasma cholesterol levels, and accelerated CVD. Given its central role in lipoprotein metabolism, abundance of the LDLR is tightly regulated. Transcription of the *LDLR* is controlled by the sterol regulatory element binding protein (SREBP) family of transcription factors and is increased

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when cellular cholesterol levels decline to allow efficient uptake of LDL-derived cholesterol.[5] In addition to transcriptional regulation of the *LDLR*, post-transcriptional regulation is emerging as a critical determinant of LDL-C metabolism. Several genetic studies identified mutations in the LDLR adaptor protein 1 (*LDLRAP1/ARH*)[6] and the SREBP target gene proprotein convertase subtilisin/kexin 9 (*PCSK9*)[7] that result in hypercholesterolaemia due to altered endocytosis, trafficking, and stability of the LDLR.

As is the case with hypercholesterolaemia, studies have also uncovered a genetic basis for hypocholesterolaemia resulting from mutations in the genes *APOB*,[8] *MTP*,[9] *ANGPTL3*,[10] *SARA2*,[11] and *PCSK9*,[12,13] as extensively reviewed in Calandra *et al.*[14] and Tarugi and Averna.[15] The case of PCSK9 is particularly intriguing as mutations in this gene can result in both gain-of-function and hypercholesterolaemia, or conversely loss-of-function and hypocholesterolaemia.[7,12,16] The effect of heterozygous loss-of-function *PCSK9* mutations on LDL-C levels is moderate yet results in a substantial reduction in the risk for coronary heart disease.[13] Two individuals carrying homozygous loss-of-function alleles have been reported.[17,18] These individuals, who lack functional PCSK9, are healthy and have very low LDL-C levels (<15 mg/dL). Supported by these findings, therapeutic strategies to target PCSK9 using siRNA-based approaches[19–21] or neutralizing antibodies[22–24] are being intensively studied and initial clinical trials are highly encouraging (reviewed in Lambert *et al.*[25]). The PCSK9 studies nicely illustrate that targeting post-transcriptional pathways controlling LDLR degradation is an attractive therapeutic approach for treating dyslipidaemia complementary to statin-based therapy.

Recent genome-wide association studies identified a relationship between circulating LDL-C levels and genetic variation in the *IDOL* locus.[8,26,27] The inducible degrader of the LDLR (IDOL, also known as MYLIP[28]) is a sterol-responsive E3 ubiquitin ligase we recently identified as a novel post-transcriptional regulator of LDLR abundance.[29,30] Acting as an E3 ligase, IDOL supports ubiquitylation of the LDLR on its protruding intracellular tail thereby targeting it for lysosomal degradation. Cell-based studies support a role for IDOL in regulating LDL uptake via the LDLR pathway; increasing expression of IDOL results in decreased LDLR abundance and LDL uptake into cells, whereas loss of IDOL expression leads to a reciprocal outcome.[29,31] The latter suggests that inhibiting IDOL expression and/or activity may offer a therapeutic venue to increase hepatic LDL-C clearance. However, our understanding of IDOL's contribution to lipoprotein metabolism in humans is limited. To address this issue we sequenced the *IDOL* coding region in cohorts with extreme LDL-C phenotypes. We report here the identification and functional characterization of *IDOL* variants in these large Dutch cohorts and discuss the implication of our finding for therapeutic targeting of IDOL for treatment of hypercholesterolaemia.

Methods

Cohorts

Through the participation of 64 lipid clinics, evenly distributed throughout The Netherlands since 1994, a representative group of over ~17 000 Dutch clinically diagnosed autosomal-dominant hypercholesterolaemia (ADH) patients has been collected using a uniform protocol and internationally accepted criteria by cardiologists and internists. From this cohort, 677 unrelated cases were selected who were negative for the presence of mutations in *LDLR*, *APOB* (amino acids 3441–3615), and *PCSK9*, and had baseline LDL-C that were above the 95th percentile for age and gender (high-LDL-C cohort).[32] Via the national genetic cascade-screening programme for familial hypercholesterolemia,[33] 560 unrelated and untreated individuals with baseline LDL cholesterol levels below the 20th percentile for age and gender and tested negative for familial ADH mutations were recruited (low-LDL-C cohort). All participants gave written informed consent.

DNA analysis

Genomic DNA was extracted from 10 mL whole blood on an AutopureLS apparatus according to a protocol provided by the manufacturer (Gentra Systems, Minneapolis, MN, USA). Primer sets were designed to analyse all exons and flanking intronic regions of *IDOL*. Primer sequences and conditions for polymerase chain reaction are available upon request. Sequence analysis was performed by direct sequencing using the Big Dye Terminator ABI Prism Kit, version 1.1 (Applied Biosystems, Foster City, CA, USA). Products of sequence reactions were run on a Genetic Analyzer 3730 (Applied Biosystems) and sequence data were analysed by the use of the Sequencer package (GeneCodes Co, Ann Arbor, MI, USA).

For mutation nomenclature, numbering was based on the cDNA with nucleotide c.1 being A of the ATG initiation codon p.1. The *IDOL* reference sequence NM_013262.3 was used.

Cell culture and transfections

HEK293T cells were obtained from the ATCC. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37°C and 5% CO_2. HEK293T cells were transfected with the indicated amounts of IDOL and LDLR expression plasmids using either Jetprime or Fugene. Transfection efficiency was monitored by co-transfecting an expression plasmid for green fluorescent protein (GFP) or E2-Crimson and was consistently >90% in HEK293T cells.

Plasmids and expression constructs

Expression plasmids for IDOL, LDLR, HA-ubiquitin, and GFP were reported previously. The E2-Crimson expression plasmid was a kind gift from Dr Benjamin Glick (University of Chicago).[34] Mutations in *IDOL* were generated by QuikChange site-directed mutagenesis and cloned using gateway-mediated recombination. DNA sequencing was used to verify the correctness of all the constructs used in this study. Plasmids used in cell transfection experiments were isolated by $CsCl_2$ gradient centrifugation.

Antibodies, immunoblot analysis, and immunoprecipitation

Total cell lysates were prepared in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% sodium dodecylsulfate, 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*. The protein concentration was determined using the Bradford assay with bovine serum albumin (BSA) as a reference. Samples (10–40 μg) were separated on NuPAGE BisTris gels (Invitrogen) and transferred to nitrocellulose. Membranes were probed with the following antibodies: LDLR (Cayman Chemical, catalog no. 10007665, 1:4000), tubulin (Calbiochem, clone CP06, 1:20 000), HA (Covance, clone HA.11, 1:20 000), FLAG-HRP (Sigma, clone M2,

1:5000), and GFP (affinity purified rabbit polyclonal anti-GFP was a gift from Dr Mireille Riedinger, UCLA, 1:5000). Secondary horseradish peroxidase-conjugated antibodies (Zymed Laboratories Inc.) were used and visualized with chemiluminescence on a Fuji LAS4000 (GE Healthcare). HA-tagged ubiquitin was immunoprecipitated as described previously.[29] All immunoblots are representative of at least three independent experiments.

Low-density lipoprotein uptake assay

DyLight488-labelled LDL was produced as previously described.[35] HEK293T cells were transfected with E2-Crimson, LDLR, and IDOL expression plasmids for 48 h as indicated. To measure LDL uptake, cells were washed twice with PBS and incubated with 5 μg/mL DyLight488-labelled LDL in DMEM supplemented with 0.5% BSA for 30 min at 37°C. Subsequently, cells were washed twice with PBS and collected in PBS-paraformaldehyde 4%. Low-density lipoprotein uptake was determined in E2-Crimson positively gated cells by FACS on a FACS Calibur. E2-Crimson was used as an indicator for transfected cells.

Statistical analysis

Data were analysed using SPSS software (version 10.1, SPSS, Chicago, IL, USA) and differences in allele frequencies were determined with the two-tailed Fisher's exact test. Differences in LDL uptake were determined with one-way analysis of variance. A probability value of $P < 0.05$ was considered statistically significant.

Results

To study the possible contribution of IDOL to lipoprotein metabolism in humans we screened Dutch hyper- and hypocholesterolaemic cohorts for the presence of functional IDOL variants. We sequenced the coding region of *IDOL* in patients with high LDL (high-LDL-C cohort, LDL-C: 5.51 ± 1.44 mmol/L). In these individuals we ruled out the presence of mutations in the *LDLR, APOB* (amino acids 3441–3615), and *PCSK9* that could explain the elevated levels of circulating LDL-C. In addition, *IDOL* was sequenced in a hypocholesterolaemic cohort with circulating LDL-C levels lower than the 20th percentile (low-LDL-C cohort, LDL-C: 2.13 ± 0.49 mmol/L). The clinical parameters of both cohorts are described in *Table 1*.

This sequencing strategy should in principle allow us to identify both gain- and loss-of-function IDOL variants and led to the identification of a total of 14 variants (*Table 2*). Of the five synonymous variants, only p.Cys391Cys (rs1060901) was predicted to have an effect on splicing, potentially introducing a new splice donor site. However, this was not observed in mRNA derived from monocytes of carriers of this variant (data not shown). In addition, one nonsense variant and eight non-synonymous missense variants were identified in our screen. The missense variants p.Ile202Leu (rs79992066) and p.Ans342Ser (rs9370867) are common variants[36] and were found in both cohorts at similar frequencies (*Table 2*). Of the remaining six rare missense variants, p.Arg193Gln, p.Val250Met, and pArg372Gln were exclusively identified in high-LDL-C individuals, while p.Gly51Ser, p.Arg.193Trp, p.Val339Ile, and the nonsense variant p.Arg.266X were restricted to low-LDL-C individuals.

The identified variants are dispersed throughout IDOL and are located in the extended N-terminal FERM domain that is essential for the interaction of IDOL with the LDLR and biological membranes (*Figure 1A*).[37,38] We employed an established co-transfection assay in which we introduce expression plasmids for LDLR and IDOL to study the consequence of the different identified coding variants on IDOL activity.[29] This cellular assay reflects the intrinsic ability of IDOL to promote degradation of the LDLR as well as its own degradation (i.e. auto-ubiquitylation and subsequent degradation). As previously reported, expressing wild-type (WT) IDOL dramatically reduced the level of the LDLR protein. Introducing the different IDOL missense variants resulted in degradation of the LDLR to a similar extent and had no substantial effect on IDOL stability, also when IDOL dosage was varied (*Figure 1B* and Supplementary material online, *Figure. S1*). Furthermore, identification of additional carriers of these missense variants by family investigation failed to show co-segregation with the LDL-C phenotype in these families (data not shown). The p.Arg266X nonsense variant was unable to promote degradation of the LDLR, and to enhance degradation of another IDOL target, the VLDLR (Supplementary material online, *Figure S2*).[39] Further evaluation of the p.Arg266X variant in the family had to be restricted unfortunately to the probands' children (*Figure 2*). The LDL-C level measured in the unaffected son of the proband was within the normal range (LDL-C:2.64 mmol/L; pLDL:65) and that of the probands' daughter, who was a heterozygous carrier of p.Arg266X, showed LDL-C levels in the lower extreme of the general population (LDL-C:1.79 mmol/L; pLDL:14). More family members were not available for analysis.

Diminished IDOL activity offers a plausible explanation for the LDL phenotype in carriers of the p.Arg266X variant. Most likely, this truncated transcript will be subject to nonsense-mediated RNA decay, precluding production of the encoded IDOL protein.[40] Further pointing towards IDOL, using targeted capture and next-generation sequencing we excluded variation in other

Table 1. Clinical characteristics of the high LDL and low LDL cohorts

Cohort	*n*	% M	Age (year)	TC (mmol/L)	LDL (mmol/L)	HDL (mmol/L)	TG (mmol/L)
High-LDL	677[a]	46	40 (18)	7.81 (1.67)	5.51 (1.44)	1.43 (0.46)	2.03 (2.49)
Low-LDL	560	47	43 (18)	4.00 (0.67)	2.13 (0.49)	1.39 (0.39)	1.06 (0.55)

M, male; Y, year; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TG, triglycerides.
[a]Individuals ($n = 52$) without a complete lipid profile at baseline due to statin treatment were not included in the clinical characteristics calculations. Standard deviations are indicated between brackets.

Table 2. Genetic variation found in the high- and low LDL cohorts

c.Nomen	p.Nomen	Splice effect	rs number	High-LDL cohort MAF	Low-LDL cohort MAF	Frequency	*P* value[a]	EVS MAF
c.30G>T	p.Ala10Ala			0	0.001	Rare		NP
c.151G>A	p.Gly51Ser		rs149696224	0	0.001	Rare		0,0002
c.577C>T	p.Arg193Trp			0	0.001	Rare		0,0002
c.578G>A	p.Arg193Gln	Acceptor site		0.001	0	Rare		NP
c.604A>C	p.Ile202Leu		rs79992066	0.009	0.014	Common	0.330	0.015
c.748G>A	p.Val250Met			0.001	0	Rare		NP
c.796C>T	p.Arg266X			0	0.001	Rare		NP
c.801G>A	p.Ala267Ala			0.001	0	Rare		0.00008
c.813G>A	p.Thr271Thr		rs34627146	0.004	0.009	Rare		0.011
c.1015G>A	p.Val339Ile		rs142124143	0	0.002	Rare		0.002
c.1025A>G	p.Asn342Ser		rs9370867	0.503	0.476	Common	0.217	0.615
c.1115G>A	p.Arg372Gln		rs150756832	0.001	0	Rare		0.0002
c.1173C>T	p.Cys391Cys	Donor site	rs1060901	0.091	0.096	Common	0.660	0.065
c.1230C>T	p.Ser410Ser			0	0.001	Rare		NP

MAF, minimal allele frequency; EVS, exome variant server (http://evs.gs.washington.edu/EVS/); NP, not present.
[a]Differences in minor allele frequencies were determined with the two-tailed Fisher's exact test. A *P* value was calculated for MAF >0.01.

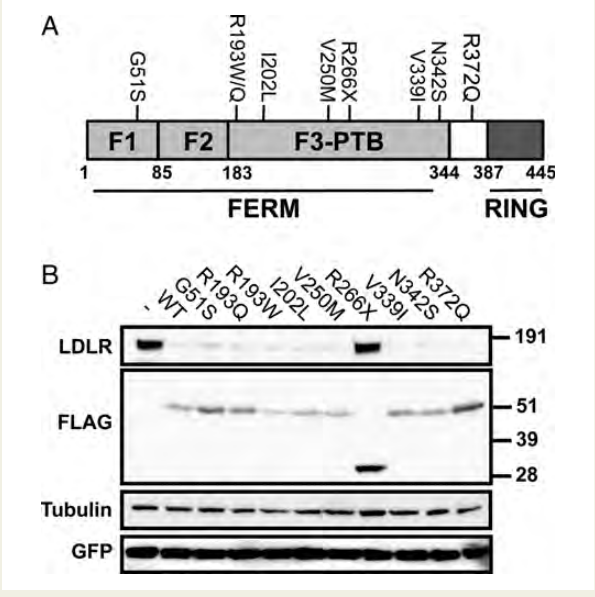

Figure 1. Functional characterization of identified IDOL variants. (*A*) Domain structure representation of the IDOL protein. The identified IDOL variants are indicated in the scheme. (*B*) HEK293T cells were transfected with expression vectors encoding low-density lipoprotein receptor and the indicated FLAG-tagged IDOL variants at a 1:1 ratio. A green fluorescent protein expression plasmid was cotransfected to monitor transfection efficiency. Total cell lysates were analysed by immunoblotting as indicated. All immunoblots are representative of at least three independent experiments with similar results.

GWAS-identified LDL-associated loci (including *PCSK9*, *APOB*, *ANGPTL3*, *SARA2*, and *MTP*) in the proband (data not shown).[8,26,27] While degradation of the abnormal IDOL transcript is a likely scenario we also considered the possibility that the truncated IDOL protein is produced and tested its function. The encoded IDOL protein lacks the complete RING domain (*Figure 1A*) that we have shown to be critical for IDOL's ubiquitylation activity. Consistent with loss of this domain, the p.Arg266X variant was unable to enhance ubiquitylation of the LDLR,[37] a prerequisite step for targeting the receptor towards the lysosomal degradation pathway (*Figure 3A*). Furthermore, unlike WT IDOL, the p.Arg266X variant was unable to attenuate LDL uptake into cells (*Figure 3B*). In these experiments, the level of heterologous IDOL substantially exceeds that of endogenous IDOL. This is, however, not the case in the individuals carrying the p.Arg266X allele in whom a second functional IDOL allele is present. In addition, since the functional IDOL unit is dimeric[41] we also considered the possibility that the mutant-encoded protein could act in a dominant negative manner to affect dimerization or IDOL function. We tested this by measuring the functional interaction between the WT and mutant encoded alleles. In the presence of a constant amount of V5-IDOL$_{WT}$, introducing increasing amounts of FLAG-IDOL$_{WT}$ enhanced LDLR degradation (*Figure 4*). In contrast to this, increasing FLAG-IDOL$_{R266X}$ dosage had no influence on degradation of the LDLR by V5-IDOL$_{WT}$. We point out that even though IDOL-mediated degradation of the LDLR is evident, we were unable to detect the V5- and FLAG-tagged IDOL in the low-dosage lanes due to IDOL's intrinsic instability.[29,37] Taken together, these assays demonstrate that the p.Arg266X variant represents a crippled protein with a complete loss of IDOL function.

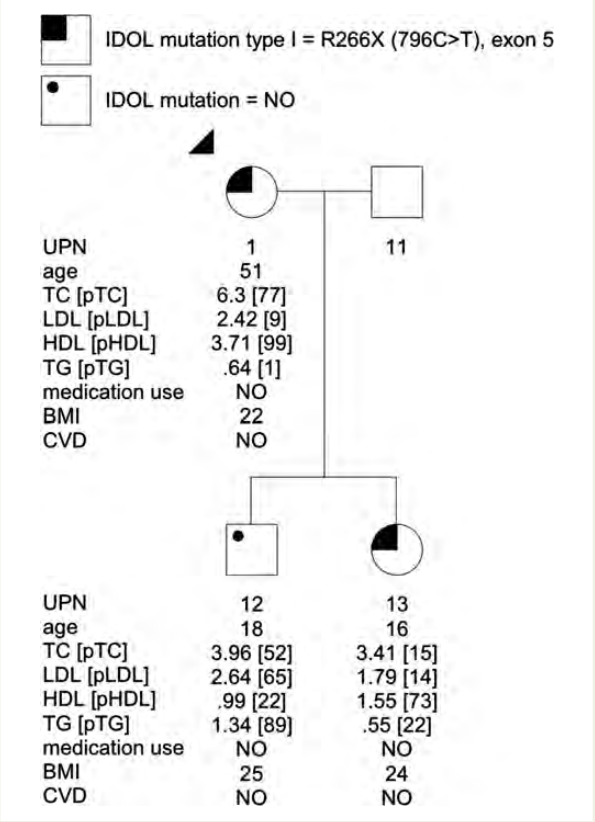

Figure 2. Pedigree and clinical parameters of the p.Arg266X proband. Pedigree of the p.Arg266X proband with clinical parameters indicated. UPN, unique personal number; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TG, triglycerides; p, percentile; BMI, body mass index; CVD, cardiovascular disease.

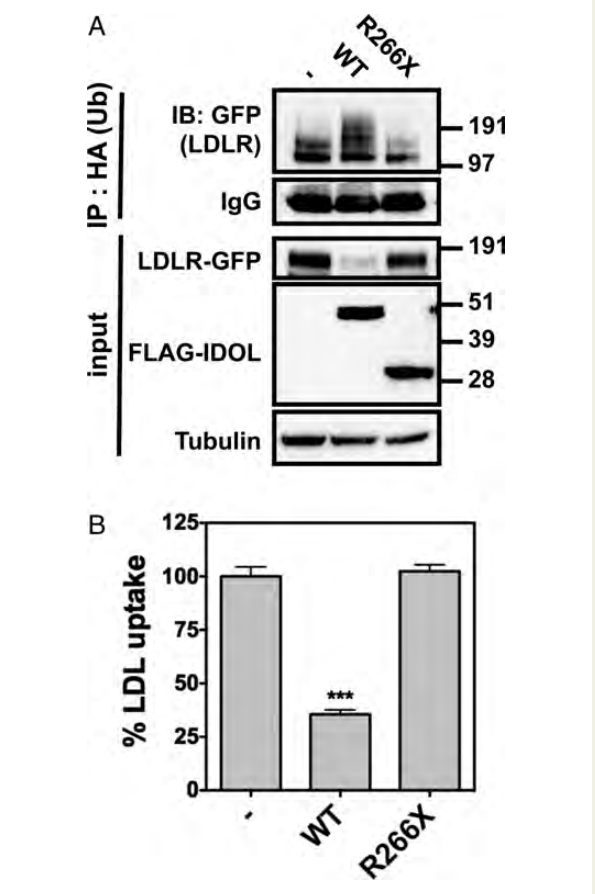

Figure 3. p.Arg266X results in a complete loss of IDOL activity. (*A*) HEK293T cells were transfected with expression plasmids for FLAG-IDOL and LDLR-GFP (1:1 ratio) and HA-ubiquitin. Samples were immunoprecipitated and analysed by immunoblotting as indicated. Immunoblot is representative of three independent experiments with a similar result. (*B*) HEK293T cells were transfected with the indicated expression plasmids for IDOL and LDLR (at a 1:1 ratio) and pE2-Crimson to monitor transfection efficiency. Forty-eight hours post-transfection cells were incubated with fluorescently labelled LDL for 30 min and uptake was assessed by FACS. Each bar and error represent the mean ± SE of three experiments done in triplicate, $^{***}P < 0.001$.

Discussion

Therapeutic interventions aimed at increasing hepatic LDLR and LDL clearance are the mainstay for treatment of hypercholesterolaemia and form the mechanistic basis for the use of statins.[42] As such, additional pathways that lead to increased LDLR abundance may represent attractive targets for treating dyslipidaemia.[43] The case of targeting PCSK9 exemplifies this concept. Recent clinical trials demonstrate that blocking PCSK9 from degrading the LDLR using antisense oligonucleotides[19–21] or neutralizing antibodies[22–24] prevent receptor degradation and result in a substantial decrease in LDL-C. Akin to PCSK9, the E3-ubiquitin ligase IDOL promotes degradation of the LDLR and attenuates LDL uptake into cells.[29] In line with its function, IDOL has been recently proposed to contribute to variation in circulating levels of LDL-C in humans.[8,26,27] To test this directly we characterized *IDOL* variation in a Dutch cohort with extreme LDL phenotypes. Our screening approach was designed to potentially identify both loss- and gain-of-function *IDOL* variants and resulted in finding of both common and rare *IDOL* variants, some of which were restricted to one extreme of the LDL phenotype. However, none of the missense variants displayed substantially altered activity and all retained their ability to promote LDLR degradation in cellular assays. In a recent report, Weissglas-Volkov *et al.* [36] reported that one of the variants we also indentified in our study, p.Asn342Ser (rs9370867), was associated with higher total cholesterol levels in a hypertriglyceridaemic Mexican population. This was attributed to distinct activity of the two encoded alleles. However, the distribution of the p.Asn342Ser alleles was different in the Dutch population and allele frequencies were comparable in our two extreme LDL cohorts (*Table 2*). Additionally, no association between this variant and LDL-C levels was reported in a Brazilian cohort,[44] despite the fact that the distribution of the p.Asn342Ser allele is comparable with that in the Mexican cohort. Therefore, whether the p.Asn342Ser variant contributes

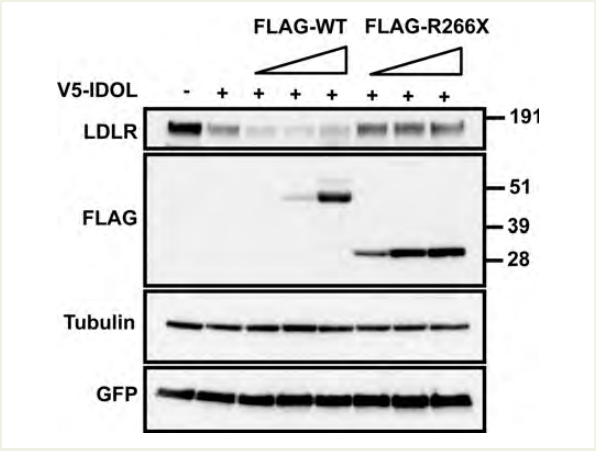

Figure 4. Analysis of the functional interaction between wild-type and p.Arg266X IDOL. HEK293T cells were transfected with expression plasmids for LDLR (350 ng), V5-IDOL (50 ng), and GFP (50 ng) to monitor transfection efficiency. Increasing amounts of FLAG-IDOL (WT or R266X; 50,150, and 350 ng) were introduced as indicated while maintaining an equal amount of total DNA. Total cell lysates were analysed by immunoblotting as indicated. All immunoblots are representative of at least three independent experiments with a similar outcome.

to circulating levels of LDL-C will require further examination in additional populations. We also identified one nonsense *IDOL* variant. The p.Arg266X allele encodes a truncated IDOL protein lacking the complete RING domain. Although the premature stop codon is distant from the natural transcription stop site and therefore likely subject to nonsense-mediated RNA decay, we considered the possibility that the truncated protein is produced. Our functional analysis indicates that the truncated protein is unable to promote ubiquitylation and degradation of the LDLR, attenuate LDL uptake into cells, or act in a dominant manner to antagonize WT IDOL. As far as we know, this represents the first description of a complete loss-of-function *IDOL* variant. We did not find mutations in other GWAS-identified loci for LDL-C, including in genes implicated in hypocholesterolaemia. Therefore, we consider that decreased IDOL activity leading to enhanced LDL clearance offers a plausible explanation for the LDL phenotype in carriers of the p.Arg266X variant.

Our current results add upon previous cell-based studies to support the idea that inhibition of IDOL activity may increase LDL clearance.[29] The mechanistic understanding of IDOL function and the availability of structural models of the IDOL–LDLR interface[37,38] can facilitate development of small molecules that inhibit degradation of the receptor by IDOL. Alternatively, as is being currently tested clinically for PCSK9,[19–21] siRNA technology could be used to reduce hepatic expression of *IDOL* potentially leading to higher levels of LDLR. While both PCSK9 and IDOL lead to degradation of the LDLR, they do this independently of each other.[30,31,45,46] Furthermore, transcriptional regulation of *PCSK9* and *IDOL* is also distinct. *PCSK9* is co-regulated with the *LDLR* by the SREBP pathway,[47,48] whereas the liver X receptor transcription factors control expression of IDOL.[29] Therefore, targeting of IDOL may provide an alternative approach to increasing LDLR abundance independent of SREBP/PCSK9 and complementary to statins.

In summary, by screening individuals with extreme LDL phenotypes we characterized variants in *IDOL* and the possible contribution of these variants to circulating levels of LDL-C. Our study is the first to report a functionally altered rare IDOL variant in individuals with low circulating levels of LDL-C. It is interesting to point out that genetic variation in IDOL has been also recently suggested to influence the clinical response to the LDL-lowering drug rosuvastatin.[49] Collectively, our observations provide further evidence for a role for IDOL in LDL metabolism in humans and support investigating therapeutic strategies to target IDOL for treatment of hypercholesterolaemia and prevention of coronary artery disease.

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Conflict of interest: none declared.

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