Letter

The E3 ubiquitin ligase c-IAP1 regulates PCSK9-mediated LDLR degradation: Linking the TNF-α pathway to cholesterol uptake

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Abstract

Proprotein convertase subtilisin/kexin type 9 (PCSK9), in addition to LDLR (low-density lipoprotein receptor) and APOB (apolipoprotein B), is one of three loci implicated in autosomal dominant hypercholesterolaemia (ADH)[1]. A number of *PCSK9* gain-of-function mutations and loss-of-function mutations have been identified from families afflicted with ADH with hypercholesterolaemia or hypocholesterolaemia, respectively[1-4]. In humans, the main function of PCSK9 appears to be the post-transcriptional regulation of the number of cell-surface LDL receptors[5-7]. To date, only LDLR and its closest family members VLDLR and ApoER2 have been shown to bind with PCSK9[8,9]. To find new binding partners for PCSK9, we used a shotgun proteomic method to analyse the protein complex pulled down by immunoprecipitation against FLAG-tagged PCSK9 protein. Among 22 potential novel binding proteins identified, we found that the cellular inhibitor of apoptosis protein 1 (c-IAP1[10]) and the TNF receptor-associated factor 2 (TRAF2[11]) complex are regulated differently in different dominant PCSK9 mutations that occur naturally. Further immunoprecipitation analysis showed that c-IAP1 is a direct binding partner for PCSK9. One of the "gain-of-function" mutants, PCSK9-S127R, which has impaired autocatalytic activity, is defective in binding to c-IAP1. The other dominant mutation, PCSK9-D374Y[12], which is 10-fold more potent in degrading the LDLR protein than wild-type PCSK9, can be significantly ubiquitinated by c-IAP1 in vitro. The ubiquitinated PCSK9-D374Y is unable to degrade LDLR, which is its main cause of hypercholesterolaemia in patients. These results indicate that there is a novel cholesterol uptake regulation pathway linking PCSK9/LDLR to the E3 ubiquitin ligase c-IAP1 in a TNF-α response pathway. This highlights the possibility of developing new treatments for human cardiovascular diseases through ubiquitin ligase–mediated ubiquitination of target proteins in cholesterol metabolism.

To identify novel binding partners of PCSK9, we generated a human T-Rex-293 stable cell line that overexpressed FLAG-tagged wild-type PCSK9[5]. The FLAG-tagged PCSK9 and associated proteins were isolated by performing anti-FLAG immunoprecipitation (IP) from cellular extracts; the eluted protein mixture was subjected to shotgun proteomic analysis[13]. Briefly, protein complexes pulled down by immunoprecipitation with FLAG-tagged PCSK9 protein were subjected to limited electrophoresis, after which 3-5 molecular weight regions were cut out and digested. Liquid chromatography/mass spectrometry/mass spectrometry (LC-MS/MS) was performed on every fraction. The lists of identified proteins for each sample (with their scores) were subjected to statistical validation and aligned for comparison

using the Scaffold program. A negative control cell line, T-Rex-293 cells transfected with the empty vector pcDNA3.1, was used for background subtraction.

In total, 22 co-IP proteins have been identified (Table 1) that contain at least 2 unique spectra (2 distinct peptides) in three independent experimental samples but are absent (0 spectrum) in all three negative control samples. Many of these proteins are ER-located proteins, such as the UDP-glucose glycoprotein glucosyltransferase 1 (UGGT-1), protein disulfide isomerase family A member 4 (PDIA4, also called endoplasmic reticulum resident protein 72, ERP72) and Calmegin (CLGN). Some of them are associated with the ubiquitination pathway, such as c-IAP1, TRAF2 and Stub1 E3 ligase. Some are molecular chaperones, such as DNJA1, DNJA2, DNJA3, DJB11, and DJC10 in the DnaJ (Hsp40) homolog subfamily. There are also several mitochondrial carriers, including SLC25 A1, A10 and A12.

We have further confirmed **6** of the 22 PCSK9 binding proteins by western blot using available antibodies. Among the 6 proteins confirmed by western blot, we observed that one of the "gain- of- function" mutants, PCSK9-S127R, which has impaired autocatalytic activity, was defective in binding to c-IAP1 and TRAF2 proteins (Fig.1). c-IAP1 and TRAF2 are known binding partners in the TNFa-mediated apoptosis pathway[10].

To further confirm the binding of c-IAP1 to wild-type PCSK9, we co-transfected wild-type FLAG-tagged PCSK9 (pCMV-PCSK9-FLAG[5]) and myc-tagged cIAP-1 in a pCMV6 expression vector (Origene Inc.) into T-Rex-293 cells. The empty vector, pCMV6-entry vector (Origene, Inc), was used as a control vector. After 24 h, the cell lysates were immunoprecipitated with anti-myc antibody. As shown in Fig. 2a, the PCSK9 protein was detected in the IP product (Lane 2). To determine which regions of c-IAP1 were involved in binding to PCSK9, we have made several deletion mutation constructs of c-IAP1 for PCSK9 binding studies. We found that only the region containing the baculoviral IAP repeat 3 (BIR3) domain of c-IAP1 could be used to pull down wild-type PCSK9 (Fig. 2a, lane 3), indicating that c-IAP1-BIR3 is the binding site for PCSK9. The binding of PCSK9 to c-IAP1 was further confirmed using surface plasmon resonance (SPR) experiments, in which the binding affinities of the wild-type PCSK9 protein for c-IAP1 were determined at pH 7.4 with kinetic constants for dissociation at a Kd of 44.3 ± 5 nM (n=3, T100 evaluation software, supplementary Fig. S1). We also used confocal microscopy to conduct immunostaining studies to confirm the colocalisation of PCSK9 and c-IAP1 in the cytoplasm of 293 cells with stable overexpression of PCSK9 (Supplementary Fig. S2).

We next determined whether TRAF2 binds to the wild-type PCSK9. We co-transfected wild-type FLAG-tagged PCSK9 and myc-tagged TRAF2-pCMV6 expression vectors into T-Rex-293 cells. After 24 h, the cell lysates were immunoprecipitated with anti-myc antibody. We were unable to detect the PCSK9 protein in the IP product (Fig. 2b, lane 2), indicating that TRAF2 was not a direct binding partner for PCSK9. c-IAP1 was the only physical binding partner of PCSK9 in the c-IAP1/TRAF2 complex. As a positive control, we co-transfected wild-type FLAG-tagged PCSK9 and myc-tagged LDLR-pCMV6 into T-Rex-293 cells. After 24 h, the cell lysates were immunoprecipitated with anti-myc antibody. We were able to detect the PCSK9 protein in the IP product (Fig. 2b, lane 3), confirming LDLR binding to PCSK9 in our system.

We next used a siRNA-c-IAP1 construct to knock down the endogenous c-IAP 1 in a human T-Rex-293 stable cell line that overexpressed FLAG-tagged wild-type PCSK9. We observed

significantly increased pro-PCSK9 bands (90% PCSK9 protein is still pro-PCSK9, only 10% is converted to the mature band) in comparison to the non-silencing RNA control (over 90% of PCSK9 is converted to the mature band), indicating that c-IAP 1 is directly involved in processing PCSK9 from a proprotein to the functionally mature protein (Fig. 3a). We have also detected high molecular weight aggregates of PCSK9 formed in c-IAP1 siRNA treated samples. Because of a very low LDLR protein level in the PCSK9-overexpressed 293 cells, we transfected the myc-tagged LDLR-pCMV6 plasmids into a c-IAP1 siRNA knocked-out cell line or a control cell line with empty vector as control. There was a more than 30% reduction of LDLR protein level in c-IAP 1 siRNA knockout 293 cells when compared to that of control cells, indicating that c-IAP1 negatively regulated PCSK9-mediated LDLR degradation (Fig. 3b).

To further investigate the role of c-IAP1 in PCSK9-mediated LDLR reduction, we transiently co-transfected wild-type FLAG-tagged PCSK9 or a 'gain of mutation' construct (PCSK9-D374Y-pcDNA3) with or without cIAP-1 into HepG2 cells. As shown in Fig. 4a, the LDLR level was decreased slightly (18% less than empty vector transfection) after wild-type PCSK9 transfection. The PCSK9-D374Y mutation was significantly more potent in reducing the LDLR protein level (over 80% less than empty vector transfection). The co-transfection with CMV6-cIAP 1 vector attenuated both PCSK9-wt- and PCSK9-D374Y-mediated LDLR decrease, indicating the c-IAP1 protein inhibits PCSK9-mediated LDLR degradation (Fig. 4a).

We next tested whether D374Y-PCSK9-mediated LDLR degradation was dependent upon lysosomal function or ubiquitin-mediated proteasomal degradation, as c-IAP1 is a well-known E3 ubiquitin ligase in the proteasome-mediated protein degradation pathway[14]. We transiently transfected a gain-of-function mutation construct of PCSK9-D374Y-pcDNA3 into HepG2 cells. After 24 h, we treated the transfected cells with either 20 μM of MG132 (a proteasome inhibitor) or 100 μM of NH_4Cl (a lysosomal inhibitor), or left the cells untreated. As shown in Fig. 4b, the LDLR level was significantly decreased after transfection with PCSK9-D374Y. Treatment with MG132 was able to increase the LDLR level back to 44% of the original level, whereas treatment with NH_4Cl was able to increase the LDLR level back to normal. The result indicated both lysosomal and proteasome-mediated protein degradation pathways were involved in mutant PCSK9-D374Y-mediated LDLR degradation.

We then tested c-IAP1's ability to ubiquitinate the PCSK9-D374Y in vivo and in vitro. T-Rex-293 cells were co-transfected with wild-type FLAG-tagged PCSK9 or a 'gain-of-function' mutation construct of PCSK9-D374Y-pcDNA3 and pcDNA3.1-(HA-ubiquitin) with or without c-IAP1-pCMV6. After 24h, the whole cell lysates were immunoprecipitated with anti-ubiquitin antibody and probed with PCSK9 antibody. As shown in Figure 4c, only the HA-UB/cIAP-1 and D374 mutant combination resulted in an appearance of multiple high-molecular weight bands representing the polyubiquitinated PCSK9 (Fig. 4c, Lane 4). For the in vitro ubiquitin assay, the FLAG-tagged D374Y-PCSK9 was purified and subjected to ubiquitination in the presence or absence of recombinant c-IAP1 (Fig. 4d). PCSK9 was found to be ubiquitinated by c-IAP1, as shown by the appearance of multiple high-molecular weight bands on an SDS-PAGE gel representing the polyubiquitinated PCSK9 in the presence of recombinant c-IAP1 (Fig. 4d, Lane 3). In control experiments, there were no detectable polyubiquitinated PCSK9 bands in the samples without c-IAP(Fig.4d, Lane 2) or without D374Y protein(Fig.4d, Lane 4).

To further elucidate the functional significance of D374Y-PCSK9 ubiquitination, we added

1 μg/ml of ubiquitinated D374Y-PCSK9 recombinant protein into DMEM (without 10% serum) to test its ability to reduce the levels of endogenous LDLRs in HepG2 cells. As shown in Fig. 4e, the LDLR level was significantly decreased in the PCSK9-D374Y-treated sample (lane 2, over 90% decrease in LDLR level compared to the untreated sample), but there was no LDLR level decrease in the samples treated with 1 μg of ubiquitinated D374Y-treated (lane 3). The ubiquitin buffer itself has no significant effects on LDLR protein levels in HepG2 cells (lane 4).

We also carried out an in vitro ubiquitin assay on the purified wild-type PCSK9 protein. We noticed that wild-type PCSK9 could also be ubiquitinated at a much higher protein concentration (4 μg, Fig. 4f, Lane 3). The other dominant mutation, S127R, which was unable to bind cIAP-1, was only very weakly ubiquitinated by c-IAP1 at the 4 μg concentration (Fig. 4f, Lane 4). In the functional analysis, there was a modest effect on the LDLR level (20% decrease in LDLR) after administration of 4 μg/ml wild-type PCSK9 in HepG2 cell culture (without serum) for 16 h (Fig. 4g. lane 2) in comparison to D374Y-PCSK9 administration (85% decrease of LDLR, Fig. 4g, lane 3). Ubiquitination of wild-type PCSK9 abrogates its ability to reduce LDLR levels in HepG2 cells (Fig. 4g. Lane 4).

Finally, we measured the uptake of LDL in ubiquitinated PCSK9-D374Y and non-ubiquitinated PCSK9-D374Y treated HepG2 cells (Fig. 4h). Paralleling the levels of LDLR on western blot, we found that the mean level of LDL uptake in the 1 μg/ml D374Y treatment was reduced on average by 50% (Fig. 4g, green peak, mean intensity 8.7±1, n=3) when compared with untreated samples (brown peak, mean intensity 17.2±1, n=3, P=0.03). In contrast, 1 μg/ml of ubiquitinated D374Y was unable to reduce LDL uptake (blue peak, mean intensity 16.5±1.2, n=3) compared with untreated sample (mean intensity 17.2±1,n=3, P=0.22). In the control experiment, the ubiquitin reaction without cIAP-1 had no significant effect on the mutant D374Y's ability to reduce LDL uptake (red peak, mean intensity 7.8±0.2, n=3) in comparison to the D374Y treatment (green peak, mean intensity 8.7±1, n=3, P=0.45, Fig. 4h).

In summary, we report here that E3 ubiquitin ligase c-IAP1, also known as Baculoviral IAP repeat-containing protein 2 (BIRC2), plays an important role in regulating PCSK9-mediated LDLR degradation. By binding to wild-type PCSK9, it promotes its maturation from its proprotein form. One of the 'gain-of-function' mutations, PCSK9-S127R, has a defect in binding to c-IAP1, and so has impaired autocatalytic activity. The precise mechanism for how the S127R mutant proprotein promotes LDLR degradation and causes hypercholesterolaemia is still unknown. However, in c-IAP1 siRNA knockout cells, in addition to the significant defect in PCSK9 maturation, there is significant LDLR depletion, indicating c-IAP1 negatively regulates PCSK9-mediated LDLR degradation. By not binding to c-IAP1, S127R could work more effectively to degrade LDLR. The other possibility is that S127R could bind to other E3 ligases to regulate the LDLR/PCSK9 pathway differently. In fact, from our preliminary results, the other E3 ligase we detected in our shotgun proteomic analysis, stub1 (STIP1homology and U-box containing protein 1)[15] has been found to bind much more strongly to S127R than to wild-type PCSK9 (Supplementary Fig S3). Stub1 is a ubiquitin ligase/co-chaperone that participates in protein quality control by targeting a broad range of chaperone protein substrates, including Hsp70, Hsc70 and Hsp90. The proteins in the DNAJ (Hsp40) subfamily we detected in the shotgun proteomic analysis are also known to form complexes with Hsp70. A close relative of LDLR, LDLR related protein 1b (LRP1b) has been shown to bind to DNAJA1[16]. Furthermore, PDIA4, also known as ERP72, has been shown to be in a chaperone complex with the DNAJ family and APOB[17]. Therefore, S127R could

potentially bind to these complexes through the Hsp70 /APOB[18] pathway to exert its effect on LDLR degradation (Supplementary Fig. S4).

Interestingly, the other dominant mutation, PCSK9-D374Y, binds to c-IAP1 and can be cleaved normally and secreted from cells. We showed that by binding to PCSK9-D374Y, c-IAP1 ubiquitinates it very effectively. Function analysis showed that ubiquitinated PCSK9-D374Y has lost its ability to degrade LDLR in culture cells in vitro. Given previous observations showing that there is a good correlation between the effect of PCK9 mutations on LDLR in cultured cells in vitro and their effect on the plasma cholesterol level of heterozygous carriers of the mutations[19], we have envisaged a novel approach, using c-IAP1 or another E3 ligase to inactive some of the most severe hypercholesterolaemic mutations, such as PCSK9-D374Y[12].

The results indicate for the first time that there is a novel cholesterol regulation pathway that links PCSK9/LDLR cholesterol uptake to the TNF-α-induced c-IAP1/TRAF2 regulatory pathway. Interestingly, knockout of the c-IAP1 homologue in Zebrafish causes endothelial cell death, blood pooling, vascular haemorrhage, and vascular regression, mediated in part by the deregulation of the NFκB signally pathway[20]. In mammalian cells, there are three homologues of c-IAP, c-IAP1, c-IAP2 and X-linked IAP (XIAP)[21]. These IAP proteins are widely expressed and inhibit the apoptosis process in a variety of circumstances. Interestingly, it has been suggested that two nonsense single-nucleotide polymorphisms (SNPs) at the PCSK9 locus, which have been associated with life-long hypocholesterolaemia, may have been positively selected in individuals of African origin as lower cholesterol may prevent some parasitic infection[22]. It is possible that PCSK9 is an evolutionary link between cholesterol metabolism and the TNF-α mediated inflammatory response.

How c-IAP1 regulates the PCSK9/LDLR pathway remains to be elucidated. The available evidence has indicated that the PCSK9/LDLR complex shuttles to the lysosome, where degradation of the LDLR occurs[23]. The wild-type PCSK9-induced degradation of LDLR was not affected by inhibitors of the proteasome in human HepG2 cells in adenoviral-mediated PCSK9 overexpression system[23]. We have found, in cells that overexpress D379Y, an inhibitor of the proteasome (MG132) did have some effect on the LDLR level, although it had much less effect than that of the ammonium chloride treatment (Fig. 4b). The discrepancy between our data and previous data[23] could be due to the level of expression of PCSK9. Our expression is relatively low in comparison with expression after adenovirus infection. We could not obtain conclusive data using a wild-type PCSK9 plasmid transfection because LDLR degradation occurs at a level reduced by 20%. Recently, LDLR has been found to be subjected to ubiquitination by an E3 ligase (Idol, inducible degrader of the LDLR)[24], indicating that both proteasomal degradation and lysosomal degradation may be involved in LDLR degradation and recycling.

Interestingly, the c-IAP1/TRAF2 complex has been shown to be degraded through the lysosomal degradation pathway in TWEAK/FN14 induced apoptosis[25]. It is possible that c-IAP1 could escort the PCSK9/LDLR complexes to the lysosome. On the other hand, c-IAP1 has also been known to be involved in proteasome-mediated degradation of TRAF2 in TNF-α dependent pathway[10]. We can envisage this as an alternative pathway that c-IAP1 uses to escort PCSK9 to proteasome-mediate degradation, allowing unbound LDLR to be recycled back to the cellular membrane (Supplementary Fig. S4). The c-IAP1/TRAF2 switch would determine the fate of LDLR in the PCSK9/LDLR complex. Interestingly, TRAF2 has been shown to be recruited directly into cholesterol-rich lipid rafts upon TNF-α stimulation[26], so

the TRAF2/c-IAP1 complex is likely to be a key switch in this novel pathway. Further clarification of this pathway will open completely new avenues for exploring new treatments for cardiovascular and infectious diseases.

Tables and Figures

Table 1 List of the potential PCSK9 binding proteins identified through affinity purification and shotgun LC-MS/MS analysis. Each protein was identified by at least two matched spectra (95% confidence minimum) in all three experiments with no spectrum identified in the control samples (empty vector).

Fig. 1. Immunoprecipitation (IP)/western blot analysis of novel PCSK9 binding proteins in PCSK9-FLAG pull-down assay. Cellular extracts from a T-Rex 293 cell line stably overexpressing FLAG-tagged wild-type PCSK9 or a negative control cell line, T-Rex-293 cells transfected with the empty vector pcDNA3.1(control) were subjected to anti-FLAG IP and blotted to nitrocellulose and probed with the indicated antibodies. The western blots shown are representative of three separate experiments.

Fig. 2. c-IAP1 interacts with PCSK9. (a) Co-IP of PCSK9-wt and c-IAP1. Myc-tagged c-IAP1 or c-IAP1-BIR3 was co-transfected with either empty vector (pCMV6-entry vector) or FLAG-tagged wild-type PCSK9 into T-Rex-293 cells. Cell lysates were immunoprecipitated with an anti-myc tag antibody followed by immunoblotting with either anti-PCSK9 or anti-c-IAP1 antibodies. (b): Co-IP of PCSK9-wt, TRAF2 and LDLR. Myc-tagged TRAF2 or LDLR was co-transfected with FLAG-tagged wild-type PCSK9 into T-Rex-293 cells. Cell lysates were immunoprecipitated with an anti-myc tag antibody followed by immunoblotting with either anti-PCSK9 or anti-TRAF or LDLR antibodies. The western blots shown are representative of three separate experiments.

Fig. 3. Silencing of c-IAP1 by pGB c-IAP1 siRNA mixture. (a) A wild-type PCSK9 overexpressed T-Rex-293 stable cell line was transfected with pGB c-IAP1 siRNA. A stable c-IAP1 siRNA clone was established with nearly 100% knockdown of endogenous c-IAP1 protein (lane 1) in comparison with empty pGB control vector (lane 2). Western blot was probed with anti-c-IAP1 or anti-PCSK9 antibodies. Scanning densitometry analysis of three western blots is shown below. Data are presented as the percentage conversion to mature PCSK9 (p63), calculated as the p63 value divided by the sum of p63 + p75 (Pro-PCSK9), divided by the tubulin, multiplied by 100. ** indicates a significant difference (P=0.009) from c-IAP siRNA treated cells from control siRNA cells.(b) Western blot analysis of the LDLR level in pGB-c-IAP1 siRNA knockout cells. Myc-tagged LDLR-CMV6 or empty vector was transfected into c-IAP1-siRNA-knockout cells or control siRNA cells. After 48 h, the cell lysates were analysed by western blot with anti-myc antibody to detect LDLR protein. LDLR amounts were quantified and normalised to the amount of α-tubulin. The ratio of LDLR/tubulin in control siRNA cells was assigned a value of 1.00. * indicates a significant difference (P =0.04) between treated cells and control siRNA cells.

Fig. 4. Identification of PCSK9 as a substrate of c-IAP1 ubiquitin ligase in vivo and in vitro**.** a. Western blot analysis of the LDLR level in transient transfection of wild-type FLAG-tagged PCSK9-pcDNA3 or PCSK9-D374YpcDNA3 with or without cIAP-1pCMV6 in HepG2 cells. LDLR was quantified and normalised to the amount of α-tubulin. The ratio of

LDLR/tubulin in the cells transfected with empty vector control was assigned a value of 1.00. P-values are from comparisons between transfection with empty vector and the PCSK9 expression vectors with or without c-IAP1 expression vector. Values are means + s.d. from three separate experiments. * denotes P<0.05; ** denotes P<0.01.

b. Proteasomal and lysosomal inhibitors prevent D374Y-PCSK9-mediated LDLR degradation. HepG2 cells were transiently transfected with a plasmid expressing PCSK9-D374YpcDNA3 or empty vector (pCDNA3.1). 24 h after transfection, cells were grown in serum-free DMEM medium for an additional 16 h and then treated with 10 μM of proteasomal inhibitor MG132 or the lysosomal inhibitor Ammonium Chloride (NH_4Cl) for 4 h in serum-free DMEM medium. Western blotting of total cell lysates using anti-LDLR antibody. Anti-tubulin antibody was used as a control. LDLR was quantified and normalised to the amount of α-tubulin. The ratio of LDLR/tubulin in the cells transfected with empty vector control was assigned a value of 1.00. The western blots shown are representative of three separate experiments.

c. Hep G2 cells were co-transfected with wild-type PCSK9 and D-374Y pCSK9 with or without c-IAP1 and the Haemagglutinin (HA-ubiquitin) expression plasmid. After 24 h, lysates were subjected to IP with anti-ubiquitin antibody and probed with anti-PCSK9 antibody. The western blots shown are representative of three separate experiments.

d. FLAG-tagged PCSK9-D374Y was subjected to a ubiquitination assay in the presence of recombinant c-IAP1. The poly-ubiquitination of D374 was detected by immunoblotting with anti-PCSK9 antibody.

e. Effect of ubiquitination of mutant D374Y on LDLR expression in whole cell extracts of Hep G2 cells. Lane 1, Untreated; Lane2, D374Y (1μg); Lane 3, c-IAP1-ubiquinated D374Y (1 μg); Lane 4, ubiquitination buffer only. LDLR was quantified and normalised to the amount of α-tubulin. The ratio of LDLR/tubulin in the untreated cells was assigned a value of 1.00. P-values are from comparisons between untreated samples and recombinant D379Y treatment and ubiquitination treatment. Values are means + s.d. from three separate experiments. ** denotes P<0.01.

f. FLAG-tagged wild-type -PCSK9, S127-PCSK9 and PCSK9-D374Y were subjected to ubiquitination assays in the presence of recombinant c-IAP1. The ubiquitinated PCSK9 was detected by immunoblotting with anti-PCSK9 antibody.

g. Effect of ubiquitination of wild-type PCSK9 on LDLR expression in whole cell extracts of Hep G2 cells. Lane 1, Untreated; Lane2, D374Y (1 μg); Lane 3, wild-type PCSK9 (4 μg); Lane4, c-IAP1-ubiquinated wt-PCSK9 (4 μg). LDLR was quantified and normalised to the amount of α-tubulin. The ratio of LDLR/tubulin in the untreated cells was assigned a value of 1.00. P-values are from comparisons between untreated samples and recombinant wild-type PCSK9 treatment and ubiquitination treatment. Values are means + s.d. from three separate experiments. * denotes P<0.05; ** denotes P<0.01.

h. Flow cytometric analysis of BODIPY-labelled LDL uptake in Hep G2 cells. Cells were incubated in the presence or absence of D374Y protein with or without ubiquitination. Graph is representative of three separate experiments with similar results.

Supplementary Fig. S1. SPR analysis of interactions of PCSK9 and c-IAP1. Representative overlays for various concentrations of purified PCSK9-wt to immobilised c-IAP1. Coloured lines represent data; black lines indicate a theoretical good fit to a simple 1:1 kinetic model with a Kd of 44.3±5 nM (n=3).

Fig. S2. Co-localisation of PCSK9 and c-IAP1 in the cytoplasm. Cells stably overexpressing PCSK9 were immunostained with mouse anti-FLAG M2 monoclonal antibody (for detection of PCSK9) using secondary anti-mouse antibody labelled with Alexa488(Green); Rabbit anti c-IAP was used to detect the c-IPA1 protein, with an anti-rabbit–cy3 labelled antibody. Cells were subjected to confocal microscopy examination. ***Green*** fluorescence indicates PCSK9; ***red*** indicates c-IAP1. In the merged images, ***yellow*** staining indicates co-localisation. Bar, 20 μm.

Fig.S3. IP/western blot analysis of interaction between the FLAG-tagged PCSK9 and stub1 protein in PCSK9-FLAG pull-down assay. Cellular extracts from the T-Rex 293 stable cell line overexpressing FLAG-tagged wild-type PCSK9 was subjected to anti-FLAG IP, blotted to nitrocellulose and probed with the stub1 antibody and PCSK9 antibody. The western blots shown are representative of three separate experiments.

Fig. S4. Proposed model of c-IAP1/TRAF2 regulation of the PCSK9-mediated LDLR degradation. Upon activation by tumour necrosis factor α, TNFR complex recruit TRAF-2 to its cytoplasmic tail, leading to recruitment of c-IAP1. c-IAP1 can bind to PCSK9 and promote its maturation. The second role of c-IAP1 is its E3-ubiquitin ligase activity. When extra-cellular PCSK9/LDLR complexes re-enter the cell, c-IAP1 binds to PCSK9, leading to its proteasomal degradation, releasing LDLR to recycle back to the membrane. There may be a switch for LDLR/PCSK9 to be shuttled either to the proteasome or to the lysosome. Due to extremely tight binding to LDLR, PCSK9-D374Y/LDLR will lead to more destruction of LDLR in the proteasome, whereas wild-type PCSK9 may depend mostly on the lysosomal pathway. Due to its inability to bind to c-IAP1, PCSK9-S127R may bind to other E3 ligases (such as Stub1 ligase) to form a complex with HSP70, DNAJ family, ERP72, or APOB, leading to the destruction of LDLR. Several mitochondrial (MT) carriers may also potentially bind PCSK9. Their roles are still unknown.

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Competing Financial Interests
The authors declare that they have no competing financial interests.

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Methods Summary

Cell culture, DNA constructs, trasfections and western blot analysis

HepG2 cells and T-Rex 293 cells were grown in DMEM containing 25mM glucos and 10% fetal calf serum, as described[27]. C-terminal flag-tagged Wild-type PCSK9, S127R-PCSK9 and F216L-PCSK9 were kindly provided by Jay D. Horton. D374Y mutation was introduced by oligonucleiotide-direct mutagenesis. C-terminal myc-tagged LDLR, cIAP1, TRAF2 in CMV6-based mammalian expression vector were obtained from Origene, Inc. PCR was used to generate deletion constructs of myc-tagged c-IAP1, containing only BIR1 or BIR2 or BIR3 or Ring domains. The PCR products were cloned in frame to the pCMV6 entry clone. All transfections were done with either T-Rex 293 cells and HepG2 cells using Superfect.

Shotgun analysis of the FLAG-tagged PCSK9 and associated proteins complex samples.

A stable expressed flag-tagged PCSK9 cell line was grown in DEME medium with 10% FBS. The flag-tagged PCSK9 protein was isolated using the flag-immunopereciptation kit(Sigma) following by elution with the Flag-peptide 3xflag peptide(final concertation 150ng/ul 3xflag peptide). The elution samples were then send to the Protein Analysis Facility, University of Lausanne, Switzerland , where protein mixtures were separated by limited electrophoresis after which 3-5 molecular weight regions are cut and digested. Analysis is performed by LC-MS/MS on every fraction. The resulting collections of spectra are pooled for every sample before database search. A negative control cell line, T-Rex-293 cells transfected with the empty vector pcDNA3.1 was used for background subtraction.

RNA interference.
c-IPA1 *siRNA* mix(*pGB*-cIAP1 *siRNA, Biovision Research Products, CA, USA*) or control *siRNA* (*pGB*-control) were transfected into a stable wild-type PCSK9 overexpress cell line using the superfect reagent(Qiagene, UK). After 48h, the cells were subjected G148 selection to obtain the stable cell lines with nearly 100% knock-out the c-IAP1 protein by western blot analysis.

***In vivo* ubiqutination analysis**
pcDNA.1Empty vector and plasmid containing HA-tagged ubiquitin gene(HA-ubiqutin , Addgene(Cambridge, MA) were co-transefected with PCSK9 expression vector with or without the pcDNA6 –c-IAP1 plasmids into T-REX 293 cells. 24h later, the lysates were immunopreciptation with anti-ubiquitin anibody(Ubiquitin enrichment kit, Thermo Fisher) analyzed by Western blotting with anti-PCSK9 antibody.

***In vitro* Ubiquitanation Assay**
Purified Flag-tagged wild-type PCSK9(1-4μg), or D374Y-PCSK9(1 μg) or S127R-PCSK9(1-4μg) and recombinant c-IAP1(1μg , R&D system, UK) were incubated in a reaction buffer(50mM Tris-HCl(pH7.5), 5mM $MgCl_2$, 2mM ATP, 0.6mM DTT) with recombinant rabbit E1(100ng), UbcH5b(250ng) and ubiquitin(6ng) at 37^{o}C for 2hr. The resulting mixtures were analyzed by immunoblotting using anti-PCSK9 antibody.

***In vitro* ubiquitination function assay on the purifed PCSK9 proteins**

HepG2 cells were seeded in 6 well tissue plates at concentration of $2x10^5$ cells/ml. After 24h, the medium was replaced with DMEM medium without FBS and added the purified D374Y protein(1μg/ml) or wild-type PCSK9 (4μg/ml). In the control experiments, only c-IAP1 ubiquitination buffer was added into medium without PCSK9 protein. After 16h, cells was washed twice in PBS and harvested for western blot analysis as previous described[28].

Full Methods

Cell culture

HepG2 cells were obtained from European collection of cel culture(Wiltshire,UK). T-Rex 293 cells were obtained from (Invitrogen, Paisley, UK). Cell were grown in DMEM containing 25mM glucos and 10% fetal calf serum, as described[27].

DNA constructs, trasfections and western blot analysis

C-terminal flag-tagged Wild-type PCSK9, S127R-PCSK9 and F216L-PCSK9 were kindly provided by Jay D. Horton(University of Texas Southwestern Medical Center, Dallas, TX,USA). D374Y mutation was introduced by oligonucleiotide-direct mutagenesis with forward primer 5'-CATTGGTGCCTCCAGCTACTGCAGCACCTGC-3' and reverse primer 5'-GCAGGTGCTGCAGTAGCTGGAGGCACCAATG-3' using QuickChange XL Mutagenesis kit(Stragenen,La Jolla,CA, USA). The intergrity of the construct was confirmed DNA sequencing. C-terminal myc-tagged LDLR, cIAP1, TRAF2 in CMV6-based mammalian expression vector were obtained from Origene, Inc. PCR was used to generate deletion constructs of myc-tagged c-IAP1, containing only BIR1 or BIR2 or BIR3 or Ring domains. The PCR products were cloned in frame to the pCMV6 entry clone(Origene, USA) with Sgf1 and Mlu1 restriction sites. The successful creation of all constructs was confirmed by DNA sequencing. HA-tagged ubiquitin plasmid was purchased from Addgene(Cambridge, MA). All the transfection were done on T-Rex293 cells or HepG2 cells using superfect(Qiagen, UK) with 1-2ug DNA.

The antibodies used were a rabbit antibody directed against amino acids 184-196 of human LDLR(Research Diagnostics Inc.) and a rabbit antibody against PCSK9(Cayman). Other antibdies, including c-IAP1, Traf2, PDIA4 and DNJA1 were from Abcam (Cambridge, UK). UGGT-1 antibody is from Santa Cruz Biotechnology, CA, USA). Anti-Stub1 antibody and anti-myc tag antibody are from Millipore, UK.

All transfections were done with either T-Rex 293 cells and HepG2 cells using Superfect(Qiagen, UK). The methods of whole cell extract and western blots were carried out as described[27]. Western blot densitometry was carried out using the VisionworksLS software (UVP, Cambridge, UK). All data were analysed by GB-Stat V5.4.4 program(written by Dr. Philip Friedman, Howard University) using student t-Test(two-tailed).

Generation of stable cell lines, immunoprecitation and protein purification.

The PCSK9 expression plasmids were co-transefected with pTK-hygromycin (BD Clontech) into T-Rex 293 cells. 48h later, cells were subjected to selection with 50μg/ml of hygromycin. The positive clones which over expressed flag-tgged PCSK9 were detected by

western blot. Flag-tagged protein immunoprciptation was carried out by using Flag Tagged protein immunoprecipitation kit(FlagIPT-1, Sigma) with final elution using 3xflag peptide(final concertation 150ng/µl 3xflag peptide). C-myc tagged protein was immunopreciptaed with Pierce Mammalian c-Myc Tag IP/Co-IP kit(Thermo scientific, UK).

PCSK9 proteins from stable expressed 293 cell lines, including wild-type, D374Y and S127R PCSK9 were purified by using FLAG-M Purification Kit(Sigma, UK) following the manufacture's instruction. The final elution were concentrated with vivaspin 6 (Artoris Stedim biotech, UK) and dialysis against PBS using Slid-A-lyzer mini dialysis unit(Thermo scientific, UK). Protein concentration was measured using Bio-Rad Protein Assay kit, Cat:500-0006, Bio-Rad. UK). The protein purity was determined by SDS-PAGE and visualized by Coomassie Blue stain with over 90% purity.

Shotgun analysis of the FLAG-tagged PCSK9 and associated proteins complex samples.

A stable expressed flag-tagged PCSK9 cell line was grown in DEME medium with 10% FBS. The flag-tagged PCSK9 protein was isolated using the flag-immunopereciptation kit(Sigma) following by elution with the Flag-peptide 3xflag peptide(final concertation 150ng/ul 3xflag peptide). The elution samples were then send to the Protein Analysis Facility, Center for Integrative Genomics, Faculty of Biology and Medecine, University of Lausanne, Switzerland , where protein mixtures were separated by limited electrophoresis after which 3-5 molecular weight regions are cut and digested. Analysis is performed by LC-MS/MS on every fraction. The resulting collections of spectra are pooled for every sample before database search. The Lists of identified proteins for each sample with their scores are subjected to statistical validation and aligned for comparison with Scaffold program. A negative control cell line, T-Rex-293 cells transfected with the empty vector pcDNA3.1 was used for background subtraction.

Surface Plasmon Resonance

Surface Plasmon Resonance studies on the binding of c-IAP1 to wild-type PCSK9 were carried out on a Biacore T100, essentially following the manufacturer's recommended conditions (Biacore, Uppsala, Sweden). Briefly, recombinant human c-IAP1 protein(R&D, UK) was immobilized to CM5 chips (Biacore) with surface densities of 100 resonance units (RU) with amine-coupling kit (BIAcore) using immobilization wizard. Purified recombinant wild-type PCSK9(isolated from whole cell extract) diluted in the Hepes buffer(Hepes, pH7.4, 150mM NaCl and 0.1 mM $CaCl_2$) were injected in a concentration range of 12.5-100nM at a flow rate of 30 µl/min. Regeneration buffer is 10mM glycine?HCl(pH2.5). Association and dissociation data from all concentrations were fit globally using T100 evaluation software with 1:1 Langmuir binding model.

RNA interference.

c-IPA1 siRNA *mix(*pGB-*cIAP1* siRNA, Biovision Research Products, CA, USA*)* or control siRNA *(*pGB-control) were transfected into a stable wild-type PCSK9 overexpress cell line using the superfect reagent(Qiagene, UK). After 48h, the cells were subjected G148 selection to obtain the stable cell lines with nearly 100% knock-out the c-IAP1 protein by western blot analysis.

Degradation of the LDLR in the presence of MG132 or Ammonium Chloride

HepG2 cells were transient transfected with plasmid expressing PCSK9-D374YpcDNA3 or empty vector(pCDNA3.1). 24h after transfection, the fresh DMEM culture medium(without serum) was added for 16h. Then cells were treated with 10 μM of proteasome inhibitors MG132 or the lysosomal inhibitor Ammonium Chloride (NH4Cl) for 4h in serum-free DMEM medium. Cells were washed twice in PBS and harvested for western blot analysis as previous described[27].

Immunofluorescence Staining and Image Analysis

PCSK9 overexpressed stable cell were grown to 50% confluence in coverslips in 6-well tissue culture plate, washed twice with PBS, and fixed with 4% paraformaldehyde in PBS for 10 min. The cells were then washed with PBS and permeabilized in PBS containing 0.1% Triton X-100 and 5% normal horse serum for 30 min. For detection of flag-tagged PCSK9, the cells were stained with a 1:1000 dilution of anti-FLAG M2 antibody (Sigma) and a 1:500 dilution of anti-mouse Alex 488-conjugated secondary antibody (Invitrogen). For detection of c-IAP1, the cells were stained with anti-c-IAP1 rabbit antibody (Abcam, Cambridge, UK) at a 1:500 dilution and a 1:500 dilution of anti-rabbit cy3 secondary antibody (Sigma). After washing three times in PBS, images were captured on a confocal microscope (Leica TCS SP, Germany).

***In vivo* ubiqutination analysis**

pcDNA.1Empty vector and plasmid containing HA-tagged ubiquitin gene(HA-ubiqutin , Addgene(Cambridge, MA) were co-transefected with PCSK9 expression vector with or without the pcDNA6 –c-IAP1 plasmids into T-REX 293 cells. 24h later, the lysates were immunopreciptation with anti-ubiquitin anibody(Ubiquitin enrichment kit, Thermo Fisher) analyzed by Western blotting with anti-PCSK9 antibody.

***In vitro* Ubiquitanation Assay**

Purified Flag-tagged wild-type PCSK9(1-4μg), or D374Y-PCSK9(1 μg) or S127R-PCSK9(1-4μg) and recombinant c-IAP1(1μg , R&D system, UK) were incubated in a reaction buffer(50mM Tris-HCl(pH7.5), 5mM $MgCl_2$, 2mM ATP, 0.6mM DTT) with recombinant rabbit E1(100ng), UbcH5b(250ng) and ubiquitin(6ng) at 37^{o}C for 1hr. The resulting mixtures were analyzed by immunoblotting using anti-PCSK9 antibody.

***In vitro* ubiquitination function assay on the purifed PCSK9 proteins**

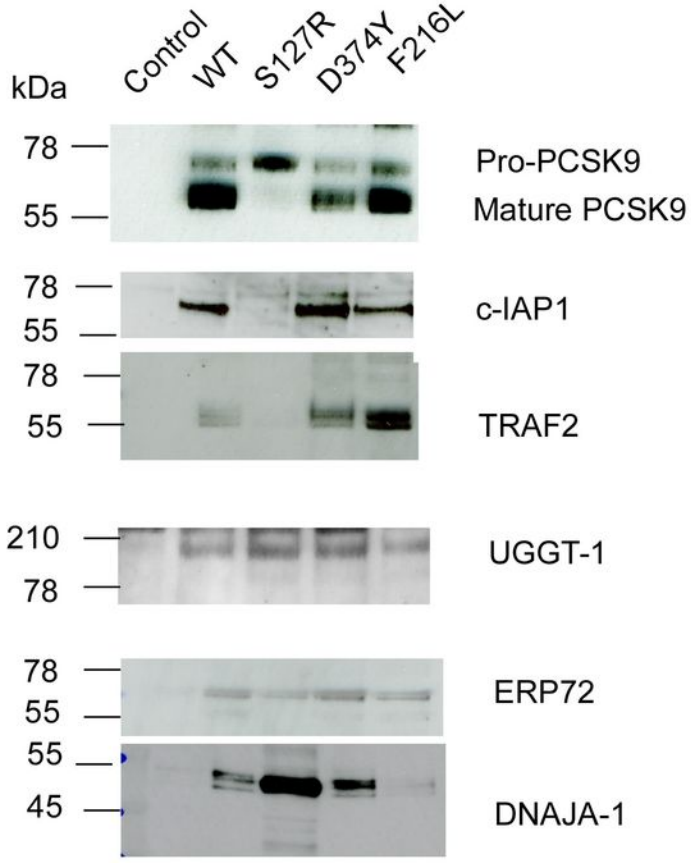
HepG2 cells were seeded in 6 well tissue plates at concentration of $2x10^5$ cells/ml. After 24h, the medium was replaced with DMEM medium without FBS and added the purified D374Y protein(1μg/ml) or wild-type PCSK9 (4μg/ml). In the control experiments, only c-IAP1 ubiquitination buffer was added in tomedium without PCSK9 protein. After 16h, cells was washed twice in PBS and harvested for western blot analysis as previous described[27].

LDL uptake assay by flow cytometry

HepG2 cells were seeded in 6 well tissue plates at concentration $2x10^5$ cells/ml. After 24h, the medium was replaced with DMEM medium without FBS and purified D374Y(1μg/ml PCSK9=13.4 nM) or 1-4μg/ml purified wild-type PCSK9. After 16h, the medium was replaced with fresh medium containing 10μg/ml Bopipy FL LDL(Invitrogen , UK). The cells

were incubated for 4 h at 37^0C. The cells were washed with PBS and trypsinized and resuspended in FAC Flow solution. At lease 10,000 cells were analyzed on a FACSCalibur (BD Biosciences. UK) using Cellquest and FlowJo software.

Gene Symbols	Gene Names	Experiment 1		Experiment 2		Experiment 3	
		Control	unique spectra	Control	unique spectra	Control	unique spectra
PCSK9	proprotein convertase subtilisin/kexin type 9	3	1466	2	1171	4	1128
UGGT1	UDP-glucose glycoprotein glucosyltransferase 1	0	9	0	12	0	15
PDIA4(ERP72)	protein disulfide isomerase family A, member 4	0	7	0	5	0	7
SLC25A1	solute carrier family 25 member 1	0	6	0	2	0	6
DNAJA1	DnaJ (Hsp40) homolog, subfamily A, member 1	0	12	0	10	0	7
CLGN	Calmegin	0	2	0	2	0	2
DNAJA2	DnaJ (Hsp40) homolog, subfamily A, member 2	0	2	0	3	0	4
c-IAP1(BIRC2)	cellular inhibitor of apoptosis protein 1	0	7	0	9	0	8
TBB6	tubulin, beta 6	0	5	0	2	0	3
TRAF2	TNF receptor-associated factor 2	0	4	0	3	0	3
SLC25A10	solute carrier family 25. member 10	0	6	0	3	0	6
DNAJB11	DnaJ (Hsp40) homolog, subfamily B, member 11	0	5	0	4	0	3
CDIPT	CDP-diacylglycerol-inositol 3-phosphatidyltransferase	0	3	0	6	0	4
DNAJC10	DnaJ (Hsp40) homolog, subfamily C, member 10	0	6	0	3	0	6
DNAJA3	DnaJ (Hsp40) homolog, subfamily A, member 3	0	2	0	3	0	3
RHOT1	ras homolog gene family, member T1	0	2	0	3	0	4
AGK	Acylglycerol kinase lipid kinase	0	2	0	3	0	2
HSPB1	heat shock 27kDa protein 1	0	4	0	5	0	2
RCN1	reticulocalbin 1	0	3	0	4	0	5
Ribosomal L28	partial ribosomal protein L28 variant	0	2	0	6	0	5
Stub1	STIP1 homology and U-box containing protein 1	0	2	0	3	0	2
PGRC1	progesterone receptor membrane component 1	0	2	0	2	0	2
SLC25A12	Solute carrier family 25 member 12	0	2	0	5	0	4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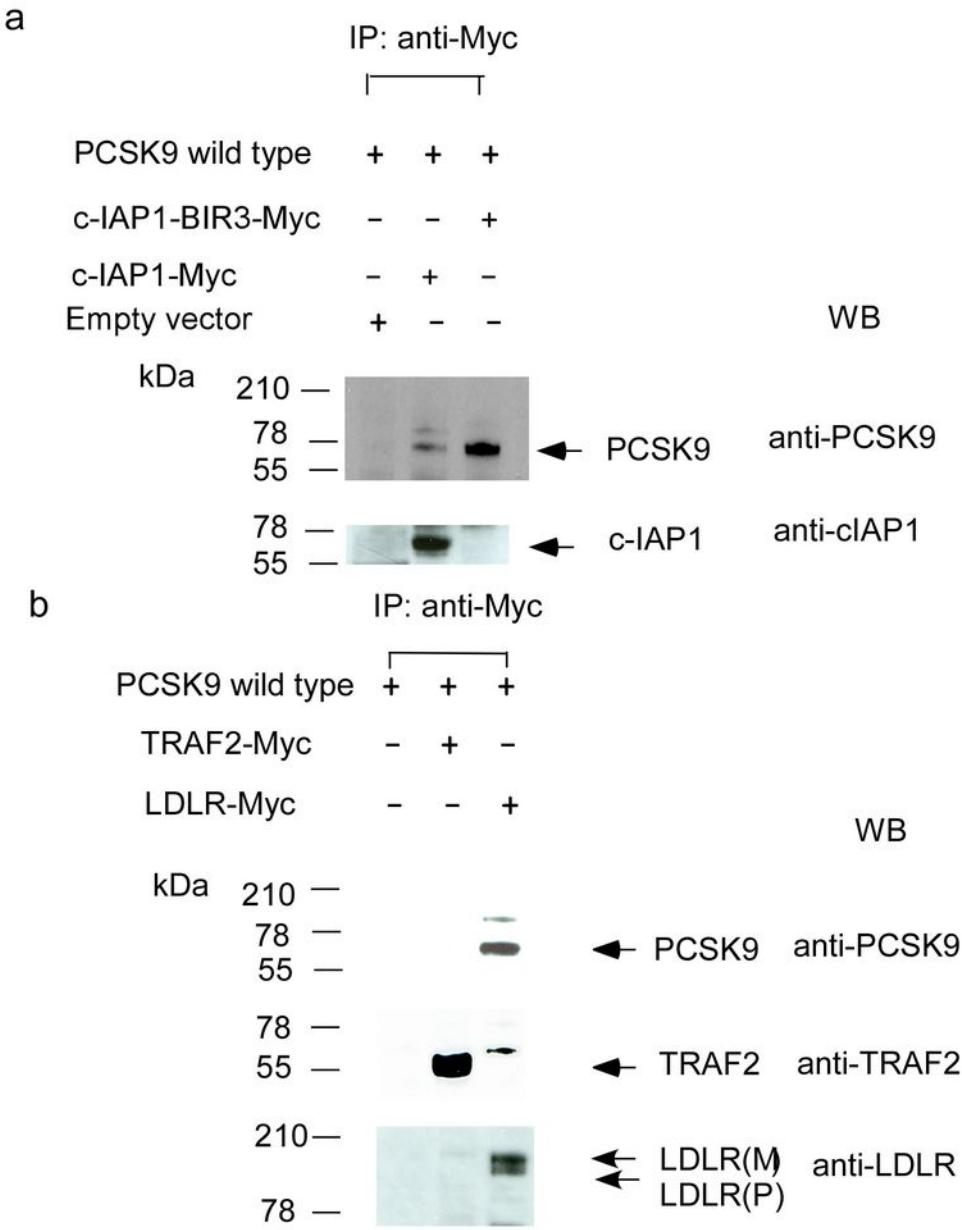
ERP72
55
45
DNAJA-1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Empty vector + − −
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kDa
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78
55
PCSK9
anti-PCSK9
78
55
c-IAP1
anti-cIAP1
b
IP: anti-Myc
PCSK9 wild type + + +
TRAF2-Myc − + −
LDLR-Myc − − +
WB
kDa
210
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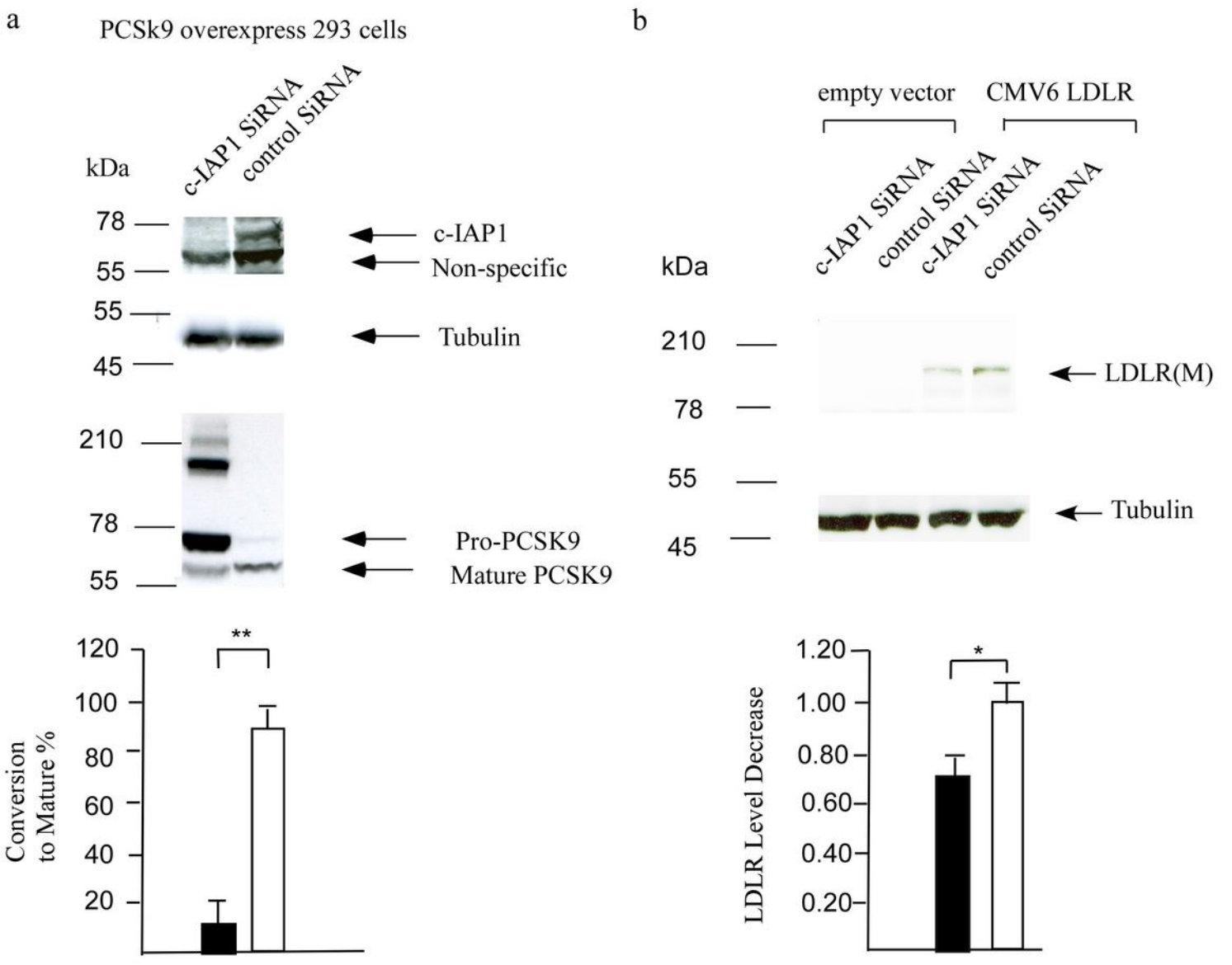
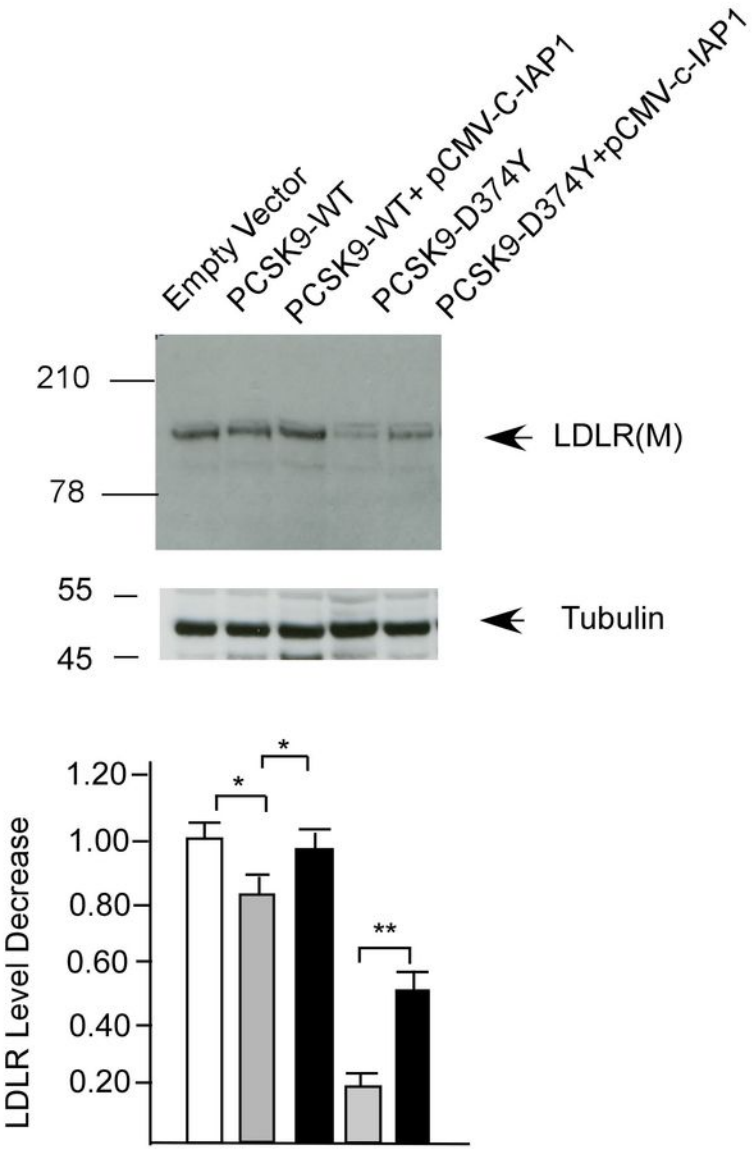
LDLR(P)
anti-LDLR


a
PCSk9 overexpress 293 cells
c-IAP1 SiRNA
control SiRNA
kDa
78
55
55
45
210
78
55
c-IAP1
Non-specific
Tubulin
Pro-PCSK9
Mature PCSK9
**
120
100
80
60
40
20
Conversion to Mature %
b
empty vector
CMV6 LDLR
c-IAP1 SiRNA
control SiRNA
c-IAP1 SiRNA
control SiRNA
kDa
210
78
55
45
LDLR(M)
Tubulin
*
1.20
1.00
0.80
0.60
0.40
0.20
LDLR Level Decrease


Empty Vector
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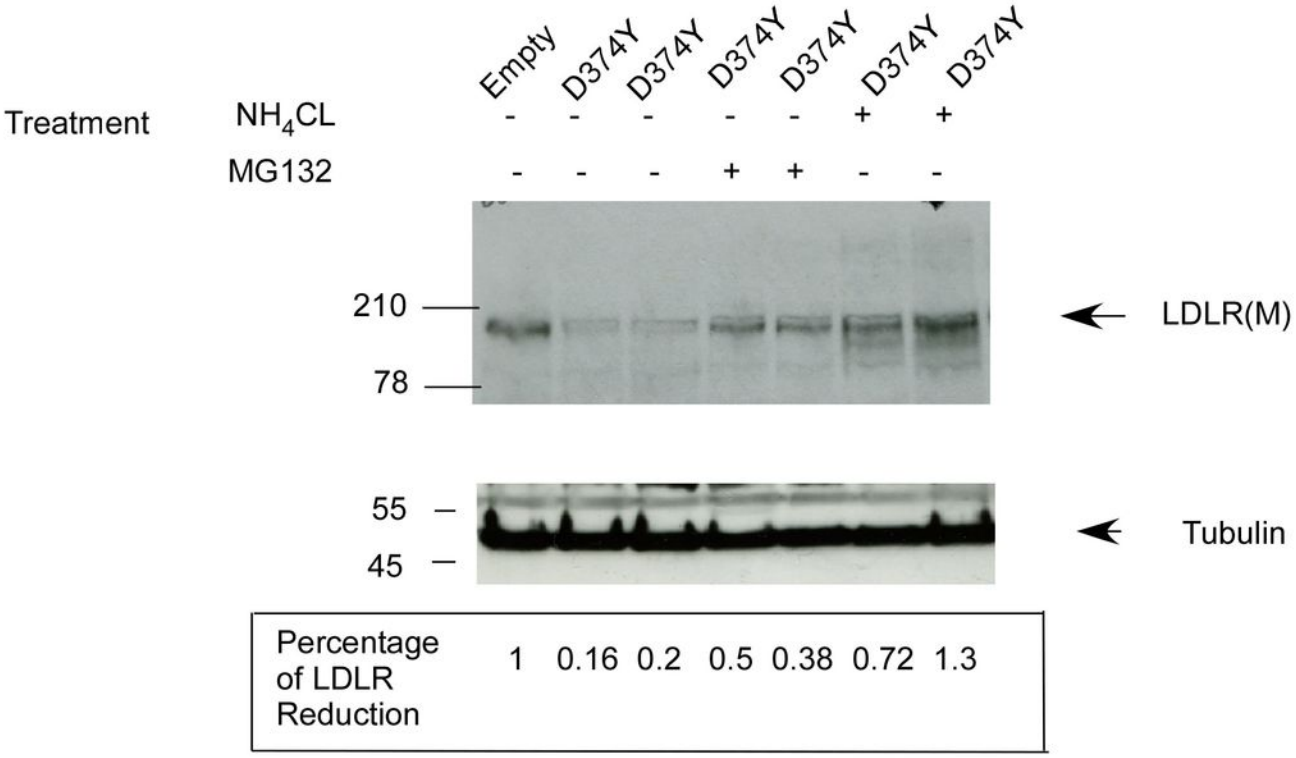
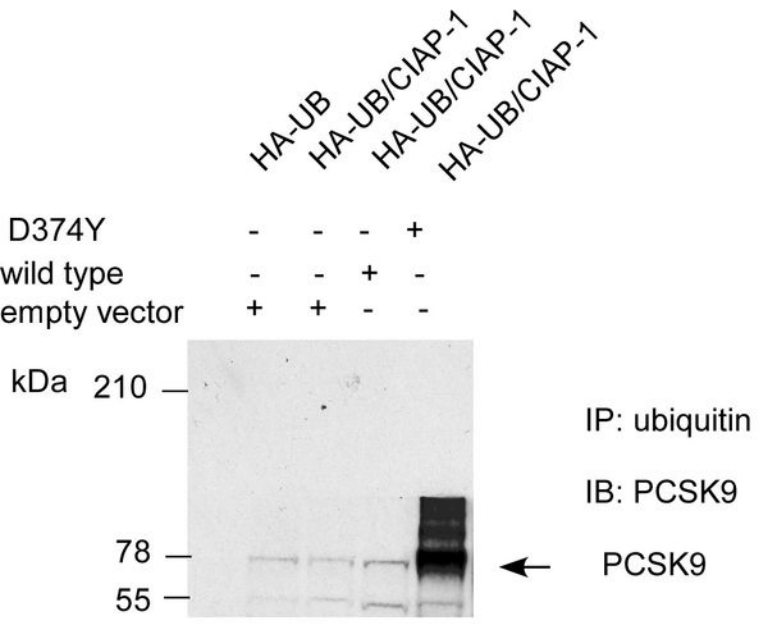
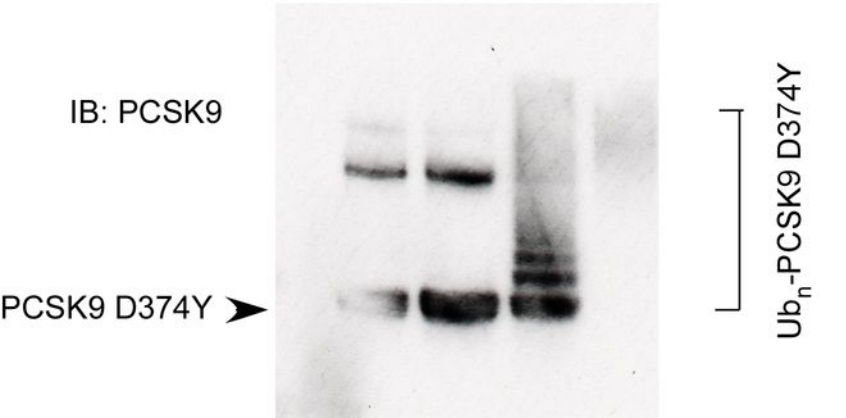
PCSK9-WT+ pCMV-C-IAP1
PCSK9-D374Y
PCSK9-D374Y+pCMV-c-IAP1
210
78
LDLR(M)
55
45
Tubulin
LDLR Level Decrease
1.20
1.00
0.80
0.60
0.40
0.20
*
*
**

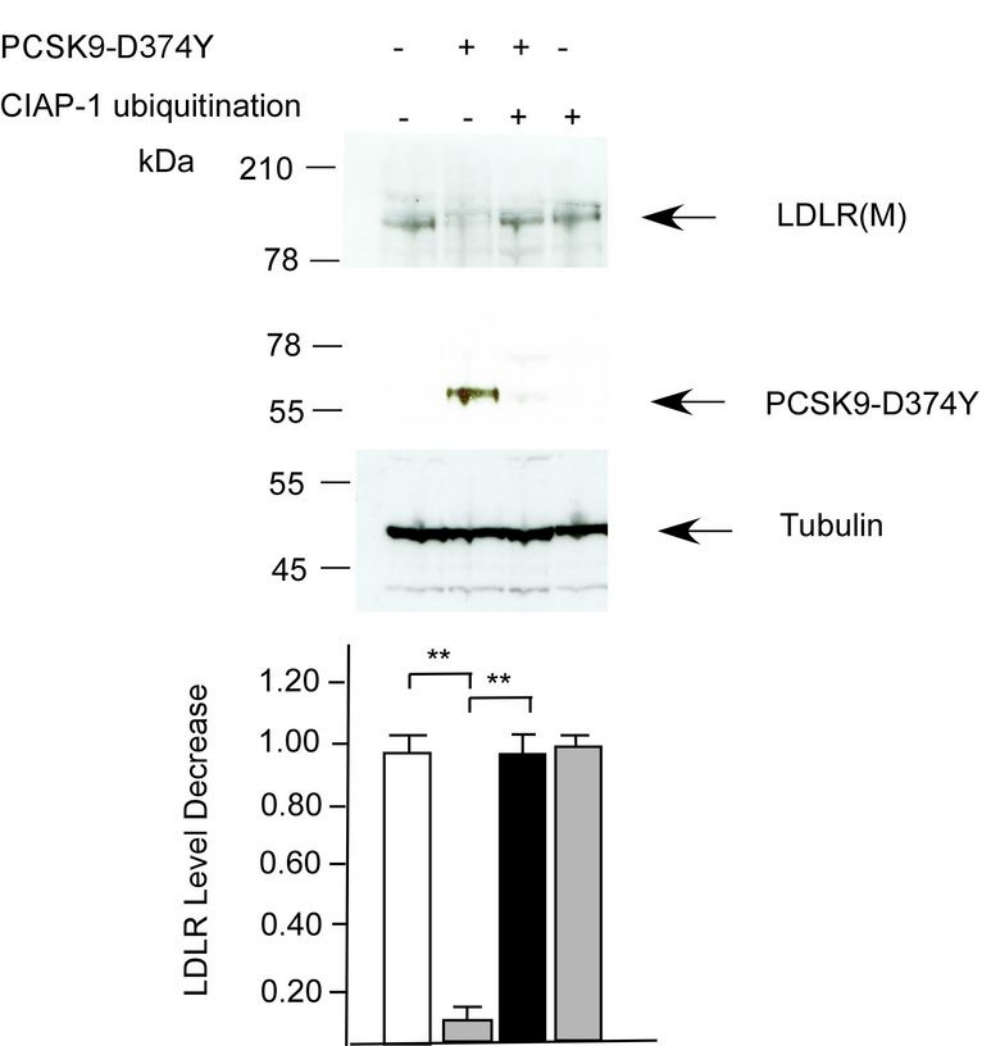


Percentage of LDLR Reduction	1	0.16	0.2	0.5	0.38	0.72	1.3


HA-UB
HA-UB/CIAP-1
HA-UB/CIAP-1
HA-UB/CIAP-1
D374Y - - - +
wild type - - + -
empty vector + + - -
kDa
210
78
55
IP: ubiquitin
IB: PCSK9
PCSK9

c-IAP1	-	-	+	+
D374Y	+	+	+	-
E1 E2	-	+	+	+

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- + + -
CIAP-1 ubiquitination
- - + +
kDa
210
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LDLR(M)
78
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PCSK9-D374Y
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Tubulin
LDLR Level Decrease
1.20
1.00
0.80
0.60
0.40
0.20
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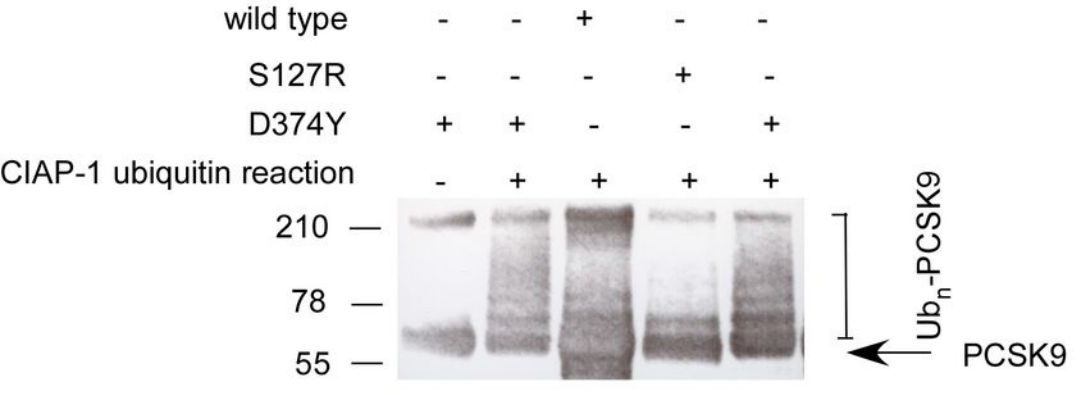
wild type - - + - -
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D374Y + + - - +
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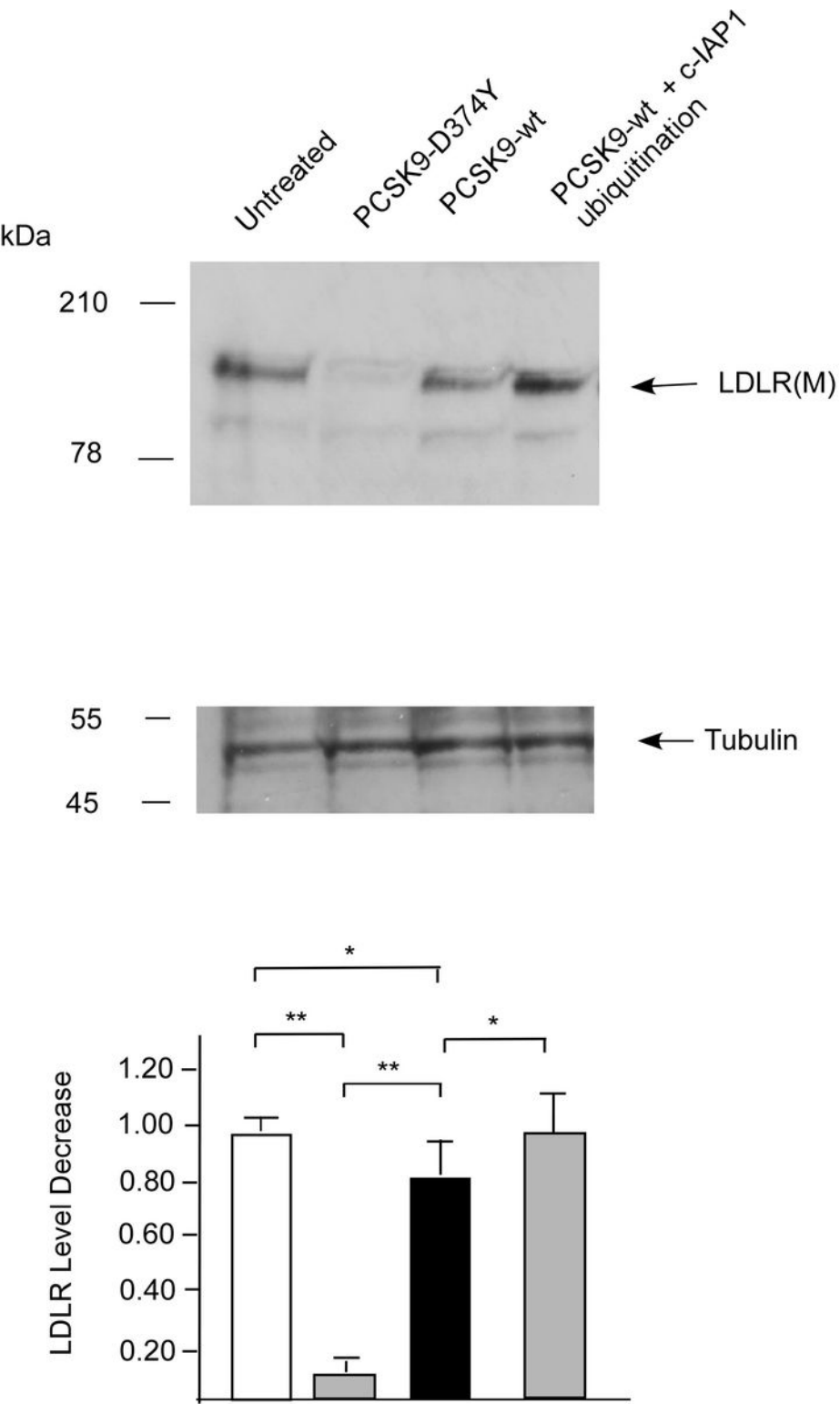
Ub$_n$-PCSK9
PCSK9


Untreated
PCSK9-D374Y
PCSK9-wt
PCSK9-wt + c-IAP1 ubiquitination
kDa
210
78
LDLR(M)
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45
Tubulin
LDLR Level Decrease
1.20
1.00
0.80
0.60
0.40
0.20
*
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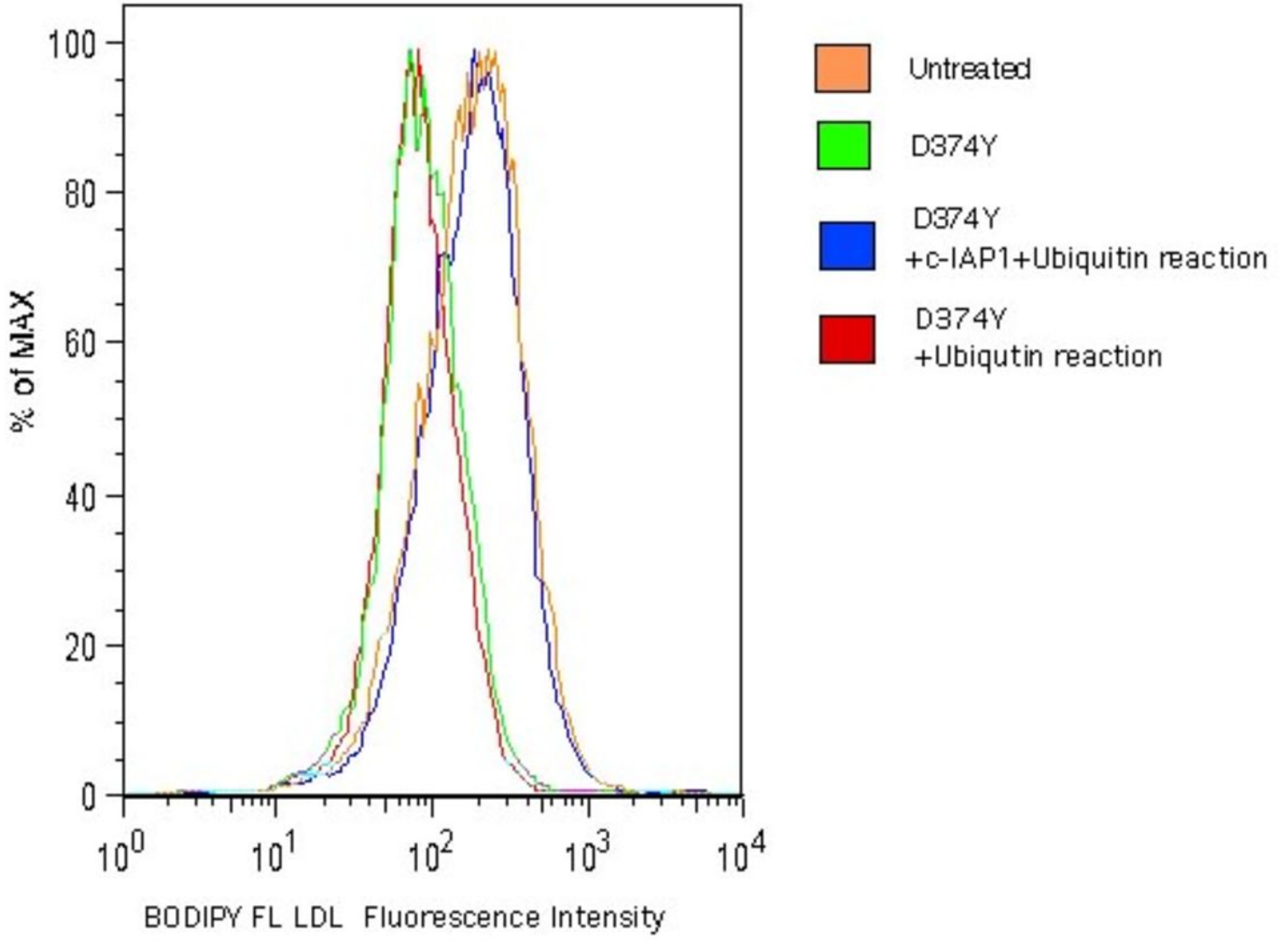
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40
20
0
% of MAX
10^0
10^1
10^2
10^3
10^4
BODIPY FL LDL Fluorescence Intensity
Untreated
D374Y
D374Y
+c-IAP1+Ubiquitin reaction
D374Y
+Ubiqutin reaction