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Molecular biology of PCSK9: its role in LDL metabolism

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Abstract

Proprotein convertase subtilisin-like kexin type 9 (PCSK9) is a newly discovered serine protease that destroys low density lipoprotein (LDL) receptors in liver and thereby controls the level of LDL in plasma. Mutations that increase PCSK9 activity cause hypercholesterolemia and coronary heart disease (CHD); mutations that inactivate PCSK9 have the opposite effect, lowering LDL levels and reducing CHD. Although the mechanism of PCSK9 action is not yet clear, the protease provides a new therapeutic target to lower plasma levels of LDL and prevent CHD.

A new regulator of cholesterol trafficking

Just when we thought all of the major molecular players in low density lipoprotein (LDL) metabolism had been identified, another burst onto the scene. In 2003, four groups reported the characterization of a new member of the proprotein convertase gene family: a transcript encoding a novel proprotein convertase (see Glossary) previously shown to be up-regulated during apoptosis in neuronal cells [1]. The link between proprotein convertase subtilisinlike kexin type 9 (PCSK9) and cholesterol metabolism rapidly followed with the discovery that selected mutations in the gene cause autosomal dominant hypercholesterolemia [2] and the observation that PCSK9 was regulated by cholesterol [3,4].

The discovery of PCSK9 has provided new insights into the metabolism of LDL and into the determinants of plasma LDL-cholesterol (LDL-C) levels. Here, we review the current understanding of the cell biology, physiology and genetics of PCSK9, and its implication for the treatment of hypercholesterolemia and coronary heart disease (CHD).

Structural features of PCSK9

PCSK9 is the ninth member of the subtilisin family of kexin-like proconvertases to be identified [5]. Like other family members, the signal sequence (amino acids 1–30) in PCSK9 is followed by the prodomain (amino acids 31–152) and catalytic domain [1] (Figure 1). PCSK9 lacks a classical P domain [6], which is required for folding and regulation of protease activity in the other proprotein convertases [7]; rather, the catalytic domain is followed by a 279-amino acid cysteine- and histidine-rich C-terminal region. The protein is synthesized as a ~72-kDa precursor that undergoes autocatalytic cleavage between the prodomain and catalytic domain [1,6]. The prodomain (~14 kDa) remains bound to the mature protein (63 kDa) as it traverses the secretory pathway. The site of intramolecular cleavage in PCSK9 (Val-Phe-Ala-Gln↓Ser-Ile-Pro) differs from most other proconvertases in that cleavage does not occur after a basic residue [5]. Obtaining a robust *in vitro* assay for PCSK9 activity has proved difficult and little

is known about the requirements for catalytic activity. In contrast to other proprotein convertases, autocatalytic cleavage of PCSK9 does not require calcium [8]. Mutagenesis studies have revealed that the sequence required for autocatalytic cleavage is degenerate, which has further complicated efforts to identify the natural substrate(s) of PCSK9 [6]. The mature PCSK9 and the associated prodomain both undergo tyrosine sulfation in the late Golgi complex before secretion [8,9]. Sulfation of tyrosine residues in other proteins enhances protein–protein interactions, but the role of this post-translational modification in PCSK9 has not been defined [10].

Gain-of-function mutations in PCSK9

Selected missense mutations in PCSK9 cause hypercholesterolemia

Plasma levels of LDL-C, the major cholesterol-carrying lipoprotein in humans, are determined by the relative rates of LDL production and clearance. Prior to 2003, only two autosomal dominant forms of hypercholesterolemia were known: familial hypercholesterolemia (FH), caused by mutations in the gene encoding the LDL receptor (*LDLR*), and familial defective Apo-B100 (FDB), caused by mutations in ApoB-100 (*APOB*) that disrupt binding of LDL to LDLR [11]. Both of these disorders decrease LDLR-mediated endocytosis in the liver, the major route of clearance of circulating LDL. Chronic elevations in plasma LDL-C levels in FH and FDB result in the accumulation of cholesterol in tissues (xanthomas) and in arteries, especially the coronary arteries (coronary atherosclerosis).

Initially, three missense mutations in the gene encoding PCSK9 were identified in families with a clinical phenotype resembling FH and FDB: S127R, F216L [2] and D374Y [12,13]. Subsequently, additional missense mutations were identified in hypercholesterolemic subjects (Figure 1). Mutations in *PCSK9* account for a much smaller percentage of dominant hypercholesterolemia than do mutations in *LDLR* and *APOB* [14–17]. Proband heterozygous for mutations in both *LDLR* and *PCSK9* have plasma levels of LDL that are ~50% higher than relatives with either mutation alone [18]. The only clinical findings that have been reported in subjects with hypercholesterolemia due to mutations in *PCSK9* are those related to lipoprotein metabolism, suggesting that PCSK9 functions primarily in the cholesterol metabolic pathway.

PCSK9 expression and LDLR levels

Most enzyme defects cause recessive disorders. The observation that *PCSK9* mutations cause dominant hypercholesterolemia suggests that the mutations confer a gain-of-function [2], either by increasing the normal activity of PCSK9 or by conferring a new activity to the protein. The first experimental evidence for a gain-of-function mechanism came from studies in which wild-type and mutant PCSK9 (S127R and F216L) were expressed at high levels in the livers of mice; hepatic LDLR protein levels fell dramatically in the mice receiving either the wild-type or mutant PCSK9 [8,19,20]. No associated reductions in LDLR mRNA levels were observed. Thus, overexpression of PCSK9, whether mutant or wild type, reduces LDLRs through a post-transcriptional mechanism.

In contrast to the *in vivo* experiments, expression of PCSK9 in cultured cells has variable effects on LDLRs. In some cell types, such as human hepatoma cells (HepG2 and HuH7) or human embryonic kidney cells (HEK-293 cells), expression of PCSK9 dramatically reduces LDLR levels [8,9,20,21]. In other cell types, including fibroblasts, Chinese hamster ovarian (CHO-K1), monkey kidney cells (COS7) and rat liver (McArdle RH7777) cells [16,20,22,23], PCSK9 does not seem to affect LDLR expression. Cells unresponsive to PCSK9 might lack a factor required for PCSK9 function. Alternatively, either the kinetics or mode of LDLR internalization might differ in unresponsive and responsive cells.

Site of action of PCSK9 in cells

The intracellular itineraries of PCSK9 and the LDLR are similar but their paths diverge at the cell surface (Figure 2). The LDLR remains associated with the cell membrane, whereas PCSK9 is rapidly and efficiently secreted into the medium [1]. PCSK9 is also secreted *in vivo*, presumably by the liver, and is present in human plasma [9,23,24]. The major cellular site at which PCSK9 acts has not been established. Stable overexpression of PCSK9 in HepG2 cells does not affect the synthesis or trafficking of LDLR out of the ER, and proteasome inhibitors do not interfere with PCSK9-mediated reduction in LDLR [25]. PCSK9 overexpression increases degradation predominantly of the mature, glycosylated form of the LDLR [25]. Addition of brefeldin A (a fungal toxin that causes disassembly of the Golgi complex) prevented PCSK9-induced degradation of the LDLR. These data suggest that PCSK9 might promote degradation of the LDLR as it migrates from the ER to the cell membrane [25].

Alternatively, PCSK9 might remain inactive while it migrates through the secretory pathway and might act on the LDLR only after it is secreted. The prodomain of PCSK9 remains tightly attached to the mature protein during its secretion, presumably inhibiting catalytic activity [1,8,23]. In other proprotein convertases, the pro-segment undergoes a secondary proteolytic processing event either in the Golgi or after secretion that relieves the inhibition and unmasks enzymatic activity [5]. The first experimental evidence that PCSK9 might function extracellularly came from the finding that addition of conditioned medium containing PCSK9 [26] or of purified PCSK9 [23] to the medium of HepG2 cells reduces the number of cell-surface LDLRs. The LDLR can be co-immunoprecipitated with PCSK9 after PCSK9 is added exogenously to cells, implying a physical association between the two proteins [23]. The presence of ARH (autosomal recessive hypercholesterolemia), an endocytic adaptor protein required for LDLR internalization, is necessary for PCSK9-mediated degradation of LDLR. In the absence of ARH, the LDLR and PCSK9 fail to be internalized and no change in LDLR number is observed [23].

The behavior of LDLR in cultured cells might not accurately reflect conditions *in vivo*. To address this possibility, we carried out parabiosis experiments in transgenic PCSK9 mice connected to wild-type mice [23]. Comparison of liver biopsies before and after parabiosis revealed a dramatic reduction in LDLR protein in livers of the recipient wild-type mice [23]. Thus, exogenous PCSK9 can reduce LDLR number *in vivo* as well as in cultured cells.

A potential artifact of the cell-culture studies, adenoviral studies in the liver and the parabiosis studies relates to the superphysiological amounts of PCSK9 used to promote LDLR degradation. Overexpression might promote an interaction between PCSK9 and the LDLR in a cellular compartment that does not usually occur. To address this issue, the circulating levels of PCSK9 in human plasma were measured and found to range from ~50 to ~600 ng ml⁻¹. The concentration of purified PCSK9 needed to promote LDLR degradation (~500 ng ml⁻¹) falls within this range [23].

The results of these studies suggest several possible mechanisms by which PCSK9 might promote LDLR degradation. PCSK9 could bind to the LDLR in a catalytically inactive state on the cell surface and then become active in the acidic environment of the endosome, resulting in LDLR degradation. Alternatively, by binding to the LDLR, PCSK9 might interfere with the normal recycling of the LDLR after internalization, redirecting the LDLR to lysosomes rather than back to the cell surface (Figure 2). If the latter hypothesis is correct, the action of PCSK9 on LDLR might not involve catalytic activity.

Is the catalytic activity of PCSK9 required for its function?

Answering this question has proved to be more challenging than expected. PCSK9 in which the catalytic histidine has been substituted to an alanine does not undergo autocatalytic cleavage and fails to exit the ER [1]. LDLR levels do not change when a catalytically dead enzyme is expressed in liver [20] or in cultured liver cells [16,25]. Thus, autocatalytic activity seems to be required for PCSK9 to leave the ER, but is it required for PCSK9-stimulated LDLR degradation? PCSK9 might cleave the LDLR directly or might function indirectly by clipping another protein that promotes degradation of the LDLR. The possible involvement of protein (s) other than PCSK9 is suggested by the finding that PCSK9 fails to promote degradation of LDLRs in some immortalized cell lines (as discussed earlier). Studies in which the actions of PCSK9 can be examined independently of its trafficking will be required to reveal the role of catalytic activity in PCSK9-associated degradation of the LDLR.

Loss-of-function mutations in PCSK9

Nonsense and missense mutations in PCSK9 cause hypocholesterolemia

To test the hypothesis that loss-of-function mutations in PCSK9 would cause hypocholesterolemia by increasing LDL clearance, Cohen *et al.* [27] sequenced the coding region of *PCSK9* in individuals with the lowest plasma levels of LDL-C (<5th percentile) in a population-based sample. Surprisingly, one out of every 50 African-Americans in the population had a nonsense mutation in *PCSK9* (either Y142X or C679X; Figure 1) that lowered LDL-C levels by ~40% [27]. Subsequently, additional *PCSK9* mutations associated with a reduction in plasma levels of LDL-C have been found, including in-frame deletions and missense mutations [26,28,29] (Figure 1).

Three loss-of-function mutations in *PCSK9* – Y142X and C679X in African-Americans, and R46L in Caucasians – were sufficiently common to address a more general question regarding the relationship between plasma levels of LDL-C and CHD: do life-long reductions in plasma levels of LDL-C confer greater protection from CHD than cholesterol-lowering therapies instituted later in life? In a large biracial 15-year prospective study, nonsense mutations in *PCSK9* that reduced LDL-C levels by 28% decreased the frequency of CHD (defined as myocardial infarction, coronary death or coronary revascularization) by 88% [30]. In the same study, Caucasians with a R46L allele had a 50% reduction in CHD despite having a mean reduction in LDL-C levels of only 15%. The reductions in CHD associated with these mutations were greater than those observed in more short-term (typically five-year) clinical trials employing statins [31]. These data indicate that earlier intervention might magnify the clinical efficacy of cholesterol-lowering therapy by attenuating the development and progression of atherosclerosis.

The mechanisms by which loss-of-function mutations in the gene encoding PCSK9 reduce plasma cholesterol levels were investigated in mice in which *Pcsk9* was inactivated. These animals have increased hepatic LDLR protein levels, accelerated LDL clearance and reduced plasma cholesterol levels [32]. Thus, PCSK9 tonically suppresses LDLR levels, thereby limiting LDLR-mediated uptake of lipoproteins.

PCSK9 and lipoprotein synthesis

An alternative mechanism by which *PCSK9* mutations might alter plasma LDL levels is by influencing the rate of secretion of ApoB-100-containing lipoproteins from the liver. Ouguerram *et al.* [33] reported that two individuals who were heterozygotes for a gain-of-function mutation in *PCSK9* (S127R) had ApoB production rates that were threefold that of controls. LDL production rates in the *PCSK9* heterozygotes were comparable to those observed in five FH heterozygotes studied concurrently. Because PCSK9 overexpression in *Ldlr*^{-/-} mice

does not increase plasma cholesterol levels [19,20,23], the combined data indicate that, if PCSK9 does alter ApoB secretion, it is likely to be a secondary event related to the reduction in LDLRs.

Very low density lipoprotein (VLDL) is the major vehicle for the secretion of triglycerides from the liver. If PCSK9 causes hypercholesterolemia by increasing VLDL production, it would be expected that loss-of-function mutations in *PCSK9* would reduce VLDL secretion. Mutations that interfere with VLDL secretion and cause hypocholesterolemia, such as those in *MTP* (microsomal triglyceride transfer protein) and *APOB*, also result in the accumulation of triglycerides in the liver [34,35]. No increase in hepatic triglyceride content was observed in 20 African-Americans heterozygous for nonsense mutations in *PCSK9* [29]. These results do not support the concept that changes in LDL levels associated with mutations in *PCSK9* are primarily due to effects on VLDL synthesis. It remains possible that *PCSK9* mutations do reduce VLDL secretion but that the magnitude of the reduction is insufficient to affect hepatic triglyceride content.

Structure–function relationship of PCSK9

Functional analysis of the naturally occurring mutations in *PCSK9* has provided insights into the mechanism of action of PCSK9. The mutations in *PCSK9* can be separated into five groups according to their effects on synthesis and secretion of the PCSK9 protein (Table 1).

Surprisingly, it is difficult to distinguish the phenotypic effects of some gain-of-function mutations from some loss-of-function mutations in cultured cells. For example, both S127R (gain-of-function) and L253F (loss-of-function) in *PCSK9* impair autocatalytic cleavage and secretion. Only one allele (Y142X) produces no detectable protein (Class 1 mutation, null alleles), presumably owing to nonsense-mediated mRNA decay [24]. Three mutations in the prodomain (Δ 97, G106R and S127R) and one in the catalytic domain (L253F) interfere with the autocatalytic cleavage and are, therefore, Class 2 mutations (processing defective). The L253F mutation resides near the catalytic triad and might disrupt the catalytic site [24]. Inasmuch as autocatalytic cleavage of PCSK9 is required for export of the protein out of the ER, all Class 2 mutations also delay transport of PCSK9 from the ER to the cell surface (Class 3). The most common nonsense mutation (C679X) in *PCSK9*, which truncates the protein by 14 amino acids, is also a Class 3 mutation. The mutant protein is cleaved normally but is misfolded and retained in the ER [9,24].

Some loss-of-function mutations might affect the stability of PCSK9 (Class 4 mutations). In cultured cells, a small fraction of the PCSK9 synthesized undergoes a second, membrane-bound furin-mediated cleavage event [9]. Benjannet *et al.* [9] suggested that some loss-of-function mutations, such as A443T, might be more susceptible to furin cleavage. Further studies to assess the half-life of the protein will be required to determine if furin cleavage is physiologically relevant.

Mutations in *PCSK9* could also affect the affinity of the protein for the LDLR, or other proteins that promote receptor degradation (Class 5 mutation). A gain-of-function mutation, D374Y, binds more avidly to the LDLR and its activity in reducing LDLR protein is approximately tenfold that of the wild-type protein [23]. Another gain-of-function mutation (F216L) is predicted to reside very close to D374Y on the outer surface of the catalytic domain [24] and might also be a Class 5 mutation.

PCSK9 as a therapeutic target

PCSK9 and LDLR are coordinately regulated

The LDLR and PCSK9 are coordinately regulated by sterol regulatory element-binding protein-2 (SREBP-2), a transcription factor that activates many genes involved in cholesterol

metabolism [3,4]. The dual regulation of the LDLR and PCSK9 by SREBP-2 might permit the exploitation of this pathway for cholesterol-lowering therapies. Statin administration lowers LDL by inducing SREBP-2 expression, which increases expression of LDLR [36,37]. PCSK9 mRNA and protein levels are also increased in response to statins [32,38]. The increase in PCSK9 would be expected to attenuate the cholesterol-lowering effect of statins. Therefore, inhibition of PCSK9 activity would be predicted to augment statin-induced LDLR expression and accelerate LDL-C clearance, as was observed when statins were administered to PCSK9 knockout mice [32]. The low plasma LDL-C levels associated with loss-of-function mutations in *PCSK9* indicate that inhibition of PCSK9 either through small molecules, antibodies or RNAi should be effective cholesterol-lowering drugs independently of statins.

Safety issues associated with PCSK9 inhibition

Would pharmacological inhibition of PCSK9 be safe in humans? PCSK9 is expressed in the kidney and cerebellum of adult mice in addition to the liver and small intestine [1]. Although inactivation of *PCSK9* in embryos of zebrafish results in disordered neuronal development and death [39], mice lacking PCSK9 develop normally and have no gross neurological defects [32]. Humans heterozygous for loss-of-function mutations in PCSK9 seem to be healthy [27] and have a normal life-span [30]. Moreover, a compound heterozygote with two inactivating mutations in *PCSK9* (Y142X and Δ R97) and no circulating PCSK9 has been recently identified [24]. This apparently healthy 31-year-old African-American mother of two consistently has very low levels of LDL-C ($14\text{--}34\text{ mg dL}^{-1}$) [24] and grossly normal renal, hepatic and neuronal function (Y. Tuakli-Wosornu, J.C. Cohen and H.H. Hobbs, unpublished). Another individual homozygous for the C679X mutation was identified in Zimbabwe; she has a plasma LDL-C of 16 mg dL^{-1} [40]. Careful clinical assessment of this individual and other subjects with inactivating mutations in *PCSK9* might reveal additional phenotypes, providing clues to substrates of PCSK9 other than the LDLR.

Physiological role of PCSK9

Role of PCSK9 in cholesterol metabolism

As reviewed previously, both the *LDLR* and *PCSK9* are transcriptionally regulated by SREBP-2 [4]. Thus, as more LDLR protein is produced, more PCSK9 is made, ultimately leading to the degradation of the LDLR protein in hepatocytes. One possible explanation for this seemingly futile regulatory cycle is that PCSK9 might act as a 'brake' to slow the uptake of cholesterol by degrading LDLRs after they have internalized LDL. PCSK9 can potentially avert excessive cholesterol accumulation within the cell by preventing the recycling of LDLRs to the cell surface.

The liver is likely to be the primary site of synthesis of circulating PCSK9. By controlling the secretion of PCSK9, the liver might regulate the levels of LDLR expression in peripheral tissues. Tissues *in vivo* might vary in their responsiveness to PCSK9, as is seen *in vitro*. Thus, PCSK9 expression might direct LDL to specific tissues. For example, in times of stress, PCSK9 might shunt cholesterol away from the liver to steroidogenic tissues. The effect of PCSK9 expression on the levels of LDLR in extra-hepatic tissues has not been examined.

PCSK9 and human evolution

The high frequency of nonsense mutations in individuals of African descent begs the question as to whether inactivation of PCSK9 confers a selective advantage [27]. We can only speculate on the nature of the selective pressure that maintained nonsense mutations in *PCSK9* in Africans. The best evidence of positive selection in Africans is the accumulation of sequence variations in various red blood cell proteins in populations exposed to malaria [41]. Inactivation of PCSK9 might interfere with the life cycle of the malaria parasite. The LDLR has been

implicated as a portal for entry into the liver for several viruses, such as a minor group of rhinovirus and possibly hepatitis C [42,43]. Increased LDLR activity in the liver might reduce the exposure of peripheral tissues to viruses or other infectious agents that circulate in association with lipoproteins. Reductions in PCSK9 activity might decrease the pathological consequences of an infectious agent. Other members of the proprotein convertase family are hijacked by viruses and bacteria to process proteins required for infection [44]. Alternatively, the high frequency of *PCSK9* nonsense mutations in Africans and not Caucasians could simply result from genetic drift. Additional population genetic studies will be required to address this question.

Concluding remarks and future perspectives

Despite the rapid progress that has been made in the past three years regarding the biological importance of PCSK9 in LDL metabolism, several important mechanistic and clinical questions remain. First, the mechanism by which PCSK9 expression and the gain-of-function mutations promote the degradation of the LDLR remains to be defined. Second, although a model for the structure of PCSK9 has been proposed, the crystal structure has not been reported. The elucidation of the 3D structure might provide important insights into the mechanism of PCSK9 action. Further studies will be required to identify the specific site(s) in the cell at which PCSK9 functions and to determine whether catalytic activity is required for LDLR degradation. Third, if catalytic activity is required, is the LDLR or another protein the substrate for the enzyme? Fourth, does PCSK9 circulate in association with other proteins and can it act on receptors in tissues other than the liver? Finally, why has PCSK9 been retained through vertebrate evolution? Although genetic deficiency of PCSK9 does not seem to be associated with obvious phenotypes independent of LDL metabolism, it remains possible that PCSK9 functions in other pathways.

Glossary

Hypercholesterolemia, a metabolic condition characterized by the presence of excessively high cholesterol levels in the blood.; Low density lipoprotein-cholesterol (LDL-C), the principal vehicle for cholesterol transport in the blood. Plasma levels of LDL-C are directly related cardiovascular risk.; Low density lipoprotein receptor (LDLR), a cell surface glycoprotein that binds and internalizes the catabolic remnants of VLDL metabolism, including intermediate density lipoproteins and LDL.; Proprotein convertase, secreted proteolytic enzymes that cleave precursor proteins into biologically active forms.; Sterol regulatory element-binding protein (SREBP), transcription factor that coordinately regulates cholesterol and triglyceride homeostasis.; Statins, a pharmaceutical inhibitor of hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase, an enzyme involved in controlling the rate of cholesterol synthesis. Use of statins increases LDL clearance from the bloodstream by stimulating expression of LDLR in the liver.; Very low density lipoprotein (VLDL), the primary lipoprotein secreted by the liver and the major triglyceride-transport particles in the blood..

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Figure 1.

A schematic of PCSK9 with the location of naturally occurring mutations associated with elevated (top) or reduced (bottom) plasma levels of LDL-C. The major domains of PCSK9 are delineated using different colors. The mutations included are limited to those associated with significant differences in plasma levels of LDL-C in at least two independent populations or those that co-segregate consistently with hypercholesterolemia in families. Mutations associated with elevated plasma cholesterol levels found only in families who also have mutations in the *LDLR* are indicated by with an asterisk (*) [18]. The mutations for which there is functional information are referenced in Table 1. The location of the aspartic acid (D), histidine (H) and serine (S) comprising the catalytic triad and the site of binding of the single N-linked sugar (Asn533) are shown [1]. The oxyanion hole is located at Asn317. Abbreviations: SS, signal sequence; Pro, prodomain.

Figure 2.

Cellular trafficking and potential sites of PCSK9 action. PCSK9 undergoes autocatalytic cleavage in the ER. The cleaved prodomain (light blue) associates with the catalytic fragment (dark blue) and functions as a chaperone permitting the mature protein to move from the ER into the secretory pathway. Current evidence indicates that PCSK9 might work at two cellular sites. The first potential location is in a post-ER compartment, depicted here as the Golgi apparatus, where PCSK9 might target the LDLRs (green) for degradation in an acidic compartment such as the lysosome. In the second possible pathway, the PCSK9 that is secreted binds to LDLRs on the cell surface. The LDLR–PCSK9 complex is internalized together with the adaptor protein ARH (orange). PCSK9 might prevent the recycling of the LDLR from the endosome back to the cell surface and/or direct the LDLR to the lysosome where it is degraded. It is currently not known whether PCSK9 directly cleaves the LDLR or whether catalytic activity is required for either pathway.

Table 1
Functional defects associated with naturally occurring loss- and gain-of-function mutations of PCSK9 in humans^a

Mutation	Class of mutation and functional defect					Refs
	Class 1 Null	Class 2 ↓ Processing	Class 3 ↓ Transport from ER	Class 4 Alters stability	Class 5 Alters affinity for LDLR	
Gain-of-function						
S127R	—	X	X	?	?	[2,8,20]
F216L	—	—	—	?	?	[2,8,20]
D374Y	—	—	—	?	X	[12,13,23]
Loss-of-function						
R46L	—	—	—	?	?	[24,28,29]
Δ97R	—	X	X	—	—	[24]
G106R	—	X	X	—	—	[28]
Y142X	X	—	—	—	—	[24,27]
L253F	—	X	X	—	—	[24,29]
A443T	—	—	—	?	?	[9,29]
C679x	—	—	X	—	—	[9,24,27]

^aPhenotypic classifications are based on expression of the recombinant protein in cultured cells. —, no defect observed; X, defective; ?, not defined.