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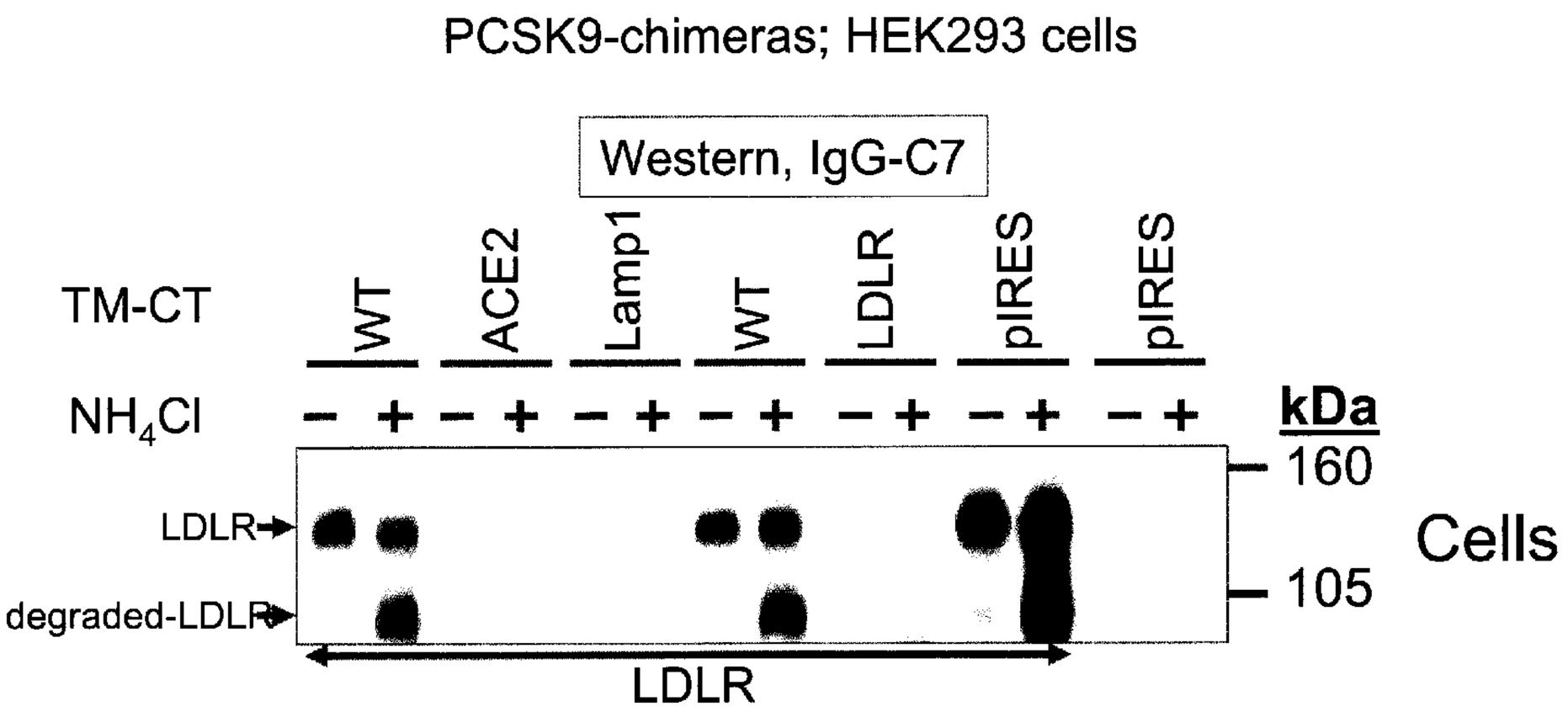
(54) Titre : PROTEINES PCSK9 CHIMERIQUES, CELLULES LES COMPRENANT ET DOSAGES LES UTILISANT
(54) Title: CHIMERIC PCSK9 PROTEINS, CELLS COMPRISING SAME, AND ASSAYS USING SAME



(57) Abrégé/Abstract:
A chimera protein comprising in the following order : a signal peptide, a proprotein convertase subtilisin/kexin type 9 preproprotein (PCSK9) sequence consisting of amino acid residues at positions 35 to 696 of SEQ ID NO: 38, a transmembrane domain and a cytosolic domain, wherein said cytosolic (CT) domain comprises a sequence able to recycle the protein from the cellular membrane to endosomes.

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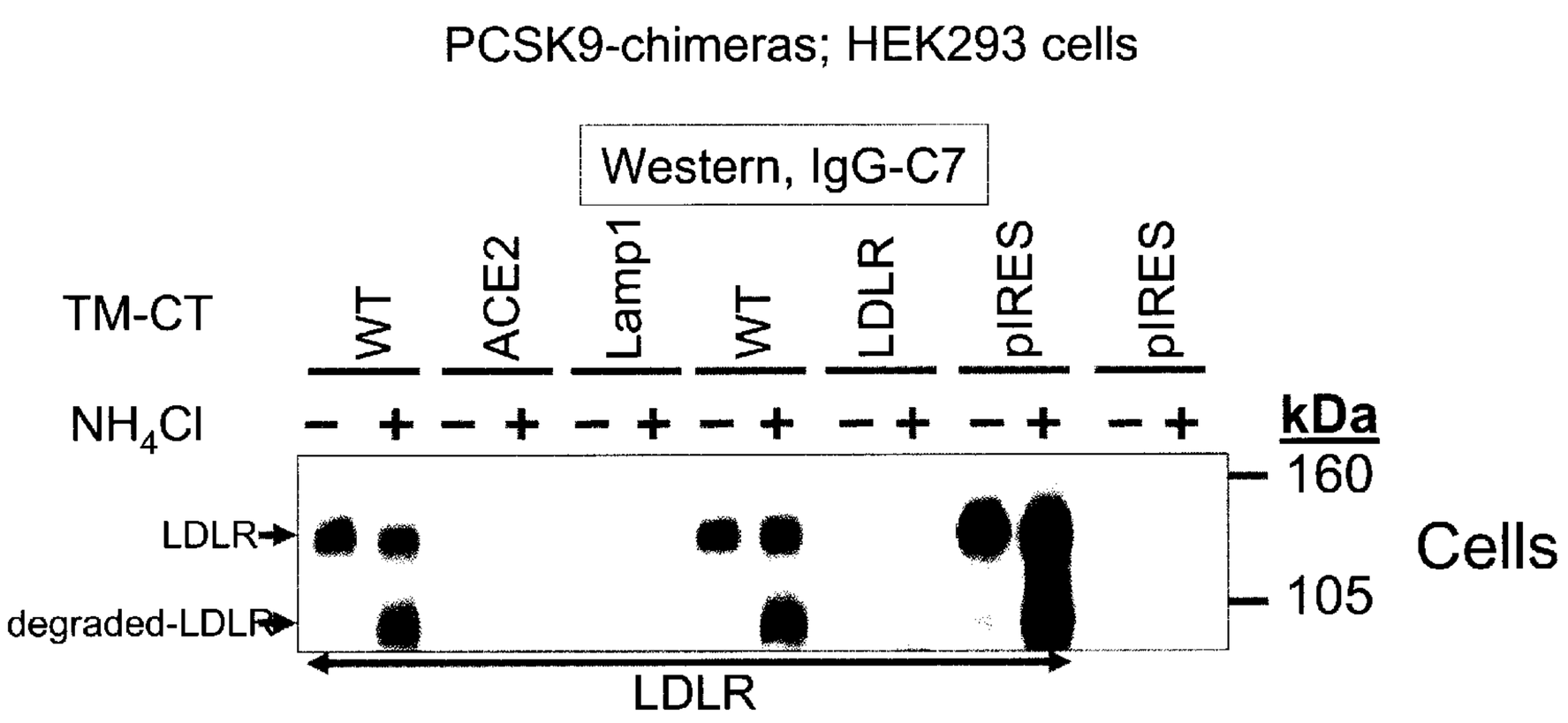
(54) **Title:** CHIMERIC PCSK9 PROTEINS, CELLS COMPRISING SAME, AND ASSAYS USING SAME



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TITLE OF THE INVENTION

CHIMERIC PCSK9 PROTEINS, CELLS COMPRISING SAME, AND ASSAYS USING SAME

FIELD OF THE INVENTION

[0002] The present invention relates to chimeric proteins, cells comprising same, and assays using same. More specifically, the present invention is concerned with cell-based assays for identifying modulators of proprotein convertase subtilisin/kexin type 9 preproprotein (PCSK9).

BACKGROUND OF THE INVENTION

The regulation of processing within the constitutive secretory pathway

[0003] The Proprotein Convertases (PCs) are responsible for the tissue-specific limited proteolysis of multiple polypeptide precursors, generating a large diversity of bioactive molecules (Seidah and Chretien, 1999; Seidah and Prat, 2002). Many cellular processing events involve an ordered cascade of cleavage events accomplished by one or more convertase(s) belonging to the PCs/SKI-1/PCSK9 mammalian subtilase family (Seidah and Chretien, 1999; Seidah and Prat, 2002; Seidah et al., 2003). This mammalian PC-family comprises nine members: PC1/3, PC2, furin, PC4, PACE4, PC5/6, PC7, SKI-1/S1P and NARC-1/PCSK9 (Seidah and Chretien, 1999; Seidah and Prat, 2002;Seidah et al., 2003). The first seven proteinases are basic amino acid specific PCs cleaving precursor proteins at single or paired basic residues within the motif (K/R)-(X)n-(K/R)↓, in which n = 0, 2, 4 or 6 (Seidah and Chretien, 1999). These proteinases are phylogenetically more closely related to each other and to yeast kexin than to SKI-1/S1P or NARC-

1/PCSK9, which belong to the pyrolysin (Seidah et al., 1999) and proteinase K (Seidah et al., 2003) subfamilies, respectively. The latter enzymes recognize the motifs R-X-(hydrophobic/aliphatic)-Z↓ (Seidah et al., 2006) and VFAQ↓ (SEQ ID NO: 127) (Benjannet et al., 2004), respectively. These enzymes have been implicated in a wide variety of functions regulating cellular homeostasis and a number of pathologies including cancer, inflammation, neurodegenerative diseases, atherosclerosis and viral infections. It was recently realized that some of these convertases play critical roles in the regulation of lipids and/or sterols (Seidah et al., 2006) either through the inactivation of lipoprotein lipases e.g., by PC5/6, PACE4 and furin (Jin et al., 2005), activation of specific membrane-bound transcription factors (SREBP-1 and -2) by SKI-1/S1P (Cheng et al., 1999), or by enhancing the degradation of the low density lipoprotein receptor (LDLR) by PCSK9 (Maxwell and Breslow, 2004; Benjannet et al., 2004; Park et al., 2004; Maxwell et al., 2005).

[0004] A number of factors regulate the processing of PCs themselves. First, convertases require removal of their inhibitory prosegment for activation (Figure 1). Analysis of the biosynthesis of furin, PACE4, PC5, PC7, SKI-1, and PCSK9 revealed that they are synthesized as zymogens that undergo autocatalytic cleavage of their N-terminal inhibitory pro-segment, which seems to act both as a chaperone and an intramolecular inhibitor (Zhong et al., 1999; Nour et al., 2003; Seidah et al., 2003). Except for PC2, primary prosegment cleavage is necessary for most convertases to exit from the endoplasmic reticulum (ER). Overexpression of furin, PC5, and PC7 prosegments as independent domains confirmed their inhibitory potency and the presence of critical elements at their C-terminus. The design of two potent and specific-inhibitors of SKI-1 based on variants of either its prosegment or α1-PDX were reported (Pullikotil et al., 2004).

[0005] The second control element is the trafficking of these enzymes to different intracellular organelles. Dependant on the cognate substrate, constitutively secreted PCs cleave in the Golgi, *trans* Golgi network (TGN), endosomes or at the cell surface (Figure 2). The modified serpin α1-PDX

(Benjannet et al., 1997; Anderson et al., 1993) and the PC-prosegments (Zhong et al., 1999) inhibit the PCs within the constitutive secretory pathway.

Regulation and Processing of PCSK9

[0006] The regulation of PCSK9 activity could be achieved by various mechanisms, which among others could act at the level of: (i) its transcription where its mRNA levels are upregulated by SREBP-2, and downregulated by cholesterol (Maxwell et al., 2003; Dubuc et al., 2004) via a reduced level of activated nuclear SREBP-2 (Horton et al., 2003;Dubuc et al., 2004); (ii) its translation which may be controlled by specific factors; (iii) its post-translational modifications including its zymogen cleavage and/or activation, glycosylation, sulfation (Seidah et al., 2003; Benjannet et al., 2004), or possibly by other processing events resulting in its degradation (Seidah et al., 2003); (iv) its cellular localization and/or sorting of mature PCSK9; (v) its level of secretion; and possibly, (vi) its subsequent cellular re-uptake.

[0007] PCSK9/NARC-1 plays a role in cholesterol homeostasis. Indeed, point mutations in the PCSK9 gene within its coding exons (Attie, 2004) are associated with either familial hypercholesterolemia (Abifadel et al., 2003; Leren, 2004; Timms et al., 2004; Allard et al., 2005; Naoumova et al., 2005) or hypocholesterolemia (Cohen et al., 2005; Kotowski et al., 2006; Berge et al., 2006) phenotypes. This led to the classification of the PCSK9 gene as the third chromosomal locus associated with autosomal dominant familial hypercholesterolemia, with the LDLR and Apolipoprotein B (Apo B) comprising the other two loci (Abifadel et al., 2003). It is thus plausible that some of the single point mutations of PCSK9 associated with autosomal dominant familial dyslipidemias could enhance or abrogate one or more of the PCSK9 regulatory events (Attie and Seidah, 2005).

[0008] It should be noted that PCSK9 is mostly expressed in adult liver hepatocytes and in small intestinal enterocytes (Seidah et al., 2003). Its exact role in these tissues is still unknown, except that this convertase possibly enhances the rate of degradation of the endogenous hepatic and possibly

intestinal LDLR. Although no PCSK9 inhibitor is yet known, potent PCSK9 siRNAs were identified that upregulate the LDLR (Benjannet et al., 2004). The lowering of the level of LDLR at the cell surface is thus a good indicator of the PCSK9 activity.

Natural mutants of PCSK9 and implication in hypocholesterolemia

[0009] It was suggested that some PCSK9 single point mutations result in a gain or enhanced function of PCSK9 on the degradation of LDLR in acidic compartments, possibly endosomes (Benjannet et al., 2004; Maxwell et al., 2005), while others would cause a loss of function (Cohen et al., 2005), and would be associated with the development of hyper- or hypo-cholesterolemia, respectively (Attie and Seidah, 2005; Kotowski et al., 2006). It was thus hypothesized that high levels of active PCSK9 are associated with a faster rate of degradation of the cell surface LDLR, resulting in increased amounts of circulating LDL-cholesterol, as the uptake of the latter in liver hepatocytes by the LDLR will be diminished accordingly, and *vice versa*. This would suggest that the level of cell surface LDLR is indirectly proportional to the level of hepatic and likely intestinal active PCSK9. This hypothesis is reinforced by the *in vivo* observations that in mice lacking a functional PCSK9 gene (PCSK9-knockout mice), the level of hepatocyte cell surface LDLR is greatly enhanced resulting in an ~50% drop in the level of circulating LDL-cholesterol (Rashid et al., 2005), whereas mice overexpressing PCSK9 result in higher levels of circulating LDL-cholesterol (Benjannet et al., 2004; Park et al., 2004; Maxwell and Breslow, 2004; Lalanne et al., 2005).

[0010] Examples of hypercholesterolemic-associated mutations include the Ser127-to-Arg (S127R) amino acid change. The S127R mutation is located between the primary and putative secondary zymogen processing sites of the PCSK9 propeptide; mutations in the catalytic subunit include Phe216-to-Leu (F216L), which is located close to the active site at His226 (Abifadel et al., 2003) and Arg218-to-Ser (R218S) (Allard et al., 2005). These and other new natural mutations reported in Table 1 below were biochemically analyzed and some of them were suggested to result in a gain of function,

likely including a gain of proteolytic activity or a better co-localization with LDLR (Benjannet et al., 2004). However, the molecular mechanisms that underlie the dominance of the dyslipidemia trait caused by PCSK9 missense mutations is still unclear.

Regulation of Other Cell Surface Receptors by PCSK9

[0011] Only the LDLR was reported to be affected by PCSK9. The LDLR is part of the 7-member LDL receptor superfamily that included amongst others, very low density lipoprotein receptor (VLDLR) (Official Symbol: VLDLR and Entry gene ID: 7436, NCBI), apolipoprotein e receptor 2 (ApoER2) (Official Symbol: LRP8 and Entry gene ID: 7804, NCBI) and LRP (Figures 11 and 12). LRP, a member of the 7-member LDL receptor superfamily that includes amongst others, LDLR, very low density lipoprotein receptor (VLDLR) (Official Symbol: VLDLR and Entry gene ID: 7436, NCBI), apolipoprotein e receptor 2 (ApoER2) (Official Symbol: LRP8 and Entry gene ID: 7804, NCBI)(Figures 11 and 12), was found not to be affected by PCSK9 (Benjannet et al., 2004). There is a need to develop a cell system that would allow the identification of novel PCSK9 targets.

Implication of PCSK9 in Human Pathologies

[0012] PCSK9 has clearly been involved in the regulation of LDL-cholesterol. Dyslipidemia is in fact the first dominant human pathology directly associated with mutations in a PC, namely in PCSK9. Since PCSK9 is also expressed in brain and gut (Seidah et al., 2003), it is plausible that mutations in PCSK9 may have other consequences aside from LDL-cholesterol regulation. Such pleiotropic effects were reported for other convertases. An example includes the β-secretase BACE1, which has been clearly implicated in Alzheimer's disease, but whose functions seems also to include memory and emotion regulation independent of its effect on the processing of β-amyloid precursor (Laird et al., 2005).

[0013] A definition of novel functions of PCSK9 would alert to potential mechanism-based side effects that may occur with PCSK9 inhibitors designed to decrease LDL-cholesterol levels. Thus, a sensitive assay for PCSK9 function is urgently needed, which may uncover new unsuspected functions of this enzyme.

In Vitro PCSK9 Assays

[0014] Most of the *in vitro* assays design for identifying proteinase inhibitors consist in the addition of the compound to a reaction mixture containing a purified enzyme and its substrate, and measuring the absence or reduction of the cleavage products observed when the mixture is incubated under similar conditions but without the inhibitory compound. However, since none of the existing methods allowed for the detection of an active enzymatic form of PCSK9, no such *in vitro* assays are yet available using PCSK9 for identifying PCSK9 inhibitors. Furthermore, some inhibitors active *in vitro* may not find utility *in vivo* because of their inability to enter the cell and reach the cellular compartments where PCSK9 is localized. There is thus a need for the development of cell-based assays specific for PCSK9 activities.

PCSK9 Cell-Based Assays

[0015] Prior art cell-based assays for identifying convertase-inhibitory compounds produce false positives. For instance, Oh *et al.* 2004 described a cell-based assay for β-secretase activity using a target chimeric protein substrate containing three domains: an amino-terminal TM domain, a beta-site and an alkaline phosphatase (AP). In this assay, the activity of BACE on the chimera results in the release of AP in the culture medium. An inhibition of the BACE activity results in the absence of AP release in the culture medium. An absence of AP in the culture medium could result not only from the inhibition of the target substrate synthesis itself, but also from a variety of irrelevant cellular mechanisms including amongst others, the absence of target chimeric protein substrate expression itself, modification of chaperones, cellular trafficking, protein folding or even a pH change within the cells, etc. It is thus difficult to determine through their use whether the absence of

detection of a specific signal resulted from enzyme inactivation or from another irrelevant reason.

[0016] Although a positive cell-based assay which targets cathepsin L in the lysosome and used for the identification of protease inhibitors was described (Belkhiri et al., 2002), this assay is not appropriate for the identification of PCSK9 inhibitors. Other positive cell-based assays measuring the increase of a signal molecule at the cell surface do not offer the appropriate sensitivity for screening due to the high background of the signal molecule already present at the cell surface. For instance, the measurement of re-appearance of LDLR at the surface of cells overexpressing wild type PCSK9 does not provide a sensitive positive screening for PCSK9 inhibitory compounds due to the LDLR background that still remains at the cell surface (Benjannet et al., 2004).

[0017] There is thus a need for an improved positive cell-based assay adapted to PCSK9.

[0018] The present invention seeks to meet these and other needs.

SUMMARY OF THE INVENTION

[0020] Five classes of proteinases are known, including the Serine (Ser), Aspartic (Asp), Metallo, Cysteine (Cys) and Threonine (Thr) proteinases, estimated to contain a total of 500-600 members in the human and mouse genomes. Different proteinases digest their substrates within different specific cell compartments or extracellularly. For instance, the proteinases present in the proteasome (Asp, Ser and Thr proteinases) are active throughout the cytoplasm and the nucleus, caspases (Cys proteinases)

are active in the cytoplasm, still other proteinases are active in the secretory and/or endocytic pathways.

[0021] The secretory and endocytic pathways of eukaryotic organelles consist of multiple compartments. Specific transport mechanisms are required to direct molecules to defined locations. The localization of proteins to specific membranes is complex and involves multiple interactions. All of the proteins that pass through the Golgi apparatus, except those that are retained there as permanent residents, are sorted in the *trans* Golgi network (TGN) according to their intended final destination. The terminology "secretory and endocytic pathways" is a generic term covering various pathways including that of proteins sorted to lysosomes (*e.g.* cathepsin B), the pathway of proteins recycled into earlier secretory compartments by recognition of a retention signal (*e.g.* KDEL (SEQ ID NO: 1) for the endoplasmic reticulum), the regulatory pathway and the constitutive secretory pathway.

[0022] The constitutive secretory pathway, is one by which proteins are secreted from the cells at a rate that is mostly limited by their rate of synthesis (Figure 2). These proteins follow a pathway that goes through the endoplasmic reticulum (ER), the Golgi, the TGN and finally through the cell surface. Some of the constitutively secreted proteins however could once at the cell surface be re-internalized via early endosomes and then directed towards either 1) the TGN once again, 2) lysosomes; or even 3) be recycled to the cell surface for another round of sorting. This trafficking is intimately associated with sorting motifs found within the cytoplasmic tail of these usually membrane-bound proteins. PCs including Furin, PC5A, PC5B, PC7, PACE4, subtilisin-kexin isoenzyme SKI-1 and PCSK9 are mostly sorted through the constitutive secretory pathway.

[0023] Depending on the cognate substrate, constitutively secreted PCs may cleave them in the Golgi, the TGN, the endosomes, the cell surface or a combination of these locations. PCSK9 seems to enhance the

degradation of the LDLR within acidic compartments, likely to be clathrin coated endosomes (Benjannet et al., 2004; Maxwell et al., 2005).

[0024] The present invention provides in a first aspect a cell-based assay for monitoring a PCSK9 activity and modulators thereof. In specific embodiments of the assay, the cell line harbors a very low to undetectable level of different cell cell surfaces molecules (herein referred as “detectors”) the absence of which depends upon PCSK9 activity. The presence of PCSK9 inhibitors are detected by the reappearance of one or more detectors. The assay can be adapted to detect, in parallel or not, the presence of different cell surface detectors.

[0025] In certain embodiments, the cell-based assays of the present invention provide an increased level of sensitivity. In certain embodiments, the cell-based assays of the present invention provide fewer false positives. In certain embodiments, the cell-based assays of the present invention allow the detection of one or several independent detectors the presence of which is dependent on the PCSK9 regulatory pathway. In certain embodiments, the cell-based assays of the present invention provide not only identify compounds that are inhibitory to the catalytic activity of PCSK9 but also identify inhibitors other steps of the PCSK9 pathway, including upstream PCSK9 regulators.

[0026] The present invention relates to chimeras comprising an amino acid primary structure containing, from the N- to C-terminal amino acid sequence: 1) the PCSK9 sequence (either wild type, mutated form, or a combination thereof) or fragments thereof having an activity on the level of LDLR at the cell surface; followed by 2) a transmembrane domain (TM) for membrane anchoring which prevents the secretion of PCSK9; and 3) a cytoplasmic also referred to as ”cytosolic” (CT) signal that allows the chimera, once it reaches the plasma membrane to be recycled through early endosomes. Figure 3 schematically presents various forms of PCSK9 chimeras of the present invention.

[0027] The chimera containing a TM-CT (e.g. the TM-CT of ACE2, Lamp1 or LDLR) that includes one or more Y-X-X-hydrophobic motifs (Jadot et al., 1992) (e.g. the Y-A-S-I sequence (SEQ ID NO: 2) present in the CT of ACE2) are sorted from the cellular membrane towards endosomes/lysosomes. Such TM-CT-containing chimera are desirable for convertases that process their substrates in endosomes/acidic compartments such as PCSK9. Measurement of a LDLR decrease at the cell surface is a good indication that the PCSK9-chimera harbor characteristics appropriate for the present invention (Figure 4).

[0028] The sequence of PCSK9 in the chimera could contain the wild type sequence or alternatively variants of PCSK9 identified as conferring to PCSK9 resistance to cleavage by other enzymes, thereby resulting in an increased PCSK9 activity. In this respect, the applicants obtained direct evidence that indeed the level of mature PCSK9 is under the control of proteolysis by one or more members of the basic-amino acid specific convertases including furin and PC5 (Figures 5 to 7).

[0029] Indeed, the Applicants observed that the secreted wild type PCSK9 could be found as an active full length protein (N1) or alternatively as a N-terminally ~8 kDa-truncated form (N2) (Figure 5). Interestingly, this N2 form was either not observed or significantly decreased in experiments using the French mutants R218S and F216L, respectively (Figure 5). This suggested that the presence of Arg218 may be critical for the production of the N2 form.

[0030] Sequence alignment of a variety of vertebrate PCSK9s showed a conservation of Arg218 which, in most cases, is found within a R-X-X-R (SEQ ID NO: 3) motif (Figure 6) typical of a basic amino acid specific PC-recognition motif that is recognized by Furin/PC5-like enzymes (Seidah and Chretien, 1999). The mutation R218S completely disrupts this motif whereas the F216L affects the P3 position (Figure 6, bottom).

[0031] Co-expression of the wild type PCSK9 with convertases as well as with β-secretase revealed that only the membrane-bound Furin form and, to a lesser extent, the PC5A are able to process the N1 form of PCSK9 into the N2 form, with the concomitant loss of the co-immunoprecipitated PCSK9 prosegment (Figure 7). The N2 form thus represents PCSK9 truncated of the first 218 amino acid sequence and is herein after referred as PCSK9-ΔN_{218}. In addition, co-expression of the serpin α1-PDX completely abolishes the processing of PCSK9 into the truncated form (Figure 7).

[0032] In agreement with these data, Furin is unable to process the R218S mutant and only partially processes the F216L mutant compared to wild type PCSK9 (Figure 8). This led the Applicants to produce a PCSK9 molecule that can be more extensively processed at Arg_{218} by the endogenous Furin, namely by replacing the wild type $RFHR_{218}\downarrow QA$ sequence (SEQ ID NO: 4) by an optimal Furin-recognition sequence $RRRR^{218}\downarrow EL$ (SEQ ID NO: 5), with the motifs $RFHR_{218}\downarrow EA$ (SEQ ID NO: 6) and $RFHR_{218}\downarrow EL$ (SEQ ID NO: 7) giving intermediate Furin-cleavability (Figure 9). Notice the absence of prosegment co-immunoprecipitating with the PCSK9-ΔN_{218} form produced with the $RRRR_{218}\downarrow EL$ (SEQ ID NO: 5) sequence (Figure 9), which would be predicted since such cleavage would remove the active site Asp_{186}.

[0033] Analysis of the activity of wild type PCSK9, of three mutants R218S, $RRRR_{218}EL$ (SEQ ID NO: 5), H226A and of a truncated form of PCSK9 that included the Cys/His-rich domain of PCSK9 (CHRD) revealed that only the wild type and the R218S PCSK9 are active in enhancing the degradation of the LDLR (Figure 10). Thus, PCSK9-ΔN_{218} is an inactive form of PCSK9 that is secreted from cells. In comparison, the active site mutant His_{229} which results in the zymogen proPCSK9 remains in the ER (Benjannet et al., 2004). These forms provide ideal controls for the activity of PCSK9 in the secretory pathway, differentiating inhibitors that affect the protease activity of PCSK9 from those affecting other cellular processes.

[0034] The cleavage of PCSK9 by Furin and/or PC5A may provide a rationale behind the hypercholesterolemia phenotype associated with the French (F216L and R218S) mutations and hypocholesterolemia phenotype in Black African Americans associated with L253F mutation (Abifadel et al., 2003; Allard et al., 2005). Thus, PCSK9 processing by other PCs is a novel mechanism regulating the level of the active form of the enzyme, and may represent a general mechanism behind other mutations resulting in either hypercholesterolemia (loss of cleavage) or hypocholesterolemia (gain of cleavage). Table 1 below presents examples of such PCSK9 mutations (Abifadel et al., 2003; Allard et al., 2005; Pisciotta et al., 2005; Kotowski et al., 2006). This does not exclude the possibility that other mechanisms may be responsible for the phenotypes behind other mutations, such as cellular sorting, post-translational modifications and zymogen activation, etc.

[0035] The present invention provides cell-based tools useful for the identification of novel PCSK9 target cell surface receptors that could be used, in addition to the LDLR, as detector molecules in the cell-based screening assays. The fact that LDLR is part of the 7-member LDL receptor superfamily that included amongst others VLDLR, ApoER2 and LRP (Figure 11) led the Applicants to test the hypothesis that PCSK9 may affect one or more of these LDLR-related proteins. While LRP was found not to be affected by PCSK9 (Benjannet et al., 2004), the two closely related LDLR-related proteins, VLDLR and ApoER2 (Figure 12), were degraded in the presence of PCSK9 (Figure 13). The expression of the [PCSK9-TM-CT] chimera of the present invention was found to be much more potent in enhancing the degradation of all three receptors, independently of the cell type tested (Figures 13 and 14).

[0036] VLDLR and/or ApoER2 have been implicated in a variety of diseases including schizophrenia and autism which implicates Reelin, the common ligand of both receptors (Fatemi, 2005), fetal growth restriction (Wang, 2006 for ApoER2), obesity (Goudriaan, 2001 for VLDLR), and the recessive form of non-progressive cerebellar ataxia found in the Hutterite population (Boycott, 2005 for VLDLR). PCSK9 could thus have implication in

such VLDLR and ApoER2-associated diseases and identify PCSK9 as a novel potential therapeutic target in such VLDLR and ApoER2-associated diseases.

[0037] The methods of classifying or stratifying the subjects of the present invention into subgroups having different phenotypes enables a better characterization of PCSK9-associated diseases and eventually a better selection of treatment depending on the subgroup to which the subject belongs.

[0038] The present invention provides powerful tools for the design of potent cell-based assays that incorporate PCSK9 and/or any of its variants alone or in combination with chimeras (Figures 3 and 15).

[0039] Transgenic expression of PCSK9 in mouse liver resulted in a line that expresses >40 fold more PCSK9 than the endogenous enzyme in hepatocytes. The transgenic protein was tagged with a V5 at its C-terminus to differentiate it from the endogenous one. Analysis of mouse plasma samples revealed that PCSK9-V5 was secreted in blood and partially processed by Furin-like enzymes to generate PCSK9-ΔN_{218} as observed in cells and in human plasma. Interestingly, analysis of VLDLR levels in the muscle of these mice versus non-transgenic control littermates revealed that the level of VLDLR was decreased at least 3-fold in muscle. This is the first evidence that circulating PCSK9 can enhance the degradation of VLDLR *in vivo* and at distant sites away from those of its synthesis.

[0040] The present invention allows the identification of novel PCSK9-associated pathways and identifies PCSK9 as a potential target in these pathways-associated diseases.

[0041] The present invention provides cell-based assays that incorporate PCSK9 associated with an increased activity (Figure 15).

[0042] The present invention provides cell-based assays that incorporate PCSK9 associated with an increased activity, and which may also incorporate chimera specifically cleaved by PCSK9 (Figure 15) such as that described in co-pending application no. WO 2007/030937 filed September 14, 2006.

[0043] The cell-based assays of the present invention advantageously mimic the environment in which inhibitors will have to work *in vivo* (i.e. using endogenous proteinases and selecting for cell-diffusible inhibitors effective in the secretory pathway). In specific embodiments, they are advantageously positive assays (i.e., selects for re-appearance of a signal molecule on the cell surface).

[0044] Cell-based assays according to specific embodiments of the present invention incorporate the use of multiple detector molecules providing to the assays a high level of sensitivity and specificity.

[0045] The assays of the present invention are able to discriminate compounds that are toxic to cells.

[0046] The present invention provides for the detection of specific PCSK9 activity through the use of one or multiple types of cell surface receptors/detector molecules.

[0047] The cell-based assays of the present invention allow for high throughput screening (HTS) of candidate compounds.

Identification of PCSK9 with enhanced activities: PCSK9 chimeric TM-CT protein

[0048] Proteins destined for location in the membrane contain a transmembrane domain comprising a stretch of 15 to 22 hydrophobic amino acids in an alpha helical secondary conformation. Several transmembrane domains are described and could be used in the present invention. TMbase™

is a database of transmembrane proteins (Hofmann K. et al. 1993) with their helical membrane-spanning (TM) domain. Without being so limited, they include that derived from the human angiotensin converting enzyme-2 (ACE2 i.e. the SARS-Corona Virus receptor), Lamp-1 and LDLR. The addition of a TM domain to the PCSK9 sequence prevents the secretion of the chimera into the extracellular medium.

[0049] Signals present within the cytoplasmic tail (CT) of several proteins determine whether or not it will be sorted through a particular secretory pathway. For example, signals determining TGN targeting of furin include amino acids of the cytoplasmic tail. Indeed, two independent targeting signals, which consist of the acidic peptide $CPSDSEEDEG_{783}$ (SEQ ID NO: 8) and the tetrapeptide $YKGL_{765}$ (SEQ ID NO: 9) (an example of Y-X-X-hydrophobic motif) were previously identified that control the recycling of the constitutively secreted Furin back from the cell surface to the TGN (Thomas, 2002). The YKGL (SEQ ID NO: 9) is a determinant for targeting from the cell surface to the endosomes, while the acidic peptide signal in the cytoplasmic tail is necessary and sufficient to localize the reporter molecule from the endosomes to the TGN. The chimera protein of the present invention combines signals present on the PCSK9 with those of a cytoplasmic tail that allow the chimera to be secreted via constitutive secretory pathway and be recycled in endosomes. The choice of a cytoplasmic tail signal relies on the ability of the protein to reach the cell surface and be recycled in endosomes. For this purpose a variety of CT in combination with different TM could be utilized such as the TM-CT from LDLR as this is one of the proteins targeted for enhanced degradation by PCSK9. Other members of the LDLR superfamily (Figure 11), including those susceptible to PCSK9 (e.g. VLDLR and ApoER2) could also be used. However, this approach is not limited to TM-CTs of proteins that are targets of PCSK9-enhanced degradation and others TM-CTs could also be used including that of the SARS coronavirus receptor the angiotensin converting enzyme-2 (ACE2) (Bergeron et al., 2005; Vincent et al., 2005) or the lysosomal proteins such as LAMP-1 (Conesa et al., 2003) (Figure 3).

N-terminal signal sequence

[0050] Proteins destined for export, for location in a membrane and more generally for the secretory pathway contain a signal peptide comprising the first 20 or so amino acids at the N-terminal end and always includes a substantial number of hydrophobic amino acids. Several peptide signals are known and could be used in the present invention. For instance, SPdb, a signal peptide database lists a number of useful signal peptides (Choo et al. 2005). Without being so limited, useful signal peptides include those of human insulin, renin as well as those of PCs themselves amongst others.

Furin/PC5-resistant and sensitive PCSK9 variants

[0051] The present invention demonstrates that the wild type PCSK9 sequence contains basic amino acid specific proprotein convertase cleavage motifs which could regulate the PCSK9 activities. PCSK9 variants described herein use specific naturally occurring (F216L and R218S mutants) or artificially modified (e.g. $RRRR^{218}\downarrow EL$ (SEQ ID NO: 5)) wild type furin/PC5 cleavage motifs, however the present invention is not limited to any of them. Any modification of the basic amino acid specific proprotein convertase cleavage motifs decreasing (e.g. F216L and R218S) or increasing (e.g. $RRRR^{218}\downarrow EL$ (SEQ ID NO: 5)) the cleavability of PCSK9 by furin, or PC5 can be used. However, this does not exclude the possibility that other mutations may hinder or enhance the cleavability of PCSK9 by other proprotein convertases.

Host Cells

[0052] Although the assays described herein use specific host cells, the present invention is not limited to any of them. Any cells, preferably human cells expressing the chimeric PCSK9 that is to be screened for modulators can be used. The use of human cells is preferred for selecting a modulator effective in human. Hence, any cells expressing a detector molecule could be used, including HuH7, HepG2, HEK293, LoVo-C5 etc...

[0053] The cell line would preferably express chimeric and/or variants of PCSK9 and present a very low to undetectable level of LDLR at the cell surface. The cells could either be used as transiently transfected cells or as stably selected clones or pools. The HuH7 cell line appears to be one of the best human cell lines to perform the assay as these cells are of hepatic origin, express endogenously PCSK9 and LDLR, and overexpression of PCSK9 in these cells causes the degradation of the LDLR. However any cell expressing LDLR and PCSK9 or an appropriate mutant thereof (e.g. PCSK9-R218S) could be used in specific embodiments of the present invention including stable HepG2. One of the advantages of using HepG2 cells is the absence of PC5 expression in these cells (Essalmani et al., 2006), although the present invention also suggests the use of a chimera resistant to PC5 (e.g. the R218S-PCSK9-[TM-CT]). In some specific assays, for instances in cases where host cells do not express PCSK9, purified recombinant PCSK9 proteins could also be added directly into the cell culture supernatant.

Cells expressing a PCSK9 with increased intracellular activities: identification of novel detector molecules

[0054] Clones of cells of the present invention express a PCSK9 with increased cellular activities either due to the addition of an appropriate TM-CT, or due to mutations conferring to PCSK9 resistance to inactivation by other convertases (e.g. PC5 and furin), or due to any combination of these features. Preferably, these cells harbor a very low to undetectable level of LDLR at the cell surface.

[0055] These cells could be used to identify novel cell surface molecules that, similarly to LDLR, are also sensitive to the presence of the PCSK9. Cells expressing a PCSK9 with increased intracellular activities are well suited for proteomics and/or genomics studies aimed at defining the pathways affected by PCSK9. Genomics and proteomics analyses may compare cells overexpressing a super active PCSK9 to cells expressing any inactive PCSK9 variant (e.g. RRRREL-TM-CT chimera). Examples of proteomic analyses include the characterization by FACS analysis (Vincent et

al., 2005) and/or Mass spectrometry (MS/MS) of the proteins that are missing at the cell surface (e.g., VLDLR and ApoER2) or endosomes/lysosomes in cells expressing super active PCSK9 (Bagshaw et al., 2005).

[0056] These novel identified cell surface molecules sensitive to PCSK9 could then be further used as detector molecules in cell-based assays. These novel detector molecules could be used either from endogenous expression or from stably or transiently transfected expressing cells.

[0057] These novel cell surface PCSK9-sensitive molecules identify PCSK9 as well as the PCSK9 pathway as novel target(s) in the treatment or prevention of their related human disease. For example amongst others, VLDLR and ApoER2 have been implicated in schizophrenia and autism because of their binding to Reelin (Fatemi, 2005), PCSK9 could also have implication in VLDLR and ApoER2-associated diseases.

Cells expressing a PCSK9 with increased cellular activities: cell-based screening assays

[0058] Clones of cells of the present invention express a PCSK9 with increased cellular activities either due to the addition of an appropriate TM-CT, or due to mutations conferring to PCSK9 resistance to inactivation by other convertases (e.g. PC5, furin), or due to any combination of these features. These cells harbor a very low to undetectable level of one or a combination of detector molecules at the cell surface (~10% or less of the level measured with a control cell expressing an inactive PCSK9, e.g., RRRREL(SEQ ID NO: 5)-PCSK9) (e.g. LDLR (Figure 10), VLDLR, ApoER2). These cells are perfectly adapted to the screening of PCSK9 inhibitory compounds.

[0059] PCSK9-[TM-CT of Lamp1] is a more powerful chimera to enhance the degradation of the LDLR, as compared to PCSK9-[TM-CT of ACE2] and PCSK9-[TM-CT of LDLR] (Figure 4), although all three are much

more effective than the wild type (WT) PCSK9. Accordingly, without being so limited, any one of them could be used in cell-based assays to screen for PCSK9 modulating molecules including PCSK9 inhibitors.

Detector molecules at the surface of cells: selection of inhibitory compounds

[0060] In the presence of compounds that inhibit the PCSK9 activities, detector molecules will reappear at the cell surface. Inhibition of the functional activity of PCSK9 implies that the compound is able to enter the cell and reach the endosomes or other compartments. This does not exclude however that some compounds may alternatively inhibit a PCSK9 activity at the cell surface or outside the cell. In any cases, such a positive selection procedure ensures that the screening identifies only compounds that are not toxic to cells.

[0061] The compounds could modify a step of the PCSK9 pathway, including the activity of an upstream regulator (e.g. by increasing the activity of furin on PCSK9 degradation). The compounds could also inhibit the catalytic site of the enzyme or other allosteric sites that impact on the productive catalytic activity or functions of the convertase. These compounds can then be tested *in vitro* to better define their exact mechanism of action.

[0062] However, it is also conceivable that some compounds will act in cellular compartments that control the folding and/or trafficking of the convertase, e.g., in the ER. It is less likely that the cell-based assays of the present invention will select such non specific PCSK9 inhibitors because they are likely to affect other proteins and likely lead to cellular stress and death. Such compounds would less likely be picked up by the cell-based assays of the present invention.

[0063] Although the assays described herein use specific detection tools, the present invention should not be so limited. Any method measuring specifically the presence of a detector molecule, or variant thereof should

work. This includes measuring one or several detector molecules including LDLR, VLDLR, ApoER2 or any other detector molecule sensitive to the action of PCSK9. For instance, using specific monoclonal antibodies, either commercially available or produced using the detector molecule sequence, the level of each detector molecule could be estimated by antibodies labelled with a variety of light emitting systems, e.g., fluorochromes or chemiluminescent probes.

DEFINITION

[0064] As used herein the terms "proteinase" refers to an enzyme that breaks down proteins into their component peptides.

[0065] As used herein the terms 'PCSK9 activity' refers to detectable enzymatic, biochemical or cellular activity attributable to PCSK9. Without being so limited, such activities include the effect of PCSK9 on reducing the level of LDLR (or VLDLR or ApoER2) at the cell surface, and/or the PCSK9 proteinase activity itself.

[0066] As used herein the terms "PCSK9-associated disease or condition" refer to diseases or conditions resulting in part from a defective PCSK9 activity and diseases resulting in part from a defective activity of a PCSK9 target such as LDLR, VLDLR or ApoER2. Similarly, as used herein the terms "LDLR-associated disease or condition", VLDLR-associated disease or condition" and "ApoER2-associated disease or condition" refer to diseases resulting in part from a defective LDLR activity, a defective VLDLR activity or a defective ApoER2 activity, respectively. For instance, as defined herein, hypercholesterolemia is an LDLR-associated condition while fetal growth restriction is a ApoER2-associated disease and the recessive form of non-progressive cerebellar ataxia found in the Hutterite population is a VLDLR--associated disease. Without being so limited, PCSK9-associated diseases or conditions include cardiovascular diseases such as hypercholesterolemia,

atherosclerosis, stroke and ischemia; schizophrenia, autism; fetal growth restriction; obesity; and a recessive form of non-progressive cerebellar ataxia.

[0067] As used herein, the term "modulator" refers to a compound that increases or decreases the PCKS9 activity. It includes proteins, peptides and small molecules.

[0068] As used herein, the term "PCSK9 inhibitor" includes any compound able to directly or indirectly reduce the transcription, translation, or activity of PCSK9. It includes intracellular as well as extracellular PCSK9 inhibitors. Without being so limited, such inhibitors include siRNA, antisense molecules, proteins, peptides, small molecules, antibodies, etc.

[0069] As used herein the term "subject" is meant to refer to any mammal including human, mice, rat, dog, cat, pig, cow, monkey, horse, etc. In a particular embodiment, it refers to a human.

[0070] As used herein the terminology "biological sample" refers to any solid or liquid sample isolated from a living being. In a particular embodiment, it refers to any solid or liquid sample isolated from a human. Without being so limited it includes a biopsy material, blood, saliva, synovial fluid, urine, amniotic fluid and cerebrospinal fluid.

[0071] As used herein the terminology "blood sample" is meant to refer to blood, plasma or serum.

[0072] As used herein the terminology "control blood sample" is meant to refer to a blood sample of a subject known not to suffer from the PCSK9-associated disease under scrutiny in the assay. In specific embodiments, it is the sample of a subject not to suffer from a PCSK9-associated disease. In particular embodiments where dyslipidemia is under scrutiny, it thus refers to a subject known not to suffer from dyslipidemia.

[0073] As used herein the term “purified” in the expression “purified polypeptide” means altered "by the hand of man" from its natural state (i.e. if it occurs in nature, it has been changed or removed from its original environment) or it has been synthesized in a non-natural environment (*e.g.*, artificially synthesized). These terms do not require absolute purity (such as a homogeneous preparation) but instead represents an indication that it is relatively more pure than in the natural environment. For example, a protein/peptide naturally present in a living organism is not "purified", but the same protein separated (about 90-95% pure at least) from the coexisting materials of its natural state is “purified” as this term is employed herein.

[0074] Similarly, as used herein, the term “purified” in the expression “purified antibody” is simply meant to distinguish man-made antibody from an antibody that may naturally be produced by an animal against its own antigens. Hence, raw serum and hybridoma culture medium containing anti-PCSK9-ΔN_{218} antibody are “purified antibodies” within the meaning of the present invention.

[0075] As used herein, the term “ligand” broadly refers to natural, synthetic or semi-synthetic molecules. The term “molecule” therefore denotes for example chemicals, macromolecules, cell or tissue extracts (from plants or animals) and the like. Non limiting examples of molecules include nucleic acid molecules, peptides, antibodies, carbohydrates and pharmaceutical agents. The ligand appropriate for the present invention can be selected and screened by a variety of means including random screening, rational selection and by rational design using for example protein or ligand modeling methods such as computer modeling. The terms “rationally selected” or “rationally designed” are meant to define compounds which have been chosen based on the configuration of interacting domains of the present invention. As will be understood by the person of ordinary skill, macromolecules having non-naturally occurring modifications are also within the scope of the term “ligand”. For example, peptidomimetics, well known in the pharmaceutical industry and

generally referred to as peptide analogs can be generated by modeling as mentioned above.

[0076] **Antibodies**

[0077] As used herein, the term "anti-PCSK9-ΔN_{218} antibody" or "immunologically specific anti- PCSK9-ΔN_{218} antibody" refers to an antibody that specifically binds to (interacts with) a PCSK9-ΔN_{218} protein and displays no substantial binding to other naturally occurring proteins other than the ones sharing the same antigenic determinants as the PCSK9-ΔN_{218} protein. The term antibody or immunoglobulin is used in the broadest sense, and covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies, and antibody fragments so long as they exhibit the desired biological activity. Antibody fragments comprise a portion of a full length antibody, generally an antigen binding or variable region thereof. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments, diabodies, linear antibodies, single-chain antibody molecules, single domain antibodies (*e.g.,* from camelids), shark NAR single domain antibodies, and multispecific antibodies formed from antibody fragments. Antibody fragments can also refer to binding moieties comprising CDRs or antigen binding domains including, but not limited to, VH regions (V_H, V_H-V_H), anticalins, PepBodies™, antibody-T-cell epitope fusions (Troybodies) or Peptibodies. Additionally, any secondary antibodies, either monoclonal or polyclonal, directed to the first antibodies would also be included within the scope of this invention.

[0078] In general, techniques for preparing antibodies (including monoclonal antibodies and hybridomas) and for detecting antigens using antibodies are well known in the art (Campbell, 1984, In "Monoclonal Antibody Technology: Laboratory Techniques in Biochemistry and Molecular Biology", Elsevier Science Publisher, Amsterdam, The Netherlands) and in Harlow et al., 1988 (in: Antibody A Laboratory Manual, CSH Laboratories). The term antibody encompasses herein polyclonal, monoclonal antibodies

and antibody variants such as single-chain antibodies, humanized antibodies, chimeric antibodies and immunologically active fragments of antibodies (e.g. Fab and Fab' fragments) which inhibit or neutralize their respective interaction domains in Hyphen and/or are specific thereto.

[0079] Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc), intravenous (iv) or intraperitoneal (ip) injections of the relevant antigen with or without an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, *e.g.,* keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N=C=NR$, where R and R^1 are different alkyl groups.

[0080] Animals may be immunized against the antigen, immunogenic conjugates, or derivatives by combining the antigen or conjugate (*e.g.,* 100 µg for rabbits or 5 µg for mice) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with the antigen or conjugate (*e.g.,* with 1/5 to 1/10 of the original amount used to immunize) in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, for conjugate immunizations, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

[0081] Monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al.*, Nature, 256: 495 (1975), or may be made by recombinant DNA methods (*e.g.,* U.S. Patent No. 6,204,023).

Monoclonal antibodies may also be made using the techniques described in U.S. Patent Nos. 6,025,155 and 6,077,677 as well as U.S. Patent Application Publication Nos. 2002/0160970 and 2003/0083293 (see also, *e.g.*, Lindenbaum et al., 2004).

[0082] In the hybridoma method, a mouse or other appropriate host animal, such as a rat, hamster or monkey, is immunized (*e.g.,* as hereinabove described) to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the antigen used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (*see, e.g.,* Goding 1986)).

[0083] The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

[0084] As used herein, the term “a” or “the” means “at least one”.

[0085] As used herein, the term “PCSK9 sequence” refers to a sequence having PCSK9 catalytic activity, and/or having the ability to traffic through its normal secretory pathway and to lower the protein level of LDLR at the cell surface. As used herein, it is not limited to the native PCSK9 sequence but encompasses any functional recombinant or mutant sequence, having preserved ability to reduce the level of LDLR (or other detector molecule) at the cell surface.

[0086] As used herein, the term "high enough" when referring the homology between a candidate surface receptor and a known cell surface receptor directly regulated by PCSK9 refers to more than 50% identity overall.

[0087] More specifically, in accordance with the present invention, there is provided a chimera protein comprising in the following order : a signal peptide, a proprotein convertase subtilisin/kexin type 9 preproprotein (PCSK9) sequence consisting of amino acid residues at positions 35 to 696 of SEQ ID NO: 38, a transmembrane domain and a cytosolic domain, wherein said cytosolic (CT) domain comprises a sequence able to recycle the protein from the cellular membrane to endosomes.

[0088] In a specific embodiment of the chimera, the PCSK9 sequence is as set forth in SEQ ID NO: 33. In an other specific embodiment of the chimera, the PCSK9 sequence consists of amino acid residues at positions 35 to 694 of SEQ ID NO: 35. In an other specific embodiment of the chimera, the PCSK9 sequence consists of amino acid residues at positions 35 to 691 of SEQ ID NO: 36. In an other specific embodiment of the chimera, the PCSK9 sequence consists of amino acid residues at positions 31 to 692 of SEQ ID NO: 37. In an other specific embodiment, the chimera further comprises at least one mutation associated with hypercholesterolemia. In an other specific embodiment, the chimera further comprises at least one mutation associated with hypocholesterolemia. In an other specific embodiment of the chimera, the PCSK9 sequence includes a basic amino acid specific proprotein convertases (PC)-recognition motif that comprises at least one mutation that reduces its recognition by furin/Proprotein convertase 5 (PC5)-like enzymes as compared to that of a wild-type PCSK9 sequence. In an other specific embodiment of the chimera, the at least one mutation is selected from the group consisting of a substitution of phenylalanine for a leucine at position 220 of SEQ ID NO: 38 and a substitution of arginine for a serine at position 222 of SEQ ID NO: 38. In an other specific embodiment of the chimera, the at least one mutation is a substitution of phenylalanine for a leucine at position 220 of SEQ ID NO: 38. In an other specific embodiment of the chimera, the at

least one mutation is a substitution of arginine for a serine at position 222 of SEQ ID NO: 38. In an other specific embodiment of the chimera, the PCSK9 sequence includes a basic amino acid specific proprotein convertases (PC)-recognition motif that comprises at least one mutation that increases its recognition by furin/proprotein convertase 5 (PC5)-like enzymes as compared to that of a wild-type PCSK9 sequence. In an other specific embodiment of the chimera, the at least one mutation is a substitution of phenylalanine for an arginine at position 220, a substitution of histidine for an arginine at position 221, a substitution of glutamine for an glutamic acid at position 223, and a substitution of an alanine for a leucine at position 224 of SEQ ID NO: 38. In an other specific embodiment of the chimera, the transmembrane domain and the cytosolic domain are as set forth in SEQ ID NO: 22. In an other specific embodiment of the chimera, the transmembrane domain and the cytosolic domain are as set forth in SEQ ID NO: 25. In an other specific embodiment of the chimera, the transmembrane domain and the cytosolic domain are as set forth in SEQ ID NO: 28. In an other specific embodiment, the amino acid sequence of the chimera is as set forth in SEQ ID NO: 20. In an other specific embodiment, the chimera is encoded by a nucleotide sequence as set forth in SEQ ID NO: 19. In an other specific embodiment, the amino acid sequence of the chimera is as set forth in SEQ ID NO: 24. In an other specific embodiment, the chimera is encoded by a nucleotide sequence as set forth in SEQ ID NO: 23. In an other specific embodiment, the amino acid sequence of the chimera is as set forth in SEQ ID NO: 27. In an other specific embodiment, the chimera is encoded by a nucleotide sequence as set forth in SEQ ID NO: 26.

[0089] In accordance with another aspect of the present invention, there is provided a cell expressing the chimera protein of the present invention. In a other embodiment, the cell expresses the chimera as a transiently transfected cell. In a other embodiment, the cell expresses the chimera as a stably transfected cell. In a other embodiment, the cell further expresses at its cell surface a low level of any one of a very low density

lipoprotein receptor (VLDLR), a low density lipoprotein receptor (LDLR) and an apolipoprotein e receptor 2 (ApoER2).

[0090] In accordance with another aspect of the present invention, there is provided a cell-based assay for identifying a proprotein convertase subtilisin/kexin type 9 preproprotein (PCSK9) inhibitor, which comprises the steps of : (a) providing the cell of the present invention; and (b) comparing the cell surface expression of at least one PCSK9 target receptor, in the presence of a candidate inhibitor and in the absence thereof, whereby a higher level of the at least one receptor at the cell surface in the presence of the candidate inhibitor as compared to in the absence thereof is an indication that the candidate is a PCSK9 inhibitor.

[0091] In accordance with another aspect of the present invention, there is provided a cell-based assay for identifying a proprotein convertase subtilisin/kexin type 9 preproprotein (PCSK9) inhibitor, which comprises the steps of : (a) providing a cell expressing a PCSK9 having an increased resistance to a proprotein convertase (PC); and (b) comparing the cell surface expression of at least one PCSK9 target receptor, in the presence of a candidate inhibitor and in the absence thereof, whereby a higher level of the at least one receptor at the cell surface in the presence of the candidate inhibitor as compared to in the absence thereof is an indication that the candidate is a PCSK9 inhibitor.

[0092] In a specific embodiment of the cell-based assay, the PC is furin. In an other specific embodiment of the cell-based assay, the PC is PC5. In an other specific embodiment of the cell-based assay, the PCSK9 is as set forth in SEQ ID NO: 38 but includes at least one mutation selected from the group consisting of a substitution of phenylalanine for a leucine at position 220 and a substitution of arginine for a serine at position 222 of SEQ ID NO: 38. In an other specific embodiment of the cell-based assay, the at least one PCSK9 target receptor is selected from the group consisting of a very low density lipoprotein receptor (VLDLR), a low density lipoprotein receptor

(LDLR); an apolipoprotein receptor 2 (ApoER2), and a combination thereof. In an other specific embodiment of the cell-based assay, the the at least one PCSK9 target receptor is LDLR. In an other specific embodiment of the cell-based assay, the at least one PCSK9 target receptor is VLDLR. In an other specific embodiment of the cell-based assay, the at least one PCSK9 target receptor is ApoER2.

[0093] In accordance with another aspect of the present invention, there is provided a method of identifying a cell surface receptor directly or indirectly regulated by PCSK9 comprising (a) providing a cell expressing the chimera protein of the present invention, and further expressing a candidate surface receptor; and (b) contacting the cell line with a PCSK9 inhibitor; whereby a higher level of expression of the candidate surface receptor in the presence of the inhibitor as compared to in the absence thereof is an indication that the candidate surface receptor is a cell surface receptor directly or indirectly regulated by PCSK9.

[0094] In accordance with another aspect of the present invention, there is provided a method of identifying a cell surface receptor for use in methods of the present invention: (a) comparing the amino acid sequence of a candidate cell surface receptor with that of at least one PCSK9 target receptor; whereby if the sequence homology between the candidate surface receptor and the PCSK9 target receptor is high enough, the candidate surface receptor is selected as a cell surface receptor for use in methods of the present invention.

[0095] In accordance with another aspect of the present invention, there is provided a method of identifying proprotein convertase subtilisin/kexin type 9 preproprotein (PCSK9) variant that has an increased activity in a cell as compared to a wild type PCSK9 comprising (a) testing a candidate PCSK9 variant for its resistance to furin, whereby a candidate PCSK9 variant having an increased resistance to furin as compared to that of the wild type PCSK9 is an indication that it is a PCSK9 variant having an increased activity.

[0096] In accordance with another aspect of the present invention, there is provided a method of identifying proprotein convertase subtilisin/kexin type 9 preproprotein (PCSK9) variant that has a reduced activity in a cell as compared to a wild type PCSK9 comprising (a) testing a candidate PCSK9 variant for its sensitivity to furin, whereby a candidate PCSK9 variant having an increased sensitivity to furin as compared to that of the wild type PCSK9 is an indication that it is a PCSK9 variant having a reduced activity in a cell.

[0097] In accordance with another aspect of the present invention, there is provided a method of identifying proprotein convertase-sensitivity proprotein convertase subtilisin/kexin type 9 preproprotein (PC-sensitivity PCSK9) variants comprising a) contacting a candidate PC-sensitivity PCSK9 variant with a PC, b) comparing the level of PCSK9 degradation or activity obtained with the candidate PC-sensitivity PCSK9 variant with that obtained with a wild type PCSK9, whereby a difference between the level of PCSK9 degradation or activity of the candidate PC-sensitivity PCSK9 variant and that of the wild type PCSK9 is an indication that the candidate is a PC-sensitivity PCSK9 variant.

[0098] In a specific embodiment, the method is a method of identifying a PC-resistant PCSK9 variant, whereby a lower level of PCSK9 degradation and/or a higher level of PCSK9 activity obtained with the candidate PC-sensitivity PCSK9 variant compared to that obtained with the wild type PCSK9 is an indication that the candidate is a PC-resistant PCSK9 variant. In an other specific embodiment, the method is a method of identifying a PC-hypersensitive PCSK9 variant, whereby a higher level of PCSK9 degradation and/or a lower level of PCSK9 activity of the candidate PC-sensitivity PCSK9 variant compared to that of the wild type PCSK9 is an indication that the candidate is a PC-hypersensitive PCSK9 variant.

[0099] In accordance with another aspect of the present invention, there is provided a method of identifying a novel target in the proprotein convertase subtilisin/kexin type 9 preproprotein (PCSK9) regulatory pathway

comprising (a) contacting a candidate proprotein convertase (PC) with a PCSK9; and (b) comparing the level of PCSK9 degradation or activity obtained in the presence of the PC and in the absence thereof, whereby a difference between the level of PCSK9 degradation or activity obtained in the presence of the PC and in the absence thereof is an indication that the PC is a novel target in the PCSK9 pathway.

[00100] In accordance with another aspect of the present invention, there is provided a purified polypeptide, the amino acid sequence of which consists of SEQ ID NO: 32. In accordance with another aspect of the present invention, there is provided a purified polypeptide, the amino acid sequence of which consists of SEQ ID NO: 31.

[00101] In accordance with another aspect of the present invention, there is provided a purified antibody that binds specifically to a polypeptide of the present invention.

[00102] In accordance with another aspect of the present invention, there is provided a kit comprising a purified ligand that specifically binds to a polypeptide of the present invention, and instructions to use the ligand for detecting in, or purifying the polypeptide from, a biological sample. In a specific embodiment, the kit further comprises a purified ligand that specifically binds to another polypeptide of the present invention. In an other specific embodiment, the kit further comprises a purified ligand that binds to a first polypeptide of the present invention and to a polypeptide as set forth in SEQ ID NO: 34. In an other specific embodiment, the purified ligand that specifically binds to a first polypeptide of the present invention is a purified antibody. In an other specific embodiment, the purified ligand that specifically binds to a second polypeptide of the present invention is a purified antibody.

[00103] In accordance with another aspect of the present invention, there is provided a method of determining whether a biological sample contains a polypeptide of the present invention, comprising contacting the

sample with a purified ligand that specifically binds to the polypeptide, and determining whether the ligand specifically binds to the sample, the binding being an indication that the sample contains the polypeptide. In a specific embodiment of the method, the ligand is a purified antibody.

[00104] In accordance with another aspect of the present invention, there is provided a method of purifying another polypeptide of the present invention from a biological sample containing the polypeptide, said method comprising: (a) contacting the biological sample with a purified ligand that specifically binds to the polypeptide, the ligand being bound to a solid support, to produce a ligand-polypeptide complex, (b) separating the complex from the remainder of the sample, and (c) releasing the polypeptide from the ligand thereby obtaining the purified polypeptide.

[00105] In accordance with another aspect of the present invention, there is provided a method of classifying a subject having a proprotein convertase subtilisin/kexin type 9 preproprotein (PCSK9)-associated disease or condition comprising measuring the concentration of a polypeptide, the amino acid sequence of which is as set forth in SEQ ID NO: 34 in a blood sample of the subject, wherein the results of the measuring step enables the classification of the subject into a subgroup. In a specific embodiment, the method further comprises measuring the concentration of a polypeptide, the amino acid sequence of which is as set forth in SEQ ID NO: 32 in the blood sample of the subject.

[00106] In accordance with another aspect of the present invention, there is provided a method of diagnosing a proprotein convertase subtilisin/kexin type 9 preproprotein (PCSK9)-associated disease or condition in a subject comprising (a) measuring the concentration of a polypeptide, the amino acid sequence of which is as set forth in SEQ ID NO: 34 and of another polypeptide of the present invention in a blood sample of a subject, wherein a ratio of the polypeptide, the amino acid sequence of which is as set forth in SEQ ID NO: 34 : the polypeptide, the amino acid sequence of which is as set

forth in SEQ ID NO: 32 that is higher than that in a control blood sample is an indication that the subject is predisposed to a PCSK9-associated disease or condition.

[00107] In accordance with another aspect of the present invention, there is provided a method of selecting a treatment for a subject comprising (a) measuring the concentration of a polypeptide, the amino acid sequence of which is as set forth in SEQ ID NO: 34 in a blood sample of the subject, wherein a concentration of the polypeptide higher in the blood sample of the subject than that in a control blood sample is an indication that a proprotein convertase subtilisin/kexin type 9 preproprotein (PCSK9) inhibitor may be a useful treatment for the subject. In a specific embodiment, the method further comprises (b) measuring the concentration of the polypeptide, the amino acid sequence of which is as set forth in SEQ ID NO: 32 in the blood sample of the subject, wherein a ratio of the polypeptide, the amino acid sequence of which is as set forth in SEQ ID NO: 34: the polypeptide, the amino acid of which is as set forth in SEQ ID NO: 32 that is higher in the blood sample of the subject than that in a control blood sample is an indication that a PCSK9 inhibitor may be a useful treatment for the subject. In an other specific embodiment, the method is *in vitro*. In an other specific embodiment, the subject is selected from the group consisting of a statin-treated subject, a subject resistant to lipid lowering treatment and a subject having a proprotein convertase subtilisin/kexin type 9 preproprotein (PCSK9)-associated disease or condition. In an other specific embodiment, the PCSK9-associated disease or condition is selected from the group consisting of a cardiovascular disease, schizophrenia, autism, fetal growth restriction, obesity, and a recessive form of non-progressive cerebellar ataxia. In an other specific embodiment, the cardiovascular disease is selected from the group consisting of hypercholesterolemia, atherosclerosis, stroke and ischemia. In an other specific embodiment, the PCSK9-associated disease or condition is hypercholesterolemia. In an other specific embodiment of the methods, the subject is a human.

[00108] In accordance with another aspect of the present invention, there is provided a method of modulating expression of a very low density lipoprotein receptor (VLDLR) or of a apolipoprotein e receptor 2 (ApoER2) at the surface of cells expressing VLDLR and/or ApoER2, comprising modulating proprotein convertase subtilisin/kexin type 9 preproprotein (PCSK9) activity, wherein the modulating of PCSK9 activity modulates the expression of VLDLR and/or ApoER2 at the surface of the cells. In an other specific embodiment, the method is for decreasing the expression of VLDLR at the surface of muscle, heart, kidney and/or brain cells, and wherein the modulating PCSK9 activity is an increasing of PCSK9 secretion. In an other specific embodiment, the method is for decreasing the expression of ApoER2 at the surface of brain, blood platelet and/or testis cells, and wherein the modulating of PCSK9 activity is an increasing of PCSK9 secretion. In an other specific embodiment, the method is for increasing the expression of VLDLR at the surface of muscle, heart, kidney and/or brain cells, and wherein the modulating of PCSK9 activity is a decreasing of PCSK9 secretion. In an other specific embodiment, the method is for increasing the expression of ApoER2 at the surface of brain, blood platelet and/or testis cells, and wherein the modulating PCSK9 activity is a decreasing of PCSK9 secretion. In an other specific embodiment of the method, the decreasing of PCSK9 secretion is achieved with a PCSK9 inhibitor.

[00109] In accordance with another aspect of the present invention, there is provided a method of increasing LDLR expression at the surface of cells of tissues other than liver comprising inhibiting proprotein convertase subtilisin/kexin type 9 preproprotein (PCSK9) secretion from hepatocytes, wherein the inhibiting of PCSK9 secretion from hepatocytes increases the expression of LDLR at the surface of the cells.

[00110] In accordance with another aspect of the present invention, there is provided a use of a proprotein convertase subtilisin/kexin type 9 preproprotein (PCSK9) modulator for modulating expression of a very low

density lipoprotein receptor (VLDLR) or of a apolipoprotein e receptor 2 (ApoER2) at the surface of cells expressing VLDLR and/or ApoER2.

[00111] In accordance with another aspect of the present invention, there is provided a use of a proprotein convertase subtilisin/kexin type 9 preproprotein (PCSK9) modulator in the making of a medicament for modulating expression of a very low density lipoprotein receptor (VLDLR) or of a apolipoprotein e receptor 2 (ApoER2) at the surface of cells expressing VLDLR and/or ApoER2. In a specific embodiment, the use is for decreasing the expression of VLDLR at the surface of muscle, heart, kidney and/or brain cells, and wherein the PCSK9 modulator is able to increase PCSK9 secretion. In a specific embodiment, the use is for decreasing the expression of ApoER2 at the surface of brain, blood platelet and/or testis cells, and wherein the PCSK9 modulator is able to increase PCSK9 secretion. In a specific embodiment, the use is for increasing the expression of VLDLR at the surface of muscle, heart, kidney and/or brain cells, and wherein the PCSK9 modulator is able to decrease PCSK9 secretion. In a specific embodiment, the use is for increasing the expression of ApoER2 at the surface of brain, blood platelet and/or testis cells, and wherein the PCSK9 modulator is able to decrease PCSK9 secretion. In a specific embodiment, the PCSK9 modulator is a PCSK9 inhibitor.

[00112] In accordance with another aspect of the present invention, there is provided a use of an inhibitor of proprotein convertase subtilisin/kexin type 9 preproprotein (PCSK9) secretion from hepatocytes for increasing LDLR expression at the surface of cells of tissues other than liver.

[00113] Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of specific embodiments thereof, given by way of example only with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[00114] In the appended drawings:

[00115] Figure 1 presents a schematic diagram of the steps involved in the zymogen activation of PCs that start in the ER where the primary autocatalytic cleavage occurs. This allows the inactive complex of prosegment-PC to exit from the ER and traffic towards the Golgi and the trans Golgi Network (TGN). The dissociation of the prosegment from the active enzyme takes place usually in an acidic compartment, which is believed to be the endosomes in the case of PCSK9, whereby it is likely that PCSK9 will cleave its prosegment a second time to liberate itself and be active *in trans* on other proteins;

[00116] Figure 2 schematically presents the cell localization where PCs cleave their substrates in the secretory and endocytic pathways. ER: endoplasmic reticulum; TGN: trans Golgi network; SG: secretory granule; s: Serine residue from the active site from the PC-like; Endo: endosome; prosegment, PC-derived inhibitory prosegment;

[00117] Figure 3 schematically presents three examples of PCSK9-transmembrane-cytosolic tail (TM-CT) chimeras used in the present invention along with a wild-type PCSK9. Herein, SP, Pro, Catalytic, and CHRD represent 4 domains of PCSK9: the signal peptide, prosegment, catalytic domain and Cys/His rich domain, respectively. In these chimeras the C-terminus of PCSK9 is fused to the TM-CT of LDLR, Lamp1 and ACE2 respectively;

[00118] Figure 4 shows the Western blot analysis of the level of LDLR (detected by a commercially available C7-mAb) in HEK293 cells transiently co-transfected with LDLR and each of the indicated wild type (WT) PCSK9 or PCSK9-TM-CT chimeras (see Figure 5), in the presence (+) or absence (-) of NH_4Cl for 24h. Note that NH_4Cl allows the rescue from degradation of an

intermediate immunoreactive ~105 kDa form of LDLR that is likely found in endosomes. The pIRES lanes represent the empty vector pIRES-2 either alone or co-transfected with LDLR (as indicated at the bottom of the panel) as controls;

[00119] Figure 5 shows the processing of PCSK9 and some of its mutants. In (A) the positions of the mutations along the PCSK9 sequence tagged with a V5 antigen at the C-terminus are schematically shown (Seidah et al., 2003) In (B) a Western blot analysis using V5 mAb. The expression of either wild type (WT) or the indicated human PCSK9 mutants (top of the lanes) was analysed in transiently transfected HEK293 cells. Notice the absence of the N2 degradation product in the media of HEK293 cells expressing the R218S mutant and its lower levels in the F216L mutant (arrows). In the cell lysates, the active site mutant H226A (an inactive enzyme form) remains as a zymogen (proPCSK9) in the ER and is not secreted into the media;

[00120] Figure 6 shows the amino acid sequences alignment of various species of PCSK9 in the region of arginine at position 218 (Arg_{218}), from the P8 to the P4' processing site: h: human; m: mouse; r: rat; xl: Xenopus laevis (SEQ ID NO: 10); zf: zebfrafish (SEQ ID NO: 11); ck: chicken (SEQ ID NO: 12); tn: Tetraodon nigroviridis (SEQ ID NO: 13); fr: Fugu rubripes (SEQ ID NO: 14). The corresponding sequences of the F216L (SEQ ID NO: 15) and R218S (SEQ ID NO: 16) mutants are shown for comparison at the bottom;

[00121] Figure 7 shows the cleavability of overexpressed human PCSK9 in HEK293 cells by the indicated proprotein convertases and the inhibition of this process by the PC-inhibitor α1-PDX. Twenty four hours following the co-transfection of PCSK9 with a PC or with an empty vector control (the pIRES-2 vector from Invitrogen), the cells were labeled with [^{35}S] [Met+Cys] for 4h (P4h). The cell lysates (Cells) and media were immunoprecipitated with a V5-mAb and the immunoprecipitates resolved by SDS-PAGE, as described (Seidah et al., 2003). sFurin represents the soluble form of furin lacking the

transmembrane-cytosolic tail (Decroly et al., 1996). The stars point to lanes where either processing occurred (with Furin and PC5A) or was inhibited (α1-PDX). From now on, N2 is defined herein as the N-terminal 218 amino acid truncated PCSK9 product (PCSK9-ΔN_{218}). The pIRES lanes represent the empty vector pIRES-2 either alone or co-transfected with PCSK9 (as indicated at the bottom of the panel) as controls;

[00122] Figure 8 shows the cleavability of overexpressed human PCSK9 mutants F216L and R218S in HEK293 cells by the indicated proprotein convertases, or by β-secretase BACE1 (Benjannet et al., 2001). Twenty four hours following the co-transfection of the PCSK9 natural mutants and each PC or with an empty vector control (pIRES). The cells were labeled with [^{35}S] [Met+Cys] for 5h (P5h) and the cell lysates (Cells) and media analyzed as in Figure 7. The stars point to lanes where either processing occurred (Furin and PC5A) or was inhibited by the presence of the mutation (R218S with furin). Cleavage sites GTRFHS^{218}↓QA (SEQ ID NO: 17); GTRLHR^{218}↓QA (SEQ ID NO: 18);

[00123] Figure 9 schematically shows the biosynthetic analysis of the processing of overexpressed human PCSK9 and its indicated mutants following a 5h pulse-labelling of HEK293 cells with [^{35}S] [Met+Cys] as described in Figure 7. The star emphasizes the effectiveness of the RRRR$_{218}$EL (SEQ ID NO: 6) mutant to allow the complete processing of PCSK9 into the PCSK9-ΔN_{218} form by endogenous Furin. PCSK9 amino acid sequence in the vicinity of position 218 RFHR$_{218}$QA (SEQ ID NO: 4) (WT); RFHR$_{218}$EA (SEQ ID NO: 6) (Q219E); RFHR$_{218}$EL (SEQ ID NO: 7) (QA219,220EL); RRRR$_{218}$EL (SEQ ID NO: 5);

[00124] Figure 10 shows the Western blot analysis of the level of endogenous LDLR in HuH7 cells transiently transfected with the indicated PCSK9 constructs, all containing a C-terminal V5-tag. The stars point to the only two constructs that significantly decreased the level of endogenous LDLR (detected by a commercially available C7-mAb). Cells expressed either full

length PCSK9 (FL-PCSK9), namely wild type sequence (WT), mutated R218S, RRRREL (SEQ ID NO: 5) or active site H226A; a truncated PCSK9 construct with the Cys/His rich domain (signal peptide fused to amino acid 455-692 of PCSK9 and ending with a V5 tag) (CHRD) or the empty vector control (pIRES-2). The levels of β-actin was used as control, as well as the level of PCSK9 intracellular proteins produced (detected by a V5-mAb);

[00125] Figure 11 presents a schematic diagram of the LDLR superfamily;

[00126] Figure 12 presents the amino acid sequences alignment of the human LDLR (SEQ ID NO: 118), VLDLR (SEQ ID NO: 119) and ApoER2 (SEQ ID NO: 120), indicating a high degree of sequence identity between the three proteins;

[00127] Figure 13 shows the Western blot analysis of the level of overexpressed ApoER2, VLDLR, and LDLR in CHOK1 cells transiently co-transfected with the indicated PCSK9 constructs (at the top of the lanes) or empty vectors (pIRES2-EGFP = IRES; pcDNA3 = DNA3; Invitrogen). The triangle indicates the position in the gel of respectively ApoER2 (first panel), VLDLR (second panel) and LDLR (third panel). The stars point to the constructs that significantly decreased the level of the receptors (detected by antibodies mentioned below the SDS-PAGE gel, where a23 and a74 are generous gifts from Dr. Joachim Nimpf, Austria and the commercial LDLR antibody was from abcam). The PCSK9 Y38F is a mutant where the Tyr-sulfation of the prosegment is eliminated. The intracellular levels of PCSK9 (detected by a specific in-house polyclonal antibody made in rabbits against human PCSK9) are shown in the corresponding cell lysates in the lower panels;

[00128] Figure 14 shows the Western blot analysis of the level of overexpressed ApoER2 in various cells, such as CHOK1, Neuro2A, HuH7 and HEKL293 cells, transiently co-transfected with the indicated PCSK9

constructs (top lanes). The stars point to the construct [PCSK9-TM-CT (Lamp1)] that significantly decreased the level of ApoER2 in all cells tested. The intracellular levels of PCSK9 (detected by a specific in house antibody against human PCSK9) are shown in the bottom panels;

[00129] Figure 15 schematically shows a multiple positive read out screening assay for the identification of compounds causing PCSK9 inhibition. In this assay, the cell contains a) one chimera of the present invention leading to the expression of a PCSK9 associated with an increase cellular activities (PCSK9-[TM-CT]) and, b) a chimeric construction containing the target site for PCSK9 enzymatic activity (CELISA). In the presented example, the increased detection at the cell surface of endogenous LDLR and of chimeric protein harbouring the HA tag are both dependent on the inhibition of the PCSK9 activity;

[00130] Figure 16 shows the cDNA nucleotide sequence (SEQ ID NO : 19) and the amino acid sequence (SEQ ID NO : 20) of a chimera protein comprising a full-length human PCSK9 (1-692) (SEQ ID NO: 21) indirectly fused to the transmembrane and the cytosolic domains (TM-CT) of LDLR. TM in bold and CT underlined (SEQ ID NO: 22). The fragment between the PCSK9 1-692 and the TM-CT is a V5-tag and is optional;

[00131] Figure 17 shows the cDNA nucleotide sequence (SEQ ID NO : 23) and the amino acid sequence (SEQ ID NO : 24) of a chimera protein comprising a full-length human PCSK9 (1-692) (SEQ ID NO: 21) fused to the transmembrane and the cytosolic domains (TM-CT) of Lamp1 (SEQ ID NO: 25). TM in bold and CT underlined (SEQ ID NO: *). The fragment between the PCSK9 1-692 and the TM-CT is a V5-tag and is optional ;

[00132] Figure 18 shows the cDNA nucleotide sequence (SEQ ID NO : 26) and the amino acid sequence (SEQ ID NO : 27) of a chimera protein comprising full-length human PCSK9 (1-692) (SEQ ID NO: 21) fused to the transmembrane and the cytosolic domains (TM-CT) of ACE2. TM in bold and

CT underlined (SEQ ID NO: 28). The fragment between the PCSK9 1-692 and the TM-CT is a V5-tag and is optional;

[00133] Figure 19 shows: 1. the amino acid sequence of human full-length PCSK9 (1-692) (SEQ ID NO: 21); 2. the amino acid sequence of Pro-protein PCSK9 (i.e. without signal peptide) (31-692) (SEQ ID NO: 33); 3. the amino acid sequence of the active full-length PCSK9 ("active form") (153-692) (SEQ ID NO: 34); 4. the N-terminal fragment of the furin/PC5-cleaved PCSK9 active form (SEQ ID NO: 31) (153-218); and 5. the C-terminal fragment of the furin/PC5-cleaved PCSK9 active form (219-692) (PCSK9-ΔN_{218}) (SEQ ID NO: 32). In these sequences, the signal peptide is underlined (SEQ ID NO: 29), the prosegment is bolded (SEQ ID NO: 30), the N-terminal fragment resulting from the cleavage of the furin/PC5 is italicized (SEQ ID NO: 31) and the C-terminal fragment (N2) resulting from the cleavage of the furin/PC5 is in regular font (SEQ ID NO: 32);

[00134] Figure 20 shows the presence of the Furin/PC5-cleaved PCSK9 form in human plasma. Human plasma was obtained from two healthy volunteers, one male and one female. Human lipoprotein-deficient serum (*LPDS*) was obtained from a commercial pool of plasma. One-hundred microliters of plasma were immunoprecipitated with Ab1-hPC9 or preimmune rabbit serum (*PI*). Immunoprecipitates were separated on 8% glycine gels, and PCSK9 forms were detected with rabbit TrueBlot according to the manufacturer's instructions. Media from HEK293 cells transfected with R218S or $RRRR^{218}EL$ (SEQ ID NO: 6) were immunoprecipitated and loaded as markers of PCSK9 forms. Note the migration difference between V5-tagged and untagged PCSK9 (*left panel*);

[00135] Figure 21 shows the degradation of the VLDLR in skeletal muscles of transgenic mice overexpressing PCSK9 in the liver. Immunohistochemistry of VLDLR (red) in skeletal mouse muscles of control Pcsk9+/+, Pcsk9-/- (Pcsk9 knockout) and transgenic mice (Tg-hPcsk9-V5). The nuclei were stained using TO-PRO-3 (blue; invitrogen). scale: 50µM; and

[00136] Figure 22 presents an alignment of human full-length PCSK9 (1-692) (SEQ ID NO: 21) (NP_777596); mouse full-length PCSK9 (SEQ ID NO: 35) (NP_705793); rat full-length PCSK9 (SEQ ID NO: 36) (NP_954862); and monkey predicted full-length PCSK9 (SEQ ID NO: 37) (XP_513430) wherein "*" denotes that the residues in that column are identical in all sequences of the alignment, ":" denotes that conserved substitutions have been observed, and "." denotes that semi-conserved substitutions have been observed. A consensus sequence derived from this alignment (SEQ ID NO: 38) is also presented. In this consensus, x can be any amino acid and in addition, at positions where the amino acid residue is absent in at least one species of the alignment (denoted by a dash in the alignment), x can also be absent. In specific embodiments of the consensus, mutations associated with diseases or conditions such as dyslipidemia are excluded from the consensus sequence. Hence, in specific embodiments, at position 50 of SEQ ID NO: 38, X is not leucine. In other specific embodiments, one or more X are defined as being any of the amino acids found at that position in the sequences of the alignment. Consensus sequences are also derived from each of the signal peptide (SEQ ID NO: 121), the pro-protein PCSK9 without signal peptide (SEQ ID NO: 122), the prosegment (SEQ ID NO: 123), the active full-length PCSK9 (without signal peptide and prosegment) (SEQ ID NO: 124), the N-terminal fragment of a furin/PC5 cleaved PCSK9 (SEQ ID NO: 125), and the C-terminal fragment of a furin/PC5 cleaved PCSK9 (SEQ ID NO: 126).

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[00137] In a first aspect of the present invention, the examples described herein present cells expressing chimeras presenting a PCSK9 sequence developed in light of an increase PCSK9-associated cellular activity (Figures 3-10). These examples also describe the use of these cells for the identification of convertases involved in the PCSK9 regulation (Figure 5-10). Other examples describe the use of these cells to identify novel surface molecules sensitive to PCSK9 and that could be used as detector molecules

in cell-based assays (Figures 11-14). The method chosen is based on positive and sensitive selection for PCSK9 inhibitors that enhance the cell surface expression of detector molecules (Figure 15).

[00138] In a second aspect of the present invention, the examples described herein present assays for the identification of PCSK9 inhibitors that induces the reappearance of multiple read out at the surface of cell (LDLR, VLDLR and/or ApoER2).

[00139] The present invention is illustrated in further details by the following non-limiting examples presenting sensitive tailor-made cell-based assays designed to isolate convertases inhibitors.

EXAMPLE 1

Identification of PCSK9 with enhanced cellular activities

Construction of the chimera

[00140] The constructions of the three presented PCSK9 chimera (Figure 3) were obtained by standard PCR and cloning techniques (Wiley, J. & Sons) and were made in the model vector phCMV3 (Invitrogen). The cDNA and amino acid sequences appear in Figures 16-18. The chimera presented contain the TM-CT domains of the human low density lipoprotein receptor (LDLR), human lysosomal-associated membrane protein 1 (Lamp1) or human angiotensin converting enzyme-2 (ACE2).

Degradation of LDLR cell surface in the presence of the chimera

[00141] HEK293 cells were transiently transfected with different PCSK9-chimera constructions (Benjannet et al., 2004). Figure 4 shows the Western blot analysis of the level of LDLR (as detected by a commercially available C7-mAb) in HEK293 cells transiently co-transfected with full length LDLR and, as indicated at the top of each lanes, with either wild type (WT) PCSK9, a PCSK9-TM-CT chimera or an empty vector (pIRES). Cells were either treated (+) or not (-) with 5 mM NH_4Cl for 24h. Note that NH_4Cl allows the rescue from

degradation of an intermediate immunoreactive ~105 kDa form of LDLR that is likely found in endosomes. The pIRES lanes represent the empty vector pIRES-2 either alone or co-transfected with LDLR (as indicated at the bottom of the panel) as controls. HEK293 cells co-transfected with LDLR in the presence of any of the three TM-CT chimera tested shows an enhanced degradation of LDLR as compared to the wild type PCSK9 control.

[00142] The LDLR's TM-CT was selected for this particular example as it is one of the proteins targeted for enhanced degradation by PCSK9. Other members of the LDLR superfamily (Figure 11) could have been used, including those that the applicants have shown herein to be degraded by PCSK9, i.e., VLDLR and ApoER2 (Figures 13 and 14). This approach is not either limited to TM-CTs of proteins that are targets for PCSK9-enhanced degradation, as it is also shown herein that the use the TM-CTs of the SARS coronavirus receptor the angiotensin converting enzyme-2 (ACE2) (Bergeron et al., 2005;Vincent et al., 2005) or even TM-CT from lysosomal proteins such as LAMP1 (Conesa et al., 2003) also induce an enhanced degradation of the LDLR at the cell surface (Figure 4).

PCSK9 mutations and their effects on PCSK9 processivity

[00143] The processing of different PCSK9 mutants associated with familial hypercholesterolemia (Figure 5A and Table 1 below) were analyzed.

[00144] TABLE 1: PCSK9 variants associated with hypocholesterolemia, hypercholesterolemia and other PCSK9 variants

Mutation names	Reference	Origin	WT sequence changed for►
Hypocholesterolemia			
R46L	Abifadel M Nature Genet 2003	French, US, Norway,Canada	LVLALRSEEDG► (SEQ ID NO: 39) LVLALLSEEDG (SEQ ID NO: 40)
A68 frameshift L82X	Fasano T ATVB 2007	Italy, Sicily	TFHRCAKDPWR► (SEQ ID NO: 41) TFHRCPRIRRGGCLAPTWWC$_{COOH}$ (SEQ ID NO: 42)
ΔR97	Zhao Z 2006	USA Black population	ERTARRKLQAQA► (SEQ ID NO: 43) ERTAR-KLQAQA (SEQ ID NO: 44)
G106R	Berge KE ATVB 2006	Norway	QAARRGYLTKI► (SEQ ID NO: 45) QAARRRYLTKI (SEQ ID NO: 46)
Y142X	Cohen J Nature genetics 2005	USA Black population	LPHVDYIEEDS► (SEQ ID NO: 47) LPHVD$_{COOH}$ (SEQ ID NO: 48)

L253F	Kotowski IK Am J Hum Gen 2006	USA	RSLRV**L**NCQGK► (SEQ ID NO: 49) RSLRV**F**NCQGK (SEQ ID NO: 50)
C679X	Cohen J Nature Genet 2005	USA	AVAICCRSRHL► (SEQ ID NO: 51) AVAIC$_{COOH}$ (SEQ ID NO: 52)
Hypercholesterolemia			
S127R	Abidafel M Nature 2003	France	FLVKM**S**GDLLE► (SEQ ID NO: 53) FLVKM**R**GDLLE (SEQ ID NO: 54)
F216L	Abidafel M Nature 2003	France	EDGTR**F**HRQAS► (SEQ ID NO: 55) EDGTR**L**HRQAS (SEQ ID NO: 56)
R218S	Allard D Hum Mutation 2005	France	EDGTRF**H**RQASK► (SEQ ID NO: 57) EDGTRF**H**SQASK (SEQ ID NO: 58)
D374Y	Leren TP Clin Genet 2004	Anglo-Saxon	IGASS**D**CSTCF► (SEQ ID NO: 59) IGASS**Y**CSTCF (SEQ ID NO: 60)
R469W	Allard D Hum Mutation 2005	Cameroun	HSGPT**R**MATAI► (SEQ ID NO: 61) HSGPT**W**MATAI (SEQ ID NO: 62)
R496W	Pisciotta L Atheroscl 2006	Italy	RSGKR**R**GERME► (SEQ ID NO: 63) RSGKR**W**GERME (SEQ ID NO: 64)
Other variants			
V4I	Shioji K J Hum Genet 2004	Japan	MGT**V**SSRRS► (SEQ ID NO: 65) MGT**I**SSRRS (SEQ ID NO: 66)
15_16insL	Abidafel M Nature 2003	France	LPL-LLLLLLLLGPA► (SEQ ID NO: 67) LPLLLLLLLLLLGPA (SEQ ID NO: 68)
15_16insLL	Chen SN J Am Coll Card 2005	USA	LPL--LLLLLLLLGP► (SEQ ID NO: 69) LPLLLLLLLLLLGP (SEQ ID NO: 70)
R46L + A53V		Canada	LVLALLSEEDG + (SEQ ID NO: 40) EEDGLVEAPEH (SEQ ID NO: 71)
A53V	Abidafel M Nature 2003	France, USA, Canada	EEDGL**A**EAPEH► (SEQ ID NO: 72) EEDGL**V**EAPEH (SEQ ID NO: 73)
E57K	Kotowski IK Am J Hum Gen 2006	USA	LAEAP**E**HGTTA► (SEQ ID NO: 74) LAEAP**K**HGTTA (SEQ ID NO: 75)
T77I	Fasano T ATVB 2007	Italy, Sicily	WRLPG**T**YVVVL► (SEQ ID NO: 76) WRLPG**I**YVVVLKEET (SEQ ID NO: 77)
V114A	Fasano T ATVB 2007	Italy, Sicily	TKILH**V**FHGLL► (SEQ ID NO: 78) TKILH**A**FHGLL (SEQ ID NO: 79)
N157K	Leren TP Clin Genet 2004	Norway	QSIPW**N**LERIT► (SEQ ID NO: 80) QSIPW**K**LERIT (SEQ ID NO: 81)
R237W	Benjannet S JBC 2004	Canada (QC) Norway	GVVSG**R**DAGVA► (SEQ ID NO: 82) GVVSG**W**DAGVA (SEQ ID NO: 83)
R357H	Allard D Hum Mutation 2005	France	GTNFG**R**CVDLF► (SEQ ID NO: 84) GTNFG**H**CVDLF (SEQ ID NO: 85)
H391N	Kotowski IK Am J Hum Gen 2006	USA Black population	SQAAA**H**VAGIA► (SEQ ID NO: 86) SQAAA**N**VAGIA (SEQ ID NO: 87)
H417Q	Kotowski IK Am J Hum Gen 2006	USA	RQRLI**H**FSAKD► (SEQ ID NO: 88) RQRLI**Q**FSAKD (SEQ ID NO: 89)
I424V	Shioji K J Hum Genet 2004	Japan	SAKDV**I**NEAWF► (SEQ ID NO: 90) SAKDV**V**NEAWF (SEQ ID NO: 91)
N425S	Pisciotta L Atheroscl 2006	Italy	AQDVI**N**EAWFP► (SEQ ID NO: 92) AQDVI**S**EAWFP (SEQ ID NO: 93)
A443T	Allard D Hum Mutation 2005	France, USA	PNLVA**A**LPPST► (SEQ ID NO:94) PNLVA**T**LPPST (SEQ ID NO: 95)
I474V	Abidafel M Nature 2003	Japan	RMATA**I**ARCAP► (SEQ ID NO: 96) RMATA**V**ARCAP (SEQ ID NO: 97)
E482G	Kotowski IK Am J Hum Gen 2006	USA Black population	CAPDE**E**LLSCS► (SEQ ID NO: 98) CAPDE**G**LLSCS (SEQ ID NO: 99)
F515L	Kotowski IK Am J Hum Gen 2006	USA Black population	RAHNA**F**GGEGV► (SEQ ID NO: 100) RAHNA**L**GGEGV (SEQ ID NO: 101)
A522T	Fasano T ATVB 2007	Italy, Sicily	GEGVY**A**IARCC► (SEQ ID NO: 102) GEGVY**T**IARCC (SEQ ID NO: 103)

H553R	Kotowski IK Am J Hum Gen 2006	USA Black population	TRVHCHQQGHV► (SEQ ID NO: 104) TRVHCRQQGHV (SEQ ID NO: 105)
Q554E	Kotowski IK Am J Hum Gen 2006	USA	RVHCHQQGHVL► (SEQ ID NO: 106) RVHCHEQGHVL (SEQ ID NO: 107)
P616L	Fasano T ATVB 2007	Italy, Sicily	KEHGIPAPQEQ► (SEQ ID NO: 108) KEHGILAPQEQ (SEQ ID NO: 109)
Q619P	Kotowski IK Am J Hum Gen 2006	USA	GIPAPQEQVTV► (SEQ ID NO: 110) GIPAPPEQVTV (SEQ ID NO: 111)
E670G	Abidafel M Nature 2003	USA	GSTSEEAVTAV► (SEQ ID NO: 112) GSTSEGAVTAV (SEQ ID NO: 113)

[00145] Wild type and mutated PCSK9 sequences linked to hypercholesterolemia were fused to a tag V5 antigen at the C-terminus as schematically shown in Figure 5A (Seidah et al., 2003). Western blot analyses using V5 mAb were performed with transiently transfected HEK293 cells and the expression and processing of either wild type (WT) or the indicated human PCSK9 mutants (top of the lanes) were compared. Notice the absence of the N2 degradation product in the media of HEK293 cells expressing the R218S mutant and its lower levels in the F216L mutant (arrows). In the cell lysates, the active site mutant H226A (an inactive enzyme form) remains as a zymogen (proPCSK9) remaining in the ER and not secreted into the media.

[00146] It was observed that following signal peptidase cleavage, the endoplasmic reticulum (ER) resident zymogen proPCSK9 (~75 kDa) autocatalytically cleaves its N-terminal prosegment resulting in a tight binding complex of PCSK9 and the prosegment (amino acid, aa, 31-152; ~15 kDa). The latter complex can then exit the ER and is secreted constitutively as a major full length mature ~60 kDa protein (N1) (Figure 5, wild type, WT). However, in many cell lines was also observed the presence of an N-terminally truncated form of N1, of about ~52 kDa called N2 (Figure 5). The loss of ~8 kDa from the N1 product occurs also in a number of natural mutants including the S127R and R237W ones, but not in the active site mutant H226A that remains in the ER as proPCSK9 (Figure 5). Interestingly, this N2 form was either not observed or significantly decreased in two natural mutants, i.e., the French mutants R218S and F216L, respectively (Figure 5).

Sequence alignment of the vertebrate PCSK9

[00147] Sequence alignment of vertebrate PCSK9 showed a complete conservation of Arg_{218}, which in most cases is found within an R-X-X-R (SEQ ID NO: 3) or KXXXXR (SEQ ID NO: 114) sequence (Figure 6), typical of a basic amino acid specific PC-recognition motif recognized by Furin and/or PC5-like enzymes (Seidah and Chretien, 1999). The mutation R218S would completely disrupt this motif as it eliminates the Arg in position P1, and the F216L would affect the P3 position of this motif (Figure 6, bottom). These observations fit with the applicants' repeated inability to obtain a N-terminal sequence of N2 using Edman degradation, as the N-terminal Gln of human PCSK9 would be expected to cyclize on the sequencer and block the reaction (data not shown).

Identification of protein convertases that process PCSK9

[00148] Was next tested the hypothesis that cleavage at Arg_{218} is performed by one or more basic amino acid PCs. Accordingly, wild type PCSK9 was co-expressed with all the convertases as well as with β-secretase BACE1 in HEK293 cells. Cells transiently transfected with vectors expressing hPCSK9-V5 and/or the different convertases (as indicated at the top of lanes) were pulse-labeled with ^{35}S-(Met+Cys) for 4h and cell extracts (Cells) and media (Media) were immunoprecipitated with a V5 antibody and the precipitates were resolved by SDS/PAGE. Data revealed that only the membrane-bound Furin (but not sFurin, the soluble one, lacking the transmembrane-cytosolic tail) and to a lesser extent PC5A are capable of processing the N1 form of PCSK9 into the N2 form, with the concomitant loss of the co-immunoprecipitated PCSK9 prosegment (Figure 7). The N-terminally truncated N2 product is thus referred to as PCSK9-ΔN_{218} (SEQ ID NO: 32) (PCSK9 lacking the first 218 amino acids; Figure 5). In addition, co-expression of the serpin α1-PDX, which inhibits most of the basic aa-specific PCs (Anderson et al., 1993; Benjannet et al., 1997), completely inhibits such processing into PCSK9-ΔN_{218} (Figure 7). In agreement, Furin can no longer process the R218S mutant, and cleaves to a lesser extent the F216L one

(compare Figures 7 and 8). The R218S mutation abrogates the Furin/PC5A cleavage at the sequence $RFHR_{218}$.

[00149] Based on the crystal structure of Furin (Henrich et al., 2003) and the analysis of its many substrates (Seidah and Chretien, 1999), the best substrates of Furin would have the motif RX(R/K)R↓(E/D)L (SEQ ID NO: 115). This led the applicants to produce mutants of PCSK9 that should greatly enhance the ability of Furin to process this molecule. Thus, endogenous Furin in HEK293 cells can completely process PCSK9 at Arg_{218} upon replacement of the wild type $RFHR_{218}$↓QA (SEQ ID NO: 4) sequence by an optimal Furin-recognition sequence $RRRR^{218}$↓EL (SEQ ID NO: 5), while the motifs $RFHR_{218}$↓EA (SEQ ID NO: 6) and $RFHR_{218}$↓EL (SEQ ID NO: 7) exhibited intermediate Furin-cleavability (Figure 9). Notice the almost absence of prosegment co-immunoprecipitating with the PCSK9-ΔN218 form produced (Figure 9), which would be predicted since such cleavage would remove the PCSK9 segment 153-218 (SEQ ID NO: 31), which contains the active site Asp^{186}. This would also suggest that the PCSK9-ΔN_{218} form is unable to tightly bind the prosegment, which in all PCs only binds the catalytically active convertase (Zhong et al., 1999; Nour et al., 2005; Essalmani et al., 2006).

Degradation of LDLR cell surface in the presence of the PCSK9 mutants

[00150] Earlier studies revealed that overexpression of PCSK9 results in an enhanced degradation of the LDLR in a number of cells lines and *in vivo*. Accordingly, the activity on the degradation of LDLR of wild type PCSK9, its R218S mutant, the $RRRR_{218}$EL (SEQ ID NO: 5) variant, the active site mutant H226A and the Cys/His-rich domain of PCSK9 (aa 454-692 CHRD) was analyzed by Western blots in HuH7 cells (Benjannet et al., 2004) (Figure 10). Results revealed that only wild type PCSK9 and its R218S mutant are active in enhancing the degradation of the LDLR, as compared to the pIRES control empty vector, while the $RRRR^{218}$EL (SEQ ID NO: 5) variant and the CHRD form are inactive, as is the active site mutant H226A (Figure 10). Thus, PCSK9-ΔN_{218} is an inactive form of PCSK9 that is secreted from cells, as compared to the active site mutant His_{226}, which results in an uncleaved

zymogen proPCSK9 that remains in the ER. Thus, the RRRR^{218}EL (SEQ ID NO: 5) variant of PCSK9 provides an ideal control for the activity of PCSK9 in the secretory pathway, as opposed to active site mutants that can no longer exit from the ER and hence do not co-traffic with the LDLR to the cell surface/endosomes.

Novel members of the PCSK9 pathway

[00151] The cleavage of PCSK9 by Furin and/or PC5A provides a rationale behind the hypercholesterolemia phenotype associated with the French (F216L and R218S) mutations and hypocholesterolemia phenotype in Black African Americans associated with L253F mutation (results not shown) (Abifadel et al., 2003;Allard et al., 2005). Thus, PCSK9 processing by other PCs is a novel mechanism regulating the level of the active form of the enzyme, and may represent a general mechanism behind other mutations resulting in either hypercholesterolemia (loss of cleavage) or hypocholesterolemia (gain of cleavage) (Table 1 above). This does not exclude the possibility that other mechanisms may be responsible for the phenotypes behind other mutations, such as cellular sorting, post-translational modifications and zymogen activation, etc. This information provides powerful tools for the design of potent cell-based assays that incorporate PCSK9 variants with enhanced cellular activities. This information also allows the identification of novel targets (e.g. PC5A, Furin) in the PCSK9 regulatory pathway.

Circulating forms of PCSK9 in human plasma

[00152] To substantiate the physiological relevance of the *ex vivo* observation of the cleavage of human PCSK9 by furin/PC5 into secretable PCSK9-ΔN_{218}, the forms of PCSK9 that are found in the normal plasma of two individuals, one female and one male, as well as in a lipoprotein-deficient serum prepared from a commercial pool of normal human plasma (Bioreclamation Inc.), were characterized. An in-house human antibody (Ab1-hPC9) obtained from rabbits injected with affinity-purified pro-PCSK9 (aa 31-

454) (SEQ ID NO: 126) expressed in *Escherichia coli* BL21 was selected for immunoprecipitation, followed by Western blotting analysis.

[00153] One-hundred microliters of plasma were immunoprecipitated with Ab1-hPC9 (1:200) or preimmune rabbit serum (PI). Immunoprecipitates were separated by SDS-PAGE on 8% glycine gels. Following transfer to a polyvinylidene difluoride membrane, PCSK9 forms were detected with the same antibody (1:3000) followed by rabbit TrueBlot™ (eBioscience) as a secondary antibody according to the manufacturer's instructions. Affinity removal of IgGs and albumin from plasma was performed using a ProteoSeek™ removal kit (Pierce). Media from HEK293 cells transfected with R218S or $RRRR_{218}EL$ (SEQ ID NO: 5) were immunoprecipitated and loaded as markers of PCSK9 forms.

[00154] The data presented in Figure 20 shows that, in all cases, PCSK9 and its PCSK9-ΔN_{218} product are both circulating in male and female plasma. The ratio between these two circulating forms varies between individuals. Furthermore, a similar result was obtained with the commercial pooled human sera. Furin/PC5 processing of PCSK9 is thus physiological.

[00155] The level of active form of PCSK9 as well as the ratio between the active and furin/PC5-cleaved forms present in plasma collected from patients with PCSK9-associated diseases are measured by known techniques such as an enzyme-linked immunosorbant assay (ELISA), immunoprecipitation followed by Western blotting and quantitative mass spectrometry. The in-house polyclonal human antibody (Ab1-hPC9) recognizes both active full-length and cleaved forms, whereas the *in house* monoclonal antibody only recognizes the furin/PC5-cleaved C-terminal PCSK9 form. An antibody that specifically binds to the active full-length can be produced by using the 153-218 (SEQ ID NO: 31) fragment as antigen (or any species equivalent e.g. monkey 153-218, etc.). The combined use of both antibodies, in ELISA for example, allows the determination of the full-length to furin/PC5-cleaved PCSK9 forms ratio. The furin/PC5-cleaved PCSK9 form

was measured to represent from 10 to 20% of total PCSK9 forms in a normal sample. These measurements are used as a diagnostic tool for the tailor-made therapeutic approach applicable to each patient. For example, a high ratio of full-length to furin/PC5-cleaved PCSK9 forms is indicative of a predisposition to hypercholesterolemia. A high ratio of full-length to furin/PC5-cleaved PCSK9 forms is also indicative of a need for a treatment targeting the PCSK9 activity. Such treatment for reducing the level of circulating LDL-cholesterol could combine, for example, statins and PCSK9-inhibitors. Variation of the ratio is also indicative of the presence of a PCSK9 variant (e.g., R218S or D374Y), or the presence of a PCSK9 upstream regulator variant (e.g. furin/PC5), or of others factors such as a specific diet, or treatment with statin.

[00156] These measurements could also be used to compare the PCSK9 profiles of different cohorts, for example, cohorts of patients treated with statins, cohorts of hypercholesterolemic and hypocholesterolemic patients as well as those that are resistant to various lipid lowering treatments or other PCSK9-associated diseases.

[00157] The present invention provides a method of measuring the ratio between full-length active PCSK9 form and its inactive PCSK9-ΔN_{218} product in the plasma and the use of such measurement as a diagnostic tool in PCSK9-associated diseases. Commercially available antibodies include rabbit anti-human PCSK9-(490–502) pAb (Cayman Chemical, catalog no. 10007185) which recognizes pro-PCSK9 and PCSK9-N218 but not full-length active form of PCSK9 (amino acids 153-692) and goat anti-human PCSK9 (679-692) (Imgenex, catalog no IMX-3786) antibodies. Taking into account the slightly higher molecular mass of V5-tagged PCSK9 compared with untagged PCSK9, it was possible to show that the plasma forms co-migrated with markers obtained from the medium of HEK293 cells overexpressing the uncleavable R218S or fully processed $RRRR_{218}EL$ (SEQ ID NO: 5) variant (Figure 20)).

EXAMPLE 2

ENHANCED CELLULAR PCSK9 ACTIVITIES AND THE IDENTIFICATION OF NOVEL CELL SURFACE DETECTOR MOLECULES

[00158] Cells which express a PCSK9 with increased cellular activities, as measured by a very low to undetectable level of LDLR at the cell surface, could be used to identify novel cell surface molecules that, similarly to LDLR, are also sensitive to the presence of the PCSK9.

[00159] While LRP was found not to be affected by PCSK9 (Benjannet et al., 2004), the present invention shows that VLDLR and ApoER2 (Figure 11 and 12), are degraded by PCSK9 (Figures 13 and 14).[PCSK9-TM-CT (Lamp1)] (SEQ ID NO: 24) was selected as the chimera that results in the highest efficacy of degradation of either LDLR, VLDLR or ApoER2.

[00160] A stable transfectant pool of [PCSK9-TM-CT (Lamp1)] was obtained in HuH7 cells that were resistant to G418. These cells were then FACS-selected for clones with the lowest levels of endogenous cell-surface LDLR. These cells form the basis for the proteomics and genomics analysis for the discovery of PCSK9-related functions.

EXAMPLE 3

CELL-BASED ASSAY FOR THE INHIBITION OF PCSK9

[00161] Stable clones expressing [PCSK9-TM-CT (Lamp1)] (SEQ ID NO: 24) formed the basis for a cell-based assay for the HTS analysis for the discovery of PCSK9-inhibitory/silencing compounds.

[00162] The sequence of PCSK9 in the chimera could contain the human full-length (SEQ ID NO: 21) or any sequence satisfying the consensus derived from the human, mice, rat and monkey PCSK9 or alternatively variants of PCSK9 identified as conferring to PCSK9 resistance to cleavage by other enzymes, e.g., the R218S mutation (Figure 8), thereby resulting in an increased PCSK9 activity due to its lower degradation.

[00163] LDLR read out may be used in HuH7 cells or any cell type of interest. The protein level of the LDLR at the cell surface is extremely low as verified by FACS analysis. Upon HTS screening, the increased level of LDLR at the cell surface was measured using either a fluorescent or HRP-tagged antibody to LDLR, or using a fluorescent ligand such as Dil-LDL (Figure 15). However, HuH7 cells stably expressing low levels of ApoER2 or VLDLR are also obtained as alternative assays for PCSK9 activity and the selection for appropriate inhibitors. These assays could also incorporate multiple read-outs, namely LDLR, VLDLR or ApoER2 (each measured with specific antibodies linked to different fluorochromes, e.g., green, magenta and red flurorescent moieties).

EXAMPLE 4

HIGH THROUGHPUT SCREENING FOR PCSK9 INHIBITORS

[00164] Candidate inhibitors are screened on the cell assays of the present invention. Compounds showing statistically significant activity in both replicates are selected as hit compounds. Hit compounds are be verified by LC mass spectrometry and 10-point titrations are performed in triplicate on each compound to determine IC50 values (concentration of 50% inhibition). In addition to the screening process itself, expression and purification of a modulated candidate convertases for *in vitro* assays, assay adaptation, and Quantitative Structure-Activity Relationship (QSAR) studies on hits are performed. Particularly, inhibitors with Kis in the nanomolar range are sought. *In vitro* and *ex vivo* validation of the lead compounds will confirm their inhibitory potency and effects.

EXAMPLE 5

MULTIPLEXED POSITIVE CELL-BASED ASSAYS FOR PCSK9 INHIBITORS COMBINED WITH A CELISA

[00165] While inhibitors of the function of PCSK9 would reflect a blockage at some point of the PCSK9 pathway, they do not necessarily represent catalytic inhibitors. For this purpose, the present invention encompasses incorporating in the cell-based assay in addition to the PCSK9 chimera (e.g., R218S-PCSK9-[TM-CT]), another bait-specific chimera that

expresses a cell-surface protein containing a PCSK9 recognition processing sequence (e.g., SSVFAQSIPWN (SEQ ID NO: 117)) (such as that described in co-pending WO 2007/030937 filed September 14, 2006). The bait-specific cells surface protein contains the following configurations: [signal peptide]-[HA tag]-[Bait region of PCSK9, e.g., SSVFAQSIPWN (SEQ ID NO: 117)]- [Fc portion of human immunoglobulin]-[TM-CT (ACE2)]. This allows a better evaluation of the effect of the selected compounds on the enzymatic activity of PCSK9 itself within a cellular context.

[00166] The chimera expressing a bait specific for PCSK9 (SSVFAQSIPWN (SEQ ID NO: 117) or a longer form) is stably transfected into cells expressing PCSK9- [TM-CT-Lamp1] (SEQ ID NO: 24). FACS-selected stable pools of HuH7 cells that do not present LDLR at the surface are selected. The absence or very low amount of LDLR could also be tested with fluorogenic LDLR ligand (DiI-LDL) or using of a monoclonal antibody to LDLR. Inhibitors of PCSK9 catalytic activity affect the appearance of the HA tag from the bait-specific chimera at the cell surface. The detection in parallel of both HA and LDLR at the cell surface could be performed using a variety of assays including CELISA assays (such as described in co-pending WO 2007/030937 filed September 14, 2006) and the use of a fluorogenic LDLR ligand or mAB to LDLR coupled to a chemiluminescent probe (Figure 15). Screening is performed to identify compounds associated to high levels of the HA tag and the LDLR at the cell surface.

EXAMPLE 6

Optimization of leads

[00167] Once inhibitor "leads" are identified, they will be further characterized for affinity, mode of inhibition and specificity using *in vitro* assays and purified PC enzymes.

EXAMPLE 7

Validation of novel PCSK9-associated pathways using an animal model

[00168] A transgenic mice specifically overexpressing PCSK9 in hepatocytes was generated. The transgene was under the control of the ApoE promoter and enhancer regulation. The mice were seemingly healthy, however their circulating LDL-Cholesterol was quite elevated. Transgenic expression of PCSK9 in mouse liver resulted in a line that expressed 40 fold higher PCSK9 than the endogenous enzyme in hepatocytes (result not shown). The transgenic protein was tagged with a V5 at its c-terminus to differentiate it from the endogenous one. Analysis of mouse plasma samples revealed that PCSK9-V5 is secreted in blood and is partially processed by Furin/PC5-like enzymes to generate PCSK9-ΔN_{218} as observed in cells and in human plasma. Immunofluorescence and Confocal Microscopy analyses of the skeletal mouse muscles were performed using a rabbit polyclonal Ab:VLDLR (a74; 1:200). Immunofluorescence analyses were performed with a Zeiss™ LSM-510 confocal microscope. Confocal immunofluorescence microscopy was performed with a Nikon Eclipse™ TE2000-U laser-scanning microscope with 408, 488, and 543-nm laser lines. Images were processed with Adobe Photoshop™ CS2, version 9.0 (Adobe Systems). Interestingly, analysis of VLDLR levels in the muscle of transgenic mice versus non-transgenic control littermates ($PCSK9^{+/+}$; $PCSK9^{-/-}$) revealed that the level of VLDR is dramatically decreased in these mice (right panel, Figure 21). In contrast, the level of VLDLR in PCSK9-knockout mice is dramatically increased compared to the control mice (middle panel, Figure 21). This is the first evidence that circulating PCSK9 can enhance the degradation of VLDLR *in vivo*. The decrease level of VLDLR in the transgenic mice overexpressing PCSK9 in the liver was also confirm by immunodetection of PCSK9 in extracts of skeletal muscle (see Figure 21).

[00169] The present invention allow the identification of novel PCSK9-associated pathways and identify PCSK9 as a potential target in these pathways-associated diseases (e.g. in the VLDLR-associated diseases).

[00170] The scope of the claims should not be limited by the preferred embodiments set forth in the examples, but should be given the broadest interpretation consistent with the description as a whole.

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CLAIMS:

1. A method of selecting a treatment for a subject having a proprotein convertase subtilisin/kexin type 9 preproprotein (PCSK9)-associated disease or condition, wherein the PCSK9-associated disease or condition is a cardiovascular disease, schizophrenia, autism, fetal growth restriction, obesity or a recessive form of non-progressive cerebellar ataxia, comprising:

(a) measuring the concentration of a polypeptide, the amino acid sequence of which is as set forth in SEQ ID NO: 34 in a blood sample of the subject,

wherein a concentration of the polypeptide higher in the blood sample of the subject than that in a control blood sample is an indication that a proprotein convertase subtilisin/kexin type 9 preproprotein (PCSK9) inhibitor is a useful treatment for the subject.

2. The method of claim 1, further comprising:

(b) measuring the concentration of a polypeptide, the amino acid sequence of which is as set forth in SEQ ID NO: 32 in the blood sample of the subject, wherein a ratio of the polypeptide, the amino acid sequence of which is as set forth in SEQ ID NO: 34 over the polypeptide, the amino acid sequence of which is as set forth in SEQ ID NO: 32 that is higher in the blood sample of the subject than that in a control blood sample is an indication that a PCSK9 inhibitor is a useful treatment for the subject.

3. A method of diagnosing a proprotein convertase subtilisin/kexin type 9 preproprotein (PCSK9)-associated disease or condition or a predisposition thereto, wherein the PCSK9-associated disease or condition is a cardiovascular disease, schizophrenia, autism, fetal growth restriction, obesity or a recessive form of non-progressive cerebellar ataxia, in a subject comprising:

(a) measuring the concentration of a polypeptide, the amino acid sequence of which is as set forth in SEQ ID NO: 34 in a blood sample of a subject,

wherein a concentration of the polypeptide higher in the blood sample of the subject than that in a control blood sample is an indication that the subject has a PCSK9-associated disease or condition or has a predisposition thereto.

4. The method of claim 3, further comprising:

(b) measuring the concentration of a polypeptide, the amino acid sequence of which is as set forth in SEQ ID NO: 32 in the blood sample of the subject,

wherein a ratio of the polypeptide as set forth in SEQ ID NO: 34 over the polypeptide as set forth in SEQ ID NO: 32 that is higher in the blood sample of the subject than that in a control blood sample is an indication that the subject has a PCSK9-associated disease or condition or has a predisposition thereto.

5. The method of claim 2 or 4, wherein the polypeptide as set forth in SEQ ID NO: 32 further comprises at least one mutation associated with hypercholesterolemia or with hypocholesterolemia.

6. The method of claim 2, 4, or 5, wherein the concentration of the polypeptide as set forth in SEQ ID NO: 32 is measured by mass spectroscopy.

7. The method of any one of claims 1 to 6, wherein the polypeptide as set forth in SEQ ID NO: 34 further comprises at least one mutation associated with hypercholesterolemia or with hypocholesterolemia.

8. The method of any one of claims 1 to 7, wherein the concentration of the polypeptide as set forth in SEQ ID NO: 34 is measured by mass spectroscopy.

9. The method of any one of claims 1 to 8, wherein the subject is a statin-treated subject, a subject resistant to lipid lowering

treatment or a subject having a proprotein convertase subtilisin/kexin type 9 preproprotein (PCSK9)-associated disease or condition.

10. The method of any one of claims 1 to 9, wherein the PCSK9-associated disease or condition is hypercholesterolemia, atherosclerosis, stroke or ischemia.

11. The method of claim 10, wherein the PCSK9-associated disease or condition is hypercholesterolemia.

12. The method of claim 10, wherein the PCSK9-associated disease or condition is atherosclerosis.

13. The method of claim 10, wherein the PCSK9-associated disease or condition is stroke.

14. The method of claim 10, wherein the PCSK9-associated disease or condition is ischemia.

15. The method of any one of claims 1 to 14, wherein the subject is a human.

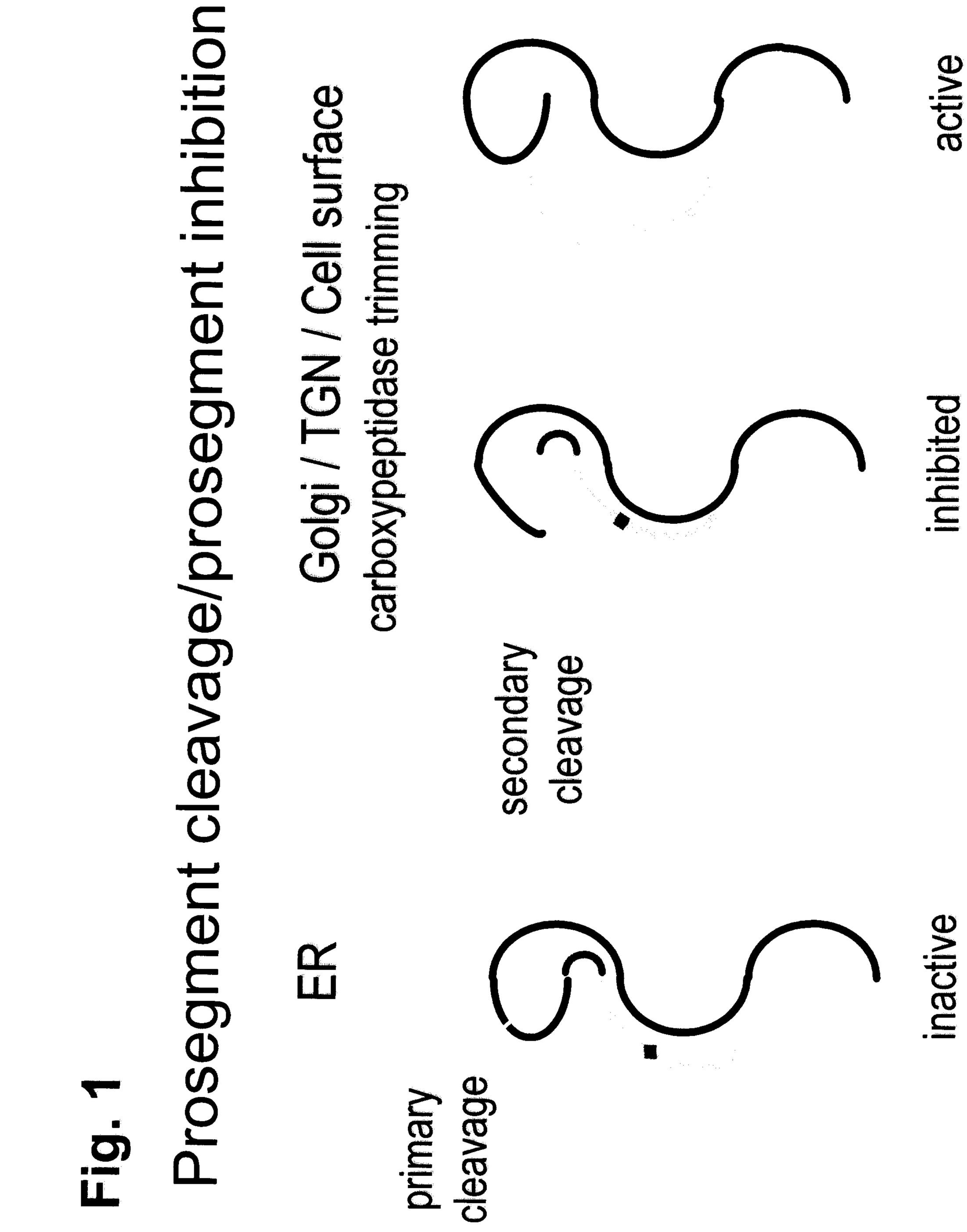


Fig. 1
Prosegment cleavage/prosegment inhibition
ER
primary cleavage
inactive
secondary cleavage
Golgi / TGN / Cell surface
carboxypeptidase trimming
active
inhibited

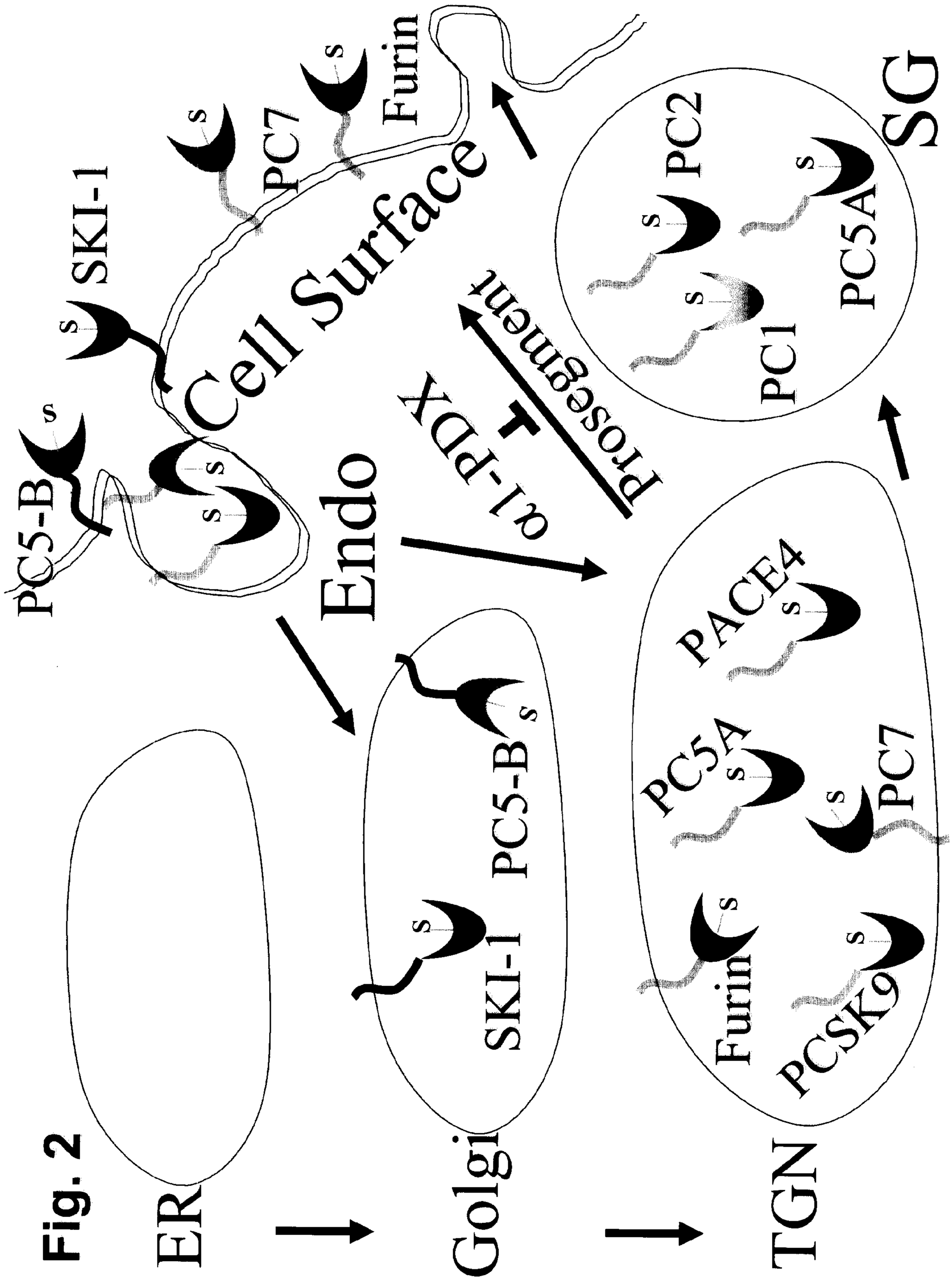
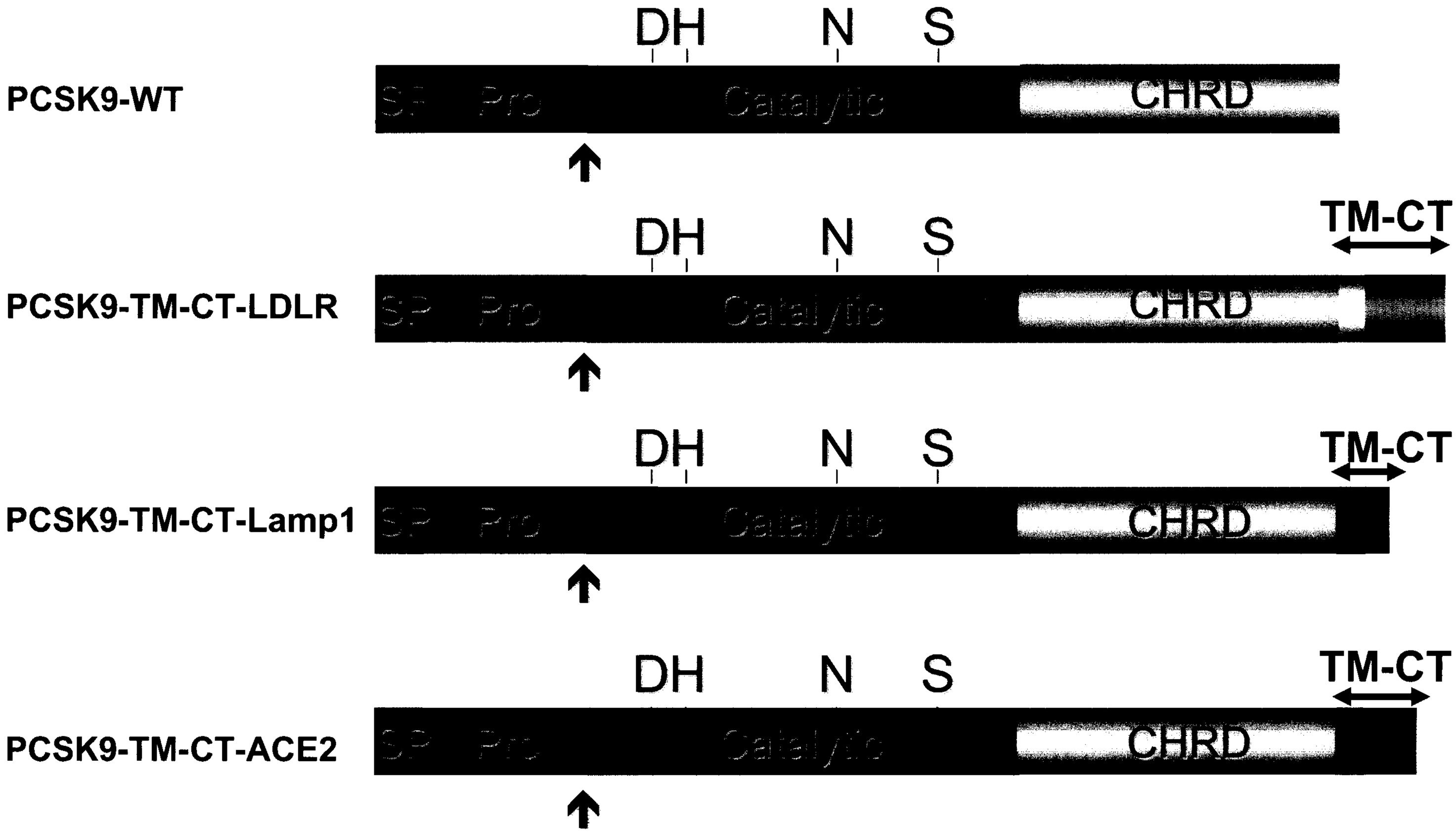

ER
Golgi
TGN
Endo
Cell Surface
Furin
PC7
SKI-1
PC5-B
PACE4
PC2
PC1
PC5A
PCSK9
α1-PDX
Prosegment
S

Fig. 2

Fig. 3

Type-I membrane bound forms of human PCSK9



WO 2007/128121
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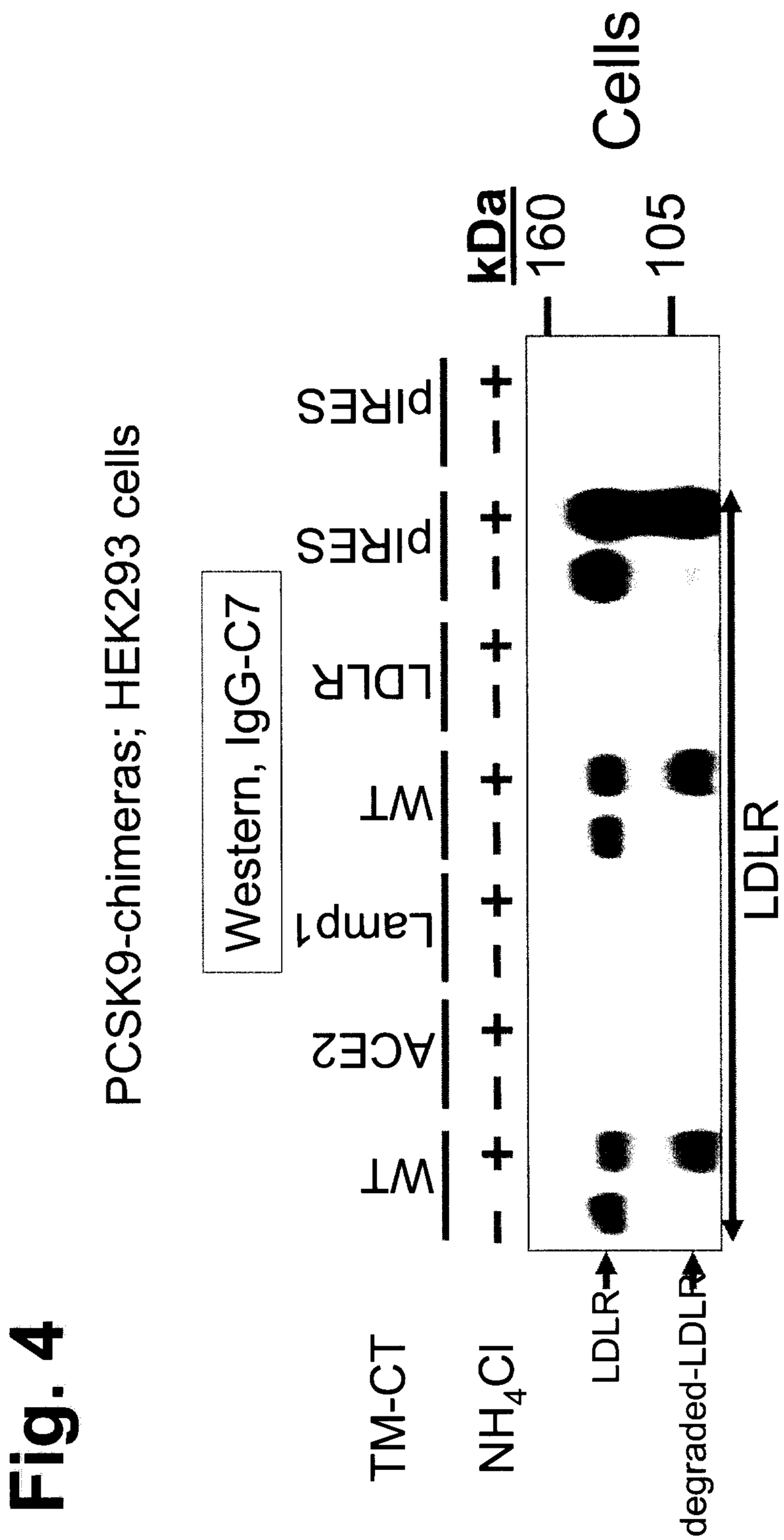
Cells
PCSK9-chimeras; HEK293 cells
Western, IgG-C7
WT
Lamp1
ACE2
WT
LDLR
pIRES
pIRES
pIRES
kDa
160
105
LDLR
LDLR
degraded-LDLR
NH4Cl
TM-CT

Fig. 4

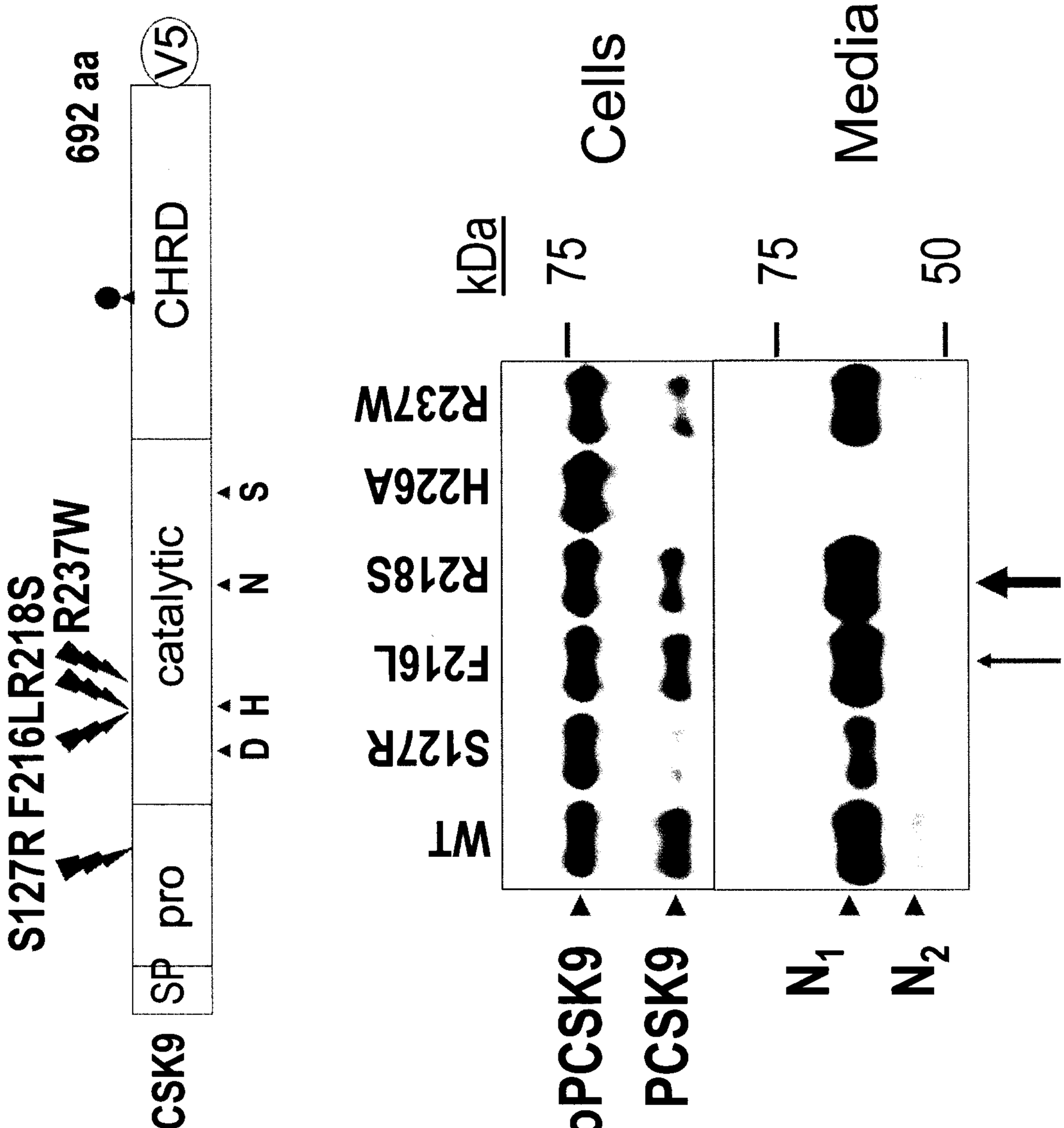
A
proPCSK9
692 aa
V5
CHRD
catalytic
pro
SP
S127R F216L R218S R237W
S
N
H
D
B
proPCSK9
Cells
Media
kDa
75
75
50
WT
S127R
F216L
R218S
H226A
R237W
N1
N2

Fig. 5

Fig. 6

Possible inactivation of PCSK9 by Furin-like convertases

Substrate		P8	P6	P4	P2	P1	P1'	P4'
(h,m,r,xl)	PCSK9	E	D	G	T	**R**	F	**H**	**R** ↓	Q	A	S	K
(zf)	PCSK9	E	D	G	T	**R**	V	**H**	**R** ↓	Q	A	S	Q
(ck)	PCSK9	E	D	S	S	**R**	F	**H**	**R** ↓	Q	A	S	K
(tn)	PCSK9	V	G	E	A	G	G	**H**	**R** ↓	E	A	S	R
(fr)	PCSK9	V	E	**K**	G	G	G	**H**	**R** ↓	E	A	S	R
(h) (F216L)	PCSK9	E	D	G	T	**R**	**L** (216)	**H**	**R** ↓	Q	A	S	K
(h) (R218S)	PCSK9	E	D	G	T	**R**	F	**H**	**S** (218) ↓	Q	A	S	K

WO 2007/128121
PCT/CA2007/000794
CA 02652247 2008-11-10
6/24

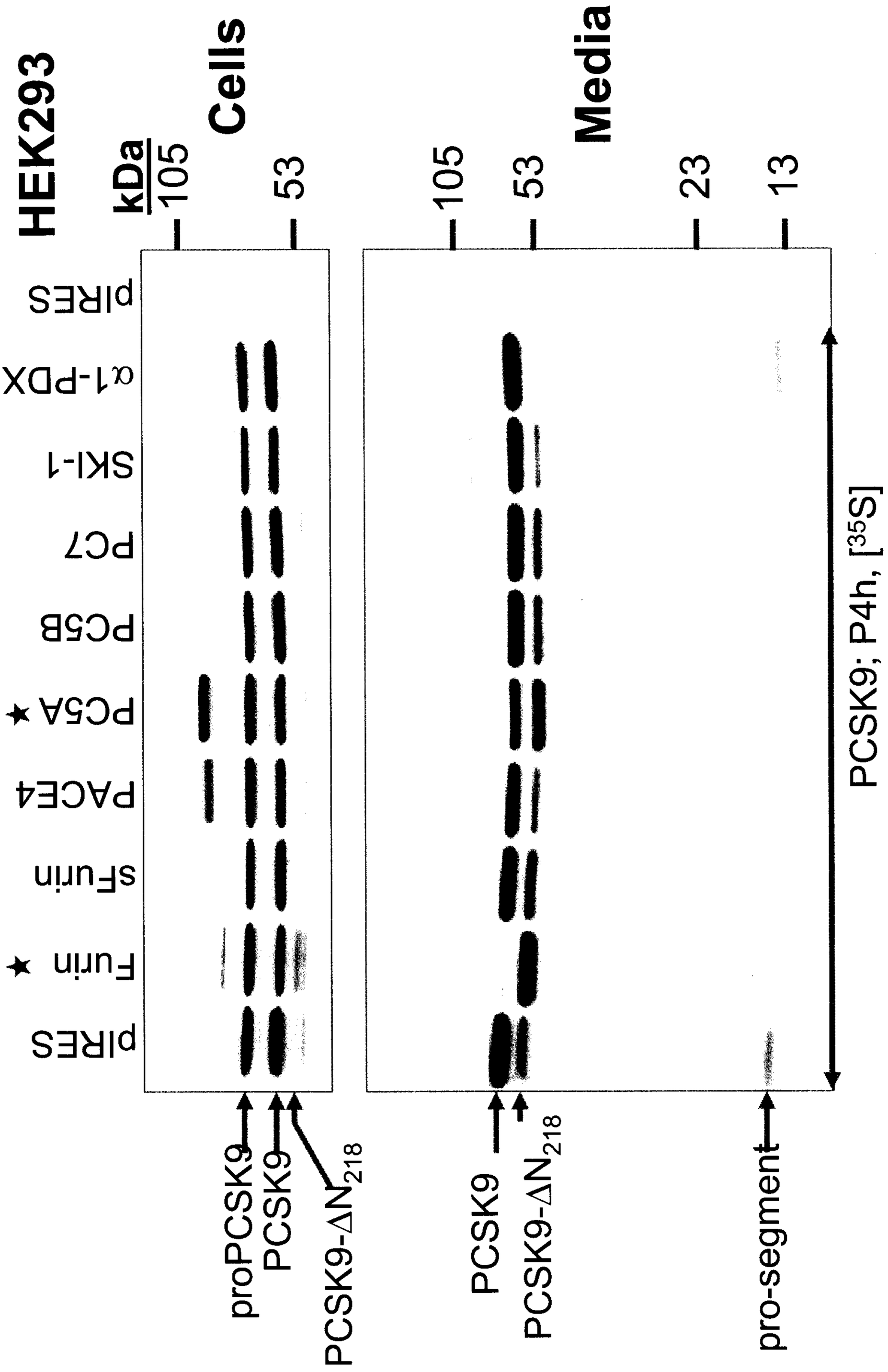
HEK293
kDa
105
53
Cells
105
53
23
13
Media
pIRES
α1-PDX★
SKI-1
PC7
PC5B
PC5A ★
PACE4
sFurin
Furin ★
pIRES
PCSK9; P4h, [35S]
proPCSK9
PCSK9
PCSK9-ΔN218
PCSK9
PCSK9-ΔN218
pro-segment

Fig. 7

HEK293

Cells

Cleavage sites
GTRFHS218- QA
GTRLHR218QA

Media

P5h, [35S]

kDa

105 53 105 53 23 13

BACE-1
SKI-1
PC7
PC5A ★
Furin ★
pIRES

BACE-1
SKI-1
PC7
PC5A ★
Furin ★
pIRES

R218S

F216L

proPCSK9
PCSK9
PCSK9-ΔN218

PCSK9
PCSK9-ΔN218
pro-segment

Fig. 8

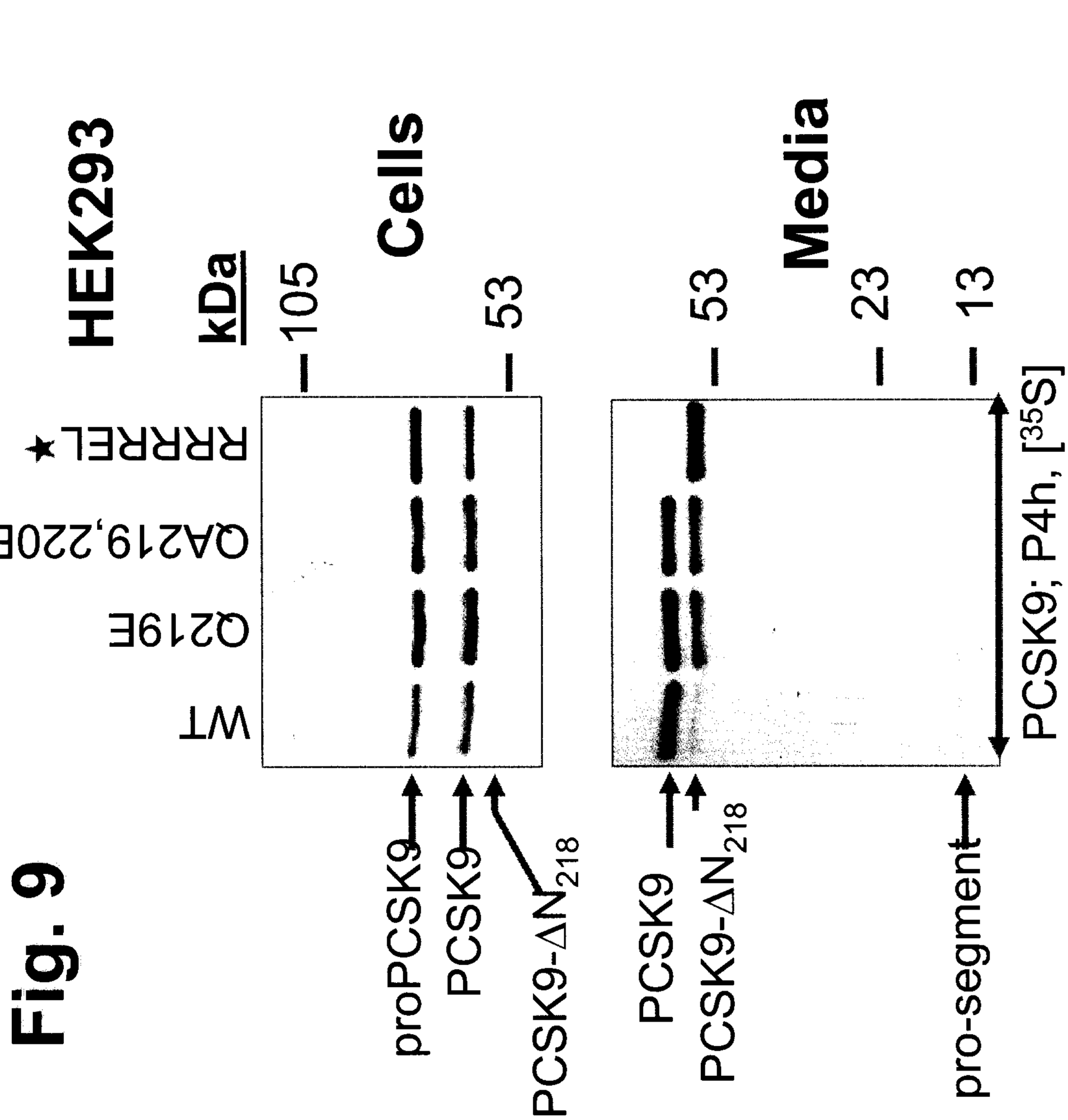
HEK293
kDa
105
53
Cells
53
23
13
Media
WT
Q219E
Q219,220EL
RRRREL★
proPCSK9
PCSK9
PCSK9-ΔN218
PCSK9
PCSK9-ΔN218
pro-segment
PCSK9; P4h, [35S]

Fig. 9

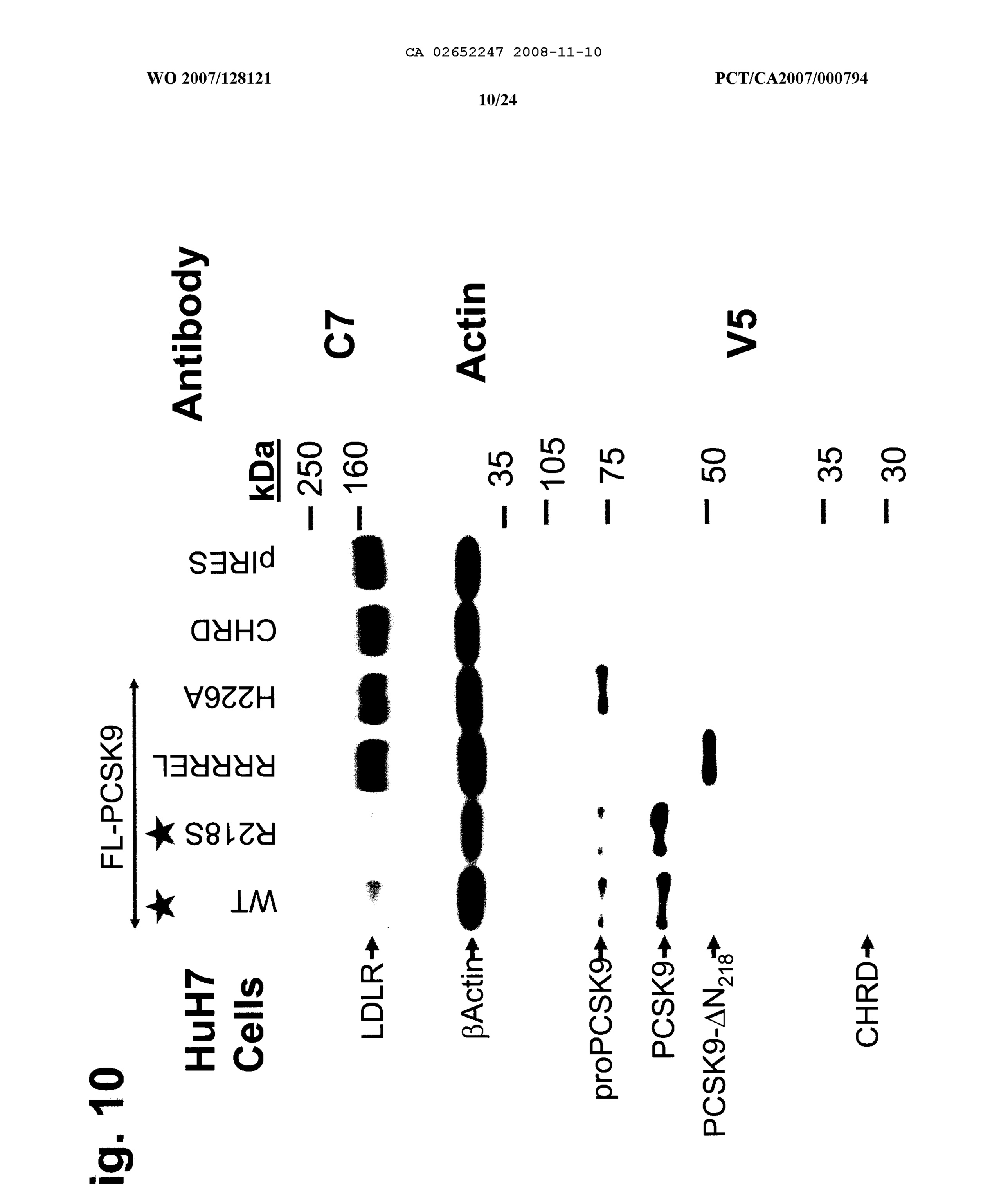
Fig. 10
HuH7 Cells
FL-PCSK9
WT
R218S
RRRREL
H226A
pIRES
CHRD
kDa
250
160
105
75
50
35
30
LDLR
βActin
proPCSK9
PCSK9
PCSK9-ΔN218
CHRD
Antibody
C7
Actin
V5

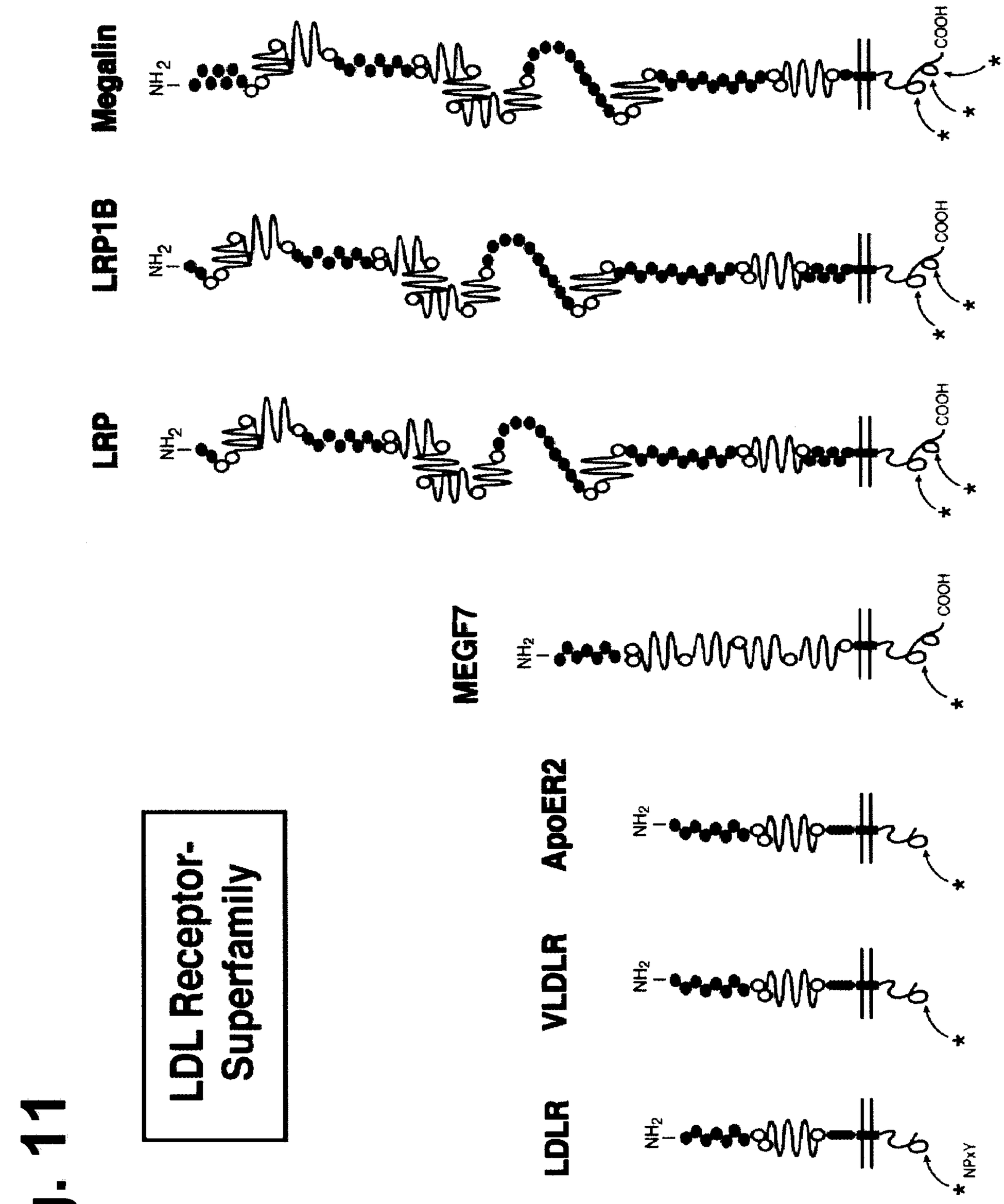
Megalin
LRP1B
LRP
MEGF7
ApoER2
VLDLR
LDLR
NH2
COOH
NPxY
LDL Receptor-Superfamily

Fig. 11

Fig. 12

```
                1       10        20        30        40        50        60        70        80        90       100       110       120       130
                |-------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
hldlr                                                                 MGPWGWKLRWTVALLLAA-AGTAVGDRCERNEFQCQDGKCISYKWVCDGSAECQDGSDESQETCLSVTCKSGDFSCGGRVNRCIPQ
hvldlr          MGTSALWALWLLLALCWAPRESGATGTGRKAKCEPSQFQCTNGRCITLLWKCDGDEDCVDGSDEKNCVKKTCAESDFVCNNGQCVPSRWKCDGDPDCEDGSDESPEQCHMRTCRIWEISCGAHSTQCIPV
hapoER2

                131     140       150       160       170       180       190       200       210       220       230       240       250       260
                |-------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
hldlr           FWRCDGQVDCDNGSDEQGCPPKTCSQDEFRCHDGKCISRQFVCDSDRDCLDGSDEASCPVLTCGPASFQCNSSTCIPQLWACDNDPDCEDGSDEWPQRCRGLYVFQGDSSPCSAFEFHCLSGECIHSSWR
hvldlr          SWRCDGENDCDSGEDEENCGNITCSPDEFTCSSGRCISRNFVCNGQDDCSDGSDELDCAPPTCGAHEFQCSTSSCIPISWVCDDDADCSDQSDESLEQCGRQPVIH---TKCPASEIQCGSGECIHKKWR
hapoER2                                                                                                 MGLPEPGPLRLLALLLLLLLLLLLQLQHLAAAAADPLLGG---QGPAKDCEKDQFQCRNERCIPSVWR

                261     270       280       290       300       310       320       330       340       350       360       370       380       390
                |-------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
hldlr           CDGGPDCKDKSDEENCAVATCRPDEFQCSDGNCIHGSRQCDREYDCKDMSDEVGCVNVTLCEGPNKFKC--HSGECITLDKVCNMARDCRDWSDEPIKECGTNECLDNNGGCSHVCNDLKIGYECLCPDG
hvldlr          CDGDPDCKDGSDEVNCPSRTCRPDQFECEDGSCIHGSRQCNGIRDCVDGSDEYNCKNVNQCLGPGKFKC--RSGECIDISKVCNQEQDCRDWSDEPLKECHINECLVNNGGCSHICKDLVIGYECDCAAG
hapoER2         CDEDDDCLDHSDEDDCPKKTCADSDFTCDNGHCIHERWKCDGEEECPDGSDESEATCTKQVCPAEKLSCGPTSHKCVPASWRCDGEKDCEGGADEAGCATWLNECLHNNGGCSHICTDLKIGFECTCPAG

                391     400       410       420       430       440       450       460       470       480       490       500       510       520
                |-------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
hldlr           FQLVAQRRCEDIDECQDPDTCSQLCVNLEGGYKCQCEEGFQLDPHTKACKAV-GSIAYLFFTNRHEVRKMTLDRSEYTSLIPNLRNVVALDTEVASNRIYWSDLSQRMICSTQLDRAHGVSSYDTVISRD
hvldlr          FELIDRKTCGDIDECQNPGICSQICTNLKGGYKCECSRGYQHDLATGVCKAV-GKEPSLIFTNRRDIRKIGLERKEYIQLVEQLRNTVALDADIAAQKLFWADLSQKAIFSASIDDKVG---RHVKMIDN
hapoER2         FQLLDQKTCGDIDECKDPDACSQICVNYKGYFKCECYPGYEMDLLTKNCKAAAGKSPSLIFTNRHEVRRIDLVKRNYSRLIPMLKNVVALDVEVATNRIYWCDLSYRKIYSAYMDKASDPKEQEVLIDEQ

                521     530       540       550       560       570       580       590       600       610       620       630       640       650
                |-------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
hldlr           IQAPDGLAVDWIHSNIYWTDSVLGTVSVADTKGVKRKTLFRENGSKPRAIVVDPVHGFMYWTDWGTPAKIKKGGLNGVDIYSLVTENIQWPNGITLDLLSGRLYWVDSKLHSISSIDVNGGNRKTILEDE
hvldlr          VYNPAAIAVDWVYKTIYWTDAASKTISVATLDGTKRKFLFNSDLREPASIAVDPLSGFVYWSDWGEPAKIEKAGMNGFDRRPLVTADIQWPNGITLDLIKSRLYWLDSKLHMLSSVDLNGQDRRIVLKSL
hapoER2         LHSPEGLAVDWVHKHIYWTDSGNKTISVATYDGGRRRTLFSRNLSEPRAIAYDPLRGFMYWSDWGDQAKIEKSGLNGVDRQTLVSDNIEWPNGITLDLLSQRLYWVDSKLHQLSSIDFSGGNRKTLISST

                651     660       670       680       690       700       710       720       730       740       750       760       770       780
                |-------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
hldlr           KRLAHPFSLAVFEDKVFWTDIINEAIFSANRLTGSDVNLLAENLLSPEDMVLFHNLTQPRGVNWCERTTLSNGGCQYLCLPAPQINPHSPKFTCACPDGMLLARDMRSCLTEAEAAVATQETSTVRLKVS
hvldlr          EFLAHPLALTIFEDRVYWIDGENEAVYGANKFTGSELATLVNNLNDAQDIIVYHELVQPSGKNWCEED-MENGGCEYLCLPAPQINDHSPKYTCSCPSGYNVEENGRDCQSTATTVTYSETKDTNTTEIS
hapoER2         DFLSHPFGIAVFEDKVFWTDLENEAIFSANRLNGLEISILAENLMNPHDIVIFHELKQPRAPDACELSVQPNGGCEYLCLPAPQISSHSPKYTCACPDTMLGPDMKRCYRAPQSTSTTTLASTMTRTVP

                781     790       800       810       820       830       840       850       860       870       880       890       900       910
                |-------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
hldlr           STAVRT---------QHTITTRPVPDTSRLPGATPG--LTTVEIVTMSHQALGDVAGRGNEKKPSS-----VRALSIVLPIVLLVFLCLGVFLLWKNWRLKNINSINFDNPVYQKTTEDEVHICHN-QD---
hvldlr          ATS----------------------GLVPGGINV--TTAVSEVSVP---------------PKG-----TSAAWAILPLLLLVMAAVGGYLMWRNWQHKNMKSMNFDNPVYLKTTEEDLSIDIG-RHSAS
hapoER2         ATTRAPGTTVHRSTYQNHSTETPSLTAAVPSSVSVPRAPSISPSTLSPATSNHSQHYANEDSKMGSTVTAAVIGIIVPIVVIALLCMSGYLIWRNWKRKNTKSMNFDNPVYRKTTEEEDEDELHIGRTAQ

                911     920       930       940       950       960       970       980       989
                |-------+---------+---------+---------+---------+---------+---------+---------|
hldlr           -GYSYPSRQMVSLEDDVA
hvldlr          VGHTYPAISVVSTDDDLA
hapoER2         IGHVYPA-AISSFDRPLWAEPCLGETREPEDPAPALKELFVLPGEPRSQLHQLPKNPLSELPVVKSKRVALSLEDDGLP
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WO 2007/128121
PCT/CA2007/000794
CA 02652247 2008-11-10
12/24

Degradation of the ApoER2, VLDLR and LDLR by PCSK9 in CHOK1

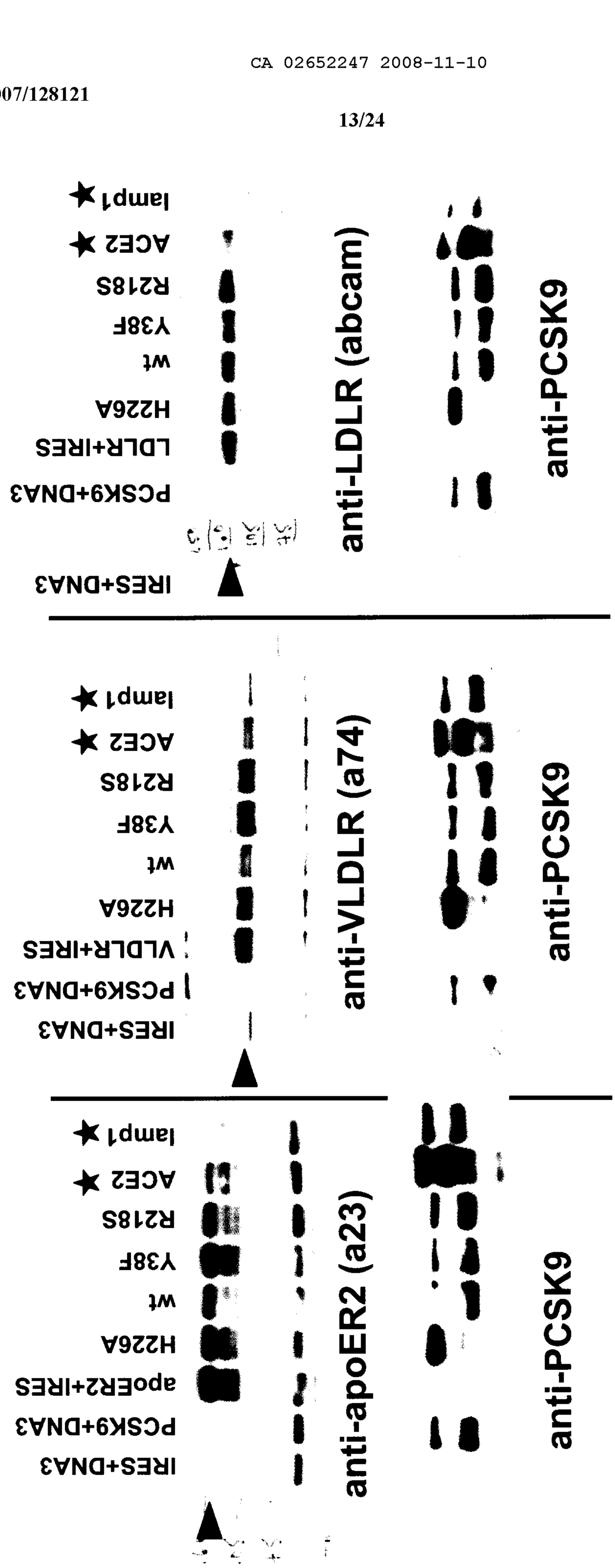
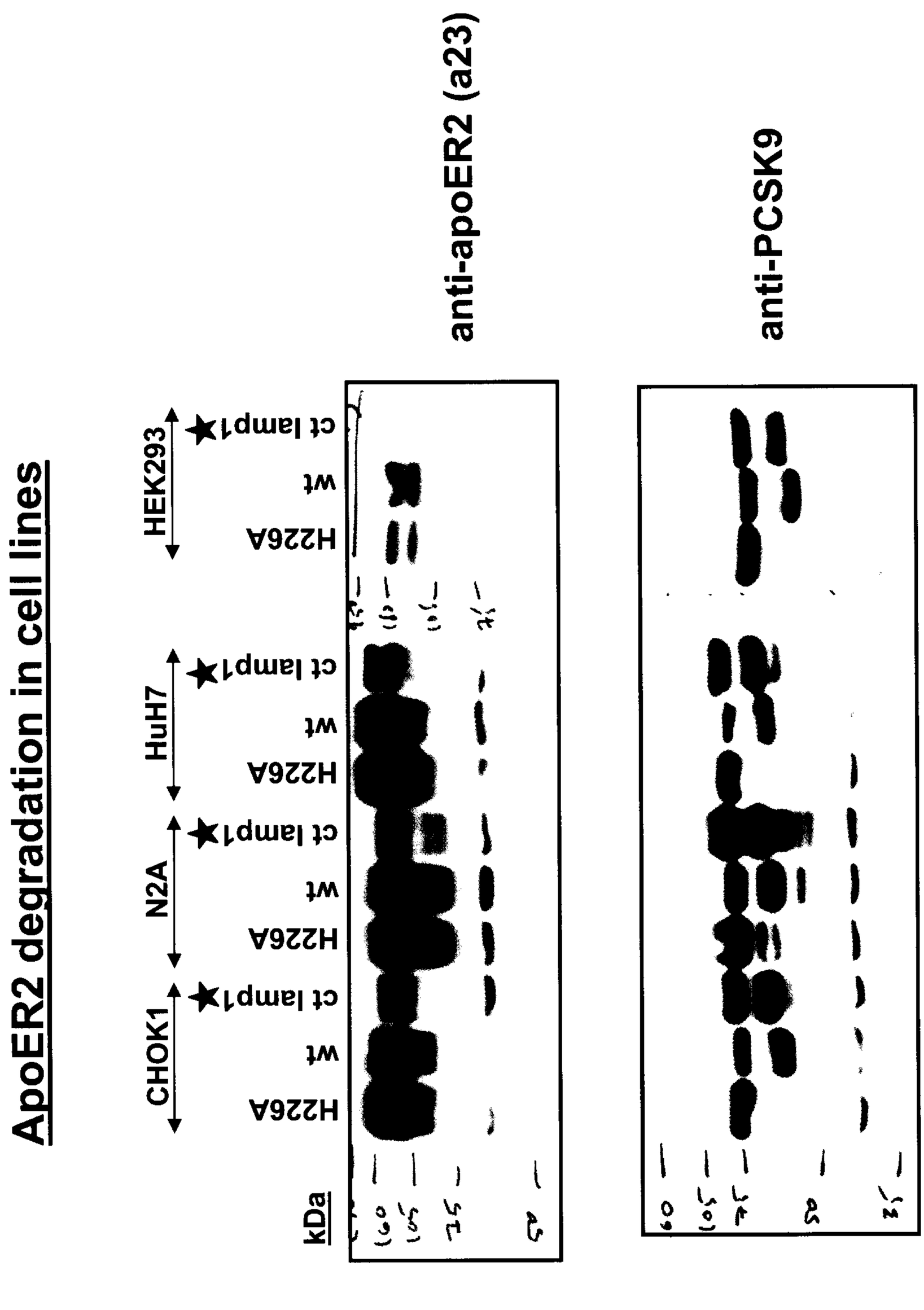


Fig. 13

Fig. 14


ApoER2 degradation in cell lines
(a23)
anti-apoER2
anti-PCSK9
HEK293
HuH7
N2A
CHOK1
ct lamp1★
wt
H226A
kDa

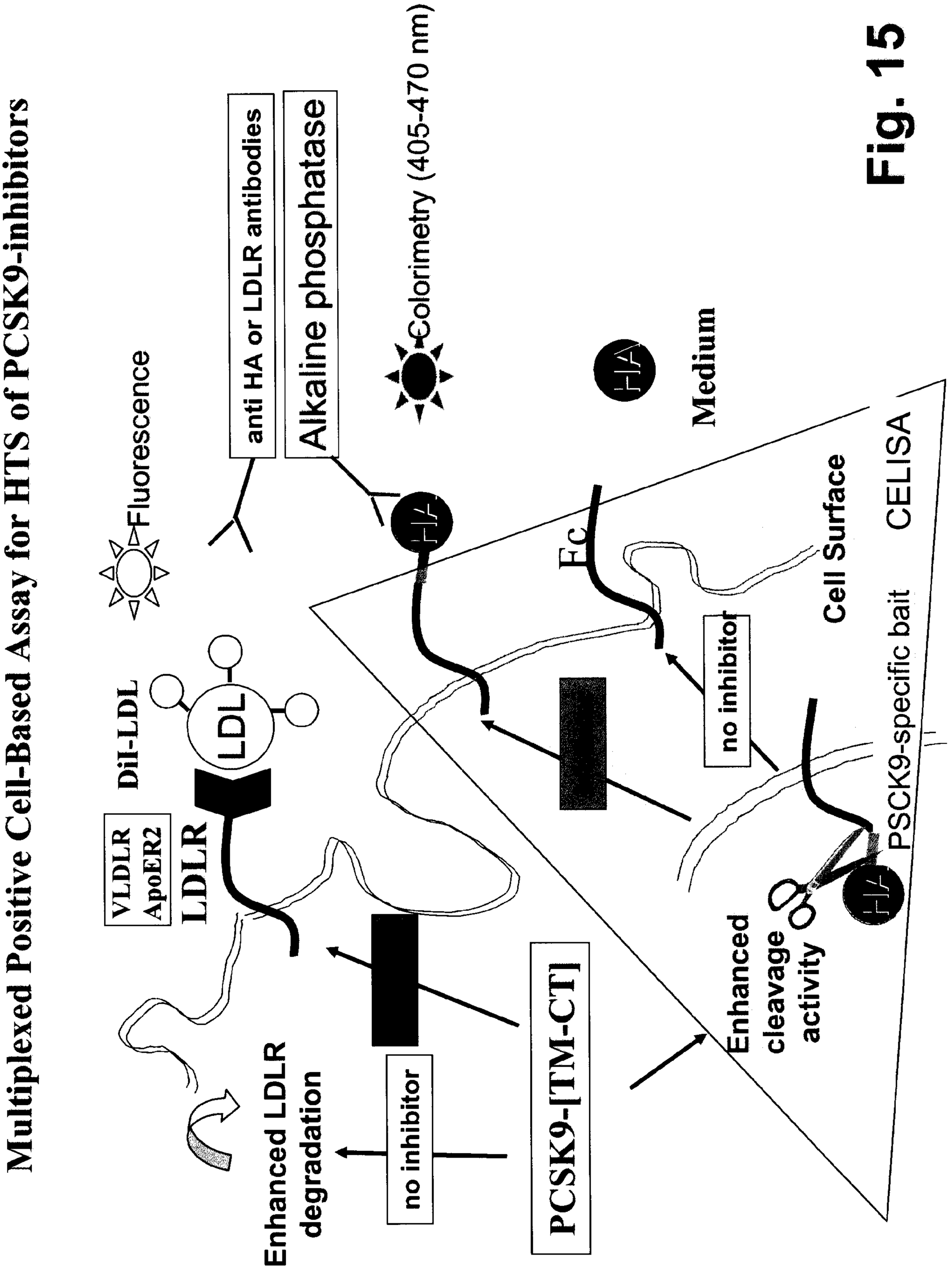

Multiplexed Positive Cell-Based Assay for HTS of PCSK9-inhibitors
Fluorescence
DiI-LDL
VLDLR
ApoER2
LDLR
LDL
Enhanced LDLR degradation
no inhibitor
PCSK9-[TM-CT]
anti HA or LDLR antibodies
Alkaline phosphatase
Colorimetry (405-470 nm)
Fc
Cell Surface
Medium
no inhibitor
Enhanced cleavage activity
PSCK9-specific bait
CELISA

Fig. 15

Human PCSK9 TM-CT ACE2

ATGGGCACCGTCAGCTCCAGGCGGTCCTGGTGGCCGCTGCCACTGCTGCTGCTGCTGCTGCTGCTCCTGGGT
CCCGCGGGCGCCCGTGCGCAGGAGGACGAGGACGGCGACTACGAGGAGCTGGTGCTAGCCTTGCGTTCCGAG
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AGGTTGCCTGGCACCTACGTGGTGGTGCTGAAGGAGGAGACCCACCTCTCGCAGTCAGAGCGCACTGCCCGC
CGCCTGCAGGCCCAGGCTGCCCGCCGGGGATACCTCACCAAGATCCTGCATGTCTTCCATGGCCTTCTTCCT
GGCTTCCTGGTGAAGATGAGTGGCGACCTGCTGGAGCTGGCCTTGAAGTTGCCCCATGTCGACTACATCGAG
GAGGACTCCTCTGTCTTTGCCCAGAGCATCCCGTGGAACCTGGAGCGGATTACCCCTCCACGGTACCGGGCG
GATGAATACCAGCCCCCCGACGGAGGCAGCCTGGTGGAGGTGTATCTCCTAGACACCAGCATACAGAGTGAC
CACCGGGAAATCGAGGGCAGGGTCATGGTCACCGACTTCGAGAATGTGCCCGAGGAGGACGGGACCCGCTTC
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ACCGCTGCCGGCAACTTCCGAGACGATGCCTGCCTCTACTCCCCAGCCTCAGCTCCCGAGGTCATCACAGTT
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GAGGAGCTGCTGAGCTGCTCCAGTTTCTCCAGGAGTGGGAAGCGGCGGGGCGAGCGCATGGAGGCCCAAGGG
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GCCTGCGAGGAGGGCTGGACCCTGACTGGCTGCAGTGCCCTCCCTGGGACCTCCCACGTCCTGGGGGCCTAC
GCCGTAGACAACACGTGTGTAGTCAGGAGCCGGGACGTCAGCACTACAGGCAGCACCAGCGAAGAGGCCGTG
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EDSSVFAQSIPWNLERITPPRYRADEYQPPDGGSLVEVYLLDTSIQSDHREIEGRVMVTDFENVPEEDGTRF
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PVLRPRGQPNQCVGHREASIHASCCHAPGLECKVKEHGIPAPQEQVTVACEEGWTLTGCSALPGTSHVLGAY
AVDNTCVVRSRDVSTTGSTSEEAVTAVAICCRSRHLAQASQELQTGKPIPNPLLGLDSTGG**IWLIVFGVVMG**
VIVVGIVILIFTGIRDRKKKNKARSGENPYASIDISKGENNPGFQNTDDVQTSF

Figure 16

Human PCSK9 TM-CT Lamp1

ATGGGCACCGTCAGCTCCAGGCGGTCCTGGTGGCCGCTGCCACTGCTGCTGCTGCTGCTGCTGCTCCTGGGT
CCCGCGGGCGCCCGTGCGCAGGAGGACGAGGACGGCGACTACGAGGAGCTGGTGCTAGCCTTGCGTTCCGAG
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AGGTTGCCTGGCACCTACGTGGTGGTGCTGAAGGAGGAGACCCACCTCTCGCAGTCAGAGCGCACTGCCCGC
CGCCTGCAGGCCCAGGCTGCCCGCCGGGGATACCTCACCAAGATCCTGCATGTCTTCCATGGCCTTCTTCCT
GGCTTCCTGGTGAAGATGAGTGGCGACCTGCTGGAGCTGGCCTTGAAGTTGCCCCATGTCGACTACATCGAG
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TAG

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PVLRPRGQPNQCVGHREASIHASCCHAPGLECKVKEHGIPAPQEQVTVACEEGWTLTGCSALPGTSHVLGAY
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GLVLIVLIAYLVGRKRSHAGYQTI

Figure 17

Human PCSK9 TM-CT LDLR

ATGGGCACCGTCAGCTCCAGGCGGTCCTGGTGGCCGCTGCCACTGCTGCTGCTGCTGCTGCTCCTGGGT
CCCGCGGGCGCCCGTGCGCAGGAGGACGAGGACGGCGACTACGAGGAGCTGGTGCTAGCCTTGCGTTCCGAG
GAGGACGGCCTGGCCGAAGCACCCGAGCACGGAACCACAGCCACCTTCCACCGCTGCGCCAAGGATCCGTGG
AGGTTGCCTGGCACCTACGTGGTGGTGCTGAAGGAGGAGACCCACCTCTCGCAGTCAGAGCGCACTGCCCGC
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GATGAATACCAGCCCCCCGACGGAGGCAGCCTGGTGGAGGTGTATCTCCTAGACACCAGCATACAGAGTGAC
CACCGGGAAATCGAGGGCAGGGTCATGGTCACCGACTTCGAGAATGTGCCCGAGGAGGACGGGACCCGCTTC
CACAGACAGGCCAGCAAGTGTGACAGTCATGGCACCCACCTGGCAGGGGTGGTCAGCGGCCGGGATGCCGGC
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CTCATAGGCCTGGAGTTTATTCGGAAAAGCCAGCTGGTCCAGCCTGTGGGGCCACTGGTGGTGCTGCTGCCC
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ACCGCTGCCGGCAACTTCCGAGACGATGCCTGCCTCTACTCCCCAGCCTCAGCTCCCGAGGTCATCACAGTT
GGGGCCACCAATGCCCAGGACCAGCCGGTGACCCTGGGGACTTTGGGGACCAACTTTGGCCGCTGTGTGGAC
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CTACCCCAGGCCAACTGCAGCGTCCACACAGCTCCACCAGCTGAGGCCAGCATGGGGACCCGTGTCCACTGC
CACCAACAGGGCCACGTCCTCACAGGCTGCAGCTCCCACTGGGAGGTGGAGGACCTTGGCACCCACAAGCCG
CCTGTGCTGAGGCCACGAGGTCAGCCCAACCAGTGCGTGGGCCACAGGGAGGCCAGCATCCACGCTTCCTGC
TGCCATGCCCCAGGTCTGGAATGCAAAGTCAAGGAGCATGGAATCCCGGCCCCTCAGGAGCAGGTGACCGTG
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PVLRPRGQPNQCVGHREASIHASCCHAPGLECKVKEHGIPAPQEQVTVACEEGWTLTGCSALPGTSHVLGAY
AVDNTCVVRSRDVSTTGSTSEEAVTAVAICCRSRHLAQASQELQTGKPIPNPLLGLDSTGG**ALSIVLPIVLL**
VFLCLGVFLLWKNWRLKNINSINFDNPVYQKTTEDEVHICHNQDGYSYPRQMVSLEDDVA

Figure 18

1. Pro-protein PCSK9 (with signal peptide) 1-692

MGTVSSRRSWWPLPLLLLLLLLLGPAGARA**QEDEDGDYEELVLALRSEEDGLAEAPEHGTTATFHRCAKD**
PWRLPGTYVVVLKEETHLSQSERTARRLQAQAARRGYLTKILHVFHGLLPGFLVKMSGDLLELALKLPHV
DYIEEDSSVFAQ*SIPWNLERITPPRYRADEYQPPDGGSLVEVYLLDTSIQSDHREIEGRVMVTDFENVPE*
*EDGTRFHR*QASKCDSHGTHLAGVVSGRDAGVAKGASMRSLRVLNCQGKGTVSGTLIGLEFIRKSQLVQPV
GPLVVLLPLAGGYSRVLNAACQRLARAGVVLVTAAGNFRDDACLYSPASAPEVITVGATNAQDQPVTLGT
LGTNFGRCVDLFAPGEDIIGASSDCSTCFVSQSGTSQAAAHVAGIAAMMLSAEPELTLAELRQRLIHFSA
KDVINEAWFPEDQRVLTPNLVAALPPSTHGAGWQLFCRTVWSAHSGPTRMATAVARCAPDEELLSCSSFS
RSGKRRGERMEAQGGKLVCRAHNAFGGEGVYAIARCCLLPQANCSVHTAPPAEASMGTRVHCHQQGHVLT
GCSSHWEVEDLGTHKPPVLRPRGQPNQCVGHREASIHASCCHAPGLECKVKEHGIPAPQEQVTVACEEGW
TLTGCSALPGTSHVLGAYAVDNTCVVRSRDVSTTGSTSEGAVTAVAICCRSRHLAQASQELQ

2. Pro-protein PCSK9 (without signal peptide) 31-692

QEDEDGDYEELVLALRSEEDGLAEAPEHGTTATFHRCAKD
PWRLPGTYVVVLKEETHLSQSERTARRLQAQAARRGYLTKILHVFHGLLPGFLVKMSGDLLELALKLPHV
DYIEEDSSVFAQ*SIPWNLERITPPRYRADEYQPPDGGSLVEVYLLDTSIQSDHREIEGRVMVTDFENVPE*
*EDGTRFHR*QASKCDSHGTHLAGVVSGRDAGVAKGASMRSLRVLNCQGKGTVSGTLIGLEFIRKSQLVQPV
GPLVVLLPLAGGYSRVLNAACQRLARAGVVLVTAAGNFRDDACLYSPASAPEVITVGATNAQDQPVTLGT
LGTNFGRCVDLFAPGEDIIGASSDCSTCFVSQSGTSQAAAHVAGIAAMMLSAEPELTLAELRQRLIHFSA
KDVINEAWFPEDQRVLTPNLVAALPPSTHGAGWQLFCRTVWSAHSGPTRMATAVARCAPDEELLSCSSFS
RSGKRRGERMEAQGGKLVCRAHNAFGGEGVYAIARCCLLPQANCSVHTAPPAEASMGTRVHCHQQGHVLT
GCSSHWEVEDLGTHKPPVLRPRGQPNQCVGHREASIHASCCHAPGLECKVKEHGIPAPQEQVTVACEEGW
TLTGCSALPGTSHVLGAYAVDNTCVVRSRDVSTTGSTSEGAVTAVAICCRSRHLAQASQELQ

3. PSCK9 amino acids 153 to 692 (active full-length form i.e. without signal peptide and prosegment)

SIPWNLERITPPRYRADEYQPPDGGSLVEVYLLDTSIQSDHREIEGRVMVTDFENVPE
*EDGTRFHR*QASKCDSHGTHLAGVVSGRDAGVAKGASMRSLRVLNCQGKGTVSGTLIGLEFIRKSQLVQPV
GPLVVLLPLAGGYSRVLNAACQRLARAGVVLVTAAGNFRDDACLYSPASAPEVITVGATNAQDQPVTLGT
LGTNFGRCVDLFAPGEDIIGASSDCSTCFVSQSGTSQAAAHVAGIAAMMLSAEPELTLAELRQRLIHFSA
KDVINEAWFPEDQRVLTPNLVAALPPSTHGAGWQLFCRTVWSAHSGPTRMATAVARCAPDEELLSCSSFS
RSGKRRGERMEAQGGKLVCRAHNAFGGEGVYAIARCCLLPQANCSVHTAPPAEASMGTRVHCHQQGHVLT
GCSSHWEVEDLGTHKPPVLRPRGQPNQCVGHREASIHASCCHAPGLECKVKEHGIPAPQEQVTVACEEGW
TLTGCSALPGTSHVLGAYAVDNTCVVRSRDVSTTGSTSEGAVTAVAICCRSRHLAQASQELQ

4. PSCK9 amino acids 153 to 218 (N-terminal fragment produced by furin/PC5 cleavage)

SIPWNLERITPPRYRADEYQPPDGGSLVEVYLLDTSIQSDHREIEGRVMVTDFENVPE
EDGTRFHR

Figure 19

5. PSCK9 amino acids 219 to 692 (PCSK9-ΔN_{218} : furin/PC5-cleaved form)

QASKCDSHGTHLAGVVSGRDAGVAKGASMRSLRVLNCQGKGTVSGTLIGLEFIRKSQLVQPV
GPLVVLLPLAGGYSRVLNAACQRLARAGVVLVTAAGNFRDDACLYSPASAPEVITVGATNAQDQPVTLGT
LGTNFGRCVDLFAPGEDIIGASSDCSTCFVSQSGTSQAAAHVAGIAAMMLSAEPELTLAELRQRLIHFSA
KDVINEAWFPEDQRVLTPNLVAALPPSTHGAGWQLFCRTVWSAHSGPTRMATAVARCAPDEELLSCSSFS
RSGKRRGERMEAQGGKLVCRAHNAFGGEGVYAIARCCLLPQANCSVHTAPPAEASMGTRVHCHQQGHVLT
GCSSHWEVEDLGTHKPPVLRPRGQPNQCVGHREASIHASCCHAPGLECKVKEHGIPAPQEQVTVACEEGW
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Figure 19 (continued)

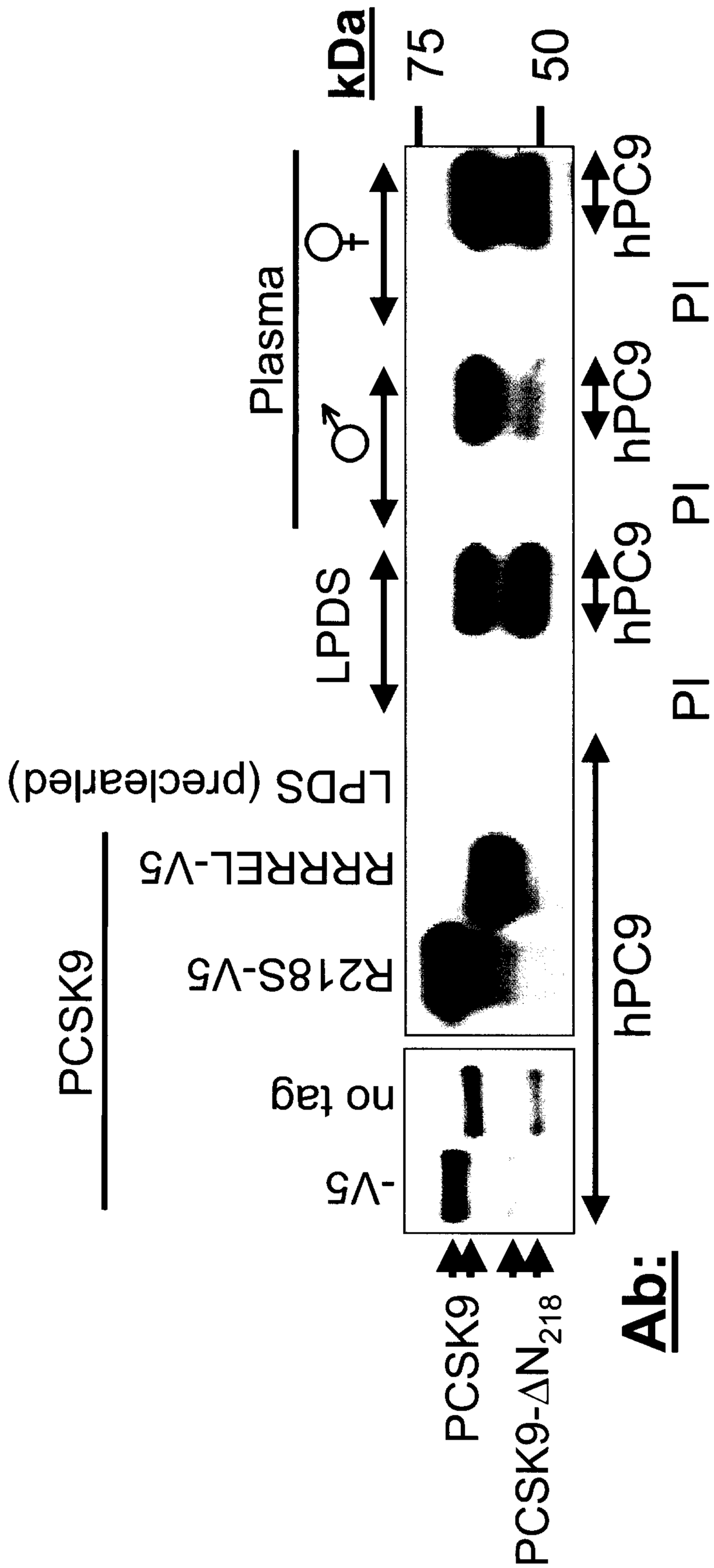

kDa
75
50
Plasma
♀
♂
LPDS
hPC9
PI
hPC9
PI
hPC9
PI
hPC9
LPDS (precleaved)
RRRREL-V5
R218S-V5
no tag
-V5
PCSK9
PCSK9
PCSK9-ΔN218
Ab:

Fig. 20

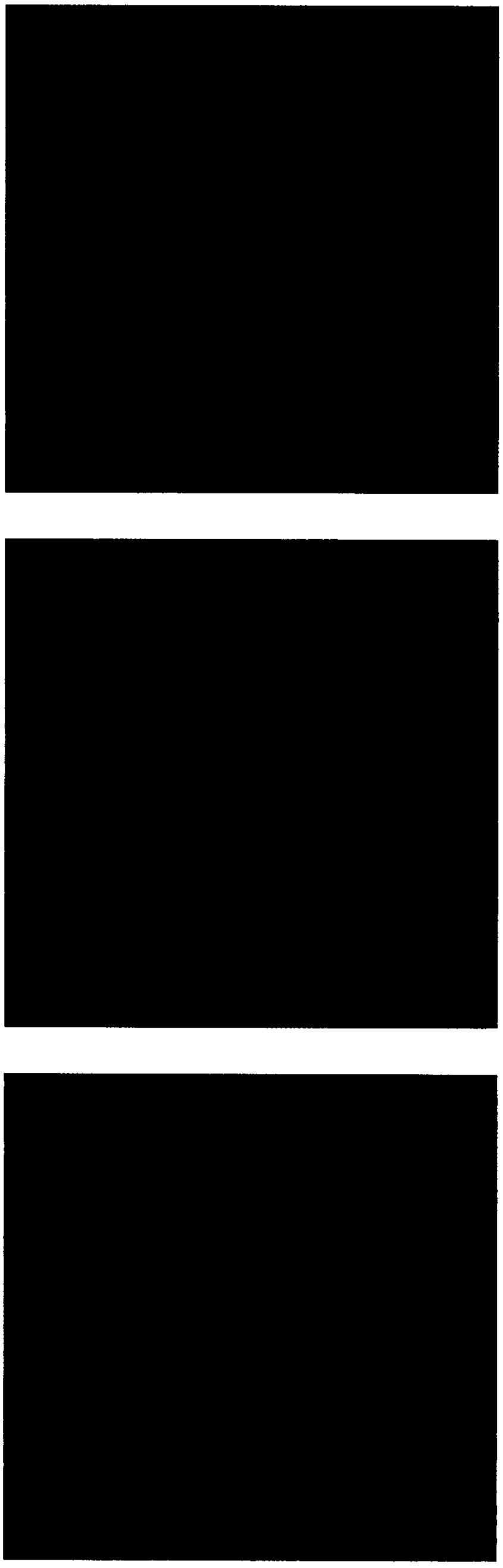


Fig. 21

```
CLUSTAL W (1.83) multiple sequence alignment


mouse           MGTHCSAWLRWPLLPLLPPLLLLLLLLCPTGAGAQ-DEDGDYEELMLALPSQEDGLADEA 59
rat             MGIRCSTWLRWPLS----PQLLLLLLLCPTGSRAQ-DEDGDYEELMLALPSQEDSLVDEA 55
human           MGTVSSRRSWWPLP----LLLLLLLLLGPAGARAQEDEDGDYEELVLALRSEEDGLAEAP 56
monkey          MGTVSSRRSWWPLP----LLLLLLLLLGPAGARAQEDEDGDYEELVLALRSEEDGLAEAP 56
                **   .*    ***       ******* *:*: ** *********:*** *:**.*.:. .
consensus       MGXXXSXXXXWPLPXXXXXXLLLLLLLLXPXGXXAQXDEDGDYEELXLALXSXEDXLXXXX 60


mouse           AHVATATFRRCSKEAWRLPGTYIVVLMEETQRLQIEQTAHRLQTRAARRGYVIKVLHIFY 119
rat             SHVATATFRRCSKEAWRLPGTYVVVLMEETQRLQVEQTAHRLQTWAARRGYVIKVLHVFY 115
human           EHGTTATFHRCAKDPWRLPGTYVVVLKEETHLSQSERTARRLQAQAARRGYLTKILHVFH 116
monkey          EHGTTATFHRCAKDPWRLPGTYVVVLKEETHLSQSERTARRLQAQAARRGYLTKILHVFH 116
                 * :****:**:*:.*******:*** ***:  * *:**:***: ******: *:**:*:
consensus       XHXXTATFXRCXKXXWRLPGTYXVVLXEETXXXQXEXTAXRLQXXAARRGYXXKXLHXFX 120


mouse           DLFPGFLVKMSSDLLGLALKLPHVEYIEEDSFVFAQSIPWNLERIIPAWHQTEEDRSPDG 179
rat             DLFPGFLVKMSSDLLGLALKLPHVEYIEEDSLVFAQSIPWNLERIIPAWQQTEEDSSPDG 175
human           GLLPGFLVKMSGDLLELALKLPHVDYIEEDSSVFAQSIPWNLERITPPRYRADEYQPPDG 176
monkey          GLLPGFLVKMSGDLLELALKLPHVDYIEEDSSVFAQSIPWNLERITPPRYRADEYQPPDG 176
                .*:********.*** ********:****** ************ *.  :::*  .***
consensus       XLXPGFLVKMSXDLLXLALKLPHVXYIEEDSXVFAQSIPWNLERIXPXXXXXXEXXXPDG 180


mouse           SSQVEVYLLDTSIQGAHREIEGRVTITDFNSVPEEDGTRFHRQASKCDSHGTHLAGVVSG 239
rat             SSQVEVYLLDTSIQSGHREIEGRVTITDFNSVPEEDGTRFHRQASKCDSHGTHLAGVVSG 235
human           GSLVEVYLLDTSIQSDHREIEGRVMVTDFENVPEEDGTRFHRQASKCDSHGTHLAGVVSG 236
monkey          GSLVEVYLLDTSIQSDHREIEGRVMVTDFENVPEEDGTRFHRQASKCDSHGTHLAGVVSG 236
                .* ***********. ******** :***:.****************************
consensus       XSXVEVYLLDTSIQXXHREIEGRVXXTDFXXVPEEDGTRFHRQASKCDSHGTHLAGVVSG 240


mouse           RDAGVAKGTSLHSLRVLNCQGKGTVSGTLIGLEFIRKSQLIQPSGPLVVLLPLAGGYSRI 299
rat             RDAGVAKGTSLHSLRVLNCQGKGTVSGTLIGLEFIRKSQLIQPSGPLVVLLPLAGGYSRI 295
human           RDAGVAKGASMRSLRVLNCQGKGTVSGTLIGLEFIRKSQLVQPVGPLVVLLPLAGGYSRV 296
monkey          RDAGVAKGASMRSLRVLNCQGKGTVSGTLIGLEFIRKSQLVQPVGPLVVLLPLAGGYSRV 296
                ********:*::*****************************:** ***************:
consensus       RDAGVAKGXSXXSLRVLNCQGKGTVSGTLIGLEFIRKSQLXQPXGPLVVLLPLAGGYSRX 300


mouse           LNAACRHLARTGVVLVAAAGNFRDDACLYSPASAPEVITVGATNAQDQPVTLGTLGTNFG 359
rat             LNTACQRLARTGVVLVAAAGNFRDDACLYSPASAPEVITVGATNAQDQPVTLGTLGTNFG 355
human           LNAACQRLARAGVVLVTAAGNFRDDACLYSPASAPEVITVGATNAQDQPVTLGTLGTNFG 356
monkey          LNAACQRLARAGVVLVTAAGNFRDDACLYSPASAPEVITVGATNAQDQPVTLGTLGTNFG 356
                **:**::***:*****:**************************************
consensus       LNXACXXLARXGVVLVXAAGNFRDDACLYSPASAPEVITVGATNAQDQPVTLGTLGTNFG 360
```

Figure 22

```
mouse       RCVDLFAPGKDIIGASSDCSTCFMSQSGTSQAAAHVAGIVARMLSREPTLTLAELRQRLI 419
rat         RCVDLFAPGKDIIGASSDCSTCYMSQSGTSQAAAHVAGIVAMMLNRDPALTLAELRQRLI 415
human       RCVDLFAPGEDIIGASSDCSTCFVSQSGTSQAAAHVAGIAAMMLSAEPELTLAELRQRLI 416
monkey      RCVDLFAPGEDIIGASSDCSTCFVSQSGTSQAAAHVAGIAAMMLSAEPELTLAELRQRLI 416
            *********:************::****************.* **. :* **********
consensus   RCVDLFAPGXDIIGASSDCSTCXXSQSGTSQAAAHVAGIXAMMLXXXPXLTLAELRQRLI 420

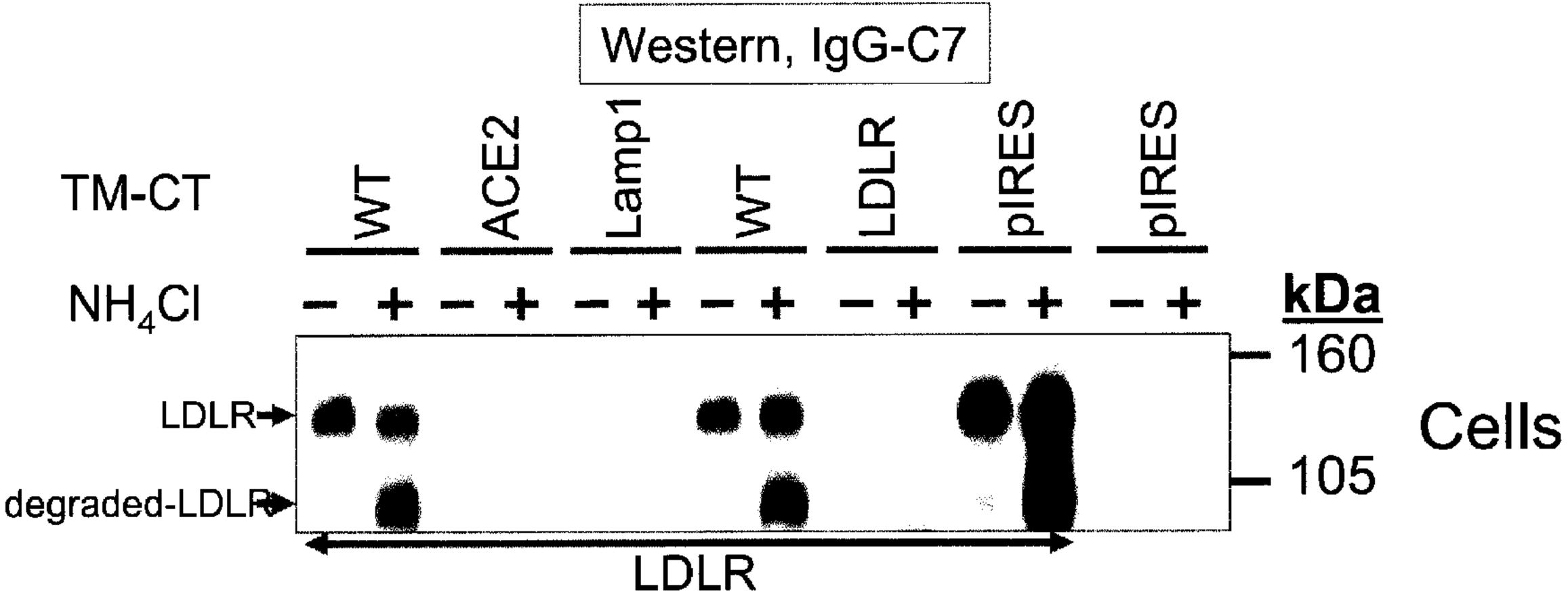
mouse       HFSTKDVINMAWFPEDQQVLTPNLVATLPPSTHETGGQLLCRTVWSAHSGPTRTATATAR 479
rat         LFSTKDVINMAWFPEDQRVLTPNRVATLPPSTQETGGQLLCRTVWSAHSGPTRTATATAR 475
human       HFSAKDVINEAWFPEDQRVLTPNLVAALPPSTHGAGWQLFCRTVWSAHSGPTRMATAVAR 476
monkey      HFSAKDVINEAWFPEDQRVLTPNLVAALPPSTHGAGWQLFCRTVWSAHSGPTRMATAVAR 476
             **:***** *******:***** **:*****: :* **:************ ***.**
consensus   XFSXKDVINXAWFPEDQXVLTPNXVAXLPPSTXXXGXQLXCRTVWSAHSGPTRXATAXAR 480

mouse       CAPEEELLSCSSFSRSGRRRGDWIEAIGGQQVCKALNAFGGEGVYAVARCCLVPRANCSI 539
rat         CAPEEELLSCSSFSRSGRRRGDRIEAIGGQQVCKALNAFGGEGVYAVARCCLLPRVNCSI 535
human       CAPDEELLSCSSFSRSGKRRGERMEAQGGKLVCRAHNAFGGEGVYAIARCCLLPQANCSV 536
monkey      CAPDEELLSCSSFSRSGKRRGERMEAQGGKLVCRAHNAFGGEGVYAIARCCLLPQANCSI 536
            ***:**************:***: :** **: **:* **********:*****:*:.***:
consensus   CAPXEELLSCSSFSRSGXRRGXXXEAXGGXXVCXAXNAFGGEGVYAXARCCLXPXXNCSX 540

mouse       HNTPAARAGLETHVHCHQKDHVLTGCSFHWEVEDLSVRRQPALRSRRQPGQCVGHQAASV 599
rat         HNTPAARAGPQTPVHCHQKDHVLTGCSFHWEVENLRAQQQPLLRSRHQPGQCVGHQEASV 595
human       HTAPPAEASMGTRVHCHQQGHVLTGCSSHWEVEDLGTHKPPVLRPRGQPNQCVGHREASI 596
monkey      HTAPPAEAGMGTRVHCHQQGHVLTGCSSHWEVEDLGTHKPPMLRPRGQPNQCVGHREASI 596
            *.:*.*.*.  * *****:.******* *****:* .:: * **.* **.*****: **:
consensus   HXXPXAXAXXXTXVHCHQXXHVLTGCSXHWEVEXLXXXXXPXLRXRXQPXQCVGHXXASX 600

mouse       YASCCHAPGLECKIKEHGISGPSEQVTVACEAGWTLTGCNVLPGASLTLGAYSVDNLCVA 659
rat         HASCCHAPGLECKIKEHGIAGPAEQVTVACEAGWTLTGCNVLPGASLPLGAYSVDNVCVA 655
human       HASCCHAPGLECKVKEHGIPAPQEQVTVACEEGWTLTGCSALPGTSHVLGAYAVDNTCVV 656
monkey      HASCCRAPGLECKVKEHGIPAPQEQVTVACEEGWTLTGCSALPGTSHVLGAYAVDNTCVV 656
            :****:*******:*****..* ******** *******..***:*  ****:*** **.
consensus   XASCCXAPGLECKXKEHGIXXPXEQVTVACEXGWTLTGCXXLPGXSXXLGAYXVDNXCVX 660

mouse       RVHDTARADRTSGEATVAAAICCRSRPSAKASWVQ- 694
rat         RIRDAGRADRTSEEATVAAAICCRSRPSAKASWVHQ 691
human       RSRDVSTTGSTSEGAVTAVAICCRSRHLAQASQELQ 692
monkey      RSRDVSTAGSTSEEAVAAVAICCRSRHLAQASQELQ 692
            * :*.. :. **  *..*.*******  *:**
consensus   RXXDXXXXXXTSXXAXXAXAICCRSRXXAXASXXXX 696
```

Figure 22 (continued)

PCSK9-chimeras; HEK293 cells