PCSK1 mutations and human endocrinopathies: from obesity to gastrointestinal disorders

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Prohormone convertase 1/3 (PC1/3), encoded by the *PCSK1* gene, is a serine endoprotease which is involved in the processing of a variety of proneuropeptides and prohormones. Humans who are homozygous or compound heterozygous for loss-of-function mutations in *PCSK1* exhibit a variable and pleiotropic syndrome consisting of some or all of the following: obesity, malabsorptive diarrhea, hypogonadotropic hypogonadism, altered thyroid and adrenal function and impaired regulation of plasma glucose levels in association with elevated circulating proinsulin-to-insulin ratio. Recently, more common variants in the *PCSK1* gene have been found to be associated with alterations in body mass index, increased circulating proinsulin levels and defects in glucose homeostasis. This review provides an overview of the endocrinopathies and other disorders observed in PC1/3-deficient patients, discusses the possible biochemical basis for these manifestations of the disease and proposes a model whereby certain missense mutations in *PCSK1* may result in proteins with a dominant negative action.

A. Proprotein convertases regulate protein function

I. Introduction

THE PROPROTEIN CONVERTASES (PCs) are calcium-dependent serine endoproteases involved in the processing of a variety of cellular precursors in the secretory pathway. Because of the homology of their catalytic domains to that of bacterial subtilisin and yeast kexin, the corresponding genes are known as subtilisin and kexin-like proprotein convertases (*PCSKs*). In mammals the PC family contains seven closely related members: PC1/3 (*PCSK1*), PC2 (*PCSK2*), furin (*PCSK3*), PC4 (*PCSK4*), PC5/6 (*PCSK5*), PACE4 (*PCSK6*), and PC7 (*PCSK7*); and two less related enzymes SKI-1 (*PCSK8*) and PCSK9 (*PCSK9*) (1, 2). The seven closely related enzymes, here referred as the PC family, catalyze proteolytic cleavage C-terminally to basic residue motifs. PCs are composed of three common domain structures, a prodomain, a catalytic domain and a P domain (also called homo B or middle domain), and a unique C-terminal region, which can be composed of several subdomains. The common domains are essential and sufficient for catalytic activity, while the C-terminal regions are important for intracellular trafficking and subcellular localization. Because of the high homology in the catalytic (50%–60%) and P (~30%-40%) domains, substrate specificity is largely overlapping, albeit by no means identical. The enzymes differ in tissue distribution, subcellular localization and pH optima, which largely determines substrate selectivity in vivo. For instance, furin, PACE4, PC5/6, and PC7 are widely expressed enzymes that process a large number of substrates (eg, growth factors, plasma proteins, viral coat proteins, and bacterial toxins) and exert their action at different compartments along the constitutive secretory and endocytic pathways (1). In contrast, PC1/3 and PC2, are only expressed in neural and endocrine tissues, where they cleave prohormones and proneuropeptides within the secretory granules of the regulated secretory pathway (3).

B. The neuroendocrine member PC1/3: General properties

Prohormone convertase 1/3 (PC1/3; also known as PC1, PC3 and SPC3) was cloned in 1991 by two laboratories independently (4, 5). The *PCSK1* gene consists of 14 exons located on chromosome 5 in humans and 13 in mice (6). Northern blot analysis of human tissues and cells re-

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vealed the presence of a dominant transcript of 6.2 kb, the major sites of expression being the pituitary, the brain, and the endocrine pancreas (7). The *PCSK1* promoter contains CRE-1 and CRE-2 transcriptional elements which can be transactivated by CREB-1 and ATF1 transcription factors (8, 9). The *PCSK1* gene encodes the 753-amino acid precursor preproPC1/3, from which the signal peptide is removed cotranslationally in the endoplasmic reticulum (ER). The resulting product is a proPC1/3 zymogen of 94 kDa which is unable to cleave substrates *in trans* but able to cleave its prodomain (also known as prosegment or propeptide) *in cis*. The structural domains of PC1/3 are depicted schematically in **Figure 1A**. The prodomain is involved in proper folding and early enzyme inhibition. The catalytic domain includes the catalytic triad Asp^{167}-His^{208}-Ser^{382}, which is conserved between bacterial subtilisin, yeast kexin, and all mammalian PCs, and the oxyanion hole Asn^{309}, which is conserved in all PCs except PC2. The P domain contributes to calcium and pH requirements and to enzyme stability (10). The C-terminal (Ct) domain of PC1/3 is involved in sorting to the secretory granules and in enzyme stability.

Like the other eukaryotic proprotein convertases, PC1/3 recognizes and cleaves precursor proteins C-terminally to a pair of basic residues. PC1/3 prefers to cleave after a Lys-Arg dibasic site, but it is also able to cleave other dibasic sites including Arg-Arg, Arg-Lys, Arg-X-X-Arg (where X is any amino acid), or even after a single Arg residue (11). Substrates containing large residues (Trp, Tyr) or Pro in the P1′ and P2′ sites are preferentially cleaved by PC2 and not by PC1/3 (11). PC1/3 enzymatic activity depends on the presence of mM of calcium in vitro, although this calcium requirement is different for full length and C-terminally cleaved PC1/3 (as discussed below) (12, 13). In terms of substrate specificity, expression and localization, PC1/3 mostly resembles PC2. Regarding substrate specificity, it has recently been shown that the single substitution of Ser357, located in the catalytic domain, by Gly in PC1/3 (a variant found in PC1/3 cloned from human lung tumor cells (14)) results in a hypermorphic PC1/3 form with PC2-like activity (15). In addition, both PC1/3 and PC2 bind to specific neuroendocrine chaperones (proSAAS in the case of PC1/3; 7B2 in the case of PC2). Despite these similarities, there are clear differences between both convertases. For example, while PC2 absolutely requires the presence of 7B2 for activation (16, 17), PC1/3 can be activated independently of the binding of proSAAS. ProSAAS, a granin-like protein discovered using proteomic studies on the brain of mice lacking carboxypeptidase E (18), is able to potently inhibit PC1/3 activity (18–20), suggesting that proSAAS is an endogenous inhibitor of PC1/3 and that the binding proSAAS-PC1/3 blocks the PC1/3 enzymatic activity at early stages of the regulated secretory pathway. Another difference is that PC2 has a much larger range of substrates then PC1/3 (3).

PC1/3 is predominantly expressed in neural and endocrine tissues. In the brain, PC1/3 expression is especially high in the hypothalamus (21), but it is also present in other areas such as cerebral cortex, hippocampus, and cerebellum (22–24). In peripheral tissues, PC1/3 is mainly located in adrenal medulla, pituitary, thyroid gland, endocrine pancreas (especially in β-cells), and small intestine (eg, enteroendocrine L and K cells) (25–31). PC1/3 expression can also be detected, albeit at very low levels, in adipocytes (32), in the proglucagon-producing α-cells of the pancreatic islets (30), in lung tumors (14, 33), and in certain types of immune cells (34, 35).

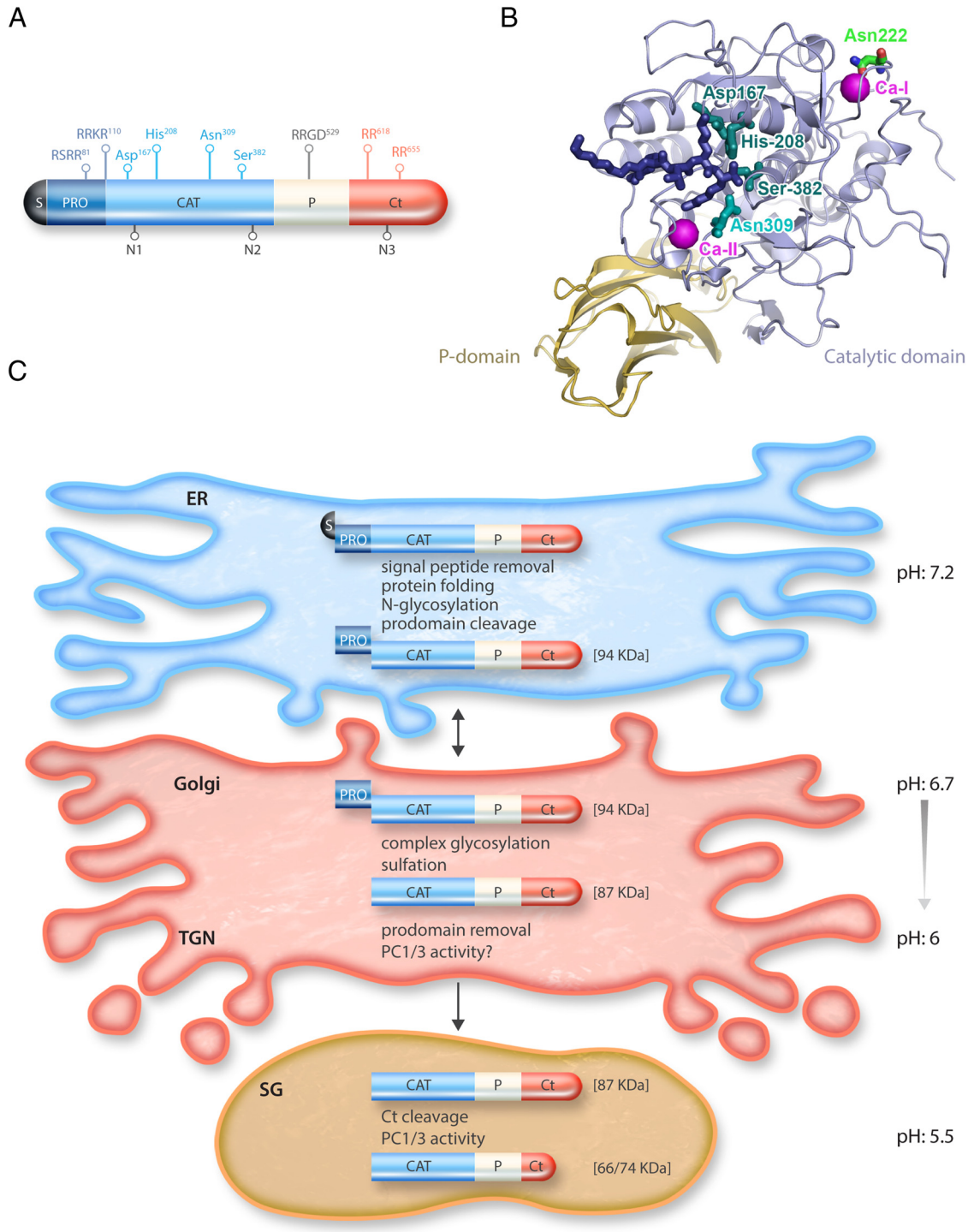
PC1/3 is crucial for the processing of a number of neuropeptides and peptide hormones such as proopiomelanocortin (POMC), proinsulin and proglucagon. Importantly, in some cases PC1/3 activity is sufficient to produce the end product from the precursor. This is for instance the case in the synthesis of adrenocorticotropic hormone (ACTH) from POMC in the anterior lobe of the pituitary gland (36, 37) and the glucagon-like peptides GLP-1, GLP-2, oxyntomodulin and glicentin from proglucagon in the small intestine (38, 39). In other cases, the generation of a given hormone or neuropeptide requires the additional action of PC2. For example, both PC1/3 and PC2 are coexpressed in the intermediate lobe of the pituitary and the hypothalamic POMC neurons, where they accomplish the proteolytic cleavage of POMC to α-melanocyte-stimulating hormone (α-MSH) (37, 40), and the islets of Langerhans in the pancreas, where both enzymes are required for the synthesis of insulin from proinsulin (41–44). In addition to PCs, the synthesis of α-MSH requires the complementary action of other enzymes including acetylases, amidases and carboxypeptidases (45).

C. Structural aspects of PC1/3

Prodomain

In PCs, the prodomain is thought to assist the correct folding and to regulate the pH-dependent activation of the catalytic domain (46, 47). In PC1/3 the prodomain is formed by 83 residues and is highly conserved between orthologues (~80% of sequence identity), although is not well conserved among paralogues of the convertase family (~30%-40%). Like other members of the PC family, except PC2 (48) and PC7 (49), proPC1/3 accomplishes autoproteolytic cleavage of the prodomain at the primary site $RSKR^{110}$ before exiting the ER (50). At this stage, the protein is referred as PC1/3, although the propeptide is

Figure 1.



PC1/3 maturation in the regulated secretory pathway. A. The catalytic domain (CAT) contains the catalytic triad, formed by Asp[167]-His[208]-Ser[382], and the oxyanion hole Asn[309]. The location of prodomain (PRO) and C-terminal (Ct) cleavage sites are indicated in dark blue and red, respectively. Glycosylation is also required for proper PC1/3 activation. In mouse PC1/3 three glycosylation sites (N1, N2 and N3) are present of which only N1 and N3 are used. In human PC1/3 only N1 and N2 sites are present of which only N1 is used (73). The RRGD motif (gray) present in the P domain is critical for proper proPC1/3 processing and further sorting to the secretory granules. B. Stereo representation of the catalytic and P domain of PC1/3, based on the crystal structure of furin (courtesy of Dr Manuel Than, Leibniz Institute for Age Research, Germany). It shows the protein backbone in cartoon representation, the dec-RVKR-CMK inhibitor in dark blue marking in the active site cleft, and the two calcium ions in purple. Also indicated are the catalytic residues Asp167, His208 and Ser382, and the oxyanion hole Asn309. The Asn222 residue, found substituted by Asp in an obese mouse model (204), is indicated in green. The figure was created using PYMOL (DeLano Scientific LLC,

still noncovalently attached. After ER exit, the compartments of the secretory pathway become progressively more acidic, which leads to partial unfolding of the prodomain and cleavage at the secondary site RRSRR[81]. Whereas the first cleavage occurs rapidly ($t^{1/2} < 2$ minutes), the second cleavage and dissociation of the prodomain occurs more slowly, as is also the case for furin (51, 52). In vitro experiments showed that these steps require a pH lower than 6.4 indicating that dissociation could take place in the *trans*-golgi network (TGN) (53). However, the exact localization where dissociation of the prodomain takes place has not been demonstrated yet. Several groups have reported that the prosegments are able to inhibit PC enzymatic activity (54–57), suggesting that this domain might prevent PC1/3 enzymatic activity in early secretory compartments. On the other hand, without a propeptide, no active enzyme is formed and the misfolded protein is retained in the ER. For furin it has been shown that expression of the propeptide *in trans* can rescue propeptideless furin, assist folding and facilitate ER exit (58). These studies demonstrate the chaperoning function of the propeptide.

The solution structure of mouse PC1/3 prodomain has been determined using heteronuclear NMR spectroscopy (59). The overall fold of the PC1/3 prodomain consists of two α-helices and four β-strands forming an antiparallel β-sheet in a β–α–β–β–α–β arrangement. It has been proposed that a hydrophobic patch present in the solvent-accessible surface of the β-sheet might be buried at the binding interface with the catalytic domain (59). No structural data about the prodomain of other members of the eukaryotic PC family have been reported to date, although it is generally assumed that other family members can also exhibit the same overall fold because of the homology among PC propeptides.

Catalytic domain

PCs contain the classical catalytic triad Asp-His-Ser in the catalytic domain at topologically conserved positions with bacterial subtilisins and yeast kexin. This domain also includes the oxyanion hole Asn which is necessary for stabilization of the tetrahedral intermediate during substrate cleavage. The catalytic domain of human PC1/3 is ~343 residues and the boundaries are based on homology with bacterial subtilisins. The catalytic domain is the most conserved region among PCs, with 50%–60% sequence similarity. In comparison to the bacterial subtilisins, PCs contains a large number of negatively charged residues in their catalytic domain, which has been shown to confer selectivity for basic substrate segments (60, 61).

The overall three-dimensional structure of mouse furin comprising the catalytic and the P domains was determined by X-ray crystallography more than a decade ago (62). This structure served as a template to model the other members of the PC family by homology modeling, including PC1/3 (**Figure 1B**) (63). More recently, the X-ray structure of human furin in complex with some noncovalent inhibitors has been reported (64). The core of the catalytic domain of mouse and human furin consists of a highly twisted β-sheet composed of seven parallel and one antiparallel β-strand, which is flanked by five adjacent and two peripheral helices and by two β-hairpin loops. Because of the high sequence similarity among PCs, this structural topology is likely conserved in all family members, as suggested by the homology modeling analyses (63). The polypeptide chain of the catalytic domain of furin is cross-connected by two disulfide bridges and it has two calcium binding sites. The cysteine residues involved in the formation of these disulfide bridges and the residues involved in the calcium binding are conserved in PC1/3, suggesting that PC1/3 has both structural elements as well.

P domain

The P domain is a well conserved region in PCs of approximately 150 residues and located C-terminal to the catalytic domain. It is unique for PCs and absent in subtilisins. In contrast to the spherical shape of the catalytic domain, the P-domain topology is barrel-like. According to the X-ray structure of furin and homology models of all other PCs, the P-domain is organized as an eight-stranded β-barrel, in which the eight β-strands are arranged in two opposing four-stranded β-sheets (**Figure 1B**) (62, 63). The C-terminal boundary of the P domain is formed by the conserved residues Gly[593] and Thr[594] which are important for the stabilization of the catalytic domain (65). The P-domain is involved in the regulation of calcium and pH dependent activation of PC1/3 (10). Furthermore the conserved sequence Arg[526]-Arg-Gly-Asp[529], also known as RRGD motif (**Figure 1A**), is crucial for proper proPC1/3

Legend to Figure 1 Continued. . .
www.pymol.org). C. In the ER, PC1/3 is synthetized as preproPC1/3. After cotranslational signal peptide (S) removal, proPC1/3 (94 kDa) undergoes protein folding, N-glycosylation and prodomain cleavage. In the early compartments of the Golgi apparatus, proPC1/3 sugar residues are modified and finally PC1/3 is sulfated in the TGN. In the mildly acidic environment of the TGN, the prodomain is removed after an additional internal cleavage, resulting in an active 87-kDa PC1/3 form. In the secretory granules (SG) 87-kDa PC1/3 is intermolecularly cleaved in the C-terminal tail at two different cleavage sites, which results in the formation of the C-terminally truncated fully activated forms of 74 and 66-kDa.

processing and further sorting to the secretory granules (66, 67).

Carboxyl terminal domain

The C-terminal domains are unique for each member of the PC family, varying both in sequence and length. The C-terminus of PC1/3 is much longer than that of PC2 (159 aa for PC1/3 and 44 aa for PC2) and is involved in the sorting of this convertase to the dense core secretory granules, as well as in its enzyme activity and stability (50, 66–68). The granule-sorting signal resides in an amphipathic α-helix present in the C-terminal 43 amino acids (68–70). Once PC1/3 is located in the secretory granules, the C-terminus can be cleaved at two dibasic sites as discussed in more detail in the next section.

D. Cell biology of PC1/3: Maturation and trafficking

After signal peptide removal, the resulting zymogen, proPC1/3 (94 kDa), requires N-glycosylation for proper folding and prodomain cleavage (71) (**Figure 1C**). Mouse PC1/3 contains three potential N-glycosylation sites of which only Asn^{173} and Asn^{645} are glycosylated (72). Glycosylation at Asn^{173} is necessary for autocatalytic activity and ER exit, while glycosylation at Asn^{645} is not. Human PC1/3 is only glycosylated at Asn^{173} (73). PC1/3 undergoes several additional posttranslational modifications in the Golgi, such as complex glycosylation and sulfation (71, 74). In the TGN, 87-kDa PC1/3 form is packaged, together with a number of prohormones and cargo proteins, into immature secretory granules. In the acidic environment of the secretory granules, 87-kDa PC1/3 is further intermolecularly cleaved at the C-terminal region, which results in the formation two truncated forms: 74-kDa and 66-kDa PC1/3 (**Figure 1C**). In contrast to the 87-kDa species, which possesses a relative low enzymatic activity compared to other convertases (12), and tends to form dimers, oligomers and aggregates (75), these truncated forms (especially 66-kDa PC1/3) are more active, exhibit higher calcium dependence and narrower pH optimum (5.0–5.5), and are mostly present as monomers (75–78). The 74/66-kDa forms, however, are much less stable than the nontruncated 87-kDa species (76–78). PC1/3 substrate processing is detectable starting at the TGN, which is in line with the pH requirements for the secondary prodomain cleavage (ie, the initial processing of POMC; (53, 79)).

E. PC1/3 substrate specificity

In the past two and a half decades a significant proportion of research in the proprotein convertase field has been focused on identifying physiological substrates for PC1/3. In vitro and *in cellulo (*experiments performed in cell cultures) strategies using overexpression of substrate and/or enzyme have been useful, but are prone to false positive results. *In cellulo* experiments using knockdown or knockout of PC1/3 are the preferred method to identify a genuine substrate. Serum samples of PC1/3 null patients have been instrumental in the identification of some physiological substrates, but with the development of knockout mouse models for PC1/3 substrate processing has been assessed in most relevant tissues, such as brain, pituitary and islets of Langerhans (3). Redundancy with other PCs, in particular PC2, exists for certain substrates, sometimes in a cell type-specific manner. A striking example is the processing of proneurotensin and neuromedin N which were reduced in whole brain extracts from *Pcsk2* knockout mice by 15% and 50%, respectively (80). The degree of processing was, however, dependent on the brain region investigated. Immunohistochemistry studies suggested region-specific redundancy by PC1/3 (80). Comparison of peptidomic analyses from brain from *Pcsk1* and *Pcsk2* knockout mice showed that PC2 has more unique substrates than PC1/3 and confirmed that PC1/3 has a preference for cleavage after Lys-Arg, Arg-Arg, Arg-Lys or Arg-X-X-Arg, whereas PC2 has a preference for cleavage after Lys-Arg and Arg-Arg (81). Other potential substrates have been identified or proposed by a variety of strategies. Supplemental Table 1 provides an overview of potential substrates and rates the supplied evidence for each substrate to be an actual substrate in vivo.

II. PCSK1 mutations in disease; a spectrum of phenotypes

A. Clinical aspects of PCSK1 deficiency

In 1995, O'Rahilly et al described a patient who presented with severe reactive hypoglycemia, evidence of impaired adrenal and thyroid function, and a history of hypogonadotropic hypogonadism and severe obesity with onset in infancy. The patient was found to have biochemical evidence of a generalized defect in prohormone conversion with plasma levels of prohormone to mature hormone ratios indicating impaired proinsulin and POMC processing (82). Subsequently, compound heterozygous mutations in *PCSK1* were identified in this patient: a deleterious mutation *PCSK1*-p.G593R and a mutation in the splice donor site c.620+4A>C causing exon skipping that leads to a frameshift and hence protein truncation (83) (OMIM: 600 955). This patient represented one of the first examples of a mutation in a single gene leading to obesity in humans. The patient had highly elevated levels of intact and 64–65 des-split proinsulin but very low levels of insulin. Proinsulin has reduced affinity for its receptor but an increased half-life (84), which is the likely ex-

planation for the postprandial hypoglycemia observed in this patient. Three additional patients have since been identified by the same group, one of which was homozygous and the other two combined heterozygous for deleterious mutations in *PCSK1* (85–87). Since these patients exhibited features of intestinal malabsorption, the early clinical history of the first PC1/3 null patient was re-evaluated, revealing that in the first decade of life, despite severe obesity, she also suffered from frequent diarrhea and was investigated by intestinal biopsy because of clinical suspicion of celiac disease. Throughout her life the patient has suffered from intermittent constipation and diarrhea.

In 2013, exome sequencing of 35 children with idiopathic malabsorptive diarrhea revealed 13 patients with PC1/3 deficiency (88). To date twenty one PC1/3-deficient patients carrying different *PCSK1* mutations have been identified, most of them located in the catalytic domain and in the P domain (Supplemental Table 2). Several nonsense mutations cause a premature stop codon leading to truncated proteins. All mutations cause a complete loss of activity measured in vitro, except for the *PCSK1*-p.P258T mutation which retains approximately 50% of activity compared to *PCSK1*-WT (88). However, the patient with the *PCSK1*-p.P258T mutation was compound homozygous for both the deleterious *PCSK1*-p.G209R mutation and the *PCSK1*-p.P258T mutation. Coexpression of the two mutant proteins in vitro showed that the catalytically inactive *PCSK1*-p.G209R mutant retains the *PCSK1*-p.P258T in the ER and therefore renders it inactive against substrates *in trans* (89). The expression of the different symptoms of the syndrome varies between patients. Thus far, 17 of 21 patients described are male suggesting the possibility of selective early mortality in females. The clinical phenotypes and diagnostic tests are depicted in Table 1.

Early-onset obesity and hyperphagia

Most the patients (17/17; four not described) were reported to suffer from early-onset obesity and hyperphagia. However, patients suffered from profound weight loss before intervention with parenteral feeding and failure to thrive. Despite the fact that some patients displayed morbid obesity and severe hyperphagia, other subjects were only moderately obese (zBMI+2.3). Some patients needed food restriction, in some cases even locking the pantry and refrigerator (90). In general, body mass index (BMI) started to rise from the age of 2, and patients became obese from early childhood and continued to be so (88, 90, 91). However, the extreme obesity of the index case at 3 years of age (zBMI+5.3; (82, 83)) has not been reported in any subsequent patients. Interestingly, in many patients a moderate increase in body weight coincided with short stature, implicating that gains in BMI are not only due to weight increases (88, 90). When tested in one patient, energy expenditure was in the normal range while the increase in food intake during an ad libitum meal was approximately twofold, comparable with patients with mutations in the melanocortin 4 receptor (MC4R) (86).

Malabsorption of fatty acids, amino acids and monosaccharides

In 2003, Jackson et al reported for the first time that deficiency of PC1/3 activity results in a severe neonatal diarrhea and intestinal malabsorption (85). All subsequent PC1/3 null patients have been reported with small-intestinal dysfunction (86, 88, 90–93). In general, the gastrointestinal (GI) complications begin very soon after birth (the first week of life), most children requiring hospitalization and parenteral nutrition due to recurrent watery diarrhea. As a consequence of the chronic diarrhea, the patients suffer weight loss, dehydration and metabolic acidosis. Because of the severity of the symptoms, several

Table 1. Overview of clinical phenotype of PC1/3-deficient patients. Abbreviations in this table: ACTH: adrenocorticotropic hormone; BMI: body mass index; FSH: follicle stimulating hormone; GLP-1: glucagon-like peptide 1; IGF-1: insulin growth factor 1; LH: luteinizing hormone; TRH: thyrotropin releasing hormone; TSH: *thyroid-stimulating hormone.*

Symptom	Clinical findings	Present	Absent	Not reported
Increased BMI	Hyperphagia	17	0	4
Polyuria/polydypsia	Low serum osmolality	7	12	2
Abnormal glucose homeostasis	Increased proinsulin, low insulin, increased 65,64 des split proinsulin	15	5	1
Malabsorptive diarrhea	Mild villous atrophy, elevated progastrin and GLP-1 precursors, normal procalcitonin	21	0	0
Decreased linear growth	Growth hormone deficiency; low IGF-1	5	9	7
Hypogonadotropic hypogonadism	Low FSH, low LH, low testosterone	7	9	5
Hypothyroidism	Low TRH, high TSH, low free T4	13	6	2
Hypocortisolism	Normal ACTH, elevated ACTH precursors	12	4	5

PC1/3 deficient patients died in early childhood (85, 88, 92). Despite the fact that intestinal biopsies did not reveal clear abnormalities in most patients, malabsorption of fat, amino acids and sugars has been confirmed in all the cases studied so far. Immunohistochemical staining of intestinal biopsies from *PCSK1* null patients showed normal staining of chromogranin A, but absence of both PC1/3 and PC2 expression (90). The severity of the malabsorption, despite the anatomical integrity of the gut and the preserved villous architecture, is remarkable and unexplained. In this regard, children with mutations in the transcription factor neurogenin 3, required for endocrine cell fate specification, have a similar syndrome (94), which illustrates the fact that the function of this scattered enteroendocrine cell population has a crucial, but as yet poorly understood role in the control of absorption by the mucosal surface of the GI tract. It is important to mention that both the malabsorptive dysfunction and chronic diarrhea tend to slightly diminish with time, concomitantly with the rapid weight gain of the children.

Hypogonadotropic hypogonadism

Several cases of hypogonadotropic hypogonadism have been reported in patients lacking PC1/3 activity. The first reported adult woman with PC1/3 deficiency exhibited low-serum estradiol, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and absence of menstruation (82, 83). She became pregnant (with quadruplets) after the administration of gonadotropins to induce ovulation (82). Since then six other different cases of hypogonadism have been reported in infants, five boys with micropenis and one girl with delayed puberty (87, 88, 90). No evidence of male hypogonadism was described in six other infants. The treatment of one male patient with micropenis with monthly testosterone injections for 3 months resulted in the normalization of his penis size (87, 88, 90).

Hypothyroidism, hypocortisolism, growth hormone (GH) deficiency and diabetes insipidus

More than half of the PC1/3-deficient patients were diagnosed to exhibit central hypothyroidism and hypocortisolism (**Table 1**), as demonstrated by their low blood T4 and cortisol levels without elevations in thyroid-stimulating hormone (TSH) and ACTH respectively. Hypothyroidism and hypocortisolism can contribute to many of the symptoms observed in PC1/3 null patients including fatigue, weight gain, constipation and abnormal menstrual cycles (82, 86, 88). Growth hormone deficiency was only diagnosed in a limited number of patients and led to reduced growth in these patients (88, 92). Interestingly, however, in a recent meta-analysis, Nead et al identified the single nucleotide polymorphisms (SNPs) rs6234-rs6235 to be significantly associated with reduced body length (95). Diabetes insipidus has been reported in a subset of PC1/3 null patients (85–88).

B. Heterozygous mutations contribute to obesity

Initially, the syndrome was described as recessive, as children of the first patient were not obese (83). Loss of one wild-type allele was not expected to result in a severe phenotype. In 2012, Froguel and coworkers identified eight novel heterozygous mutations in extremely obese patients (73). These mutations were not present in a control population of 6000 nonobese individuals. The limitation of this study was that most mutations were unique and not replicated in neither obese nor in control populations. For instance the *PCSK1*-p.N180S mutation identified in an extremely obese patient was recently identified in a lean patient (96). Only one mutation, *PCSK1*-p.Y181H, was convincingly replicated in the obese cohort, suggesting its association with obesity. Conversely, despite the location of this mutation in the catalytic domain, it did not alter the enzymatic activity using a fluorogenic substrate in in vitro experiments, indicating that enzyme activity is not the only determinant for *PCSK1* related obesity.

In 2014, Philippe et al reported on a family with dominant Mendelian inherited obesity (96). Sequencing of genes linked to known monogenic obesity, revealed a nonsense mutation in the second exon of *PCSK1*. The truncated protein contained the N-terminal propeptide and was shown to partly inhibit the wild-type enzyme in vitro (96). This indicates that expression of mutant PC1/3 can have dominant negative effects on the wild-type protein function. However, the dominant inheritance did not result in the full scope of endocrinopathies associated with PC1/3 null patients, confirming residual PC1/3 activity. As described above, the inhibitory function of PC1/3 prodomain was already established in vitro (54, 56, 57). The earlier described *PCSK1*-p.G209R mutant which was shown to retain the *PCSK1*-p.P258T hypomorph in the ER, could have a similar limited dominant negative effect (89). Aside from a clear relation with increased BMI in the reported patients, it remains unclear whether heterozygosity can lead to other endocrinopathies which are reported for null patients. Some, but certainly not all, carriers of PC1/3 null mutations were reported to be obese (Dr. M. Martin, UCLA, CA - USA; personal communication).

C. Single nucleotide polymorphisms in PCSK1 increase the risk of obesity in the population

In a study by Benzinou et al three nonsynonymous SNPs, rs6232, rs6234-rs6235, in *PCSK1* were identified

to be associated with extreme obesity in seven independent European case-control studies (97). The SNPs encode the *PCSK1*-p.N221D (rs6232), *PCSK1*-p.Q665E (rs6234) and *PCSK1*-p.S690T (rs6235) amino acid changes, respectively, of which the latter two SNPs are in linkage disequilibrium. The minor allele frequencies (MAF) in a global population for rs6232 and rs6234-rs6235 are 2% and 26% respectively (dbSNP database, average across ethnicities). However rs6232 is extremely rare in non-Caucasian ethnicities (~5% in Caucasian). In vitro assays showed that the *PCSK1*-p.N221D variation causes a significant decrease in enzymatic activity (89, 97). The *PCSK1*-p.Q665E-p.S690T variation was not shown to decrease enzymatic activity but could have an effect on protein sorting and stability as it is located in the C-terminal domain (98). The results by Benzinou et al were subsequently only partly confirmed in different case-control studies from different ethnic origins (99–106). Similarly, in individual studies for BMI or genome wide association studies (GWAS) for BMI, either weak associations or no association were identified (99, 102, 105, 107–112). To challenge the evidence on the associations of *PCSK1* SNPs with obesity and BMI, two meta-analyses were conducted independently (95, 113). Both studies confirmed an association of rs6232 and rs6234-rs6235 with obesity with odds ratios of 1.15 (1.06–1.24) and 1.07 (1.04–1.10), respectively. For rs6232, the association was only present for obesity grade I subgroups, but not for obesity grade II and III subgroups. For rs6234-rs6235, the association was significant for all subgroups. No association was identified in Asian cohorts. Both SNPs associated with increased BMI and waist circumference (WC), albeit with only a small effect size (increase of 0.02–0.03 BMI units or 0.2–0.4 cm for WC per allele). The effect of the minor alleles on obesity traits was larger in childhood and adolescent cohorts compared to adult cohorts. Interestingly, Nead et al reported that the SNPs rs6234-rs6235 are associated with a decreased height (95). No association was found for rs6232, which could be because of the low power of the analysis to detect a small difference.

Pickett et al identified another less frequent SNP in *PCSK1* to have a moderate effect on PC1/3 function (114). The nonsynonymous SNP rs1799904 encodes *PCSK1*-p.R80Q which is located at the secondary cleavage site in PC1/3 prodomain. The mutation was shown to reduce enzyme activity in an in vitro activity assay (38%–48% decrease). This SNPs is however very rare as current MAF is estimated at 0.74% (dbSNP database).

Three additional SNPs located within or in close proximity of *PCSK1* have been associated with obesity or BMI. In intron 6 of *PCSK1*, rs155971 has been associated with obesity in a Chinese population (99). In addition, rs261967 was associated with BMI in East Asians and rs2570467 was weakly associated with waist circumference in a population of African ancestry (115, 116), both SNPs lie in close proximity of *PCSK1*. Furthermore, different SNPs in or near *PCSK1* have been associated with growth traits, body weight and fat deposition in different animal species, such as pigs, bovine, chicken and goats (117–120). Many of these SNPs were either synonymous or located in introns or in the 5′UTR of *PCSK1* gene in the respective animal genome. Although these SNPs did not alter protein sequence, it is possible that mutations in introns or 5′UTR influence the expression and or splicing of the gene (117–120). Synonymous SNPs could decrease mRNA stability or influence protein function as was for instance shown for the multidrug resistance 1 gene MDR1 (121, 122), yet no evidence exists that this is the case for *PCSK1*.

III. PC1/3 substrates which contribute to the PC1/3 null phenotype

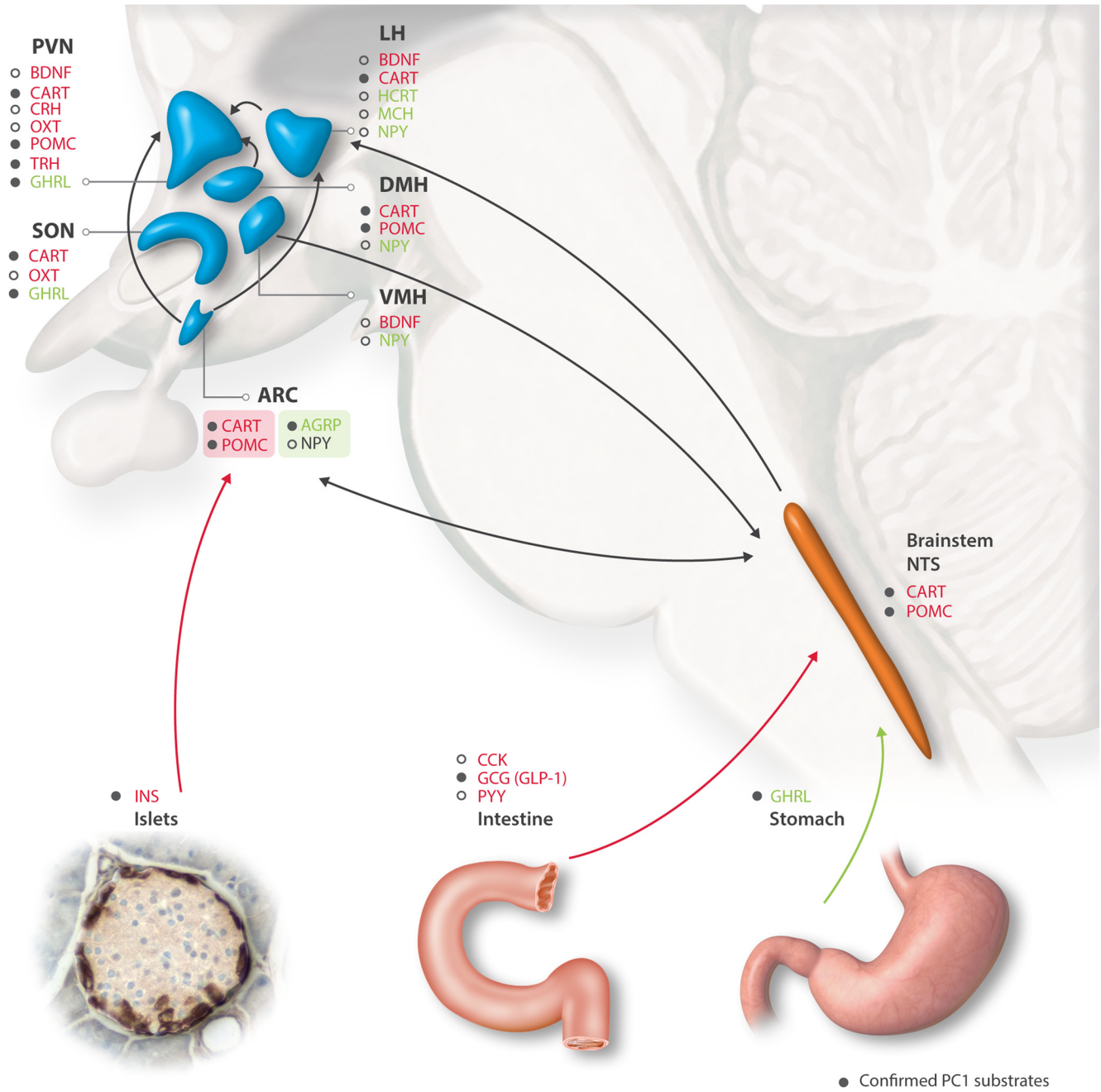
A. PC1/3 activates key players in central energy homeostasis pathway

The identification of PC1/3 deficiency in monogenic obesity suggested that substrates of PC1/3 must regulate feeding behavior and energy homeostasis. As described above, PC1/3 is expressed in different regions of the brain which are important for food consumption and metabolism. In the hypothalamus, PC1/3 expression is particularly high in the arcuate nucleus (Arc), more specifically in leptin-responsive POMC and agouti related peptide (AGRP)/neuropeptide Y (NPY) neurons. Besides the Arc, PC1/3 is expressed in several other nuclei of the hypothalamus including the magnocellular neurons within the paraventricular (PVN) and supra-optic nuclei (SON), the ventromedial hypothalamus (VMH), and the lateral hypothalamus (LH) (**Figure 2**) (21). PC1/3 cleaves both orexigenic and anorexigenic substrates which make it difficult to interpret how the complex interplay of hormonal cues tips the balance toward increased energy intake and reduced energy expenditure observed in PC1/3-deficient patients. In addition to appetite regulation, PC1/3 has been recently proposed as a new player of the adipose tissue browning (32). In this section we will discuss different known and potential PC1/3 substrates with a role in energy homeostasis and feeding behavior.

POMC, AGRP and NPY are expressed in distinct neuronal populations of the arcuate nucleus

PC1/3 is expressed in various hypothalamic nuclei known to function as centers for energy homeostasis (**Figure 2**). POMC is a well-established PC1/3 substrate which

Figure 2.



PC1/3 processes many hormones implicated in central and peripheral regulation of feeding behavior and thermogenesis. This figure includes the important nuclei and endocrine tissues implicated in feeding behavior and thermogenesis. In blue: different nuclei of the hypothalamus are depicted (ARC: arcuate nucleus; DMH: dorso medial hypothalamus; LH: lateral hypothalamus; PVN: paraventricular nucleus; SON: supra-optic nucleus; VMH: ventromedial hypothalamus). The nucleus tractus solitarii (NTS), an important peripheral neuronal nucleus in the brainstem which relays information from the periphery to the hypothalamus, is indicated in orange. Pancreatic islets, intestinal endocrine cells, and the stomach produce peripheral hormones which can influence feeding behavior by local and central mechanisms. All peptides listed are either confirmed (gray circles) or potential (white circles) PC1/3 substrates. Note that substrates are confirmed in one tissue only and tissue-specific redundancy may exist. Orexigenic and anorexigenic peptides are indicated in green and red, respectively. Abbreviations: AGRP, agouti-related peptide; BDNF, brain-derived neurotrophic factor; CART, cocaine- and amphetamine-regulated transcript; CCK, cholecystokinin; CRH, corticotropin-releasing hormone; GCG, glucagon; GHRL, ghrelin; GLP-1, glucagon-like peptide 1; HCRT, orexin precursor; INS, insulin; MCH, melanin-concentrating hormone; NPY, neuropeptide Y; OXT, oxytocin; POMC, pro-opiomelanocortin; PYY, peptide YY; TRH, thyrotropin-releasing hormone (TRH).

is expressed in distinct neuronal cell populations of the Arc (40, 123–126). As described above, PC1/3 and PC2 act in concert to process POMC to different neuropeptides (as reviewed in (127, 128)). One such cleavage product is α-MSH, an anorexigenic hormone that exerts its function by binding to the melanocortin receptors MCR3 and MCR4. POMC neurons project to over 100 different brain nuclei, including the PVN and the LH where α-MSH can bind the MCR4 leading to a decrease of food intake and an increase of thermogenesis (129). Deficiency of β-MSH, another POMC-derived peptide, also predisposes to obesity in humans, but β-MSH is a product of PC2 cleavage only (130). Conversely, AGRP, which is produced mainly by PC1/3 from its precursor proAGRP in AGRP/NPY-expressing neurons, is an orexigenic hormone that antagonizes the α-MSH effects by direct competition for the binding to the MCR4 receptors (126). Therefore, given that both AGRP and α-MSH are products of PC1/3 action, it is complex to fully understand how PC1/3 deficiency favors increased food intake. Patients with congenital POMC deficiency and *Pomc* knockout mice suffer from hyperphagia and early-onset obesity (131, 132). Ablation of AGRP/NPY neurons in mouse neonates or *Agrp* gene knockout, however, does not alter feeding behavior indicating that developmental compensatory mechanisms exist (133, 134). Removal of AGRP/NPY-expressing neurons at adult age does, however, cause severe hypophagia in mice (133, 134). NPY, which is most likely a PC2 substrate (135–139), is coexpressed with AGRP in the same neuronal population of the Arc. NPY exerts orexigenic actions through the depolarization of POMC neurons, which results in an inhibition on the action of α-MSH. Unlike AGRP, NPY does not bind to the MCR4 receptor but to NPY1R and NPY5R (140).

CART and BDNF

Cocaine- and amphetamine-regulated transcript (CART) is a neuropeptide activated by PC1/3 (141), which is expressed in several nuclei of the hypothalamus. The receptor for CART has not been identified, troubling the identification of its function. When infused in the ventricle, CART has an anorexic effect (142). Although it is coexpressed with POMC in the Arc, some evidence shows that its anorexic effect is mediated through the nucleus tractus solitarii (NTS) (143). This nucleus, located in the dorsal vagal complex (DVC) in the brainstem, is the primary site for receipt of vagal afferent innervation from the gut and also expresses POMC (**Figure 2**).

Brain derived neurotrophic factor (BDNF) is coexpressed with PC1/3 and therefore qualifies as a potential PC1/3 substrate (144). BDNF is primarily expressed in the VMH and to lesser extent in the PVN and LH (145). Different reports have described that the infusion of BDNF leads to hypophagia, and that $Bdnf^{+/-}$ mice are hyperphagic (146). Given that there is no or little expression of the BDNF receptor in the Arc, it is unlikely that BDNF directly regulates POMC or AGRP/NPY expression. In the VMH, BDNF-expressing neurons project to various regions in the brain including the NTS, which also expresses POMC (145). Thus, it might be conceivable that other interfaces between the leptin-melanocortin signaling pathway and BDNF exist. Recent evidence has demonstrated that the loss of PC7 in mice reduces proBDNF processing by 36% (147). Both the *Pcsk7* and *Bdnf* knockout mice, and heterozygous patients carrying BDNF inactivating mutations exhibit impaired cognitive function. However, the *Pcsk7* knockout mouse model was not reported to be obese nor hyperphagic. The moderate reduction of proBDNF processing in *Pcsk7* knockout mice and the absence of an obese phenotype suggests that PC7 only processes proBDNF in distinct cell types related to cognitive impairment. Possibly PC1/3 accounts for processing of proBDNF in nuclei related to feeding behavior (83, 146–149).

Melanocortin concentrating hormone, Orexin A and B, and Oxytocin

Both melanin concentrating hormone (MCH) and orexins (A and B) are thought to influence reward and motivational cues for feeding. Both peptides colocalize with PC1/3 in the LH (150). ProMCH is processed by members of the convertase family to MCH and neuropeptide EI (NEI). The latter is a product of PC2 cleavage while the convertase responsible for the first cleavage resulting in MCH has not been yet identified (151). Intracerebroventricular (icv) injection of MCH leads to hyperphagia and MCH knockout mice were hypophagic and 28% lighter than wild type littermates (152). Administration of exogenous orexin A to the LH, DMH and PVN led to hyperphagia, but not when injected in other hypothalamic regions or the brainstem (153). Oxytocin is mainly expressed in the SON and PVN and is a potential PC1/3 substrate, although the knockout mouse models suggest complete redundancy with PC2 (154, 155). Besides other well established functions of oxytocin, as a stimulus for sex drive and lactation, this neuropeptide also has an anorexic effect. Possibly it influences appetite through the amygdala, VMH, DMH or NTS (156).

B. Peripheral feeding cues are regulated by PC1/3 processing

Different peripheral hormones have been reported to influence feeding behavior. Several of these hormones are secreted by the gut in response to food intake and relay to

the nervous system via the vagal nerve to the DVC (**Figure 2**). PC1/3 is the enzyme responsible for the activation of some of these hormones that function as peripheral feeding cues and signal in various metabolic processes.

Ghrelin

Ghrelin has convincingly been shown to be a PC1/3 substrate in mice (157, 158). For its function, the precursor must be octanylated and cleaved to yield the 28 amino acid ghrelin. The peptide requires both modifications for binding to the ghrelin receptor. Ghrelin is not only expressed in the stomach and duodenum but also in certain hypothalamic areas including Arc, SON and PVN (159, 160). Ghrelin release promotes food intake, gastric motility, and GH secretion, and has a trophic effect on intestine endothelium. In the Arc it activates AGRP/NPY neurons and thereby promotes feeding behavior. Besides this direct action as orexigenic hormone, it is hypothesized that ghrelin is implicated in reward and motivation (161).

Cholecystokinin

Cholecystokinin (CCK) is a gut hormone secreted by the small intestine mucosae cells, enteric nerves or neurons of the central nervous system (CNS). In the intestine, its secretion is stimulated by certain amino acids and fat content in the chyme when entering the duodenum. In the CNS CCK is most likely processed by PC2, but PC1/3 could be the physiological convertase in the GI tract (162–166). CCK acts primarily through CCK receptors on primary vagal afferents wiring to the NTS (167). In addition it aids digestion by inhibiting gastric emptying, gastric acid secretion and by promoting secretion of pancreatic enzymes. It also increases the production and secretion of hepatic bile which is necessary for fat absorption.

Glucagon-like peptides

Proglucagon is processed in the intestinal L cells to GLP-1, GLP-2 and oxyntomodulin, mainly, if not only, by PC1/3 (85, 148). The plasma levels of GLP-1 and GLP-2 were found to be decreased in several PC1/3-deficient subjects, although it was not determined for all patients (85, 86, 91). In *Pcsk1* knockout mice intestinal proglucagon processing was absent (148). GLP-1, which is an incretin, reduces appetite and glucagon secretion, and inhibits gastric emptying. GLP-1 can also be synthesized locally in neurons that project to the brainstem and hypothalamus (168). GLP-2 has a trophic effect in the intestine and delays gastric emptying. Oxyntomodulin decreases food intake and increases thermogenesis, albeit it is unclear which receptor is responsible for these effects. Proglucagon is also expressed in the pancreatic α-cells, where the PC1/3 levels are very low. Here, proglucagon is predominantly processed by PC2 to yield glucagon, which is released into the bloodstream. Glucagon is important for increasing glycemia by stimulating hepatic gluconeogenesis. Increased levels of glucagon have been described in some patients (91), but this increase could be due to unspecific detection of proglucagon.

Insulin

Insulin is produced by the pancreatic β-cells and is the product of PC1/3 and PC2 mediated cleavage of proinsulin (41, 42, 169–171). In PC1/3-deficient patients the levels of proinsulin and 64,65-des-split proinsulin are abnormally high, whereas in control samples this cleavage product is almost undetectable (82, 85, 86, 88, 91, 92). Proinsulin has only 2%–5% of the activity of insulin which is compensated for by secretion of vast amounts. However, proinsulin has a 4–6 times longer half-life than insulin, which is probably the cause of the postprandial hypoglycemia reported in several patients. It is well-established that human proinsulin is first cleaved by PC1/3 after residue 32 and subsequently by PC2 after residue 65 (82, 172, 173). This almost obligate sequential cleavage is less constrained in rodents, where two nonallelic genes for proinsulin, proinsulin I and II, exist. Unlike mouse proinsulin II, mouse proinsulin I can be processed at both positions by PC2 (172). The primary function of insulin is promoting glucose uptake, glycogen synthesis, and inhibition of lipolysis. Insulin processing defects cause diabetes and can in turn contribute to obesity. In addition, it has been reported that deletion of the insulin receptor from the brain influences hepatic insulin sensitivity (174, 175). Further research showed that both leptin and insulin use similar signaling pathways potentially explaining the effects of insulin on feeding behavior and thermogenesis (176).

C. PC1/3 processing of gut hormones in the GI system

In the GI tract, PC1/3 is highly expressed in diverse hormone-producing cells of the small intestine. For instance, PC1/3 colocalizes with the vast majority of cells which express the gut hormones CCK, proglucagon, and substance P, and in a high percentage of glucose-dependent insulinotropic polypeptide (GIP)-expressing cells (29, 177, 178). PC1/3 is also colocalizes with progastrin in antral G-cells (179). Whereas the processing of proglucagon (as discussed above), progastrin and proGIP by PC1/3 in the endocrine intestinal L, G and K cells, respectively, has been well demonstrated, its participation in the processing of proCCK and prosubstance P remains to be demonstrated. In the endocrine intestinal K cells of the upper intestine the generation of the incretin hormone GIP is exclusively dependent on the PC1/3 activity (178). Despite the ability of PC2 to properly produce GIP from proGIP in

several endocrine cell lines, the processing of proGIP was unaltered in intestinal extracts from mice lacking PC2, suggesting that PC2 is not essential for the production of GIP (178). Furthermore, PC1/3 is the major convertase involved in the production of gastrin in antral G-cells (179, 180), albeit PC2 has been also proposed to participate in the cleavage of progastrin, consistent with the reduced but not absent gastrin in the null patients (85, 181, 182).

D. Other substrates associated with PC1/3-related endocrinopathies

Hypogonadotropic Hypogonadism

For a complete sexual maturation and successful reproduction, a number of neuropeptides, hormones and sex steroids work in a coordinated fashion across the hypothalamic-pituitary-gonadal (HPG) axis. Among this variety of hormones, the only one that can potentially be proteolytically activated by PC1/3 is progonadotropin releasing hormone (pro-GnRH). Accordingly, PC1/3 is well expressed in the hypothalamus, which contains GnRH-enriched areas, supporting the idea that it could participate in the activating cleavage of pro-GnRH in GnRH neurons (183). However, evidence exist that PC2 is also able to activate proGnRH (183). Aside from a possible role in the processing of pro-GnRH, PC1/3 could be also participating in the regulation of the HPG axis through the proteolytic activation of KISS1 or tachykinin B in the hypothalamus. It has recently been described that PCs may be required for the processing KISS1 to kisspeptin peptides (88, 90, 184). Whether PC1/3 is responsible for the KISS1, pro-GnRH or tachykinin B processing in the respective neurons, remains to be established.

Hypothyroidism

In the early nineties prothyrotropin releasing hormone (proTRH) was put forward as a PC1/3 substrate by in vitro studies (185). This was recently confirmed by peptidomic analysis of mouse brain and by direct comparison of proTRH processing in PC1/3 and PC2 knockout mice (81, 186, 187). Although PC1/3 was found to be the main physiological proTRH converting enzyme, PC2 cleaves it as well, albeit it to a lesser extent (187). ProTRH is produced in the PVN and its expression and secretion is stimulated by neuropeptides related to the leptin-melanocortin pathway. For instance, α-MSH stimulates and AGRP inhibits proTRH expression. TRH is released into the blood via the portal vessels of the median eminence. In the pituitary, TRH induces the secretion of TSH, which in turn stimulates the thyroid gland. The active hormones T4/T3 released by the thyroid gland are important to drive the metabolic rate. The implication of PC1/3 deficiency on the secretion of TRH is twofold: firstly, reduced production of α-MSH leads to a decrease of TRH expression which may, at least in part, be compensated by reduced amounts of AGRP; and secondly proTRH has been identified as a PC1/3 substrate (185, 187).

Hypocortisolism

The processing of POMC by PC1/3 and PC2 is well established and it is known that only PC1/3 is expressed in the ACTH-producing corticotroph cells of the anterior pituitary. Plasma ACTH stimulates adrenal expression of cortisol. Strikingly, in *Pcsk1* knockout mice, no ACTH was produced, yet corticosterone levels were normal (148). How mice can remain eucorticosteronemic in the absence of ACTH is unknown. Since the initial observation has not been confirmed in other studies, it is possible that either the physiological ACTH concentration was underestimated or the corticosterone concentration was overestimated in *Pcsk1* knockout mice. Similarly in patients, reduced plasma ACTH, increased ACTH precursors, and near normal cortisol levels were observed (85). Potentially, ACTH precursors might retain some affinity for the ACTH receptor explaining the near normal cortisol levels in patients. Alternatively, another endoprotease or proprotein convertase could process POMC to ACTH in the absence of PC1/3. In addition, PC1/3 could be the putative convertase responsible for the activation of pro-corticotropin releasing hormone (CRH) (188) since the production of CRH is not altered in the hypothalamus of *Pcsk2* knockout mice (136, 189). However, no direct evidence exist that CRH is a substrate for PC1/3.

Growth hormone deficiency

Several studies have demonstrated that progrowth hormone releasing hormone (proGHRH) processing to GHRH is mediated by furin and PC1/3 (190, 191), which can explain the growth retardation observed in several human patients (**Table 1**). Interestingly, *Pcsk1* null mice display severe dwarfism due to low levels of GHRH (148). Given that not all patients exhibit GH deficiency, most likely another convertase provides limited redundancy in human but not mice. Mouse proGHRH is cleaved at two positions (RMQ***R***^{30} and RLS***R***^{73}) to yield GHRH$^{31–73}$ (191). For both sites the P2 positions are not conserved in humans (RM**R**R^{31} and RLG**R**^{77}). In particular, the RM**R**R^{31} site is an improved cleavage site, making it a more likely substrate for additional convertases.

Diabetes insipidus

Vasopressin colocalizes with PC1/3 in the magnocellular neurons of the SON (21). Therefore, aberrant provasopressin processing is a likely cause for diabetes insipidus

in PC1/3 null patients. In fact, PC1/3 can process provasopressin at the neurophysin/glycopeptide boundary and the vasopressin/neurophysin boundary in vitro (154). However, brain neuropeptidomic studies in *Pcsk1* and *Pcsk2* null mice showed no alterations in provasopressin expression (81, 192, 193). *PCSK1* null patients with diabetes insipidus were successfully treated with desmopressin, a vasopressin analog, substantiating the importance of PC1/3 in the processing of provasopressin (85–88, 90, 92, 93, 194). Sequence comparison indicates that the vasopressin/neurophysin cleavage site is conserved between mice and men, but not the neurophysin/glycopeptide cleavage site (RRAR^{125}) in human vs RLTR^{129} in mice).

IV. PC1/3 deficiency: a clinical perspective

A. Clinical presentation

The clinical manifestations of *PCSK1* deficiency show considerable interindividual variation. This presumably reflects the impact of genetic background on the ability of other convertases to compensate for particular defective proprotein conversion events and/or variability in the extent to which elevated levels of incompletely processed precursors can continue to have bioactivity at their cognate receptors.

PCSK1 deficiency does not appear to impair prenatal growth and development and all children described thus far have been born at full term after an uneventful pregnancy. It is now clear that by far the most common clinical presentation of *PCSKI* deficiency is severe malabsorptive diarrhea becoming clinically evident within the first three months of life. This can be so severe as to lead to a metabolic acidosis. The failure of this diarrhea to resolve, usually leads to early specialist referral and investigations and, not infrequently, the need for nutrition to be delivered parenterally (83, 85, 86, 88, 90, 92, 93). After the age of two years, the severity of the malabsorption appears to spontaneously improve and many children can discontinue parenteral feeding. To date, there is no therapy that can reliably accelerate that spontaneous improvement. Despite the improvement, patients appear prone to persistent diarrhea and bloating which can be life-long. The index case also suffered from episodes of constipation, but this has never been reported since (82).

Guidelines for differential diagnosis of chronic infantile diarrhea have been developed (as reviewed in (195, 196). Patients presenting with idiopathic pediatric chronic diarrhea with generalized malabsorption but normal or near normal histology of the intestines should be considered for *PCSK1* deficiency. Using these criteria, Martin et al identified that approximately 25%–30% of the selected patients were homozygous or compound heterozygous for deleterious *PCSK1* mutations (88).

Over the subsequent childhood years the clinical manifestations which most frequently emerge, are persistent diarrhea, obesity, diabetes insipidus (86), and reactive hypoglycemia, albeit with variable severity. The obesity is usually associated with hyperphagia. The reactive hypoglycemia can be severe and lead to neuroglycopenic episodes several hours after a meal. Despite the fact that proinsulin processing is markedly abnormal, sometimes resulting in little or no mature insulin in the circulation, the biological activity of the highly elevated levels of proinsulin means that diabetes mellitus is not a frequent early clinical issue in patients. However, as patients age, diabetes can emerge as a clinical problem, presumably at least partly attributable to "beta cell exhaustion", and insulin treatment may be required (observation made for the index patient by Dr. S. O'Rahilly, University of Cambridge, UK).

Problems of impaired linear growth and short stature are not a hallmark feature, yet reduced GH levels has been observed in at least five patients (Table 1). The finding that the polymorphism rs6234-rs6235 is mildly, yet significantly ($\beta = -0.0224 \pm 0.0033$, $P = 5.4 \times 10{-}11$, N = 251 342), associated with decreased length seems to indicate mild impairment of the GHRH-GH axis (discussed further below) (95). Reduced growth could partially account for increased BMI of some of the heterozygous and homozygous patients.

Hypogonadotropic hypogonadism with failure of pubertal development is a striking feature in many patients (indeed the index case was amenorrheic until treated with gonadotropins for infertility in her early twenties (82)).

Almost all patients show, when appropriately tested, biochemical evidence of impairment of the hypothalamic-pituitary-adrenal and hypothalamic-pituitary-thyroid axes although severe, clinically apparent deficiencies or either cortisol or thyroxine are rarely seen. Anecdotally, some patients report highly abnormal sleep/wake cycles.

B. Diagnosis

PC1/3 deficiency is a very rare genetic disorder; thus far only 21 patients have been reported (82, 83, 85–88, 90–93). It is very likely, however, that more patients miss diagnosis or die soon after birth. Therefore, guidelines are needed for rapid differential diagnosis and proper treatment. The normal conversion of proinsulin to insulin is absolutely dependent on PC1/3 function in human pancreatic islets. The measurement of the circulating ratio of proinsulin to insulin is therefore a sensitive biochemical screen for the presence of *PCSK1* deficiency. Targeted sequencing of *PCSK1* exons and intron exon boundaries is

undertaken in several laboratories with a particular interest in this condition. The rapidly decreasing cost of whole exome and whole genome sequencing will probably result in this soon becoming the main route for analysis (90, 92, 197).

C. Management

As it is a germline genetic disorder expressed throughout many endocrine cell types in the body there is currently no specific targeted therapy for *PCSK1* deficiency. Management in early life is largely focused on supplying adequate nutrition in the face of severe diarrhea and complete parenteral nutrition is often required. It is of interest that a GLP-2 analog, teduglutide is now licensed for the treatment of the diarrhea of the short bowel syndrome (198). As proglucagon processing is severely disrupted in *PCSK1* deficiency the examination of the effects of this agent in affected patients with severe diarrhea would be of considerable interest.

Early evaluation of the thyroid, adrenal axes is recommended with appropriate correction of any deficiencies. A high index of suspicion for symptoms suggestive of diabetes insipidus or reactive hypoglycemia should be maintained and appropriate investigations undertaken if relevant symptoms present themselves. Hypogonadotropic hypogonadism is common and the reproductive axis should be evaluated around the time of normal puberty and, when necessary, appropriate sex steroids should be used to induce and maintain pubertal development. Affected patients are likely to require assisted conception if this issue arises.

Obesity can be a challenging problem for patients. No clinical trials of antiobesity strategies or weight loss promoting drugs have been specifically undertaken in patients with *PCSK1* deficiency and therefore experience is entirely anecdotal. It is likely that the lack of α-MSH tone in the hypothalamus is a contributor to the obesity and therefore patients with *PCSK1* deficiency might benefit from agents that act centrally to reduce appetite. Several such agents (Orlistat, Lorcaserin, Phentermine/Topiramate, Naltrexone/Bupropion, and Liraglutide (199, 200)) are now licensed for the treatment of obesity, though all have limited therapeutic efficacy and a range of side effects. To our knowledge, bariatric surgery has been used as a treatment for obesity in only one patient with *PCSK1* deficiency. At ~50 years of age the index case reported in (82, 83) underwent Roux en Y gastric bypass surgery. This was remarkably effective with a loss of more than 25% of body weight and a reversal of diabetes which had previously required over 200 U of insulin daily. Normoglycemia without treatment has persisted for more than 10 years.

V. The PCSK1 mouse models conundrum

A. Pcsk1 knockout mice: an unexpected phenotype

Two *Pcsk1* knockout mouse models have been developed so far. The first and most used KO model was developed by the Steiner group and reported in 2002 (148). The gene knockout was established by replacing of a 900 bp fragment of exon 1 with the Neomycin resistance gene at this location. The homozygous knockout mice suffered from prenatal lethality and difficulty to thrive as only ~20% of the mice survived beyond the first week. The *Pcsk1* knockout mice were growth retarded (40% reduction in body weight) due to a lack of proGHRH processing leading to secondary GH deficiency. Heterozygous animals were not growth retarded, but became mildly obese with age.

Proinsulin processing was severely impaired and led to glucose intolerance in homozygous mice, but not heterozygous mice. Immunoelectron microscopy revealed increased proinsulin/insulin ratio resulting in secretory granules with less electron-dense cores and a smaller halo between the core and the limiting membrane (172). Furthermore, large moist stools were noticed in older *Psck1*$^{-/-}$ mice (148), suggesting that, like most PC1/3 null patients, they suffer from GI disturbances. As described above, *Pcsk1* null animals also exhibited lower circulating GLP-1 and GLP-2, albeit the association of the deficiency of these hormones with the malabsorption problems remains elusive. The lack of PC1/3 activity not only causes malabsorption of fatty acids in mammals, but also in invertebrates such as *C. elegans* (201). An interesting phenotype in mice, but not still reported in humans, is that *Pcsk1* deficiency causes innate immune defects and uncontrolled cytokine secretion by macrophages (202). The *Pcsk1* knockout mice had an average spleen size which was almost double the size of control mice. Moreover the mice were more sensitive to LPS stimulation, suggesting a role of PC1/3 in innate immunity.

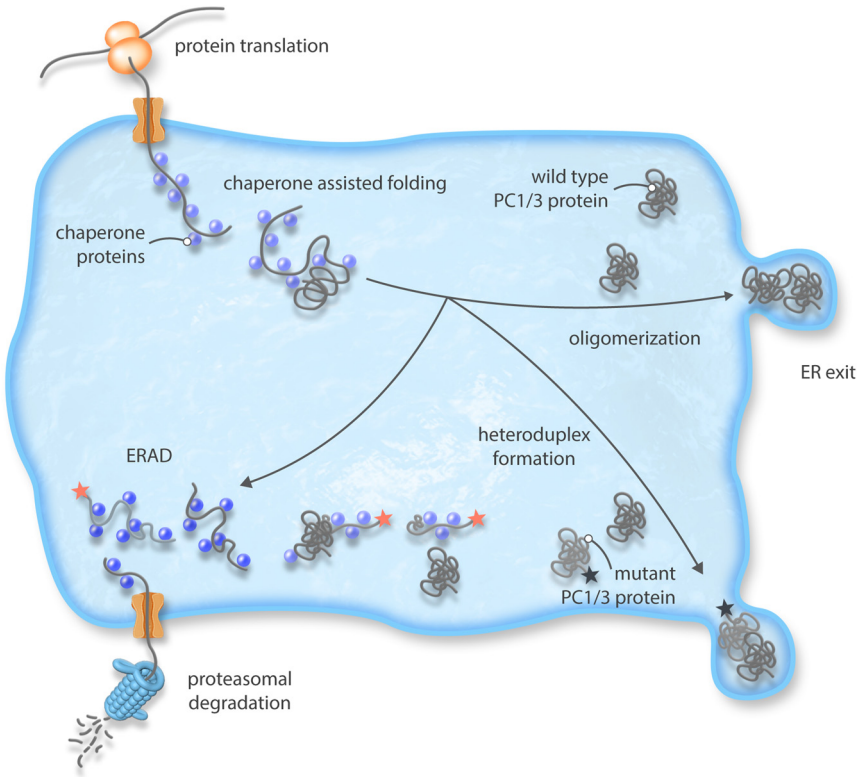
The second *Pcsk1* knockout model was developed by the Seidah group (203). This mouse model was created by homologous recombination with a disruption vector which results in insertion of the Neo gene and excision of exon 3 to 9, but suffered from preimplantation lethality. Further investigation showed that a fusion protein was expressed which contained an N-terminal 85 amino acid sequence from PC1/3 fused to 46 amino acids from the Neo gene. This fusion protein, containing most of the inhibitory propeptide of PC1/3 was shown to be able to inhibit other PCs in vitro as well. The broad inhibition of this fusion protein is likely to cause the more severe phenotype observed in the mouse model, compared to the first

mouse model and prevents its use to identify physiological substrates.

B. The Pcsk1-N222D hypomorph mouse as a model of obesity

The hypomorphic N222D mutation in the *Pcsk1* gene was identified in an obese mouse line (204). This mouse model was generated using a forward genetic screen with N-ethyl-N-nitrosourea as mutagen. The mouse model was reported to have augmented body weight and a dominant increase in body fat content when given a high fat diet (204). Accordingly, the PC1/3-N222D mice are glucose intolerant (but insulin sensitive), hyperphagic, have increased fat gain efficiency, and have a normal resting energy expenditure (204), like observed in some patients (86). The $Pcsk1^{N222D/N222D}$ mice are also less fertile as a consequence of reduced levels of plasma gonadotropins and testosterone (204), suggesting an alteration in the hypothalamic-pituitary-gonadal axis of these animals. In vitro experiments using a fluorogenic substrate demonstrated that whereas the human *PCSK1*-N222D construct showed only a reduction of roughly 50% (204), the mouse *Pcsk1*-N222D construct was virtually inactive (205). Accordingly, this mutation resulted in a decrease in the processing of substrates such as proinsulin (204, 206) and hypothalamic and pituitary POMC (204). In contrast to the *Pcsk1* knockout mice, the PC1/3-N222D animals were able to properly process other physiological substrates including in the GHRH-GH-IGF1 axis, resulting in normal size. Additionally, the N222D mutation affected the autocatalytic C-terminal processing of PC1/3 as demonstrated by the absence of the 66-kDa form in different tissues (205). The loss of function of PC1/3-N222D has been associated to its partial retention in the ER, which results in its rapid degradation by the proteasome via ER associated degradation (ERAD) (206). Since autocatalytic propeptide cleavage is not severely affected in βTC-3 cells, this suggests that the N222D mutation hampers further folding of the protein after cleavage at the primary site. This might results in an unstable protein, prone to unfolding and largely unable to pass the ER quality control (QC).

Figure 3.



Proposed model for the dominant negative effect of certain ER-retained PC1/3 mutants on wild-type PC1/3. Some *PCSK1* mutations are known to cause misfolding and ER retention of PC1/3. In addition to the inactivation of this ER-mutants itself, recent evidence suggest that these mutations (indicated with red star) negatively impact the levels of wild-type PC1/3 through the formation of heteroduplexes that are targeted for proteasomal degradation via ERAD. Other mutations (indicated with black star) that are properly folded, however, would also dimerize with wild-type PC1/3, but in this case the heteroduplex is not degraded and both proteins are able to exit the ER. The extent of UPR and ERAD, elicited by the expression of a mutant PC1/3 protein, most likely correlates with the metabolic demand of the cell.

VI. Functional consequences of human PCSK1 variants: hints from mice

A. PC1/3 variants and obesity; the calcium binding site as the culprit?

The identification of extremely obese patients heterozygous for *PCSK1* mutations and the association of *PCSK1* SNPs with obesity challenged the recessive nature of the *PCSK1*-related syndrome (73, 96, 97, 113). Some of these mutations and variations do not alter PC1/3 activity in vitro and have only mild defects on autocatalytic processing in the ER. Interestingly, five of the identified mutations (M125I, T175M, N180S, Y181H, G226R), the mutation identified in the obese mouse model (N222D), and the SNP rs6232 (N221D) affect residues that cluster around the calcium-1 (Ca-1) binding site (**Figure 3**), which has

been shown to be important for structural stability in bacterial subtilisin (73, 207). Creemers et al proposed that mutations in close proximity of this calcium-binding site could alter enzyme stability which could lead to protein misfolding and thereby decreasing the amount of active enzyme (73). Recent evidence from our group and the Lindberg group confirmed that some *PCSK1* mutations lead to protein instability (89, 205). In particular, our results indicate that several of the identified heterozygous mutations caused a delayed exit of the mutant protein from the cell (205). However, this effect was not restricted to the mutations that are close to the Ca-1 binding site.

B. Endoplasmic reticulum-retained PC1/3 mutants; dominant negative effect and ER-associated degradation

Many proteins need to oligomerize as a prerequisite for ER exit (208). Both endogenous and recombinant PC1/3 have been shown to be present in multimeric forms in CHO and AtT20 cells, and bovine chromaffin granules (75). PC1/3 is present as monomers, dimers, and oligomers/aggregates, the latter having no activity. Upon dilution, the oligomers dissociate and activity increases (75). The dimers and oligomers are constituted of 87 kDa PC1/3 and incubation with substrate stabilizes PC1/3 activity. Interestingly, Blanco et al recently demonstrated that some ER-retained PC1/3 mutants inhibited PC1/3-WT secretion and activity (89). The proposed mechanism to explain this effect was that ER-retained mutants can oligomerize with PC1/3-WT in the ER lumen, and the resulting accumulated heterocomplex can be targeted to degradation via ERAD (**Figure 3**). A similar dominant-negative effect has been already found for other mutant proteins, such as mutations in proinsulin found in the Akita mice (173, 209).

As mentioned above, mouse PC1/3-N222D was shown to be partially retained in the ER, ubiquitinated and degraded by the proteasome, which is evidence for ER associated degradation (206). Taking into account that this degradation contributes to loss of function of PC1/3, we can speculate that it could be an important factor for the etiology of the multiple endocrinopathies associated with PC1/3 deficiency. In fact, it might explain why *PCSK1* variants which appear to partially retain enzyme activity, are associated with obesity. Generally, PC1/3 activity is depicted relative to the amount of PC1/3 protein secreted in the medium. Therefore, the functional defects of mutations in PC1/3 which cause the protein to be partially secreted and partially retained in the ER might be underestimated by a conventional activity assay if the correctly folded mutant protein performs well in an in vitro assay against a synthetic substrate.

C. Loss-of-function mutants and the unfolded protein response

The hypothesis that *PCSK1* mutations cause PC1/3 misfolding and ER stress, is a recently emerging concept. The heterozygous mutations previously identified in extremely obese patients showed an increased intracellular retention compared to PC1/3-WT (205). In agreement with these results, Blanco et al demonstrated that the human ER-retained *PCSK1*-p.G209R and *PCSK1*-p.G593R variants caused low grade ER stress, identified by increased expression of the ER-resident chaperone BiP and the ER-stress marker X-box binding protein 1 (XBP-1) (89). This is in line with our recent results where we observed an increased coimmunoprecipitation of BiP with some human *PCSK1* mutants partially retained in the ER (205). Expression analysis in islets of Langerhans of $Pcsk1^{N222D/N222D}$ mice showed an enrichment of genes associated with the proteasome and the unfolded protein response (more specifically the XBP1-pathway) (205). Taken together, these studies suggest that certain human PC1/3 variants cause mild ER stress. The severity of the ER stress may vary per tissue and is likely to be highest in cells under high metabolic demand, such as β-cells after a high-fat meal. It is known that ER stress can cause hypothalamic leptin resistance and β-cell dysfunction by various mechanisms (210, 211). Whether PC1/3 mutations associated to ER stress are sufficient to cause either leptin resistance or a diabetic phenotype remains to be proven.

VII. Conclusions and future directions

In the last two decades research on *PCSK1* variations and mutations in patients and mouse models has made important contributions to our current understanding of mono- and polygenic *PCSK1*-related endocrinopathies. It is now clear that PC1/3 deficiency causes a severe endocrine disease marked by failure to thrive, severe malabsorptive diarrhea, early onset obesity and other endocrinopathies to a varying degree. Improved knowledge on the syndrome will facilitate early diagnosis which will be necessary to promote survival and wellbeing of the patients. Advanced genetic diagnostics, such as exome sequencing, will have a future role in early diagnosing this severe inheritable disease (197). Current treatments are focused on hormone replacement therapies. However, the severe GI phenotype has not been fully addressed despite the fact that this phenotype is most likely the largest contributing factor to the failure to thrive observed in PC1/3-deficient neonates. GLP-2 analogs, such as teduglutide, which have been FDA approved for short bowel syndrome, are a promising candidate drugs to improve the GI phenotype (198).

Besides PC1/3 deficiency, heterozygous mutations and

SNPs have also been reported to contribute to increased obesity parameters (73, 95, 96, 113). The identified nonsynonymous SNPs rs6232 and rs6234-rs6235 could contribute to obesity by reducing PC1/3 function and/or mildly impairing PC1/3 trafficking in the cell. Other noncoding SNPs in or nearby *PCSK1* have been associated with obesity parameters and proinsulin disorders (99, 115, 116). It is unclear whether these SNPs alter *PCSK1* expression, RNA stability or PC1/3 function. The effects of both *PCSK1* SNPs and mutations on PC1/3 function and expression still require more research. In addition to activity measurements, all aspects of PC1/3 biology should be investigated: propeptide removal, ER exit, C-terminal processing and PC1/3 oligomerization. Measuring cleavage of endogenous substrates *in cellulo,* or preferably in vivo, should become the new standard of characterizing PC1/3 enzymatic function in addition to the in vitro use of synthetic substrates. Therefore, *PCSK1* knockout cell lines are needed, which nowadays can be easily created using CRISPR-Cas9 technology (212). Furthermore, this would allow the characterization of PC1/3 variants and mutants in at physiological levels. To disseminate converging and diverging substrate specificities of PC1/3 and PC2 the generation of an *in silico* tool for substrate cleavage prediction, like ProP 1.0 for furin (213) or Neuropred (214), would be useful.

The effect of synonymous SNPs and noncoding SNPs are more challenging to investigate. RNA stability and RNA splicing can be investigated for synonymous SNPs. For assessing the effect of noncoding SNPs in *PCSK1* or near the *PCSK1* gene chromatin conformation capture techniques can be employed (215). This technique allows the study of chromatin looping and genome architecture. This can be useful to identify whether SNPs identified by GWAS are located *in cis* or *in trans* regulatory regions. Alternatively, cellular or animal models can be constructed using CRISPR-Cas9 technology (212) to introduce the specific variation, given that the region is conserved between species. To improve the *in silico* prediction of the effect of *PCSK1* mutations on enzyme function and biology, a crystal structure is needed. This would allow accurate prediction of the effect of amino acid substitutions and protein stability using algorithms to predict protein folding and aggregation properties (216–218). This could be of interest in the context of heterodimerization of PC1/3-WT with mutant proteins.

In conclusion, research on *PCSK1* mutations and variations focused on better functional characterization in a physiologically relevant context is dearly needed. This would be facilitated by improved knowledge on substrate specificity, three-dimensional structure, and cellular biology. Patients can benefit from this gained knowledge as it allows for the design of specific drugs aimed at restoring PC1/3 function or at replacing hormone deficiencies. In the short term, it is imperative to establish procedures to diagnose *PCSK1* deficiency more rapidly. This will allow to reduce PC1/3 deficiency related mortality and improve patient well-being.

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