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PCSK1 Variants and Human Obesity

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Abstract

PCSK1 , encoding prohormone convertase 1/3 (PC1/3), was one of the first genes linked to monogenic early-onset obesity. PC1/3 is a protease involved in the biosynthetic processing of a variety of neuropeptides and prohormones in endocrine tissues. PC1/3 activity is essential for the activating cleavage of many peptide hormone precursors implicated in the regulation of food ingestion, glucose homeostasis, and energy homeostasis, for example, proopiomelanocortin, proinsulin, proglucagon, and proghrelin. A large number of genome-wide association studies in a variety of different populations have now firmly established a link between three *PCSK1* polymorphisms frequent in the population and increased risk of obesity. Human subjects with PC1/3 deficiency, a rare autosomal-recessive disorder caused by the presence of loss-of-function mutations in both alleles, are obese and display a complex set of endocrinopathies. Increasing numbers of genetic diagnoses of infants with persistent diarrhea has recently led to the finding of many novel *PCSK1* mutations. *PCSK1*-deficient infants experience severe intestinal malabsorption during the first years of life, requiring controlled nutrition; these children then become hyperphagic, with associated obesity. The biochemical characterization of novel loss-of-function *PCSK1* mutations has resulted in the discovery of new pathological mechanisms affecting the cell biology of the endocrine cell beyond simple loss of enzyme activity, for example, dominant-negative effects of certain mutants on wild-type PC1/3 protein, and activation of the cellular unfolded protein response by endoplasmic reticulum–retained mutants. A better understanding of these molecular and cellular pathologies may illuminate possible treatments for the complex endocrinopathy of *PCSK1* deficiency, including obesity.

1. INTRODUCTION

Neuropeptides and prohormones require proteolytic activation prior to release for subsequent action in various tissues. This activation occurs within the regulated secretory pathway of both neurons and endocrine cells. The enzymes responsible for the initial proteolytic cleavages are known as *prohormone* convertases (PCs). PCs are calcium-dependent serine endoproteases that undergo final maturation in the acidic environment of

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the secretory granules, where they acquire full catalytic activity.[1] PC1/3 (also known as PC1, PC3, and SPC3) and PC2 are abundantly expressed in neuroendocrine cells, although the two enzymes do not always colocalize within the same cells.[2] The processing of protein precursors in nonendocrine cells is mediated by other widely expressed enzymes (in this case *proprotein* convertases), which act within the constitutive secretory pathway. The best characterized proprotein convertase is furin, which has been reported to process a variety of substrates such as growth factors and receptors, plasma proteins, proteases, bacterial toxins, and viral coat glycoproteins.[3] The other mammalian members included within this family are PC4, PC5/6, PACE4, and PC7.

Although PC1/3 usually initiates precursor cleavage, either PC1/3 or PC2 can process prohormones and neuropeptides by cleaving at conserved dibasic sites, most frequently Lys-Arg or Arg-Arg.[2] Genetically modified mice lacking convertase expression[4,5] have provided considerable information on convertase-mediated processing of substrates. Human studies have also provided considerable information on PC1/3 function, beginning with early studies of an individual with compound heterozygous mutations in the gene that encodes PC1/3, *PCSK1*.[6] This patient exhibited multiple endocrinopathies as well as morbid obesity.[6] *PCSK1* has subsequently become recognized as one of the first genes causing rare forms of monogenic obesity.[7] More recently, many index cases have been described with severe biallelic variants of the *PCSK1* gene that result, in most cases, in the total lack of PC1/3 activity.[8,9] In addition, numerous genome-wide association studies (GWAS) show a convincing association between certain polymorphisms in the *PCSK1* gene that are exceedingly common in the population and increased risk of obesity.[10,11] In this chapter, we review the biochemistry and cell biology of PC1/3 and its prohormone substrates related to obesity and glucose metabolism. A specific goal of the chapter is to address the possible mechanism(s) by which the total or partial lack of PC1/3 activity in humans may contribute to obesity.

2. PROHORMONE CONVERTASE 1/3: GENERAL PROPERTIES

PC1/3 was the third member of the proprotein convertase family to be cloned from mammalian organisms, after furin and PC2.[12,13] The PC1/3 protein is encoded by the *PCSK1* gene, which is located on chromosome 5q15–21 in humans, and chromosome 13c in the mouse.[12,14] PC1/3 mRNA is translated by ribosomes located on the rough endoplasmic reticulum (ER) into a 753-residue protein, the signal peptide-bearing inactive precursor preproPC1/3. Like other members of the subtilisin superfamily (which includes bacterial subtilisin and the yeast subtilase kex2), the catalytic triad is formed by the amino acids Asp, His, and Ser, arranged in this order (as opposed to the Ser, His, Asp order present in trypsin-like proteases). The domain structure of proPC1/3 consists of four well-defined domains: a prodomain, a catalytic domain, a P domain specific to this superfamily (including yeast kexin), and a carboxy-terminal domain. The catalytic domain is highly conserved among various species and paralogs. The propeptide domain is essential to the proper folding and ER exit of the protein.[15] The P domain plays an important role in the regulation of the calcium and pH dependence of PCs,[16] while the carboxy-terminal domain participates in the sorting of PC1/3 into secretory granules.[17] The carboxy-terminal domain may also assist the

oligomerization of PC1/3, since the truncated form of PC1/3 is less prone to oligomerization.[18]

Fig. 1 depicts the cellular maturation of pre-proPC1. After signal peptide removal in the ER, the 94 kDa proPC1/3 zymogen is multiply N-glycosylated. While the PC1/3 prodomain is known to undergo early and rapid cleavage at the primary cleavage site by autocatalysis,[19,20] it is still unclear whether the cleaved prodomain remains bound to the catalytic domain, to act as an intramolecular inhibitor, until the enzyme reaches the trans-Golgi network (TGN), as occurs for the closely related enzyme furin.[21] The furin prodomain remains associated with the catalytic domain until a Golgi compartment of lower pH is reached, at which time a "histidine switch" is activated which results in secondary site cleavage, dissociation of propeptide fragments, and enzyme activation.[22,23] This type of study has not yet been performed for PC1/3, though it is clear that PC1/3 can also operate within TGN[24]; indeed, the initial cleavages of POMC are known to occur in the Golgi.[25] Within the TGN, proPC1/3 undergoes terminal glycosylation, and also becomes sulfated.[19,20,26]

The 87 kDa active PC1/3 form undergoes further intermolecular autocatalytic cleavage of the carboxyl terminal domain within dense-core secretory granules[27] (Fig. 1). These truncated forms (74 and 66 kDa PC1/3) are much more catalytically active than the parent 87 kDa species, and also require higher calcium concentrations and lower pH (5–5.5) to exhibit maximal activity.[27,28] Although both of these smaller forms are much more unstable than the 87 kDa form,[27] impaired processing to the smaller forms is associated with weaker tissue activity against various substrates.[29]

PC1/3 expression is restricted to the central and peripheral nervous systems and endocrine and neuroendocrine organs. In brain, PC1/3 levels are particularly high in certain regions of the hypothalamus, such as the arcuate nucleus,[30] an area known to be involved in the regulation of food intake and body weight. In the periphery, PC1/3 is also highly expressed in the pancreas, pituitary, stomach, and intestine, as well as in the adrenal and thyroid glands.[31–34] PC1/3 expression is limited to specific ghrelin-expressing endocrine cells in the stomach; the α- and β-cells of the islets of Langerhans in the pancreas; and various enteroendocrine cells present in the intestine.[31,32,35] As discussed later, these specialized cells play an important role in appetite, glucose homeostasis, and nutrient assimilation by secreting several PC1/3 products including ghrelin, insulin, and proglucagon-derived peptides such as GLP-1.

3. PC1/3 SUBSTRATES INVOLVED IN THE REGULATION OF ENERGY HOMEOSTASIS AND FOOD BEHAVIOR

Many if not all circulating and hypothalamic peptide hormones involved in appetite regulation require PC processing, for example, the production of ACTH, β-endorphin, and α-MSH from POMC.[36–38] Both cell studies and peptidomic analyses of tissues from mice lacking PC1/3 activity support the idea that there are a large number of PC1/3 substrates (see Table 2[2] and Table 1[39]). In this section we will describe only the PC1/3 substrates most likely to be involved in the control of appetite, glucose homeostasis, and nutrient assimilation. Table 1 shows a set of such anorexigenic and orexigenic peptide hormones

whose biosynthesis is thought to involve PC1/3 action; this should not be taken as a comprehensive list of PC1/3-synthesized peptides involved in these processes, as the biosynthesis of many peptides (for example, neuromedin U) is not yet understood.

3.1 PC1/3 Activation of Neuropeptides in the Hypothalamus: A Key Role in the Melanocortin Pathway?

The hypothalamus is an important brain region that contains distinct neuronal populations which control energy homeostasis and feeding behavior. As mentioned earlier, hypothalamic PC1/3 expression is especially high in the arcuate nucleus, where it resides in two leptin-sensitive neuronal populations: proopiomelanocortin (POMC)-expressing neurons, and neuropeptide Y (NPY) and agouti-related peptide (AgRP)-expressing neurons.[40,41]

Alpha-melanocyte-stimulating hormone (α-MSH) is a potent POMC-derived anorexigenic hormone that reduces food intake, increases energy expenditure, and regulates glucose metabolism through the activation of melanocortin 4 receptor (MC4R) in the hypothalamic paraventricular nucleus[42,43] (Fig. 2), among other target areas in the central nervous system. α-MSH is produced within POMC-expressing neurons by a proteolytic process consisting of PC1/3-mediated generation of adrenocorticotropic hormone (ACTH) followed by PC2-specific cleavage to ACTH 1–18, and then terminal modification (dibasic trimming, acetylation, and amidation) to result in α-MSH.[44] While pituitary α-MSH levels were not altered in a peptidomics study of PC1/3 null mice,[45] reduced hypothalamic α-MSH levels were detected in a PC1/3-deficient mouse model[29] (discussed later). The mechanism underlying this decrease is not yet clear and represents an intriguing area for further study.

POMC processing is clearly impaired in cases of biallelic human *PCSK1* deficiency, with the generation of large ACTH-containing fragments rather than mature ACTH.[6,8,9,46] While in some cases this lack of authentic ACTH results in adrenocortical insufficiency,[8] other subjects exhibit normal cortisol levels.[46] Jackson et al. provided evidence for the presence of authentic ACTH in the plasma of a human *PCSK1* null patient, suggesting redundancy in the enzymatic processing of POMC.[46] It seems likely that further processing of large ACTH-containing intermediates can occur in plasma. Whether *PCSK1*-deficient patients exhibit reduced circulating α-MSH levels has not yet been examined; this might be expected based on results obtained in mice.

3.2 Other PC1/3-Generated Peptide Hormones Involved in Feeding Behaviors

In addition to α-MSH, the cocaine- and amphetamine-regulated transcript (CART) is an anorexigenic peptide expressed in POMC-positive hypothalamic neurons that clearly requires PC1/3 action for its synthesis.[47] AgRP is a PC1/3-synthesized[48] orexigenic neuropeptide that stimulates appetite and decreases energy expenditure, thereby antagonizing the effects of POMC within the arcuate nucleus. AgRP suppresses MC4R and MC3R activity by competing with α-MSH for these receptors (Fig. 2). NPY is a second orexigenic peptide hormone known to be involved in the hypothalamic control of food intake[49] whose synthesis depends on PC1/3.[50] Lastly, PC1/3 colocalizes with orexin-positive neurons in the hypothalamus,[51] though no biochemical studies have been performed to demonstrate whether its synthesis is PC1/3-dependent. Alterations in the processing of

proCART, proorexin, proAgRP, and proNPY have not yet been examined in cases of human *PCSK1* deficiency, for example, by measuring CSF levels.

3.3 PC1/3 Processing of Proinsulin to Insulin in Pancreatic β-Cells

Given the key role of peripheral insulin in energy balance and glucose homeostasis it is important to understand how this anabolic hormone is produced in the pancreas. Like the majority of secreted proteins, insulin is initially synthesized as an inactive precursor (proinsulin) within pancreatic β-cells. When proinsulin reaches the TGN and is packaged into the secretory granules, it undergoes maturation to active insulin via the joint action of PC1/3 and PC2; here, PC1/3 plays the larger role.[52,53] The absence of either PC1/3 or PC2 causes incomplete processing from proinsulin to insulin, as demonstrated by the severe deficiency of active insulin found in both *Pcsk1* and *Pcsk2* null mice.[4,54] Human studies show enormous increases in circulating proinsulin in *PCSK1* deficiency.[6,8,9,46,55–58] Interestingly, while neither hyperglycemia nor diabetes mellitus has been reported in either humans or mice bearing severe loss-of-function *PCSK1* variants, common polymorphisms are associated with increased risk of these conditions (see discussion later).

3.4 PC1/3 Activity in the Gastrointestinal System

Proglucagon, encoded by the *Gcg* gene, is expressed in the α-cells of the pancreatic islets, in certain brain areas, and in enteroendocrine cells within the small intestine; it is cleaved in a tissue-specific manner by PC1/3 and PC2, resulting in different profiles of bioactive peptides. For example, in pancreatic α-cells, which express PC2, proglucagon is specifically processed to glucagon.[59–61] In contrast, PC1/3-mediated processing of proglucagon in intestinal L cells, where PC2 is lacking, results in the production of glucagon-like peptides such as GLP-1 and GLP-2, but not of glucagon itself.[62–64] Whereas GLP-1 regulates food intake behavior and glucose and energy homeostasis, GLP-2 is mostly involved in the regulation of intestinal absorption of nutrients and gut permeability.[65,66] While studies of *Pcsk1* null mice clearly show a profound deficit in proglucagon processing,[4] human studies have been ambiguous in this regard,[46,56] and further human studies may be warranted.

Glucose-dependent insulinotropic polypeptide (GIP) is a 42-amino acid gut hormone produced by PC1/3[67] in K-cells, a population of enteroendocrine cells mainly located in the upper intestine. GIP is secreted by K cells in response to food ingestion and plays an important role as a modulator of energy homeostasis. GIP receptors are widely distributed in peripheral tissues, including fat, gut, pancreas, bone, and brain.[68] While it is well accepted that GIP stimulates glucose-dependent insulin secretion, other proposed GIP functions, for example, the induction of GLP-1 secretion in L cells, or the regulation of food intake through specific hypothalamic circuits, are still controversial.[69]

Ghrelin is a potent orexigenic hormone secreted under fasting conditions from specialized endocrine cells within the stomach. Proghrelin is processed to ghrelin only by PC1/3.[70,71] In the hypothalamus, ghrelin activates NPY/AgRP neurons, inducing food intake.[72] Ghrelin is also associated with increased GLP-1 secretion by enteroendocrine L cells[73]; thus, loss of PC1/3 activity could conceivably affect GLP-1 secretion both directly and indirectly.

PC1/3 also mediates the processing of several other gut peptide hormone precursors, for example, procholecystokinin (proCCK)[74] and progastrin[75] and likely others.[76,77]

In summary, PC1/3 activity is required for the biosynthesis of a variety of hypothalamic, pancreatic, and gut peptide hormones implicated in the control of food intake, both orexigenic and anorexigenic, as summarized in Table 1. While direct links between a specific PC1/3-synthesized peptide and alterations in food intake have not yet been demonstrated, the known involvement of PC1/3 in the synthesis of so many different appetitive peptides supports the idea that PC1/3 deficiency alters the balance of anorexigenic/orexigenic peptidergic signals. Of note, appetitive changes could arise either as the direct loss of a PC1/3-made anorexigenic peptide, or an indirect mechanism, that is, the loss of negative peptidergic input (presumably a PC1/3 product) to a tonic orexigenic system. Indeed, the influence of other hypothalamic peptides, for example, β-endorphin, corticotropin-releasing hormone (CRH) and/or neurotensin, on the PC1/3-mediated obesity phenotype remains unexplored.

4. PC1/3 DEFICIENCY AND DISEASE

4.1 PC1/3 Knockout Mouse and the PC1/3-Deficient N222D Mouse

Mouse models of PC1/3 deficiency illustrate the critical importance of PC1/3-mediated processing of peptide hormones. A *Pcsk1* null mouse was constructed in which the *Pcsk1* gene lacks the promoter and exon 1.[4] While these targeted *Pcsk1* null mice exhibit highly increased proinsulin levels as well as greatly impaired processing of proglucagon to glucagon-like peptide-1 and -2, they do not show differences in glucose tolerance or develop diabetes; nor are these mice obese.[4] The *Pcsk1* null mouse has a high postnatal mortality rate, with only one-third surviving beyond 7 days of life, and most succumbing by the second day (Ref. [4]; M. Martin, unpublished observations). In the few animals that survive into adulthood, despite grossly impaired processing of POMC to ACTH, corticosterone levels are normal.[4] The small size of these mice was ascribed to the lack of processed growth hormone-releasing hormone (GHRH); in agreement, growth hormone and insulin-like growth factor 1 (IGF1) levels are very low. While this was postulated to represent a species-specific effect,[4] impaired growth is also seen in a subset of *PCSK1*-deficient humans (see further).

A second *Pcsk1* null was constructed which involved a 32.7-kb chromosomal deletion with insertion of a neomycin cassette and produced aberrant *Pcsk1* gene products. While no homozygous mice were obtained, female heterozygote mice exhibited stunted growth under a low fat diet, and catchup growth under a high-fat diet.[78]

A *Pcsk1*-deficient mouse was created by chemical mutagenesis of C57BL/6 inbred mice, which resulted in a PC1/3 N222D missense mutation.[29] Homozygotes show glucose intolerance and become obese; obesity is associated with increased food intake. Heterozygotes show an intermediate phenotype. Despite the fact that proinsulin processing is impaired, neither diabetes nor insulin resistance were observed. Processing of 87 kDa PC1/3 to the 66 kDa form is clearly reduced in these mice.[29] In contrast to the proteomics results showing no change in pituitary α-MSH in the *Pcsk1* null mouse,[45] hypothalamic α-

MSH levels are clearly reduced in the *Pcsk1* N222D mouse.[29] Heterozygous mutant N222D mice also show a robust obesity phenotype that is completely lacking in heterozygous PC1/3 null mice. These differences support the idea that the N222D mutation may produce alterations in hypothalamic neuronal cell biology that are not seen in haploinsufficient PC1/3 null mice. In support of this idea, we have observed a dominant-negative deleterious effect of the N222D PC1/3 mutant protein on wild-type PC1/3 trafficking and on proinsulin processing in cell lines and intact islets.[79] More recent data from our laboratory show that the expression of human PC1/3 mutant proteins which are ER-retained (eg, G593R and G209R) reduces wild-type PC1/3 trafficking to secretory granules, and also results in the induction of ER stress markers.[80] While N222D PC1/3 was not examined in this latter work, it is likely that this mutant mouse protein induces similar ER stress, potentially explaining the decrease in hypothalamic α-MSH in the N222D mutant mouse. In agreement, another group has shown that both N222D PC1/3 and certain human ER-retained PC1/3 forms coimmunoprecipitate with the ER stress marker BiP (J. W. Creemers, personal communication).

The modulation of PC1/3 message levels by glucose, and in response to feeding has been previously reviewed.[2]

4.2 Common and Rare Human Haploinsufficient PCSK1 Polymorphisms are Also Linked to Obesity

The strong involvement of *PCSK1* in obesity was established by a variety of GWAS that have shown that several common nonsynonymous polymorphisms in this gene constitute a large risk factor for obesity. Indeed, *PCSK1* constitutes the third most prevalent monogenic contributor to the risk of obesity in a variety of different human populations.[81–84] The minor allele frequency of N221D (rs6232) ranges from 3–5% in different ethnic populations, while that of the linked Q665E/S690T variants (which nearly always appear together; rs6234/6235) is 24%.[85] The presence of an N221D allele has been correlated with decreased insulin sensitivity, decreased oral glucose tolerance, and increased proinsulin levels, while the Q665E/S690T variation has been found to be associated with decreased fasting glucose and an increased insulin level.[86,87] However, obesity risk data regarding the Q665E/S690T allele are not always consistent for different populations.[83,88,89] Aside from ethnicity, this may also have to do with group sizes analyzed; a larger effect in children and younger adults[83]; sex differences, with larger effects in men versus women[89,90]; and/or other factors as yet unknown, for example, varying diets, activity, or hormonal status. Additional recent data in the form of two very large metaanalyses show that the N221D polymorphism is more strongly associated with childhood obesity than with adult obesity[10]; and confirm that both N221D and Q665E/S690T are significantly associated with the risks of both common and extreme obesity-risks that are strongly modulated by both age and ethnicity.[11]

The biochemical basis for effects of common polymorphisms on PC1/3 activity is unclear. The linked Q665E/S690T polymorphisms are found in the carboxyl terminal tail of PC1/3, a domain linked to protein targeting as well as enzyme inhibition.[2] Mbikay et al. have proposed that the secondary structure of this domain is significantly affected by these two mutations.[91] Although this group found that the joint presence of these three common

polymorphisms (N221D and Q665E/S690T) conferred a significant increase in PC1/3 carboxy-terminal processing, no differences in POMC processing could be identified in these cell culture studies.[91] This leads to the question of whether this paired polymorphism biologically affects PC1/3-mediated peptide processing; produces cellular effects by another route; or requires intact endocrine tissue (rather than a tumor cell line) in order to manifest a biological effect. In contrast, the N221D mutation was proposed to affect catalysis due to its probable location adjacent to a calcium binding site[81]; however, early studies showed very little effect on enzyme activity.[85] We have recently reexamined the activity of human PC1/3 proteins containing the common polymorphisms N221D or the linked Q665E/S690T pair in cell culture studies. While the linked polymorphisms were not able to influence PC1/3 activity, the N221D polymorphism decreased the specific activity of the secreted protein by about 30%.[80] This large difference was not detected in the prior study[85] that employed a background vector containing an additional S357G mutation, which represents an activating mutation.[92]

In addition to the common mutations, nearly 1% of obese populations exhibit rare *PCSK1* mutations, and some of these are associated with profound effects on metabolism.[81] Eight rare heterozygous mutations resulting in partial loss of PC1/3 activity have been identified in obese patients.[81] Despite not displaying the severe endocrine phenotype of PC1/3-lacking subjects (described further), carriers of these mutations exhibit an 8.7-fold increased risk of obesity over subjects lacking these mutations.[81] When analyzed in cell culture, seven of the eight mutant PC1/3 proteins were found to exhibit impaired maturation and secretion, while one propeptide mutation seemed benign; again, this may be due to the inherent limitations of transfection analyses in constitutively-secreting cells rather than endocrine tissues.

The rare and common human *PCSK1* polymorphisms that have been genetically linked to obesity are shown on the top of Fig. 3. While most of the rare heterozygous mutations found in obese populations clearly impact the cell biology and enzymatic activity of PC1/3,[81] the physiological mechanisms underlying the obesity phenotype of the Q665E/S690T polymorphisms are as yet unclear. Obesity may result from changes in appetite; differences in energy metabolism; and alterations in glucose handling subsequent to peptide precursor processing effects; or, most likely, a combination of these factors. Somewhat paradoxically, the S690T polymorphism was found to be associated with higher levels of GIP and glucagon during a meal test.[90] An unexplored factor is the possibility of interactions between *PCSK1* polymorphisms with common substrate polymorphisms, for example, proglucagon.[93]

4.3 Rare Human Biallelic PCSK1 Mutations Cause Enteric and Systemic Endocrinopathies and Obesity

The first human case of *PCSK1* deficiency, identified over 18 years ago, was a patient with a loss-of-function G593R missense mutation on one allele, and a premature stop codon which resulted in an inactive truncated form on the other allele.[6] Several subsequent studies have confirmed that the total or near-total loss of PC1/3 activity observed in patients with rare inactivating mutations in both *PCSK1* alleles results in a complex age-dependent clinical phenotype that includes severe diarrhea, many other endocrine dysfunctions, and obesity.[6,8,9,46] These rare mutations are shown on the bottom portion of Fig. 3. Most of the severe

human mutations shown in Fig. 3 were identified in infants via *PCSK1* exon sequencing[8,9,46,57,90] or total exome sequencing,[55] although the first case was identified in an adult.[6] This list of rare mutations is clearly not complete; as *PCSK1* insufficiency is increasingly recognized as a genetic cause of pediatric intestinal failure, the number of children being diagnosed with mutations in this gene is growing.

The earliest clinical phenotype in humans with loss-of-function mutations in *PCSK1* is severe generalized malabsorptive diarrhea that always begins in the immediate postnatal period.[8] All forms of nutrients induce the diarrhea in a dose-dependent manner, and infants present with severe weight loss and diarrhea that ceases only with fasting or the consumption of water. Endoscopic biopsies are generally normal and do not reveal any of the other classical findings seen in children with other forms of generalized malabsorptive diarrhea.[94] More importantly, the early clinical phenotype mirrors what is seen in children with loss-of-function mutations of *NEUROG3*, a transcription factor required for enteroendocrine and islet cell development.[95] Interestingly, children with both forms of enteric endocrinopathies require parenteral nutrition in addition to reduced enteral feeds during their first 18 months of life in order to thrive. Curiously, the requirement for parenteral nutrition is significantly diminished beyond 2 years of age, and the children can thrive despite the presence of persistent diarrhea that is lifelong. Unlike children with *NEUROG3* deficiency, patients with *PCSK1* mutations develop a profound appetite that results in significant weight gain and eventually obesity beyond 2 years of age.[8] Overall, our experience suggests that children with loss of PC1/3 activity will experience persistent diarrhea and malabsorption throughout life, and early in life will require intravenous support that may be tapered off as the child ages.

While children with *PCSK1* deficiency develop obesity as they age, there are important differences that distinguish them from children with other forms of monogenic obesity.[8] Children with mutations in the leptin–melanocortin pathway experience profound obesity that becomes apparent within the first several months of life.[96] These disorders include mutations of leptin and its receptor; POMC; and MC4R, in which all patients exhibit obesity that persists throughout adulthood, with class III morbid obesity.[96,97] In contrast, as outlined earlier, patients with defects in PC1/3 have significant malnutrition and failure to thrive that requires intravenous support to manage.[8] Given PC1/3's extensive role in processing many of the peptidergic components of pathways regulating energy balance and appetite, we might have anticipated more profound obesity in probands with *PCSK1* deficiency. However, unlike other disorders that are associated with central forms of hyperphagia, *PCSK1* is also expressed in enteroendocrine cells, where it processes prohormones that presumably have an essential role in facilitating nutrient assimilation. Despite their early phase of poor growth—which is limited to children with *PCSK1* deficiency—these children eventually develop hyperphagia that results in significant weight gain despite their persistent diarrhea.[56] This diarrhea certainly results in malabsorption of nutrients that, if fully absorbed, would have likely resulted in profound weight gain and morbid obesity. Therefore, unlike other forms of monogenic obesity, significant alterations of PC1/3 action have both a central and enteric endocrinopathy that balances the weight gain as children age.

The malabsorption that occurs in subjects with both *PCSK1* and *NEUROG3* deficiency suggests that enteroendocrine cells must secrete peptide hormone(s) that normally facilitate nutrient assimilation. The murine counterparts of these disorders mimic some of the changes associated with the human disease, including early postnatal mortality.[4,98] Interestingly, none of the murine models that exhibit selective depletion of an enteric hormone or its receptor—including several of the more well-studied enteric hormones such as GLP-1 and 2, ghrelin and PYY—are associated with immediate postnatal complications.[77] These findings suggest either the existence of an uncharacterized gut hormone that requires PC1/3 processing; or that several of the currently established hormones have a redundant role in augmenting nutrient absorption. Several PC1/3-synthesized gut hormone agonists are used in clinical practice, including GLP-1 and 2 analogs that are used to treat diabetes mellitus and short bowel syndrome.[99,100] It is certainly plausible that one of these or other agonists can be used off-label to manage patients with PC1/3 deficiency; however, therapy should likely be limited to the first several years of life, since prolonged therapy could result in severe weight gain due to reversal of malabsorption. Overall, these various disorders highlight the important role of enteroendocrine cells and PC1/3 processing in the nutrient absorptive capacity of the gut.

Several *PCSK1*-deficient children exhibited linear growth abnormalities and received therapy for growth hormone (GH) deficiency. Very few of the other nonsyndromic monogenetic disorders that are associated with obesity also exhibit evidence of poor linear growth. For instance, patients with leptin deficiency exhibit normal height,[9] while those with haploinsufficient *MC4R* mutations actually show accelerated linear growth.[97,101,102] While GH deficiency was in fact reported in the few surviving *Pcsk1* null mice, as discussed earlier, the presence of an active furin consensus site in human proGHRH was thought to negate the requirement for PC1/3 processing.[4] In agreement, impaired linear growth has not been previously reported in prior reports of human *PCSK1* deficiency.[6,9,57] Given our recent findings of lowered GH in a few rare cases of biallelic PC1/3 deficiency,[8,55,103] we speculate that reduced linear growth might also contribute to elevated BMI in subjects carrying one of the common *PCSK1* polymorphisms known to be associated with obesity risk. Indeed, while the N221D polymorphism is not associated with decreased height, the more common Q665E/S690T variants are strongly associated with impaired growth in large cohorts of obese patients.[11] The exceedingly common frequency of this paired variant in the general population (heterozygous ~39%, and homozygous ~7%), suggests that it could contribute to impaired growth velocity, a highly heritable trait[104]; however, *PCSK1* has not thus far been identified as a gene contributing to height in the general population.[105] Collectively, these recent findings suggest that abnormalities in PC1/3 function might lead to reduced linear growth that can contribute to the degree of obesity as measured by BMI.

The rare missense human mutations fall into three general classes. The first class, exemplified by G593R, includes PC1/3 proteins that are retained within the endoplasmic reticulum (see red-colored mutants in Fig 3.) and always result in a severe clinical phenotype; these PC1/3 proteins are poorly folded and are likely all subject to endoplasmic reticulum-associated degradation. In the middle are mutant proteins which are somewhat impaired in secretory pathway trafficking, but are still able to traffic to the secretory granule; the mouse N222D PC1/3 mutant exemplifies this category,[79] although it is likely that certain

rare human variants also fall into this class. In the third class are mutant proteins which are clearly well folded and well secreted, but exhibit impaired catalytic activity, such as N309K, an oxyanion hole mutant lacking all *in-trans* enzymatic activity.[103] However, many catalytic domain mutants do exhibit some degree of misfolding, as judged by severe impairments in secretion.[6,8,9] This is important because ER-retained mutant proteins both negatively impact the trafficking of wild-type PC1/3 and cause ER stress,[80] which is increasingly recognized as a contributing factor to obesity.[106,107] Interestingly, a dominant-negative effect on proPC1/3 processing was found in an obese patient heterozygous for a propeptide mutation resulting in a truncated protein,[108] further supporting the idea that dominant-negative interactions of PC1/3 mutations play an important role in human obesity. However, in a study of the four heterozygous children of the first *PCSK1* null patient, the proinsulin to des31,32 proinsulin ratios were found to vary between 0.75 and 5.25 (with a parental ratio of 2.8),[46] indicating a profound influence of unknown modifier genes on the proinsulin processing phenotype.

In obese patients undergoing bariatric surgery, levels of PC1/3 mRNA in jejunal mucosal samples were significantly reduced in patients who had type 2 diabetes as compared to those who did not.[109] PC1/3 expression is regulated by the transcription factor Pax6, and a GWAS study has shown that a polymorphism in this gene is associated with lower islet expression of PC1/3.[110]

Patients with rare inactivating *PCSK1* mutations share certain similarities with patients bearing loss-of-function POMC mutations, including early-onset obesity and adrenal insufficiency.[111–114] A similar phenotype is also observed in mouse POMC knockouts,[115] highlighting the critical importance of the POMC system in adrenal development and feeding behavior. Patients with *PCSK1*-inactivating mutations possess both similarities and differences with a recently described homozygous patient bearing a truncated *CPE* gene (encoding carboxypeptidase E) who not only is morbidly obese and has hypogonadotropic hypogonadism, but also exhibits diabetes mellitus and developmental delay.[116] In agreement, male $Cpe^{fat/fat}$ mice, who contain an inactivating *Cpe* mutation, are not only obese,[117] but also have lower testicular weights.[118] Interestingly, both these mice[119] and leptin-deficient mice[51] exhibit reduced PC1/3 levels in several brain regions, including the hypothalamus, where the lack of certain PC1/3-processed neuropeptides might plausibly result in obesity and hypogonadotropic hypogonadism. No individuals have yet been identified with inactivating mutations of *PCSK2*; based on mouse data,[5] these patients would be expected to be hypoglycemic, with little circulating glucagon, and also hyperproinsulinemic.

4.4 ProSAAS and Obesity

ProSAAS is a 30 kDa PC1/3 binding protein that was originally identified during a mass spectrometry search for novel brain peptides.[120] Homologous sequences (which are at best 30% conserved) have been identified only in vertebrates,[121] though PC1/3 has been found in invertebrates.[122] The proSAAS protein, abundantly distributed within neural and endocrine tissues,[123,124] consists of two domains separated by a furin cleavage site; both the carboxy- and the amino-terminal domains can be cleaved off to generate bioactive peptides, while the

interior domain remains intact.[125–127] The C-terminal 41-residue peptide represents a nanomolar inhibitor of active PC1/3[128,129] and contains the inhibitory hexapeptide sequence Leu-Leu-Arg-Val-Lys-Arg, originally discovered as a PC1/3 inhibitor during a peptide library screen.[130] Interestingly, proSAAS transgenic mice exhibit an obesity phenotype,[131] while male proSAAS knockout mice are lean.[132] It is unclear at this point if either phenotype is related to the differential production of PC1/3-cleaved peptides, as the levels of most PC1/3-generated peptide products do not differ in proSAAS knockout mouse brains.[132]

Recent data obtained in mice show that proSAAS expression is down-regulated by the transcription factor Pax6, while PC1/3 expression is upregulated.[133] *Pax6* heterozygote mice exhibit increased proSAAS expression, reduced PC1/3 expression, reduced proteolytic conversion to the 66 kDa form, and lower islet PC1/3 activity.[134,135] These effects clearly translate into differences in proinsulin conversion and glucose handling[134]; however, weight differences were not examined in these studies. No human proSAAS polymorphisms or mutations have been reported to date.

ProSAAS has often been identified in proteomics screens of cerebrospinal fluid in patients with various neurodegenerative diseases.[136] The internal unprocessed domain of the proSAAS protein potently blocks the aggregation of various fibrillating proteins, such as beta amyloid,[136] islet amyloid polypeptide[137] and synuclein (T. Jarvela and I. Lindberg, unpublished results). Obesity effects seen in proSAAS transgenic and knockout mice may be mediated in part through its antiaggregation bioactivity, rather than directly through PC1/3 inhibition.

5. CONCLUSIONS

The discovery that *PCSK1* mutations and polymorphisms represent an underlying cause of human obesity has provided considerable insight into a genetic cause of metabolic differences between humans. At the same time, many questions remain as to the physiologic mechanisms which underlie the powerful effects of certain *PCSK1* mutations on endocrine physiology. While human studies clearly demonstrate that rare inactivating mutations in *PCSK1* strongly impact PC1/3-mediated peptide hormone processing, heterozygotes show a variety of phenotypes that range from normal to obese.[46,108] Indeed, heterozygote *Pcsk1* null mice show no obesity phenotype,[4] suggesting that haploinsufficiency cannot explain the profound metabolic effects seen in certain *PCSK1*-deficient patients. Dominant-negative effects of mutant proteins on the disposition of wild-type PC1/3 protein, as well as a possible negative impact on proteostasis within various peptide hormone-producing cells, must be considered as possible contributing reasons to their metabolic phenotype.

An enduring puzzle remains the biochemical basis for the pediatric malabsorptive diarrhea typical of homozygote loss-of-function *PCSK1* patients. Whether this is due directly to the lack of specific PC1/3-synthesized peptide products arising from the lack of enzyme activity, or is secondary to general enteroendocrine cell dysfunction evolving from pro-teotoxicity of *PCSK1* mutants, is not yet clear. A possible role for proSAAS, the known PC1/3 binding protein, should also be examined in models of intestinal dysfunction. Another interesting future area for study is to investigate the developmental factors that enable infants with

PCSK1 mutations to overcome the severe malabsorptive syndrome between 2 and 3 years of age.

The contribution of the highly common *PCSK1* polymorphisms to human obesity risk is also puzzling, given their relatively benign effects on enzyme activity when expressed in cell culture. It is likely that a better grasp of their contribution to metabolism would come from studies of animal models of these polymorphisms, in which possible alterations in peptide hormone processing would take place in the same types of endocrine cells as in humans (rather than in the constitutive and endocrine tumor cell models traditionally used). Thus, the creation of an N221D mutated mouse, or of a mouse knock-in expressing a humanized *PCSK1* gene containing the Q665E/S690T paired polymorphisms, would permit a detailed analysis of peptide hormone processing in endocrine tissues and plasma, and enable studies of the interaction of these polymorphisms with diet. Analysis of the status of ER stress markers in the hypothalami of these mice could potentially explain the reduction in hypothalamic α-MSH in mice bearing *Pcsk1* mutations. Alternatively, the creation of human iPSC lines could enable the investigation of the physiological consequences of rare and common variants in an *in vitro* setting.

In summary, while many questions remain regarding the physiological mechanisms contributing to *PCSK1*-mediated obesity, the work of the last decade has clearly increased our understanding of the role of *PCSK1* in metabolism. With the recent identification of over 2 dozen human missense and nonsense variants that provide a range of enzyme inactivation from apparently nondetectable (Q665E/S690T) to total loss (ER-retained and truncated mutants), we are well poised to elucidate the precise relationship between enzyme activity and level of clinical impairment. The discoveries of dominant-negative impairment of wild-type enzyme function, as well as of the clear ER stress evoked by ER-retained mutant PC1/3 proteins, provide additional avenues for future investigation of the cell biology of peptide hormone processing in various peptidergic tissues. Accumulating data on pediatric cases of *PCSK1* deficiency will continue to provide clinicians with improved therapeutic regimens, while work in culture and in other animal models will deliver answers to many of the questions posed previously.

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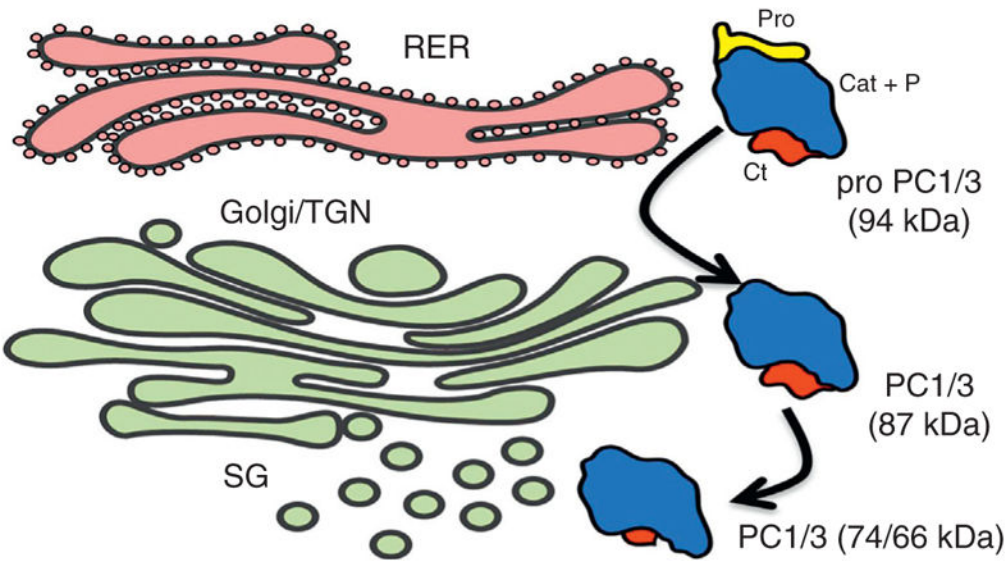

Figure 1.

PC1/3 trafficking in the regulated secretory pathway. During translocation the signal peptide is removed, resulting in the inactive zymogen proPC1/3. In the ER, proPC1/3 is folded, N-glycosylated and undergoes rapid initial prodomain cleavage. In the Golgi compartments, the protein undergoes additional posttranslational modifications, including further addition glycosylation, sugar trimming; and sulfation. Once in the TGN, PC1/3 is sorted into dense-core granules, and the Ct domain is autocatalytically cleaved, resulting in complete maturation. *RER*, rough endoplasmic reticulum; *TGN*, trans-Golgi network; *SG*, secretory granules; *Pro*, prodomain; *Cat*, catalytic domain; *Ct*, carboxy-terminal domain.

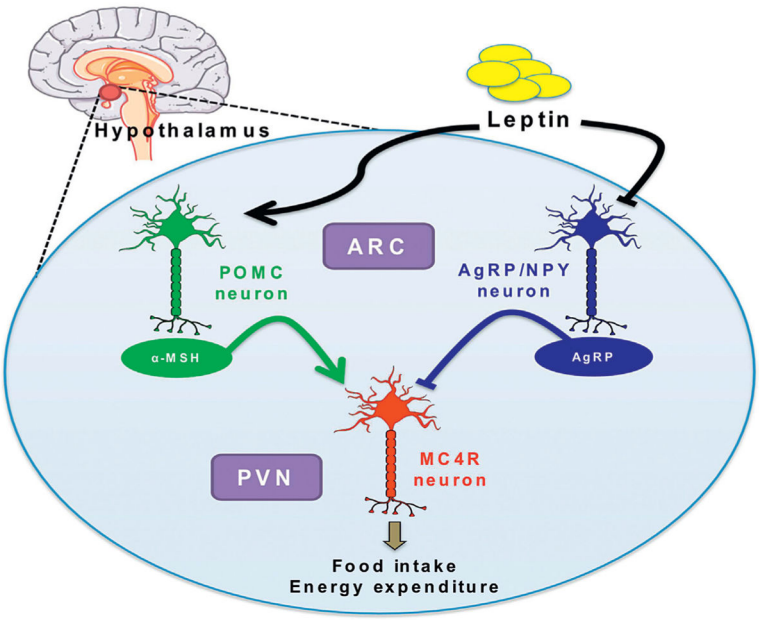

Figure 2.

PC1/3 activity is essential to the leptin–melanocortin pathway. The hypothalamus is a key regulator of food intake and energy expenditure, responding to the action of peripheral hormones. Leptin is an anorexigenic hormone, produced and secreted by white adipose tissue, whose circulating levels are proportional to the mass of body fat. Leptin binds to leptin receptors on AgRP/NPY- and POMC-expressing neurons in the ARC of the hypothalamus. Leptin binding inhibits the synthesis of AgRP and stimulates the synthesis of POMC. Acting together, PC1/3 and PC2 accomplish the proteolytic processing of POMC to α-MSH within POMC-expressing neurons. PC1/3 is specifically required for the processing of proAgRP in NPY/AgRP-producing neurons. α-MSH and AgRP compete for the MC4R in the paraventricular nucleus *(PVN)* of the hypothalamus. Whereas increased receptor activity by α-MSH binding generates an anorexigenic signal, AgRP reduces receptor activity, generating an orexigenic signal.

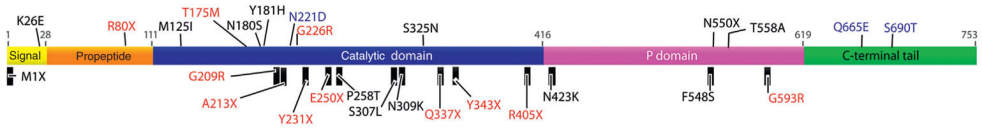

Figure 3.

Human PC1/3 mutations and variations identified to date. Over two dozen different missense and nonsense mutations in *PCSK1* have been identified in infants with diarrhea and endocrine patients (shown below panel)[6,8,9,57] or in obese populations[81] (shown above panel). The three most frequent polymorphisms (N221D, Q665E/S690T) are shown in dark gray (blue in the web version); note that these may coexist with other mutations. Mutant proteins that are not secreted and likely are ER-retained are shown in light gray (red in the web version) *Diagram modified from Ref.* [8].

Table 1

Main PC1/3 Substrates Involved in Feeding Behavior and Energy Homeostasis.

Hormone/Neuropeptide	Peptide Precursor	Feeding Regulation	Tissue Distribution
α-MSH	POMC	Anorexigenic	Hypothalamus
CART	proCART	Anorexigenic	Hypothalamus
NPY	proNPY	Orexigenic	Hypothalamus
AgRP	proAgRP	Orexigenic	Hypothalamus
Orexin/hypocretin	proOrexin/hypocretin	Orexigenic	Hypothalamus
Ghrelin	proghrelin	Orexigenic	Stomach
Insulin	proinsulin	Anorexigenic	Pancreas
CCK	proCCK	Anorexigenic	Small intestine
GLP-1	proglucagon	Anorexigenic	Small intestine

The processing of POMC and proinsulin to α-MSH and insulin requires the sequential action of PC1/3 and PC2. The bioactive peptides CART, NPY, AgRP, ghrelin, CCK, and GLP-1 are generated by PC1/3-mediated processing of their respective peptide precursors; proorexin/hypocretin processing has not yet been investigated, although PC1/3 is expressed in orexin-positive neurons.