Chapter 3

The Proprotein Convertases, 20 Years Later

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Abstract

The proprotein convertases (PCs) are secretory mammalian serine proteinases related to bacterial subtilisin-like enzymes. The family of PCs comprises nine members, PC1/3, PC2, furin, PC4, PC5/6, PACE4, PC7, SKI-1/S1P, and PCSK9 (**Fig. 3.1**). While the first seven PCs cleave after single or paired basic residues, the last two cleave at non-basic residues and the last one PCSK9 only cleaves one substrate, itself, for its activation. The targets and substrates of these convertases are very varied covering many aspects of cellular biology and communication. While it took more than 22 years to begin to identify the first member in 1989–1990, in less than 14 years they were all characterized. So where are we 20 years later in 2011? We have now reached a level of maturity needed to begin to unravel the mechanisms behind the complex physiological functions of these PCs both in health and disease states. We are still far away from comprehensively understanding the various ramifications of their roles and to identify their physiological substrates unequivocally. How do these enzymes function in vivo? Are there other partners to be identified that would modulate their activity and/or cellular localization? Would non-toxic inhibitors/silencers of some PCs provide alternative therapies to control some pathologies and improve human health? Are there human SNPs or mutations in these PCs that correlate with disease, and can these help define the finesses of their functions and/or cellular sorting? The more we know about a given field, the more questions will arise, until we are convinced that we have cornered the important angles. And yet the future may well reserve for us many surprises that may allow new leaps in our understanding of the fascinating biology of these phylogenetically ancient eukaryotic proteases (**Fig. 3.2**) implicated in health and disease, which traffic through the cells via multiple sorting pathways (**Fig. 3.3**).

Key words: Proprotein convertases, limited proteolysis, secretory proteins, single and pairs of basic residues, cancer metastasis, viral infections, neural and endocrine disorders, gene knockout, cholesterol metabolism, dyslipidemia.

1. Introduction

Whenever an important breakthrough in a given scientific discipline has been achieved, it becomes critical to summarize the historical perspectives of the discovery and to put those into context

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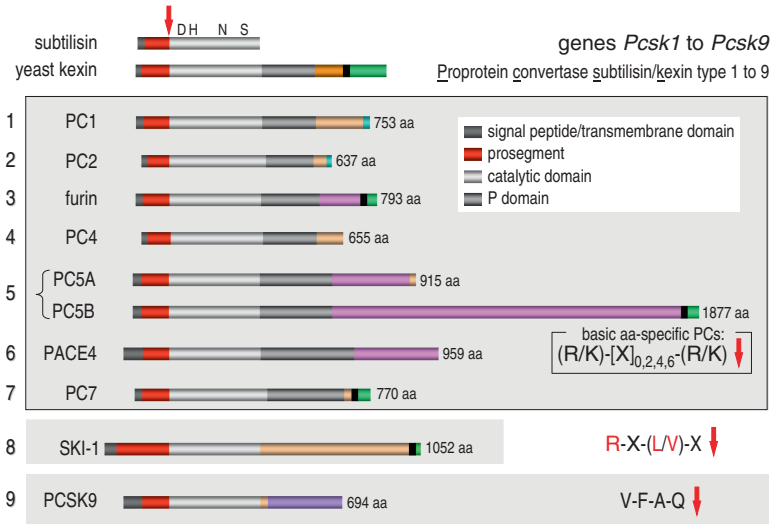

Fig. 3.1. The proprotein convertase family.

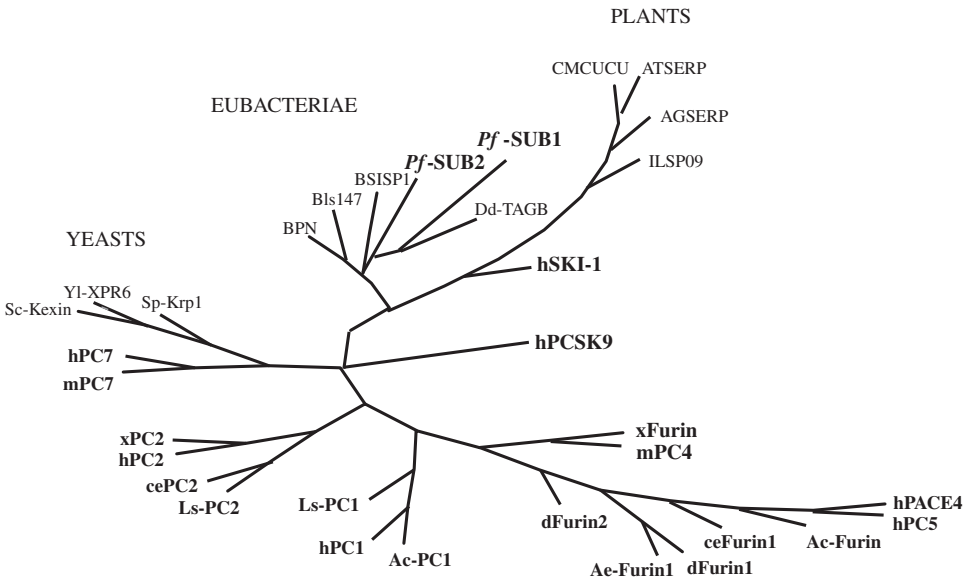

Fig. 3.2. Phylogenetic analysis of mammalian proprotein convertases.

with the present knowledge in this particular field. This is well exemplified by the discovery over the last 33 years of the proteases, their substrates, and post-translational modification (PTM) enzymes implicated in the shaping of the active form(s) of secretory polypeptides and proteins. The diversification of the genome information provided by such modifications is enormous and has played a major role in the evolution of the species.

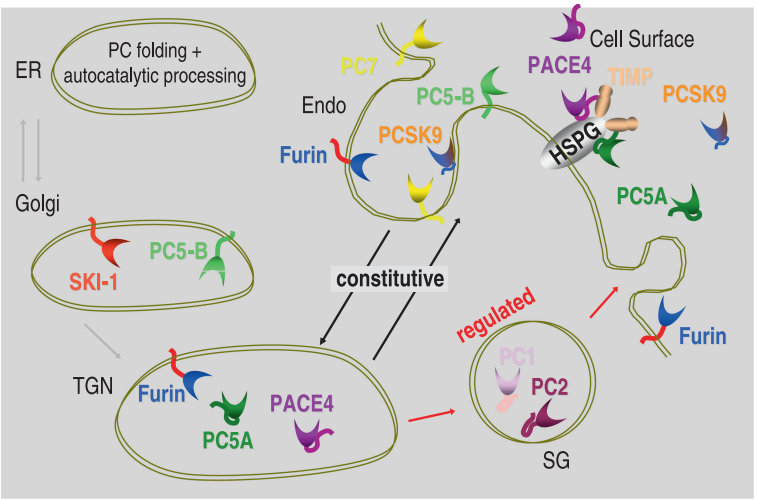

Fig. 3.3. Proprotein convertases in the secretory pathway.

Ever since the mid-1960s, a large effort was devoted toward the definition of the biosynthetic pathway and primary structural analysis of various precursors, including neural and endocrine peptides and their PTMs, coupled with the analysis of the cellular morphology and definition of the functions of the various organelles within the secretory pathway. All these monumental efforts by many talented international investigators in the field culminated with a general model in which polypeptide hormones and active proteins are often initially synthesized as relatively inactive precursors, which for maximal activation require one or more PTMs, including site-specific proteolytic cleavage, C- and N-terminal amino acid (aa) trimming, C-terminal amidation, and multiple residue modifications such as N- and O-glycosylation, Tyr and oligosaccharide sulfation, disulfide bridge formation, N-terminal acetylation, Ser/Thr phosphorylation, and Ser octanoylation. All these analyses led to the concept that within the secretory pathway there must exist a specific proteolytic machinery that results in the limited initial cleavage of proprotein and prohormone precursors, usually C-terminal to single or pairs of basic residues, of the type LysArg and ArgArg and less frequently LysLys and ArgLys. However, it was also realized that cleavage after hydrophobic or small amino acids also occurs in some cases, suggesting the presence of more than one type of proteases and/or similar enzymes with different specificities.

Where do such cleavages occur? Biosynthetic and immunocytochemical analyses of various precursor processing suggested that depending on the substrate this can occur in at least four different compartments, including the trans-Golgi network (TGN), cell surface, endosomes, and secretory granules. It turns out that the

first three processes mostly occur in proteins trafficking through the so-called constitutive secretory pathway, whereas the last one concerns those proproteins that are processed in the regulated secretory pathway (**Fig. 3.3**), which involves most neural and endocrine polypeptide hormones (1). This spatial segregation of proteins before their processing turned out to be a very refined filtering process to allow for controlled limited proteolysis in a time- and space-dependent manner.

The isolation of the processing enzymes turned out to be a very arduous process that took over 22 years to begin to identify the cognate mammalian proteases implicated in the process of protein precursor activation. During these long and arduous years hunting for the correct processing enzymes, many false positives were reported, to unfortunately be relegated to the side once they were tested and scrutinized by biochemical and cellular criteria. The limitations of the methods used to purify the enzymes and the sensitivity and specificity of the substrates used to follow these activities were often behind the limited success achieved during the 1970s–1980s. Indeed, finding a needle in a haystack has always been a challenge, requiring a lot of effort, technological advances, and often serendipity. All of these ingredients were fundamental in the discovery of the nine-membered proprotein convertase (PC) family (**Fig. 3.1**), a saga that lasted almost a quarter of a century before the first light at the end of the tunnel appeared.

2. The First Glimpse of Light

In 1984, the identification of the yeast convertase kexin (also known as Kex2p) was indeed the culminating point that led to the molecular and genetic identification of the first prototype of the mammalian proprotein convertases. Kexin cleaves the precursor of pro-K1 killer toxin and pro-α-factor of the yeast *Saccharomyces cerevisiae* at the C-terminus of pairs of basic residues of the type ArgArg↓ and LysArg↓ (2, 3). In addition pro-K1 killer toxin is also cleaved after a single basic residue in the motif ProArg↓, by one or more enzymes (3). Kexin turned out to be a serine proteinase best related to bacterial subtilases rather than eukaryotic trypsin-like enzymes (4). This unexpected result explained many of the unfruitful attempts to identify the cognate mammalian PCs based on RT-PCR analyses using degenerate oligonucleotides derived from the consensus sequence of active sites of serine proteinases of the trypsin–chymotrypsin family (5).

How relevant is the type-I membrane-bound kexin to the mammalian proprotein convertases? Answers to this question

quickly came from analyses of the processing of the mammalian pro-opiomelanocortin (POMC), the precursor of ACTH and β-endorphin, in mammalian cells overexpressing kexin (6). The data clearly showed that the yeast enzyme is capable of processing POMC into a set of products similar to those found in vivo, including β-LPH, γ-LPH, and β-endorphin, but not α-MSH. Furthermore, evidence was presented that kexin could not generate somatostatin-14 from mammalian pro-somatostatin, but rather kexin and another yeast enzyme, the aspartyl protease YAP3, cleaved in between the Arg↓Lys site to generate a Lys-extended somatostatin-14 (7). Interestingly, the monobasic cleavage generating somatostatin-28 was performed by YAP3. Finally, kexin was also shown to be able to act as a proalbumin converting enzyme (8). These data thus suggested that while kexin could be considered a prototype of the as yet undiscovered mammalian proprotein convertases, differences in specificity must exist that would require critical structural differences in mammalian PCs to produce the observed products in vivo.

3. Identification of Furin: The Beginning of an Active Era of Research

The suggestion in 1986 that the *fur* gene (*fes/fps* upstream region) cloned in the group of Wim Van de Ven by Anton Roebroek (9) was a mammalian homologue of kexin first appeared at the end of the discussion of a seminal manuscript by Robert Fuller et al. in 1988 (3). The striking similarity of furin to kexin (10, 11), especially in the catalytic serine subtilase domain, became apparent once the sequence of kexin became public (4). Quickly a number of groups began to analyze the specificity of the type-I membrane-bound furin and found that it can process intracellularly at the TGN, cell surface, and/or endosomes a large number of constitutively secreted substrates that include growth factors and their receptors, enzymes, surface glycoproteins of mammalian and viral and parasitic origin, blood coagulation factors, and even some polypeptide hormones (for comprehensive reviews, *see* (12–16)) The membrane-bound furin cycles from the cell surface back to the TGN through endosomes, a pathway regulated by various signals in its cytosolic tail (15). The furin gene (*PCSK3*) is localized to human chromosome 15 close to the *fes/feps* region (9).

Analysis of a large number of substrates processed and the various bonds cleaved suggest that furin best recognizes the sequence (R/K)-$[X]_{0,2,4,6}$-(R/K)↓P1′-P2′, with a large preference for a P1 Arg, and P1′ is usually a small amino acid with a preference for Asp and Glu, and an aliphatic aa (Ile, Val, Leu)

in P2′ is best. Indeed, the crystal structure of furin first reported in 2003 confirmed this prediction (17). One of the best furin substrates contains the sequence RX(R/K)R↓EL, which has been inserted at strategic sites in mammalian proteins and viral glycoproteins for cleavage by endogenous furin (18, 19). Similar engineering strategies have been described for the production of active insulin from proinsulin in muscle (20).

Looking at the latest PubMed literature related to furin, it is amazing that in less than 20 years since its first discovery and that of the following members of the PC family, more than 1,630 reports have appeared that mention furin or directly relate to its activity and/or functions. What has come out is that furin is ubiquitously expressed at various levels in all tissues, thereby rationalizing its widespread role in the processing of various proteins, usually resulting in their activation (12–16), but sometimes in their inactivation as is the case of lipoprotein and endothelial lipases (21) and the proprotein convertase PCSK9 (18).

The varied physiological functions of furin suggested that animals lacking this enzyme may present serious developmental problems and/or anomalies. Indeed, mice lacking furin through inactivation of its gene, *Pcsk3* (*p*roprotein *c*onvertase *s*ubtilisin *k*exin type 3), die at about embryonic day 11 (E11). Embryos fail to undertake axial rotation and ventral closure needed to form a looping heart tube and a coherent primitive gut (22). Although only a few specific furin substrates have yet been identified in vivo, the overlap in the distribution of furin mRNA and that of some members of the transforming growth factor (TGF)β family (23, 24), such as TGFβ1 (25) and BMP4 (26) often best processed by furin (27, 28), is striking. A liver-specific furin KO and other tissues from adult mice, using the inducible Mx1-Cre transgene, resulted in mice with no phenotype, demonstrating redundancy with other PCs in these tissues (29). In contrast, in vivo studies showed that furin can uniquely process the Ac45 subunit of the vacuolar-type H^+-ATPase in pancreatic β-cells (30). Furthermore, conditional deletion of furin in T cells allowed for normal T-cell development but impaired the function of regulatory and effector T cells, which produced less TGFβ1 (31).

Whether inhibition of furin in the adult using protein (32) or small molecule (33, 34) inhibitors, RNAi or antibody approaches could be a useful therapy against certain pathologies such as cancers (35), and associated metastasis (36, 37) and/or viral/parasitic infections (38, 39) are under careful examination for possible short- and long-term toxicity effects. It must be mentioned here that heterozygote mice lacking one copy of furin are alive and show no obvious anomalies (22), and hence therapies aimed at reducing 50–70% of furin activity may still be viable. Furthermore, inhibition of cell-surface furin may not be as toxic as complete furin inhibition. Hence, the use of cell-impermeable

approaches, such as inhibiting monoclonal antibodies or single-chain antibodies, may well turn out to be a feasible and therapeutically useful alternative to control some of the above deadly diseases. However, a word of caution should be taken into account. Namely, in some pathologies furin-like activity may be protective to the cell, as was recently demonstrated with the ability of furin-like enzymes to neutralize the HIV accessory protein Vpr and hence limit viral spread (40).

4. The Neural and Endocrine Convertases PC1/3 and PC2

Processing of most polypeptide hormone precursors occurs in immature secretory granules at acidic pHs (41–43). Therefore, it was expected that the cognate processing enzyme(s) implicated would be active in acidic conditions between pHs 5 and 6. While many attempts were made to isolate the cognate enzyme(s) using biochemical techniques, unfortunately these have all failed, mostly due to the low levels of the proteases and the lack of robust specific substrates sensitive to trace levels of enzymes. In my own laboratory, we had dissected 20,000 porcine pituitaries at a slaughterhouse in Saint-Hyacinthe close to Montreal, Quebec, with the hope of isolating enough enzyme for characterization by biochemical methods. This has led to the isolation and characterization of one of the many false positives (44), plasma kallikrein (45), which did cleave proenkephalin peptides correctly in vitro (46). One of the first reports closing in on the possible nature of the real proinsulin convertases used insulinoma granules as a source of enzymes, and the authors deduced that two proteases, possibly present in distinct subcellular compartments, may be involved in the generation of active insulin (47). However, the low levels of enzymes available precluded their characterization biochemically.

Technological advances are often behind new leaps in biology, and it is of no surprise that the introduction of the reverse transcriptase polymerase chain reaction (RT-PCR) played a major role in the identification of the two convertases implicated in the processing of most regulated polypeptide hormone precursors, as well as all the other PCs. This technique amplifies a single or few copies of a piece of DNA (generated for example by the action of reverse transcriptase on an mRNA pool) across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. This method was first conceptualized by Kary B. Mullis in 1983 and later optimized and improved (48). For this discovery, he shared the 1993 Nobel Prize in Chemistry with Michael Smith who in 1978 had first introduced the

use of oligonucleotides for site-directed mutagenesis (49). The automation of this technique only appeared in 1986–1987. This was just the time when I was spending a sabbatical year at the Pasteur Institute in Paris (1987, 1988) at the laboratory of François Rougeon who had first cloned the cDNA of the protease renin-2 from mouse submaxillary glands.

When I became familiar with the PCR technique and realized its potential, I decided to exploit it using both pituitary and heart atria extract as a source of mRNA. The choice of the pituitary was dictated by the production from pro-opiomelanocortin (POMC) of ACTH and β-LPH in the anterior lobe and α-MSH and β-endorphin in the intermediate lobe, likely by different convertases (50–52). In heart atria, we had shown that the precursor of atrial natriuretic factor (proANF) was processed at a single basic residue AGPR↓AL to produce the active atrial natriuretic factor (ANF; 53). The proANF convertase was reported much later to be corin, a type-II membrane-bound serine protease of the trypsin type (54).

The next leap was the use of sense and antisense degenerate oligonucleotides around the active site of proteases, a region found to be highly conserved between members of a single family. The thinking was that if the basic aa-specific processing enzyme(s) was of a trypsin type then use of degenerate oligonucleotides mimicking the active sites Ser, His, or Asp should by RT-PCR lead to the amplification of a DNA fragment of one or more processing enzymes. This technique was applied to DNA isolated by reverse transcriptase treatment of dissected rat pituitary (anterior and neurointermediate lobes) and heart atria. When we used degenerate oligonucleotides based on trypsin or serine proteases of blood coagulation factors, we invariably isolated DNAs similar to various trypsin-like enzymes, including plasma kallikrein and tryptase (unpublished results). It was thus, after frustrating 6 months of work, that the paper of Robert Fuller appeared, which alerted us on the possibility that a mammalian homologue of kexin could be furin (3). However, the genomic DNA sequence just published by Anton Roebroek suggested that the gene they had cloned could be an oncogene (9). Upon translating the 3′ end of the reported sequence, we quickly realized that the supposedly intronic sequence actually coded for a potential Asn of the oxyanion hole of subtilisin-like enzymes. We therefore designed degenerate oligonucleotides surrounding this Asn and possible Ser of active site subtilases and rapidly isolated from rat heart atria a furin sequence and from pituitary anterior and neurointermediate lobes two DNA fragments that could potentially code for part of the catalytic domain of two novel subtilisin-like enzymes. These *p*ituitary *c*onvertases (now known as *p*roprotein *c*onvertases, PCs) were given the names of PC1 and PC2, in the order in which their cDNAs were cloned (5). While the complete sequence of

PC2 was obtained from our cDNA library of AtT20 cells, that of PC1 was not possible to obtain, and we had to further clone the rest of the sequence from another AtT20 library and from two mouse insulinoma libraries (55). The chromosomal assignment of the genes coding for these convertases revealed their presence on mouse chromosomes 13 (*Pcsk1*, the PC1 gene) and 2 (*Pcsk2*, the PC2 gene), respectively (55), and the orthologous human chromosomes 5 (*PCSK1*) and 20 (*PCSK2*) (56).

The most inspiring result was obtained upon analyses of their mRNA expression by in situ hybridization. It became clear that PC1 is mostly expressed in the anterior lobe of the pituitary, including the corticotrophs where it co-localized with ACTH, while PC2 was highly concentrated in the intermediate lobe together with α-MSH (5, 55). This immediately suggested a model whereby PC1 would be critical for the formation of ACTH in the anterior lobe of the hypophysis, and PC2 must participate in the generation of α-MSH in the *pars intermedia*. This prediction based on cellular localization proved to be right upon biosynthetic analysis of cells co-expressing POMC and each of PC1 or PC2 (57).

Unknown to us and by a different approach, the group of Donald F. Steiner in Chicago independently cloned PC2 from a human insulinoma using degenerate oligonucleotides based on the kexin sequence (58) and later on also isolated PC1 (which they originally called PC3) (59). While it is an amazing coincidence that both groups named the second enzyme PC2, we now agreed to call the first enzyme PC1/3. Both enzymes later on were proven to be the processing enzymes of proinsulin (60) and implicated in POMC processing (61), validating the concept of distinct enzymes responsible for the cleavage at the B–C and A–C junctions of proinsulin (47) and agreeing with our own independent data (57).

These first exciting discoveries of PC1/3 and PC2 and the validation of their properties led to more than 500 publications to study their localizations, activation, functions in various organisms, cells, and their processing of a multitude of substrates. In essence, what has come out is that both PC1/3 and PC2 are sorted to mature secretory granules (62), likely via specific secondary structures in their C-terminal domains (63, 64), and that they are responsible for the processing of most pro-neural and endocrine peptides in a complex combinatorial fashion. It seems that PC1/3 gets activated first and hence acts on substrate before PC2. Furthermore, what also became apparent is that both PC1/3 and PC2 are under the control of endogenous inhibitors/chaperones. In the case of PC1/3 it seems that proSAAS could be processed by PC1/3 into a polypeptide product that acts as a potent inhibitor of the enzyme, thereby regulating its *in trans* enzymatic activity on other substrates (65, 66).

However, this may not be valid in all tissues (67). Interestingly, during our protein extractions of human pituitaries in search for the processing enzymes (68), we stumbled on a peptide we originally called 7B2 for its elution position on the HPLC (69, 70). While 7B2 was discovered in 1982, almost 8 years before PC2, we gained a lot of information on its *pan neuronal* and endocrine expression and its multifunctional role in the central nervous system (71–73). However, work in frogs by Gerard J.M. Martens re-discovered 7B2 and suggested that it may negatively regulate the activity of PC2 in the *pars intermedia* (74). This was confirmed by Iris Lindberg (75, 76) and by us (77, 78). It now seems that 7B2 is first needed as a chaperone to assist the folding of proPC2 in the ER. The complex proPC2–pro7B2 then exits the ER and the pro7B2 is first cleaved at $\boldsymbol{RRKRR}_{182}\downarrow$S*V*N by furin in the TGN (79), generating a C-terminal 31 aa CT-peptide that is a potent inhibitor of PC2. The proPC2 is then autocatalytically processed into PC2 within the acidic environment of immature secretory granules and in turn cleaves the CT-peptide at VVA$\boldsymbol{KK}_{189}\downarrow$S*V*P, generating an inactive form to finally liberate the active enzyme PC2, which can then act *in trans* on other substrates (73, 80).

The physiological importance of PC1/3 (81) and PC2 (82) was deduced from studies of the phenotypes of their gene knockout in mice and the discovery of two human patients with defects in PC1/3 (83, 84). In all cases mice were viable, thus suggesting that individually the genes of these convertases are not essential for life. Nevertheless, even though PC2-null mice appear normal at birth, they exhibit retarded growth. Analysis of these mice reveals chronic fasting, hypoglycemia, and a deficiency in circulating glucagon. PC2 is known to process various neuroendocrine precursors, and many of these were not fully processed in PC2-null mice, including prosomatostatin, neuronal proCCK, neurotensin, neuromedin N, prodynorphin, proorphanin FQ/nociceptin, and POMC-derived peptides.

Contrary to PC2 (82), PC1/3 gene disruption results in severe developmental abnormalities (81). The PC1/3-null mice exhibit growth retardation. The adult mutant mice are about 60% of the normal size and phenotypically resemble those that have mutant growth hormone-releasing hormone (GHRH) receptor. Interestingly, insulin growth factor 1 (IGF-1) and GHRH levels were significantly reduced along with pituitary GH mRNA levels, suggesting that this reduction contributes to the growth retardation observed in these mice. Similarly, analysis of several protein precursors known to be processed by PC1/3 revealed that these mice, like PC2 mutant mice, exhibit multiple defects in multiple hormone precursor processing events. These include the hypothalamic GHRH, pituitary POMC, proinsulin, and intestinal proglucagon. In contrast to PC2-null mice, PC1/3-null

mice process normally pituitary POMC to adrenocorticotropic hormone (ACTH) and have normal levels of blood corticosterone. Like PC2-null mice, they also developed hyperproinsulinemia.

Since PC2 is the major convertase that cleaves POMC and proenkephalin to generate the morphinomimetic peptides β-endorphin and Met- and Leu-enkephalins, respectively, it was important to investigate the role of PC2 in pain perception. Unexpectedly, after a short forced swim in warm water, PC2-null mice were significantly less (rather than more) responsive to the stimuli than wild-type mice, an indication of increased opioid-mediated stress-induced analgesia (85). The enhanced analgesia in PC2-null mice may be caused by an accumulation of opioid precursor processing intermediates with potent analgesic effects or by loss of anti-opioid peptides. Thus, the presence of abnormal cocktails of pain neuropeptides in the brain of PC2 KO mice is likely to disturb pain perception mechanisms in ways that remain to be fully elucidated.

PC1/3 deficiency in a female patient compound heterozygote for both splicing and non-synonymous mutations resulted in very low expression of the protein (83). This subject exhibited neonatal obesity and abnormal glucose homeostasis, as well as the presence of other endocrine defects, including the presence of very high circulating levels of proinsulin and multiple forms of partially processed POMC (intermediate ACTH precursors), low-serum estradiol, follicle-stimulating hormone (FSH), and luteinizing hormone (LH). Another PC1/3 deficiency female subject presented severe diarrhea, which started on the third postnatal day. Clinical investigations revealed a defect in the absorption of monosaccharides and fat, revealing the role of PC1/3 in the small intestinal absorptive function (84). Although the phenotypes of the PC1/3-null mice differ from those observed in these patients (PC1/3-null mice are not obese), the findings confirmed the importance of PC1/3 as a key neuroendocrine convertase. Interestingly, obesity, hyperphagia, and increased metabolic efficiency were recently identified in PC1/3 mutant mice exhibiting a homozygote mutation N222D/N222D that results in ~60% decrease in PC1/3 activity, suggesting that it is the dose of PC1/3 and possibly reduced hypothalamic α-MSH that may define the obesity phenotype (86). Finally, a single nucleotide polymorphic (SNP) variation in PC1/3 resulting in a N221D mutation and partial loss of function has been linked to monogenic obesity in children and adults (87).

Peptidomic analyses of PC1/3 (88) and PC2 (89) KO mice showed that loss of PC1/3 is often compensated for by PC2, but the reverse is not always true. Thus, although PC1/3 deficiency results in severe neonatal abnormalities and a reduction in litter size, many neuropeptides can still be processed in its absence. This

suggests that few PC1/3-specific substrates play major roles in mouse development, and that some are critical in the control of human metabolic diseases such as obesity (87).

Interestingly, mice lacking proSAAS (90) provided evidence that it is involved in the *prenatal* regulation of neuropeptide processing in vivo. However, *adult* mice lacking proSAAS have normal levels of all peptides detected using a peptidomics approach, suggesting that PC1/3 activity is not affected by the absence of proSAAS in adult mice. The data also showed that in adults proSAAS has other functions, e.g., body weight regulation, and these are not related to PC1/3 inhibition. Mice lacking 7B2 suggested that 7B2 is indeed required for activation of PC2 in vivo but that it has additional important functions in regulating pituitary hormone secretion (91). However, steroidal control of pituitary function is mouse strain dependent (92) and is therefore not a general phenomenon. Whether 7B2 may exhibit other functions in other mouse strains is yet to be discovered.

5. The Germ Cell-Specific PC4

In 1991, during our search by RT-PCR for other members of the PC family in mRNA extracts of various tissues, we identified in rat testis three different ~600 bp cDNAs potentially coding for three different PCs, now known as PC4, PC5/6, and PACE4. Shortly thereafter, a paper appeared that reported the complete cDNA sequence of PACE4 from a human hepatoma HepG2 cell line and an osteosarcoma cDNA library (93). We therefore concentrated on the characterization of PC4 (94) and PC5/6 (95). PC4 is expressed exclusively in male testicular germline pachytene spermatocytes and round spermatids, suggesting that it may play a specific physiological function in reproduction. In agreement, PC4 was detected in the acrosomal granules of round spermatids, in the acrosomal ridges of elongated spermatids, and on the sperm plasma membrane overlying the acrosome (96). In female mice, PC4 was expressed in macrophage-like cells of the ovary, and its levels are downregulated in activated macrophages, such as in inflammation (97). Later on, PC4 was also shown to be expressed in human placenta (98).

It took a lot of effort from my colleague Majambu Mbikay during a sabbatical year at the Jackson laboratories to obtain mice that lacked PC4 expression (99). This manuscript that appeared on June 24, 1997, was the first report on any convertase KO mouse. The in vivo fertility of homozygous mutant males was severely impaired in these mice, without any evident spermatogenic abnormality. In vitro, the fertilizing ability

of *Pcsk4*-null spermatozoa was also found to be significantly reduced. Moreover, eggs fertilized by these spermatozoa failed to grow to the blastocyst stage. Sperm physiologic anomalies likely contribute to the severe subfertility of PC4-deficient male mice (96). These results suggested that PC4 in the male may be important for achieving fertilization and for supporting early embryonic development in mice.

PC4 is a very special convertase whose C-terminus is species specific (94). This may be necessary to ensure no cross-species fertilization. So far, one of the identified specific substrates of PC4 in the testis is pituitary adenylate cyclase-activating polypeptide (PACAP) and PC4 is its sole processing enzyme in the testis and ovary of mice (100). In vitro studies with purified enzyme concluded that the most probable sequence motif for recognition by PC4 is ***K***X***K***XX***R***↓ or ***K***XX***R***↓, where X is any amino acid other than cysteine and that it prefers proline at P3, P5, and/or P2′ positions. It was also revealed that PC4 is a good candidate processing enzyme for the growth factors IGF-1 and -2 and several ADAM proteins such as ADAM-1, -2, -3, and -5 (101). Intrauterine fetal growth restriction is a leading cause of perinatal mortality. Recent work has unraveled an unusual property of PC4 in the processing of IGF-II, which has been shown to be an important regulator of fetoplacental growth. Thus, PC4 cleaves pro-IGF-II to generate the intermediate processed form, IGF-II (aa 1–102), and, subsequently, mature IGF-II (aa 1–67), thereby regulating fetoplacental growth (98). In the future, SNP variant PC4 that could affect its activity may explain some of the pathophysiology of fetoplacental growth restriction. Specific inhibitors of PC4, such as those recently reported in flavonoids (102), may one day serve as male contraceptives.

6. The Widely Expressed Convertases PC5/6 and PACE4

The identification of PC5A was done in my laboratory in 1993 (95) at a similar time to that made by Nakayama's group (103), who called the enzyme PC6. We now agreed to call it PC5/6. The convertases PC5/6 (95, 104) and PACE4 (93) seem to form a class of their own based on their primary structures and their ability to bind the cell surface via their C-terminal Cys-rich domains (CRD), which bind tissue inhibitors of metalloproteases (TIMPs) and heparin sulfate proteoglycans (HSPGs) (105, 106) and in many cases inactivate HSPG-bound proteins such as endothelial and lipoprotein lipases (21) and possibly adhesion molecules. In the CNS, it was shown that PC5/6 can process the neural adhesion molecule L1 assisting in neuronal repair and migration (107).

The specific physiological substrates of PC5/6 and PACE4 need to be unraveled in vivo, since in cellular experiments and in vitro many of the substrates processed by either enzyme can also be cleaved by furin and/or PC7.

PC5/6 is expressed as two mRNA transcripts, PC5/6A (soluble 915 aa) (95) and PC5/6B (type-I membrane bound, 1877 aa) (103), generated by differential splicing of its exons. Quantitative analysis of the tissue distribution of PC5/6 mRNA by qPCR revealed that the small intestine and kidney are the richest source of PC5/6B, whereas all other tissues express mostly PC5/6A. Both transcripts share the first 20 exons encoding the signal peptide, prosegment, catalytic domain, P-domain, and the cysteine-rich domain (CRD). The 21st exon of PC5/6A, coding for its last 38 residues, is replaced by 18 additional exons encoding the last 1,000 residues for PC5/6B (104, 108). Thus, while the CRD of PC5/6A contains 44 cysteine residues arranged in five tandem repeats of the consensus motif Cys-X_{2-3}-Cys-X_{3-4}-Cys-X_{2-7}-Cys-X_{5-10}-Cys-X_2-Cys-X_{9-13}-Cys-X_{3-5}-Cys-X_{7-16} (as it is also found in its closest homologue PACE4), the extended CRD of PC5/6B contains 22 repeats of this cysteine-rich motif. As for furin and PC7, PC5/6B also has a transmembrane domain and a cytosolic tail and cycles from the cell surface back to the TGN through endosomes (109). This regulated transit into multiple compartments is controlled by several sorting signals in their cytosolic tails and their interactions with specific sorting adaptors.

Evidence has been presented that, different from the other convertases, PC5/6A and PACE4 are activated at the cell surface while in contact with HSPGs (106). Here, the second cleavage of the prosegment, needed for zymogen activation, occurs at the cell surface, thereby limiting the functions of active PC5/6A and PACE4 to the cell surface and/or extracellular matrix, thereby favoring substrates that are also bound to HSPGs.

The present data strongly suggest unique tissue-specific functions of PC5/6 and PACE4. Thus, PC5/6 mRNA was detected only in neuronal cells, whereas PACE4 mRNA was expressed in both neuronal and glial cells. In areas that are rich in neuropeptides such as cortex, hippocampus, and hypothalamus, mRNA levels of PC5/6 were high but those of PACE4 were low or undetectable (110). In regions such as the amygdaloid body and thalamus, distinct but complementary distributions of PC5/6 and PACE4 mRNAs were observed. The medial habenular and cerebellar Purkinje cells expressed very high levels of PACE4 mRNA. Ontogeny and tissue distribution analysis showed that PC5/6 expression is detected early during embryonic development, appearing first in extra-embryonic tissues (111). By E9, it is also specifically expressed in cells of the maternal–embryonic junction, where no other convertase is expressed (112). What are

the precursors that need such specific processing events and what is the role of TIMPs and HSPGs in this process are open questions that may be resolved by tissue-specific KOs and by careful analysis of the cell-surface proteome of various tissues in the presence or absence of these convertases.

The complete knockout (KO) of PACE4 and PC5/6 genes in mice resulted in different phenotypes. Thus, while the PACE4 KO results in a 75% viable phenotype with bone morphogenesis defects (113), that of PC5/6 causes embryonic death at birth, with mice exhibiting multiple morphogenic defects likely related to impaired homeotic transformations (114, 115). Thus, newborns exhibited major defects in the anteroposterior axis with extra-thoraxic and -lumbar vertebrates (18 and 8 instead of 13 and 6, respectively) and a lack of tail (114). This phenotype had been reported for mice lacking the TGFβ-like factor, Gdf11, also known as BMP11. Both Gdf11- and PC5/6-deficient mice lack kidneys, although the phenotype was more penetrant in PC5/6 KO mice (100% agenesis versus 86% lacking one or two kidneys). We showed that Gdf11 is a favorite substrate of PC5/6, in part due to an Asn residue at the first position after the cleavage site (P1′) ($RSRR_{296}\downarrow NL$).

While PC5/6 deficiency perfectly mimics the Gdf11 one, it also results in other phenotypes, suggesting the lack of processing of other substrates: *Pcsk5*$^{-/-}$ newborns died earlier, in the first 2 h, versus the first 2 days following birth for *Gdf11*$^{-/-}$ mice, likely by asphyxiation (lung alveoli were collapsed). They also exhibited smaller size than WT, retarded ossification, severe hindlimb hypoplasia, abdominal herniation, and superficial and tissue hemorrhages, suggesting vascular fragility (114).

In collaboration with S. Batthacharya (115), magnetic resonance imaging revealed severe phenotypes reminiscent of those observed in patients exhibiting VACTERL (vertebral, anorectal, cardiac, tracheoesophageal, renal, limb) malformations. Finally, exon sequencing of control and VACTERL patients linked mutations in the human PC5 gene (*PCSK5*) to this syndrome (115). We proposed that PC5/6, at least in part via Gdf11, coordinately regulates caudal Hox paralogs, to control anteroposterior patterning, nephrogenesis, and skeletal and anorectal development.

We showed a downregulation of PC5/6 mRNA in human colon tumors at various stages (116). Since PC5/6 is very rich in intestine, we evaluated its role in tumorigenesis by crosses with an $Apc^{Min/+}$ mouse model, which develops numerous adenocarcinomas along the small intestine and fewer in the colon. Since PC5/6-deficient mice die at birth, we generated mice lacking or not lacking PC5/6 specifically in enterocytes (Villin-Cre transgene) and analyzed the number and size of the tumors. The lack of PC5/6 in enterocytes results in a significantly higher

tumor number in the duodenum and a premature mortality of $Apc^{Min/+}$ mice, suggesting that intestinal PC5/6 is protective toward tumorigenesis, especially in mouse duodenum, and possibly in human colon (116).

Recent studies revealed that in the adult PACE4 plays an important role in myogenic differentiation through its association with the IGF-II pathway (117). Thus, while PC4 processes proIGF-II in testis (98), PACE4 seems to be its cognate convertase in muscle. Finally, it was also reported that PACE4 could process the TGFβ-like substrate Nodal and that its intracellular traffic is dictated by the 18 kDa EGF-containing GPI-anchored proteoglycan Cripto that directs its traffic through an unconventional secretory pathway directly from the ER to the cell surface and sorting to detergent-resistant membrane microdomains (118). Cripto is the first receptor that binds both a PC and its substrate (Nodal), thereby enhancing the processing step.

7. The Ubiquitously Expressed PC7

In 1996, the last and still least studied member of the basic aa-specific PCs was identified in our lab and called PC7 (119). Its gene (*PCSK7*) was found to be on human chromosome 11 and mouse chromosome 9. Independently, PC7 was also cloned from a human lymphoma library and called LPC, for lymphoma PC (120). However, the name PC7 is now retained. Tissue distribution analyses revealed it to be ubiquitously expressed in most tissue and cell lines analyzed (119). It must be noted that PC7 is the most ancient of the basic aa-specific convertases (**Fig. 3.2**) and yet the most conserved phylogenetically.

Biosynthetic analyses of rat or human PC7 revealed that the enzyme is first synthesized as a zymogen which within the endoplasmic reticulum (ER) rapidly undergoes an autocatalytic cleavage at K***R***A***KR***$_{140}$↓ (rat) (119) or R***R***A***KR***$_{141}$↓ (human) (120), thereby releasing the active protease that exits the ER and is then competent to process substrates *in trans*. Further studies revealed that PC7 undergoes a number of post-translational modifications including N-glycosylation (119) and cytosolic tail Cys-palmitoylation (121). A number of investigations aimed at defining the sequence recognition of PC7 versus furin or other convertases suggested that PC7 can also cleave overexpressed substrates at Arg↓ residues both in vitro (122–125) and in cell lines (126–130). Although furin and PC7 have been proposed as the major gp160 processing convertases, rat liver microsomal gp160 processing activity was essentially resolved from furin and

only partially overlapped with PC7, and density distribution studies revealed that PC7 resides in lighter subcellular fractions than those containing furin (131). Interestingly, while overexpression of the prosegments of furin, PC5, and PC7 resulted in potent inhibitors of substrate cellular processing (132, 133), only the prosegment of PC7 is secreted into the medium (123, 132). The C-terminal KRA***KR***$_{140}$ motif in the prosegment of PC7 was critical for its observed inhibitory activity (134). Finally, the in vivo substrates of PC7 will remain to be defined, since the KO of PC7 results in viable mice (N.G. Seidah and D. Constam, unpublished results).

The function of the peptide-loading complex (PLC) is to facilitate loading of MHC class I (MHC-I) molecules with antigenic peptides in the ER and to drive the selection of these ligands toward a set of high-affinity binders. When the PLC fails to perform properly, as frequently observed in virus-infected or tumor cells, structurally unstable MHC-I peptide complexes are generated, which are prone to disintegrate instead of presenting antigens to cytotoxic T cells. Recently, it was reported that PC7, which is highly expressed in the immune system (119), may be implicated in antigen presentation, as the knockdown of its mRNA leads to lysosomal degradation of MHC-I (135). It has also been reported that PC7 may play a role in tumorigenesis (129, 136). It has yet to be proven if in vivo these are physiological functions of PC7, and what the degree of redundancy is with other members of the PC family.

8. SKI-1/S1P Activates Membrane-Bound Transcription Factors

The ubiquitously expressed SKI-1 (137) (also known as S1P) activates membrane-bound transcription factors implicated in the endoplasmic reticulum (ER) stress response (ATF6) (138) or the regulation of cholesterol and fatty acid synthesis (sterol regulatory element-binding protein (SREBP)-1 and -2) (139, 140). ProSKI-1/S1P is autocatalytically cleaved into a mature ~106 kDa membrane-bound form (137) and a secreted ~98 kDa shed form (141). Its *PCSK8* gene, ubiquitously expressed (137), is located on human chromosome 16 and mouse chromosome 8 (142). In contrast to basic-aa-specific PCs, SKI-1/S1P cleaves substrates in the general motif ***R***X(***V,L***)(K,F,L)↓ (137, 141, 143). In the absence of sterols, SKI-1/S1P cleaves the membrane-bound transcription factors sterol regulatory element-binding proteins (SREBPs) in their luminal loop (144), leading to release of a cytosolic basic helix-loop-helix transcription factor. In the

nucleus, this activates transcription of LDLR and all the genes involved in cholesterol and fatty acid synthesis (144). In the presence of sterols, SREBP cleavage is inhibited and hence transcription of its target genes is reduced, while the reverse is true in the absence of sterols (144). Other transmembrane transcription factors cleaved by SKI-1/S1P include the ER stress response factor ATF6 and CREB-like transcription factors Luman and CREB4 (143–151). We developed in vitro fluorogenic assays and inhibitors of cellular SKI-1 activity (143, 152–155). Aside from transcription factors, the other known SKI-1/S1P substrates are viral glycoproteins, brain-derived neurotrophic factor (BDNF), and somatostatin (143–151).

Recently, novel functions of SKI-1/S1P have been identified:

- Global μ-array analysis of HepG2 cells stably expressing the specific SKI-1/S1P inhibitor R134E prosegment (152) revealed that SKI-1/S1P inhibition causes widespread changes in key metabolic pathways other than those involving cholesterol and fatty acid synthesis (156).
- Small molecule inhibitors of SKI-1/S1P have been developed and shown to reduce cholesterol and fatty acid synthesis in vivo and, therefore, represent a potential new class of therapeutic agents for dyslipidemia and for a variety of cardiometabolic risk factors associated with diabetes, obesity, and the metabolic syndrome (157, 158).
- Using various protease inhibitors our data revealed that SKI-1/S1P plays a direct and/or indirect role in assembly of functional nucleation complexes in primary bone mineralization (159).
- We, and others, have shown that SKI-1/S1P is critically important in the activation of hemorrhagic fever viruses such as Lassa virus (143, 146), lymphocytic choriomeningitis virus (160), and Crimean-Congo hemorrhagic fever virus (143, 161) glycoproteins.
- Very little information is available on the in vivo physiological roles of SKI-1. Lethality occurs at the blastocyst stage in *Pcsk8*$^{-/-}$ (the SKI-1/S1P gene) mice with the absence of inner cell mass formation (162). However, liver and cartilage conditional knockouts are viable: loss of SKI-1/S1P in liver causes ~50% reduction in the levels of circulating LDL-cholesterol (LDL-C) and fatty acids (148); cartilage-specific *Pcsk8* KO mice exhibited chondrodysplasia, lack of endochondral ossification, disorganization of the collagen network, and the engorgement/fragmentation of the ER in chondrocytes in a manner characteristic of ER stress (163).

9. PCSK9 Regulates LDL-Cholesterol Levels: Implication in the Metabolic Syndrome

Complications resulting from cardiovascular disorders are the main cause of death worldwide, affecting ~13 million individuals/year, as compared to ~6 million/year due to various forms of cancer (http://www.poodwaddle.com/clocks/worldclock/). The incidence of cardiovascular pathologies is expected to increase dramatically in the next two decades. Elevated plasma cholesterol levels result in excess cholesterol deposition in arterial vessel walls and are a major risk factor for atherosclerosis and premature death by coronary artery disease. In the blood, cholesterol is transported in lipoprotein particles, ~70% of which in human are low-density lipoproteins (LDL). LDL is constantly cleared by internalization into cells by the LDL receptor (LDLR), which binds and internalizes LDL via its unique apolipoprotein B (apoB) protein. Mutations in *LDLR* or *APOB* genes are major causes for the frequent autosomal dominant genetic disorder known as familial hypercholesterolemia (164, 165). Among important cholesterol-lowering drugs are "statins," which inhibit cellular cholesterol synthesis (166). However, more efficient strategies to further decrease levels of circulating LDL-C are needed (167, 168).

Originally named NARC-1 for "neural apoptosis-regulated convertase," PCSK9 was first discovered and characterized in our laboratory (169). In collaboration with C. Boileau in Paris, we established the association between single-point mutations in the *PCSK9* gene and autosomal dominant hypercholesterolemia in two French families (170). Thus, *PCSK9* is the third gene associated with familial hypercholesterolemia (170, 171), with *LDLR* and *APOB* as the other two (164, 165). Later, Cohen et al. showed that nonsense PCSK9 mutations are associated with hypocholesterolemia in ~2% of black subjects (172, 173). Up to ~7% of black Africans living around the equator exhibit the loss of one allele of PCSK9 (174). In summary, point mutations in PCSK9 (171) are associated with either familial hypercholesterolemia (18, 170, 175–178) (gain of function of PCSK9; GOF) or hypocholesterolemia (172, 173, 179, 180) (loss of function of PCSK9; LOF). Two women lacking functional PCSK9 exhibited an ~85% reduction in circulating cholesterol associated with LDL (LDL-C) (180, 181). *Pcsk9*$^{-/-}$ mice are also viable and exhibit an ~80% drop in circulating LDL-C (182, 183), emphasizing the therapeutical potential of a PCSK9 inhibitor/silencer.

PCSK9 is mostly expressed in hepatocytes and small intestinal enterocytes (169). By an as-yet unknown mechanism(s), and independent of its enzymatic activity, PCSK9 enhances the degradation of cell-surface LDLR (180, 184–187) in endosomes/

lysosomes (188), resulting in increased circulating LDL. Statins, the best cholesterol-lowering drugs (166), reduce cholesterol synthesis by inhibiting the rate-limiting HMG-CoA reductase. The resulting cellular cholesterol depletion leads to transcription of genes involved in cholesterol metabolism, including those of PCSK9 and LDLR (189). While upregulation of LDLR reduces circulating LDL, that of PCSK9 counterbalances it through degradation of the LDLR (189, 190). PCSK9 inhibition is thus a promising complement to statin therapy to lower LDL-C (167, 180, 190).

PCSK9 (692 aa in human) comprises a signal peptide (aa 1–30) followed by prosegment (Pro; aa 31–152), catalytic (aa 153–407), hinge region (HR; aa 408–452), and C-terminal Cys-His-rich domain (CHRD; aa 453–692) segments. Following translocation into the endoplasmic reticulum (ER), the prosegment is autocatalytically cleaved at the $VFAQ_{152}\downarrow$SIP site (185). In PCs, the prosegment is an essential intramolecular chaperone and inhibitor, which is usually removed intracellularly to yield a fully active protease. Different from other PCs, PCSK9 is secreted as a stable, enzymatically inactive, non-covalent complex [Pro≡PCSK9] (169, 171, 185). In accordance, enhanced degradation of the LDLR (184–186) induced by PCSK9 does not require its catalytic activity (191, 192). In human (18) and mouse (183) plasma, both full-length PCSK9 (aa 152–692) and a truncated form PCSK9-ΔN_{218} (aa 219–692) can be detected (18, 193). The latter, which has no activity on LDLR, is likely generated by furin, since it efficiently cleaves PCSK9 ex vivo at $RFHR_{218}\downarrow$ (18). Interestingly, the human GOF R218S, F216L (177), and R215H (194) mutations associated with hypercholesterolemia prevent such a cleavage (18) and presumably result in increased levels of active PCSK9. To optimize PCSK9 inactivation by furin, we designed a PCSK9-$RRRR_{218}$EL mutant, which resulted in the secretion of only the inactive PCSK9-ΔN_{218} (18). We also contributed to the setup of two ELISA assays of circulating human PCSK9 (193, 195, 196), revealing a good correlation between levels of PCSK9 and LDL-C in human plasma (193). Sequencing of *PCSK9* exons from individuals at the extremes of the PCSK9 distribution provided a database of PCSK9 mutations, which are valuable tools in structure–function analyses. Indeed, we have recently identified a novel LOF variant, R434W, associated with low levels of circulating PCSK9 and LDL-C (193). This mutation, which occurs in an exposed loop of the hinge region, does not prevent LDLR binding, but drastically reduces the ability of PCSK9 to enhance the degradation of the LDLR (193).

9.1. Structure of PCSK9 and Deduced LDLR-PCSK9 Interacting Domain

The crystal structure of PCSK9 revealed three separate domains: the prosegment and catalytic domain in tight complex and the spatially separated CHRD (197–199). In all three crystal structures, aa 31–60 of the prosegment and portions of the CHRD

were unresolved, indicating their unstructured nature. Biochemical (200) and co-crystal structure (201) studies revealed that aa 153–156 and 367–381 directly interact with the EGF-A domain of the LDLR. The most severe mutation associated with hypercholesterolemia, D374Y (176), is within aa 367–381 and results in an ~25-fold higher affinity of PCSK9 toward LDLR (197). The shallow binding surface on PCSK9 is distant from its catalytic site, and the EGF-A domain of LDLR makes no contact with either the prosegment or the CHRD.

9.1.1. The Prosegment of PCSK9

Even though the prosegment does not bind the EGF-A domain of LDLR (201), it negatively regulates this interaction. The removal of its N-terminal acidic stretch (aa 31–53), which exhibits Tyr_{38} sulfation (169) and Ser_{47} phosphorylation (202), enhances the binding of PCSK9 to LDLR by ~sevenfold (201). Whether this unstructured acidic stretch (197–199) binds another domain of PCSK9 and/or interacts with another protein is yet to be defined.

9.1.2. The HR-CHRD of PCSK9

The HR is an exposed loop structure connecting the catalytic domain and the CHRD (193). The latter is composed of a six β-strand structure repeated three times and hence forming three subdomain modules M1, M2, and M3 (197). In the reported crystal structures (197–199), disordered segments include aa 573–584 (in M2), 660–667 (in M3), and the C-terminal aa 683–692 exhibiting Ser_{688} phosphorylation (202). A number of GOF (R469W, E482G, R496W, F515L, and H553R) and LOF (Q554E and the new one R434W) (193) mutations within the HR-CHRD were identified, but their underlying mechanisms are unknown.

9.2. Cellular Biology of PCSK9

Over 20 years experience with PCs led us to predict that PCSK9 should have more than one target (108). We thus first tested PCSK9 ex vivo activity on other members of the LDLR-like protein family. While LRP was not affected (185), the closest members to LDLR, i.e., VLDLR and ApoER2, were degraded faster in the presence of PCSK9 in a cell-type dependent fashion (203). We discovered that PCSK9 also enhances the degradation of the major hepatitis C virus (HCV) receptor, the tetraspanin protein CD81 (204).

9.2.1. PCSK9 Targets

9.2.2. PCSK9-Enhanced Degradation of the LDLR

The cell-surface localization of PCSK9 is dependent on the presence of the LDLR (205). The two proteins also co-localize in early and late endosomes (185). We previously developed an approach in which the fusion of a secretory protein of interest with the transmembrane domain and cytosolic tail (TM-CT) of the lysosomal protein Lamp1 results in an efficient degradation of its partners (105, 206). This strategy was applied to PCSK9 to better target its partners, including the LDLR, to degradative

compartments. Accordingly, fusion of PCSK9 to the TM-CT of Lamp1 (PCSK9-Lamp1) resulted in super-active forms of PCSK9 capable of depleting cells from its targets (203).

9.2.3. Extracellular Versus Intracellular Pathways

The extracellular pathway is defined by the ability of extracellular PCSK9 to target the LDLR. Indeed, incubation of cells with PCSK9, but not PCSK9-ΔC, enhances the degradation of cell-surface LDLR in endosomes/lysosomes (207). Since the PCSK9-ΔC still interacts with the LDLR and is internalized, the CHRD is likely essential for the trafficking of [PCSK9≡LDLR] to endosomes/lysosomes. Internalization of cell-surface LDLR requires the adaptor protein ARH that binds its cytosolic tail on the cytoplasmic side of clathrin heavy chain-coated vesicles (196, 208). In accordance, the pharmaceutical company Amgen developed a clinically relevant monoclonal antibody that inhibits PCSK9 interaction with LDLR and results in an ~80% reduction of LDL-C that lasted for 2 weeks in monkey (209). However, we recently demonstrated the existence of an intracellular pathway (187). First, PCSK9 can degrade the LDLR in vivo (186) and ex vivo (210) in the absence of ARH. Second, siRNA knockdown of both a and b chains of clathrin light chains, which block exclusively the intracellular pathway by preventing the trafficking from the TGN to lysosomes (211), resulted in a drastically decreased LDLR degradation (210).

9.2.4. HR-CHRD Binding Proteins

In view of the critical importance of the HR-CHRD for targeting [PCSK9≡LDLR] to lysosomes, we hypothesized that it binds directly or indirectly a membrane-associated protein that would sort the complex to lysosomes. Accordingly, a Far-Western screen of endogenous interactors of PCSK9 revealed that annexin A2 (AnxA2) binds the HR-CHRD and inhibits the ability of PCSK9 to enhance the degradation of the LDLR (212). AnxA2 lacks a signal peptide but is found at the cell surface of endothelia (213), keratinocytes (214), and epithelial (215, 216) and tumor cells (217).

9.3. In Vivo Studies

9.3.1. PCSK9 Mouse Models

Knockout (KO; *Pcsk9*$^{-/-}$) mice exhibit higher levels of LDLR protein in liver and 42% less circulating total cholesterol, with an ~80% drop in LDL-C (182, 183). In contrast, transgenic mice overexpressing PCSK9 exhibit 5–15-fold higher levels of LDL-C (183, 196, 218). We also developed mice carrying conditional floxed alleles, in which the proximal promoter and exon 1 of *Pcsk9* are *fl*anked by *loxP* sites (*Pcsk9*$^{f/f}$). In mice expressing the Cre recombinase under the control of the albumin promoter, *Pcsk9* was specifically inactivated in hepatocytes by *loxP* sites recombination (*Pcsk9*$^{f/f}$ *Alb-cre*). Total KO and liver-specific KO (LivKO) mice exhibited 42 and 27% less circulating total cholesterol, respectively, indicating that hepatic PCSK9 is

responsible for ~two-thirds of the phenotype. This suggested that the role of PCSK9 in cholesterol homeostasis is primarily mediated by its activity on LDLR, since liver accounts for ~70% of the body LDL-C clearance (219). Analysis of [*Pcsk9*$^{f/f}$ *Alb-cre*] livers demonstrated that PCSK9 expression is restricted to hepatocytes, from where circulating PCSK9 mostly originates. We generated double KO mice lacking both PCSK9 and LDLR (dKO; *Pcsk9*$^{-/-}$ *Ldlr*$^{-/-}$) and showed that their plasma lipid profile was identical to that of *Ldlr*$^{-/-}$ mice, confirming that PCSK9 activity on LDLR mediates most of its role in cholesterol homeostasis.

9.3.2. Circulating PCSK9

Human plasma contains ~100–200 ng/ml of PCSK9 (193, 196, 220). Its physiological role remains undefined, as well as that of its truncated form PCSK9-ΔN_{218} that represents ~50% of the PCSK9 species in mouse plasma. Analysis of transgenic lines that overexpress low or high levels of mouse PCSK9 in the liver indicated that only supra-physiological levels of circulating PCSK9 (~30-fold higher) increased circulating cholesterol (+60%); a threefold increase had no significant impact on circulating cholesterol. Moreover, data from transgenic mice expressing very high levels of human PCSK9 in kidney (218) or liver (196) or continuous infusions of recipient wild-type (WT) mice with recombinant human PCSK9 (221) indicated that microgram per milliliter amounts of circulating PCSK9 are required to significantly affect liver LDLR protein levels. Transgenic expression in kidney (32-fold the endogenous liver levels) led to 100% loss of LDLR protein in liver, but to only 50% loss in kidney (218). Thus, even at high levels, circulating PCSK9 reduces primarily liver LDLR with little effect on extrahepatic tissues, e.g., adrenals (196, 218).

9.3.3. Partial Hepatectomy (PHx)

To better understand the role of PCSK9 in liver, its major site of expression, we challenged this tissue by performing sham or PHx operations in WT and KO mice. Hepatectomized KO, but not WT, mice developed lesions, still visible 10 days after the liver had recovered its original mass. In addition, the proliferation of KO hepatocytes was delayed (183). Critically low levels of cholesterol may impede efficient liver regeneration. Indeed, HMG-CoA reductase mRNA levels were increased in KO regenerating livers only (2.5-fold at 72 h post-PHx). Also, when fed a high-cholesterol diet 1 week prior to PHx, KO mice no longer exhibited necrotic lesions.

9.3.4. Total Absence of PCSK9 May Affect β-Cell Function and Predispose to Diabetes

It was originally observed that the pancreatic insulin-producing β-TC3 cells express high levels of PCSK9 (169). LDLR is also highly expressed in insulin-producing pancreatic islet β-cells, possibly affecting the function of these cells. We recently showed that, compared to control mice, PCSK9-null male mice over

4 months of age carried more LDLR and less insulin in their pancreas; they were hypoinsulinemic, hyperglycemic, and glucose intolerant; their islets exhibited signs of malformation, apoptosis, and inflammation. Collectively, these observations suggested that PCSK9 may be necessary for the normal function of pancreatic islets (222).

10. Conclusions and Future Perspectives

The nine-membered family of the proprotein convertases (PCs) comprises seven basic amino acid-specific subtilisin-like serine proteinases, related to yeast kexin, known as PC1/3, PC2, furin, PC4, PC5/6, PACE4, and PC7, and two other subtilases that cleave at non-basic residues called SKI-1/S1P and PCSK9 (**Fig. 3.1**). The long and arduous task of identification of these processing enzymes is now over, as analysis of the genomes available failed to identify other potential members. While most PCs exert their functions through cleavage of substrates at either basic or non-basic aa, it is amazing that the last member PCSK9 only needs its enzymatic activity to autocatalytically process its prosegment in ER, which remains tightly associated with the catalytic subunit, resulting in an inactive protease. The absence of enzymatic activity may well explain the dominant pathological consequences of the lack or excess of PCSK9, which may be due to modified stoichiometric levels of protein–receptor complexes, such as PCSK9-LDLR. It is now the time to define the physiological functions of each PC, their substrates, and partners and to devise specific therapies aimed at controlling their levels. The development of specific inhibitors/modulators of convertases may find future applications in the control of some pathologies, e.g., hypercholesterolemia, cancer/metastasis, and viral infections.

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