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Knock-out mouse models of proprotein convertases: unique functions or redundancy?

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TABLE OF CONTENTS

1. ABSTRACT

The members of the proprotein convertase family play a central role in the processing and/or activation of various protein precursors involved in many physiological processes and various pathologies. The proteolysis of these precursors that occur at basic residues within the general motif (K/R)-(X)-(K/R) is mediated by the proprotein convertases PC1/3, PC2, Furin, PACE4, PC4, PC5 and PC7, whereas the proteolysis of precursors within hydophobic residues performed by the convertase S1P/SKI-1 and the convertase NARC-1/PCSK9 seems to prefer cleavages at the motif LVFAQSIP. Here we provide a comprehensive overview of their remarkable complex roles as revealed by disruption of their genes individually using generalized or conditional approaches.

2. INTRODUCTION

The generation of a biologically active protein is often a multi-step process. Secreted proteins are in most cases co-translationaly translocated into the lumen of the endoplasmic reticulum with concomitant cleavage of the signal peptide, followed by folding and a number of posttranslational modifications like glycosylation, disulfide-bridge formation and sulfation, before it reaches the plasma membrane. For many proteins, an essential step for activation is limited endoproteolysis; cleavage at one or more specific sites in the protein (1,2) (Figure 1). For other proteins, the unprocessed forms are biologically active and in certain cases oppose the biological action of their processed forms through specific receptors or inhibit their functions (reviewed in 2). In certain cases, the processing

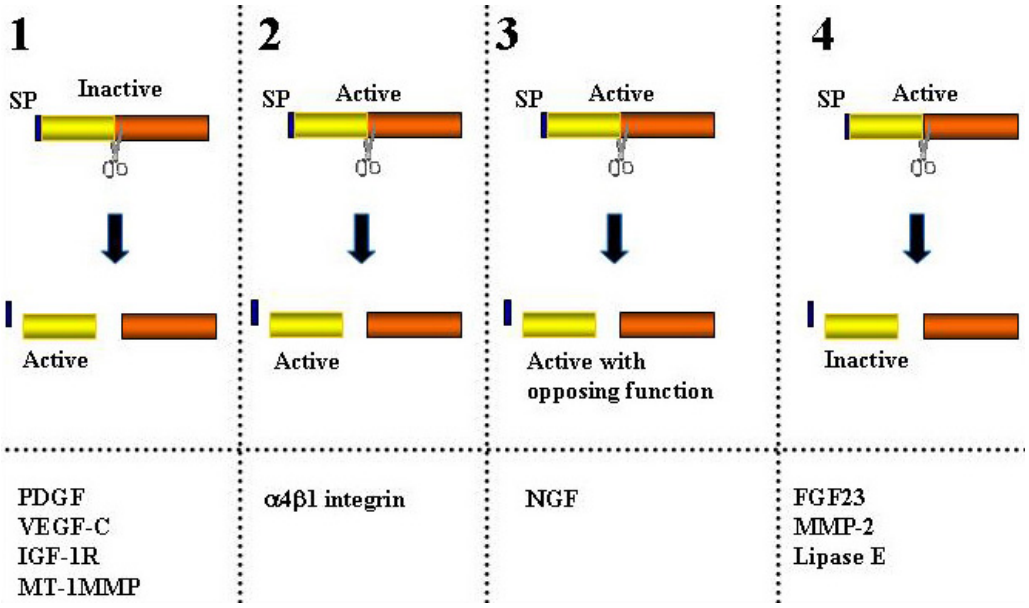

Figure 1. Schematic representation of protein precursors processing and/or activation by the proprotein convertases**.** The processing of several protein precursors is essential for the generation of active molecules (1) (e.g. PDGF, VEGF-C, IGF-1R, MT-1-MMP. The processing of other proteins doesn't affect their functions (2) (e.g. α4β1. In certain cases the unprocessed forms are biologically active and oppose the action of their processed forms through specific receptors (3) (e.g. NGF) or inhibit their functions (3) (e.g. FGF-23, MMP-2, Lipase E).

of other molecules revealed to have no effect on their biological functions (2). A large variety of these so-called proproteins are found in mammals, including neuropeptides, peptide hormones, growth and differentiation factors, receptors, enzymes, adhesion molecules, blood coagulation factors, extracellular matrix proteins, and plasma proteins. In addition, viral and bacterial pathogens utilize the proteolytic machinery of their host for activation of their proproteins (e.g. many viral coat proteins and bacterial toxins) (1).

Proprotein convertases (PCs) are a family of seven mammalian serine endoproteases (clan SB, family S8B) and constitute the major group of enzymes involved in the proteolytic cleavage carboxyterminal of the consensus cleavage site of (K/R)-$(X)_n$-(K/R), where X is any amino acid and n=0,2,4, or 6 (1-3). Subsequently, basic amino acid residues are often removed by carboxypeptidases (4) and glycine-extended peptides amidated by peptidylglycine α-amidating monooxygenase (PAM) (5). The seven PCs, Furin, PACE4, PC1/3 (previously named either PC1 or PC3), PC2, PC4, PC5/6, and PC7, have different, albeit partly overlapping expression pattern and subcellular localization. They have conserved aminotermini with highest homology in the subtilisin-like catalytic domain (6). The catalytic domain is preceded by a propeptide and followed by a P-domain (also called middle or Homo B domain) (Figure 2). These three domains are essential and sufficient for catalytic activity (7), the carboxyterminal domains control intracellular trafficking (8). The propeptide is cleaved through an intramolecular autocatalytic mechanism and is a prerequisite for exit out of the endoplasmic reticulum (ER) (9). However, the propeptide remains associated to the enzyme until it reaches the trans-Golgi network (TGN), where the local pH and Ca^{2+}-concentration facilitate a second autocatalytic internal cleavage and dissociation (10) (Figure 3). The exception to the rule is PC2, which exits the ER as a zymogen and, furthermore, the internal cleavage of the propeptide can be performed *in trans* by other PCs but not PC2 (11). SKI-1 and NARC-1 are two functionally related members of subfamily S8A, which also cleave and activate proproteins, but at non-basic motifs. The enzyme SKI-1 recognizes the motif (R/K)-X-(L,V)-Z, where Z is any aa except Pro, Cys, Glu, and Val (12) and NARC-1 prefers the motif LVFAQ (13).

Recently, the crystal structures of Furin and the yeast homologue kexin have been solved and used to model the structures of all PCs (14-16). The P-domain forms an eight-stranded jellyroll associated with the catalytic domain. The structure of the catalytic domain explains the selectivity for basic substrate segments by identifying negatively charged amino acids lining the substrate binding pockets. Furthermore, the overall charge compensation and matching of the detailed charge distribution is likely to form the basis for the observed preference of the different PCs for distinct substrates. The above-mentioned consensus cleavage site does not allow the identification of propeptides in proteins, because of the importance of the context in which it is located. However, propeptide cleavage sites can be predicted with reasonable confidence using an ensemble of neural networks (17). On the other hand, it remains impossible to predict which PC cleaves which substrate, even with the availability of crystal structures and homology models. *In vitro* cleavage studies

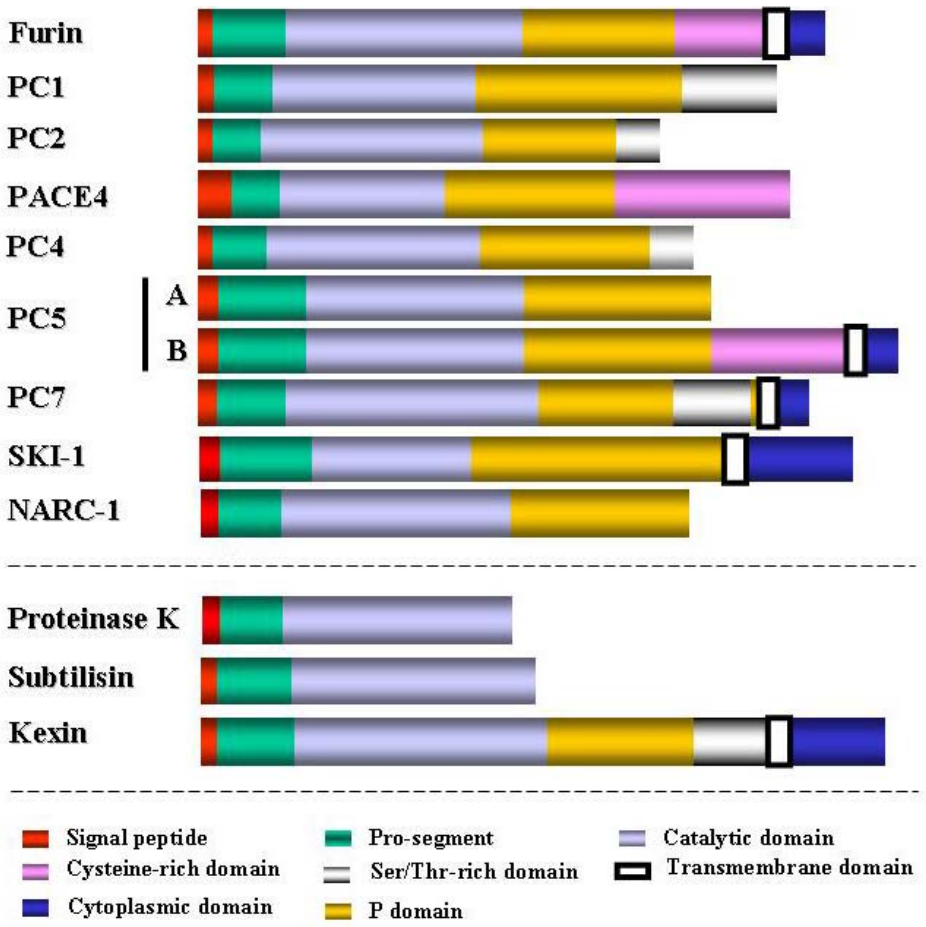

Figure 2. Schematic representation of the proprotein convertases. Represented are the structure of the convertases PC1, PC2, Furin, PACE4, PC4, PC5 (A and B isoforms) and PC7 a well as the convertases SKI-1 and NARC-1. The schematic representation for Kexin and subtilisin are given for comparison.

are of limited use for this purpose too, as they tend to generate false positives due to non-physiological stoechiometries and the absence of the cellular context. Some of these problems can be circumvented by using specific inhibitors. However, since most currently used inhibitors are pseudo-substrates, they have a tendency to cross-react with different family members (18). In addition, it is hard to exclude the possibility that they inhibit other basic amino acid-specific enzymes as well. Knockdown of a specific PC using gene silencing methods have been shown to be useful alternatives (3, 19-21), although complete knockdown might not be achieved resulting in the under appreciation of the role of a PC.

In vivo, expression patterns, subcellular localization, microenvironment or interacting proteins might preclude substrates from being processed by a specific PC. It has for instance been shown for pro-neurotensin/neuromedin that processing by PC2 varied in different regions of the brain, correlating with expression levels of PC2 but probably also depending on regionally available compensatory PCs (21). A study on the processing of α4 integrin, has demonstrated the importance of pH and therefore of subcellular localization (22). In this study it was shown that Furin performs α4 integrin processing best at slightly acidic pH (TGN-like environment) and by PC5A at neutral pH (plasma membrane). The importance of microenvironment was recently shown for the type II transmembrane collagen XXIII. This collagen is protected from Furin cleavage and subsequent shedding by its localization in lipid rafts and tissue-specific regulation of the amounts of cell surface-bound and secreted collagen XXIII is therefore controlled by a cholesterol-dependent mechanism (23). For TGF-β is has been shown that Emilin 1 inhibits TGF-beta signaling by binding specifically to the proTGF-β precursor and preventing its maturation by Furin in the extracellular space (24). These examples highlight the difficulty in the identification of a specific substrate for specific PCs when using *in vitro* or *ex vivo* approaches suggesting the use of knockout mouse models as an additional tool for the confirmation of the identity of these substrate. The remainder of this review will therefore focus on recent advances in the understanding of the biological role of each PC using mouse models. However, it should be noted here that knockout models for PCs are likely to be compound

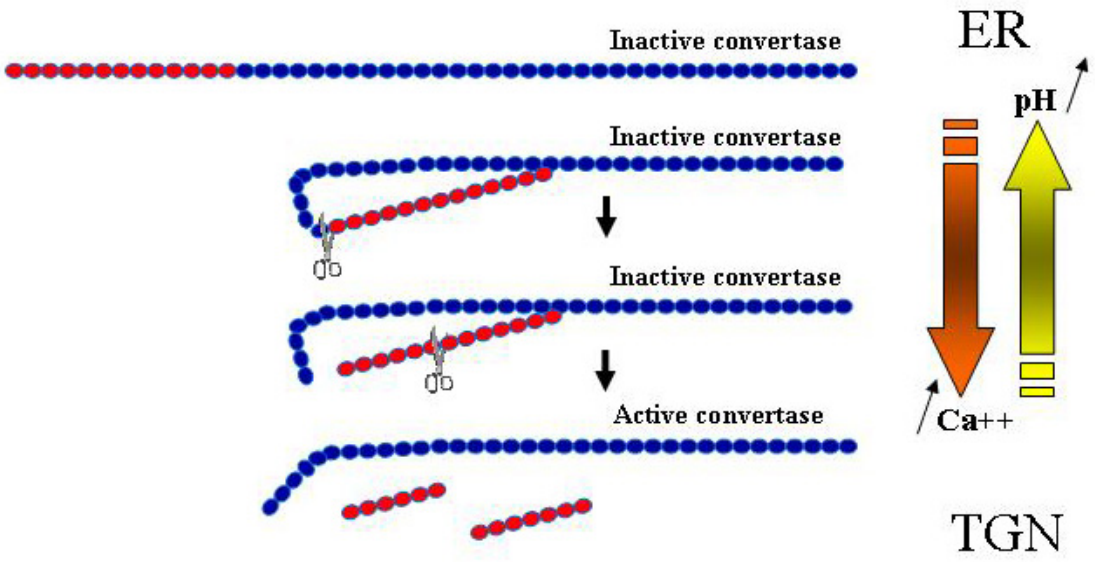

Figure 3. Convertase propetides as inhibitor. At the endoplasmic reticulum, the convertase propeptide acts as an intramolecular chaperone to facilitate the folding of the catalytic domain into the active conformation. The convertase undergoes autoproteolytic intramolecular cleavage of its propeptide. The latter remains associated with the mature fragment of the convertase and functions as a potent autoinhibitor. At low pH and higher [Ca^{2+}], the propeptide is cleaved a second time leading to a rapid dissociation of the propeptide fragments and convertase activation.

phenotypes; processing of many substrates can be affected, which all contribute to the observed phenotype. Some substrates might work synergistically and aggravate the effect, whereas others might have opposite effects of each other, potentially leveling out a more severe phenotype (3). Identification of a physiological substrate is therefore well possible; care needs to be taken to link it to the phenotype.

3. PHENOTYPES OF PC DEFICIENT MOUSE MODELS

To determine the biological role of individual PCs, mouse models have been generated for all PCs (reviewed in 3). They have proven to be extremely informative with respect to determining enzyme-substrate pairs, but also for (tissue-specific) redundancy provided by other enzymes, most likely other PCs. Furthermore, they emphasize the diversity and complexity of proproteins processing in the context of a mammal. In all cases the heterozygotes are normal. The phenotypes range form early embryonic lethality to no apparent phenotype. Early embryonic lethality indicate a non-redundant role during specific stages of embryonic development, but do not exclude extensive redundancy for substrates during adult life, as has been demonstrated for Furin and will be discussed below (25).

3.1. Furin

Furin is the prototype and probably most studied member of the PC family. It was also the first PC for which a knockout mouse model has been described and up till now the only one for which a conditional tissue-restricted knockout has been described (25, 26). It is often considered to be the workhorse of the family since it is expressed in most cells and can cleave a large number of proproteins *in vitro*. The FURIN gene (*fur*) is located on the human chromosome 15 and on mouse chromosome 7 (Table 1). The coding product is a type I transmembrane protein, initially produced as a 104 kDa pro-FURIN precursor and by an autocatalytic process is converted into a 98 kDa form (9).

Expression of Furin is detected at embryonic day e7.5 in the extraembryonic endoderm and mesoderm, anterior visceral endoderm, and in precardiac mesoderm (26). A day later, expression is found throughout the heart tube and in the lateral plate mesoderm, notochordal plate and definitive gut endoderm. Furin null mice die between e10.5-e11.5 due to hemodynamic insufficiency and cardiac ventral closure defects. Knockout embryos show multiple defects including failure of chorioallantoic fusion, abnormal yolk sac vasculature, and lack of axial rotation. Endothelial cell precursors were present, but the embryo failed to develop large vessels (26). No specific Furin substrate in the cardiovascular system has been identified, but expression of the potential substrate TGFβ1 coincides with that of Furin during this developmental stage (27). TGFβ1 null mice have strikingly similar phenotypic features, further substantiating the hypothesis that impaired processing of the proTGFβ1 precursor is at the basis of this aspect of the phenotype in the Furin null mice (28). Failure of the chorion to fuse with the allantois has been observed in several other knockout mice including α4 integrin null mice and the double knockout of bone morphogenetic protein 5 and 7 (BMP5 and BMP7) (29, 30). Members of the BMP family are substrates of PCs and are therefore good candidates to be involved in this aspect of the phenotype. However, their instability and low expression

Table 1. Amino acid sequences of the autocatalytic sites of the PCs

Convertase	Chromosomes		Amino acid number	Autocatalytic site	Accession number
	H	M			
Furin	15	7	794	[101]A-K-R-R-T-K-R-D	NP_002560
PC1	5	13	751	[105]K-E-R-S-K-R-S-V	P21662
PC2	20	2	638	[103]G-F-D-R-K-K-R-G	P16519
PACE4	15	7	969	[141]Q-E-V-K-R-R-V-K	P29122
PC4	19	10	654	[105]R-R-R-V-K-R-S-L	A54306
PC5	9	19	1870	[109]V-K-K-R-T-K-R-D	Q04592
PC7	11	9	785	[134]R-L-L-R-R-A-K-R	NP_004707
SKI-1	16	8	1052	[131]K-V-F-R-S-L-K-Y	NP_003782
NARC-1	1	4	692	[148]V-F-A-Q-S-I-P	NP_777596

Prior activation, like their substrates, the pro-segments of the PCs are removed at sites cleaved by the PCs. Indicated are the chromosome localization, number of amino acid and accession number for every PC.

levels have so far precluded direct confirmation. Recently, using an inducible knock-out system to inactivate the *fur* gene only in liver allowed the investigation of the importance of Furin in adult mice in the presence of the other PCs such as PC5, PC6 and PC7 (25). Interestingly, regardless of the complete inactivation of Furin expression in liver, no morphological abnormalities could be found. In addition, the processing of all the substrates analysed in the liver of these mice revealed to be unaffected or impaired, but never completely blocked. Of these substrates are insulin receptor, albumin, α5 integrin, lipoprotein receptor-related protein, vitronectin and α1-microglobulin/bikunin (25). Their processing despite the absence of Furin activity suggests the existence of considerable redundancy of proprotein processing activity in liver. These results that contrast the importance of Furin in early embryonic development of which the absence cause lethality indicate the non-redundant function of Furin during early embryogenesis that is lost at the adult stage a least in the liver.

Aside the crucial function of Furin during embryonic development; it's implication in various diseases such as cancer is now well established (31, 32). Using *in vivo* and *in vitro* assays, various studies revealed that specific inhibition of Furin could be used as a potential therapeutic strategy against tumor growth and metastasis (31-33). Although many protein precursors involved in tumor progression were found to be directly activated by Furin, these proteins were also found to be processed and activated by other PCs such as IGF-1R, MT1-MMP and several integrins (reviewed in 31). Thus, here again, to understand the specific role of Furin in pathology, particularly neoplasia, the identification of specific Furin substrates implicated in these processes is required.

3.2. PACE4

Localization of the PACE4 gene revealed its approximate position to *fur* gene on the human chromosome 15 and mouse chromosome 7 (Table 1), suggesting a probable common ancestry by gene duplication (34). Like Furin, PACE4 is expressed in most tissues where it processes a variety of substrates (35). The maturation of proPACE4 occurs also via an intramolecular autocleavage of its propeptide. This is the rate-limiting step for the secretion of the mature PACE4. Unlike Furin, the broadly expressed PACE4 does not possess a transmembrane anchor, but instead it contains a large cysteine-rich domain with a repeated cysteine motif, first observed in Drosophila dfurin2 (36). This domain is essential for cell surface tethering and binds heparin and TIMP-2 (37, 38). Furthermore, the secretion and the maturation of PACE4 are also controlled by the carboxy terminal sequence of PACE4 (34, 35).

Like Furin, PACE4 is expressed early during development. About a quarter of the PACE4 knockout mice die prenatally, the surviving mice appear normal (39). This is consistent with a non-redundant function during embryogenesis and a redundant function in post-natal life, although the observation that the phenotype is not fully penetrant seems to suggest some redundancy, even at early stages. The malformations in the null mice are comprised of situs ambiguous combined with left pulmonary isomerism and complex craniofacial malformations including cyclopia (39). This indicates an important role for PACE4 in patterning the early mouse embryo. Situs defects are found in heart, liver, lung, pancreas, spleen, and gut. The affected embryos eventually die between e13.5 and e15.5, probably as a consequence of cardiac malformations. The heart defects vary; the heart tube may loop in the wrong direction and have fused chambers or defective connections to the vascular system such as truncus arteriosus. Potential substrates of PACE4 involved in the specification of left/right and anteroposterior axes are member of the TGFβ superfamily including Nodal, Lefty and BMPs. However, the phenotypes of the corresponding knockout mice are much more severe than that of PACE4, again suggesting partial redundancy (40, 41). Like Furin, the alteration in PACE4 levels was also linked to carcinogenesis, however, opposing views in the literature argue the relevance of PACE4 expression in carcinogenesis. Some studies demonstrated that over-expression of PACE4 in non-tumorigenic cells increased their invasiveness (42); whereas other studies linked the absence or reduced PACE4 expression levels to ovarian cancer (43). These conflicting data demonstrate that PACE4 expression varies in a tumor-specific fashion, and raise the possibility that alteration in PCs expression may positively or negatively regulate human tumor biology.

3.3. PC1/3

Three different mouse models for PC1 deficiency have been published, all with different phenotypes (44-46). They were generated by ENU mutagenesis (44) or homologous recombination (45, 46). In addition, three

human patients with congenital PC1 deficiency have been published, with yet another phenotype (47-49). Despite their differences, they confirm the important neuroendocrine function of this enzyme localized in dense-core granules of the regulated secretory pathway.

The first published mouse model of PC1/3 was characterized by dwarfism caused by a complete block of pro-growth hormone releasing hormone (proGHRH) processing (46). The lack of mature GHRH results in low pituitary growth hormone (GH) and hepatic insulin-like growth factor-1. In addition, multiple defects in processing of many hormone precursors, including pituitary proopiomelanocortin to adrenocorticotropic hormone, proinsulin to insulin and intestinal proglucagon to glucagon-like peptide-1 and -2 (46, 50). By now, many other substrates have been analyzed using both a candidate substrate analysis and proteomics/peptidomics approaches, confirming that PC1/3 plays a key role in the processing of neuroendocrine protein/peptide precursors but also reveal the presence of a redundant system (51, 52).

In the second mouse model, a point mutation (N222D) in the catalytic domain of PC1/3 was identified after ENU mutagenesis (44). This model is of particular interest because it best resembles the human syndrome associated with PC1/3 deficiency. This N222D mutation renders PC1/3 50% catalytically less active. The homozygous mutant mice are obese, at least in part due to increased energy intake and a more efficient metabolism. Proinsulin processing is defective, leading to glucose intolerance, but neither insulin resistance nor diabetes was developed despite obesity. The obesity is associated with impaired hypothalamic α-MSH production from POMC. Remarkably, the heterozygotes have an intermediate phenotype. These mice have retained 75% PC1 activity, whereas heterozygotes from the above-described knockout mice only possess 50% activity and have no pronounced phenotype. The main difference between both models is that the null mice do not produce any PC1/3, while the N222D mutant mice have normal expression levels of (an at least partly) correctly folded and secreted protein. This suggests that N222D PC1/3 might have a dominant negative effect on an unknown pathway, or that PC1/3 has a non-catalytic function as well. Consistent with this is the observation of Mbikay and coworkers (53) that PC1/3 is localized throughout the cytoplasm of unfertilized eggs. After fertilization, PC1/3 is concentrated in pronuclei, probably mediated by its prodomain. This raises the possibility of a nuclear function for PC1/3 during zygote formation.

Recently, a third model for PC1/3 deficiency has been published which is embryonically lethal (45). The heterozygote female mutant mice exhibited stunted growth under a low fat diet, and catch-up growth under a high-fat diet. The complex phenotype may be due to the relatively large deletion introduced by the homologous recombination (32.7 kb) but might also be aggravated by expression of aberrant gene products from the mutant allele composed of the aminoterminal half of the PC1/3 propeptide fused to neomycin selection marker. This truncated propeptide might aggravate the phenotype by a more generalized inhibition of PCs as peptides derived from the propeptide of PC1/3 have been shown to inhibit Furin, PC5/6 and PC7 as well (54). Preceding all mouse models, in 1997 the first patient with compound deleterious mutations in PC1/3 was reported, later followed by two additional patients (47-49). The multihormonal syndrome is characterized by severe early-onset obesity, abnormal glucose homeostasis, impaired intestinal function and hypoadrenalism. The childhood obesity can be largely explained by hyperphagia, the degree of which is comparable with that of patients with heterozygous mutations in the melanocortin 4 receptor gene (55). It is likely that disrupted POMC processing in the hypothalamus plays a role in the development of obesity, due to reduced melanocortin signaling in the hypothalamus. Processing of proinsulin to insulin, POMC to ACTH, proglucagon to GLP1 and GLP2 were impaired. All mutations rendered PC1/3 complete inactive towards substrates *in trans*, but (residual) autocatalytic activity was still observed for two mutant alleles.

3.4. PC2 and 7B2

The other neuroendocrine-specific member of the family is PC2. This enzyme is unique in its activation mechanism that requires the molecular chaperone 7B2 (11, 56). The secretory protein 7B2 is a bifunctional molecule with an aminoterminal domain involved in proPC2 transport as well as activation and a carboxyterminal peptide that inhibits PC2 at nanomolar concentrations (57, 58). Because PC2 is not activated in the absence of 7B2, both PC2 null and 7B2 null mice are models for PC2 deficiency (59, 60). These models demonstrate once again the importance of background as initially the phenotypes differed profoundly but where highly similar after backcrossing onto the same genetic background (61). In a 129/SvEv background, knockout mice die within 9 weeks after birth of severe Cushing's syndrome arising from pituitary intermediate lobe ACTH hypersecretion. When the mice were transferred onto the C57BL/N6 background they survived and showed greatly decreased circulating corticosterone and increased blood glucose levels, most likely due to the comparatively higher adrenal resistance of the B6 strain to ACTH stimulation. Beside this remarkable phenotype, analysis of several protein precursors revealed that these mice, like PC1 mutant mice, exhibit multiple defects in other hormone precursor processing events. These include the hypothalamic GHRH, pituitary POMC, proinsulin and intestinal proglucagon. In contrast to PC1/3-null mice, PC2-null mice process normally pituitary POMC to adrenocorticotropic hormone (ACTH), and have normal levels of blood corticosterone. Like PC1-null mice, PC2 were found also to developed hyperproinsulinemia, albeit to a lesser extent. Similarly, recent analysis of the processing of proCCK in brain extracts from PC2 and 7B2-null mice revealed the presence of an exaggerated increase of cerebral proCCK and reduced CCK active form.

3.5. PC4

The expression of PC4 is mainly restricted to testicular and ovarian germ cells. In round spermatids PC4 was detected in the acrosomal granules, in the acrosomal ridges of elongated spermatids, and on the sperm plasma

membrane overlying the acrosome (62). Genetic ablation of PC4 in mice reduces fertility, as might have been expected (63). Sperm from knockout males were less able to fertilize eggs *in vitro*, and eggs that were fertilized were not viable, failing to develop to the blastocyst stage, while the ovaries of knockout females showed delayed folliculogenesis. PC4 deficient sperm underwent capacitation at a faster rate and was induced to acrosome react by lower concentrations of zona pellucida. Furthermore, their egg-binding ability was only half that of wild-type sperm (62). Several potential PC substrates are expressed in testicular germ cells, and it was shown that processing of the precursor to pituitary adenylate cyclase-activating peptide (proPACAP) is entirely dependent on PC4 in testis and ovaries (64). In pituitary, where there is no expression of PC4, proPACAP is most likely cleaved by PC1 and PC2, providing an interesting example of tissue-specific redundancy (65). The fertility observed in PACAP knockout mice is less severely affected, and is mainly due to a decrease in mating frequency (66). This suggests that additional substrates of PC4 are contributing to the phenotype. It has for instance recently been shown that PC4 is also expressed in placenta where it cleaves the insulin-like growth factor II (IGF-II) (67)

3.6. PC5/6

The PC5/6 gene is localized on human chromosome 9 and mouse chromosome 19. The human PC5/6 gene encodes a protein with high similarity to PACE4, especially (68). PC5/6 is expressed in various tissues and cell types. It consists of two isoforms, A and B that differ at the carboxyterminus. PC5/6A is a soluble protein with a cysteine rich region like PACE4, which also interacts with heparin and TIMP-2 at the cell surface (37), while PC5/6B contains an extended cysteine-rich region, a transmembrane anchor and a cytoplasmic tail that directs sorting to the TGN en endosomal compartments (69). As a consequence of the different subcellular localization, differences in substrate selectivity are observed between the splice variants, for instance in case of the lefty precursor, which is cleaved by PC5/6A but not PC5/6B (69). Expression of PC5/6 starts early in development and at e9.5 is found in somites, bulb of umbilical cord, and lung bud and a day later also in bronchial arch, the wall of bulbus cordis, and the nasal pit. At later stages and in adulthood expression is highest in small intestine, kidney and lung (71, 72). PC5/6 null mice (lacking both isoforms) die between embryonic days 4.5 and 7.5 (71). *In vitro*, PC5/6 can cleave a number of developmentally important factors, but which causes early embryonic lethality in the knockout mice is at present unclear.

3.7. PC7

The last discovered and least related member of the family is PC7 (73). Autoprocessing is slow compared to other members of the family and its cytoplasmic tail is palmitoylated (74, 75). Distinct from Furin and PACE4 genes, which both map to chromosome 15, PC7 maps to chromosome 11 (Table 1), suggesting that PC7 may have resulted from either Furin or PACE4 gene duplication, or on the contrary, that Furin and PACE4 genes arose from PC7. Various analyses demonstrated significant expression of PC7 mRNA in the colon and lymphoid-associated tissues and in various tissues, PC7 seemed to co-localize with Furin, suggesting the widespread proteolytic functions of PC7 and its participation with Furin in the activation of several substrates. Despite its ubiquitous expression pattern, starting early in development, the knockout mice do not display any abnormalities (72). On the one hand this is surprising, because *in vitro* many substrates can be cleaved by PC7. On the other hand, the same substrates are usually also cleaved by Furin and PACE4, and the expression pattern of Furin overlaps to a large extend. It is possible that PC7 is largely redundant, or that only a subset of non-essential substrates is dependent on PC7.

3.8. Subtilisin/kexin-like isozyme-1 or SKI-1

In contrast to the PCs, SKI-1 called also Site-1 protease (S1P) (76) appears to prefer processing precursors at hydophobic residues within the general motif ***RX(V,L)(K,F,L)↓*** (12). Initially, this enzyme was reported to be involved in controlling lipid metabolism by mediating the cleavage of Sterol Regulatory Element-binding Proteins (SREBPs) in its ER luminal loop (12, 76). Based on this ability, it was anticipated that the lack of SKI-1/S1P activity would induce a defect in the lipid homeostasis in SKI-1/S1P null mice (77). Although the use of the Cre recombinase system failed to induce complete disruption of SKI-1/S1P in mice liver, the partial disruption of SKI-1/S1P was able to cause a reduction in the rates of cholesterol and fatty acids synthesis (77), suggesting that the disruption of SKI-1/S1P by inducible Cre recombinase system was not sufficient to abolish SKI-1/S1P functions, or alternatively, that another protease substituted for SKI-1/S1P in the liver of these mice. Although the role of SKI-1/S1P in the homeostasis of cholesterol and fatty acids is now well established, this enzyme seems to be also involved in the morphology of cartilage as revealed by the irregular chondrocyte morphology in zebrafish following phosphomorpholino knockdown zebrafish SKI-1 (78). As for SKI-1/S1P liver null-mice, these fish present also abnormal distribution of lipids.

3.9. Neural apoptosis-regulated convertase 1 or Narc-1

Like all the convertases, NARC-1 (also called proprotein convertase subtilisin/kexin type 9 (PCSK9) is synthesized as a zymogen that undergoes autocatalytic intramolecular processing in the ER. This cleavage occurs within the motif ***LVFAQ↓SIP*** (13). Genetic analysis of several families with high risk of coronary heart disease due to increased levels of the LDL, exhibited a mutation in the *PCSK9* gene (79), suggesting a possible role for this convertase in cholesterol homeostasis and its implication in a dominant form of familial hypercholesterolemia FH3 (79, 80). Like SKI-1/S1P, PCSK9/NARC-1 is mainly expressed in the liver and small intestine, two organs involved in cholesterol homeostasis. Recently, various studies demonstrated the importance of PCSK9 in regulating the levels of circulating LDL-cholesterol in the human population, whereupon certain mutations are directly associated with the development of hypercholesterolemia with a gain of function of PCSK9, while others implicate a loss of function and result in a hypocholesterolemia phenotype (79, 80). Recently; analysis of *PCSK9* knockout

mice revealed that the animals showed a ~50% reduction in circulating LDL-cholesterol (81). Administration of HMG-CoA reductase inhibitors known as "statins" resulted in a further ~50% reduction of their circulating LDL levels. These mice found to manifest increased LDLR protein responsible for the increased clearance of circulating lipoproteins and decreased plasma cholesterol levels. Based on human PCSK9 mutations and PCSK9 knockout mice phenotype, specific inhibitors of PCSK9 may is now suggested as new potential strategy to act synergistically with statins to enhance LDLRs and reduce plasma cholesterol.

4. PERSPECTIVES

Several important lessons have been learned from the knockout mouse models. First of all, it has demonstrated the discrepancy between *in vitro* and *in vivo* studies. Furthermore, it has shown the importance of PCs in the orchestration of many biological processes. But most importantly, it has shown the dynamics of processing; both redundancy and uniqueness exist, which can be species or even tissue-specific. Most studies have been performed on a candidate-substrate basis, but recently proteomics/peptidomics approaches have been initiated as an unbiased method to identify new substrates (82-84). Despite the plethora of new insights gathered from these mouse models several questions have not been answered and need further investigation.

Furin, PACE4, PC5/6 and SKI-1/S1P nulls display embryonic lethality (Table 1). PC4 null mice are infertile or subfertile and KO mice of *PC1/3*, *PC2* , PCSK9 genes are viable despite the manifestation of hormonal and/or neuroendocrine deficiency (Table 1). This establishes non-redundant roles for the processing of certain developmental factors. However, it precludes analysis of substrates in adult mice, where redundancy might be different. This is most evident in *PC1/3*, *PC2* , PCSK9 null mice and in 25% of PACE4 null mice, where the majority of the embryos survive and develop normal (3). This suggests that in adult mice, the largely expressed PCs, which have overlapping expression patterns, are able to compensate for the missing enzyme. Tissue-specific conditional KO allowed the analysis of the function of some PCs in these tissues. Thus, liver specific conditional SKI-1/S1P null mice exhibit disorganized lipid and fatty acid homeostasis due to the lack of SREBP-1 and SREBP-2 processing. In liver-specific Furin knockout only mild biochemical phenotype was revealed (25)**.** To address the importance of redundancy, double or even triple knockout might be necessary. This will for certain require tissue-specific knockouts, to prevent embryonic lethality. These studies will also reveal whether PC7 play a redundant role in the processing of (essential) substrates, or whether it is specific for a subset of non-essential substrates since mice with disrupted *PC*7 gene failed to show any apparent abnormal phenotype.

PCs are interesting potential therapeutic targets as they cleave a number of bacterial pro-toxins and viral coat proteins, but the involvement in tumorigenesis and neurodegeneration has also been demonstrated in a number of studies (for reviews see (1, 2, 31). Proof of principle that the administration of a PC inhibitor can prevent lethality invoked by a bacterial toxin in mice was given a few years ago (85). Importantly, no dramatic side-effects were observed during short-term administration of a PC inhibitor. Crossing of PC null mice with disease models will provide insight into which PC needs to be inhibited for which disease. In certain cases, the crossing of inducible knock-out mice that target specific tissue may be required to avoid lethality that may derive from several null mice crossing. This will facilitate the development of narrow-spectrum inhibitors, which still achieve full effect but with minimal side-effects.

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Abbreviations: PCs; Proprotein convertases, PAM;

peptidylglycine α-amidating monooxygenase, ER; endoplasmic reticulum, TGN; trans-Golgi network, BMP; bone morphogenetic protein, IGF-II; binsulin-like growth factor II, SREBPs; Sterol Regulatory Element-binding Proteins.

Key Words: Knock-out Mice, Proprotein Convertases, Therapeutic Targets, Proteolytic Cleavage, Review

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