

## 60 YEARS OF POMC

# From POMC and $\alpha$ -MSH to PAM, molecular oxygen, copper, and vitamin C

Dhivya Kumar<sup>1</sup>, Richard E Mains<sup>2</sup> and Betty A Eipper<sup>1,2</sup>

<sup>1</sup>Departments of Molecular Biology and Biophysics, University of Connecticut Health Center, Farmington, Connecticut, USA

<sup>2</sup>Department of Neuroscience, University of Connecticut Health Center, Farmington, Connecticut, USA

Correspondence should be addressed to R E Mains or B A Eipper

**Email**  
mains@uchc.edu or  
eipper@uchc.edu

## Abstract

A critical role for peptide C-terminal amidation was apparent when the first bioactive peptides were identified. The conversion of POMC into adrenocorticotrophic hormone and then into  $\alpha$ -melanocyte-stimulating hormone, an amidated peptide, provided a model system for identifying the amidating enzyme. Peptidylglycine  $\alpha$ -amidating monooxygenase (PAM), the only enzyme that catalyzes this modification, is essential; mice lacking PAM survive only until mid-gestation. Purification and cloning led to the discovery that the amidation of peptidylglycine substrates proceeds in two steps: peptidylglycine  $\alpha$ -hydroxylating monooxygenase catalyzes the copper- and ascorbate-dependent  $\alpha$ -hydroxylation of the peptidylglycine substrate; peptidyl- $\alpha$ -hydroxyglycine  $\alpha$ -amidating lyase cleaves the N–C bond, producing amidated product and glyoxylate. Both enzymes are contained in the luminal domain of PAM, a type 1 integral membrane protein. The structures of both catalytic cores have been determined, revealing how they interact with metals, molecular oxygen, and substrate to catalyze both reactions. Although not essential for activity, the intrinsically disordered cytosolic domain is essential for PAM trafficking. A phylogenetic survey led to the identification of bifunctional membrane PAM in *Chlamydomonas*, a unicellular eukaryote. Accumulating evidence points to a role for PAM in copper homeostasis and in retrograde signaling from the lumen of the secretory pathway to the nucleus. The discovery of PAM in cilia, cellular antennae that sense and respond to environmental stimuli, suggests that much remains to be learned about this ancient protein.

## Key Words

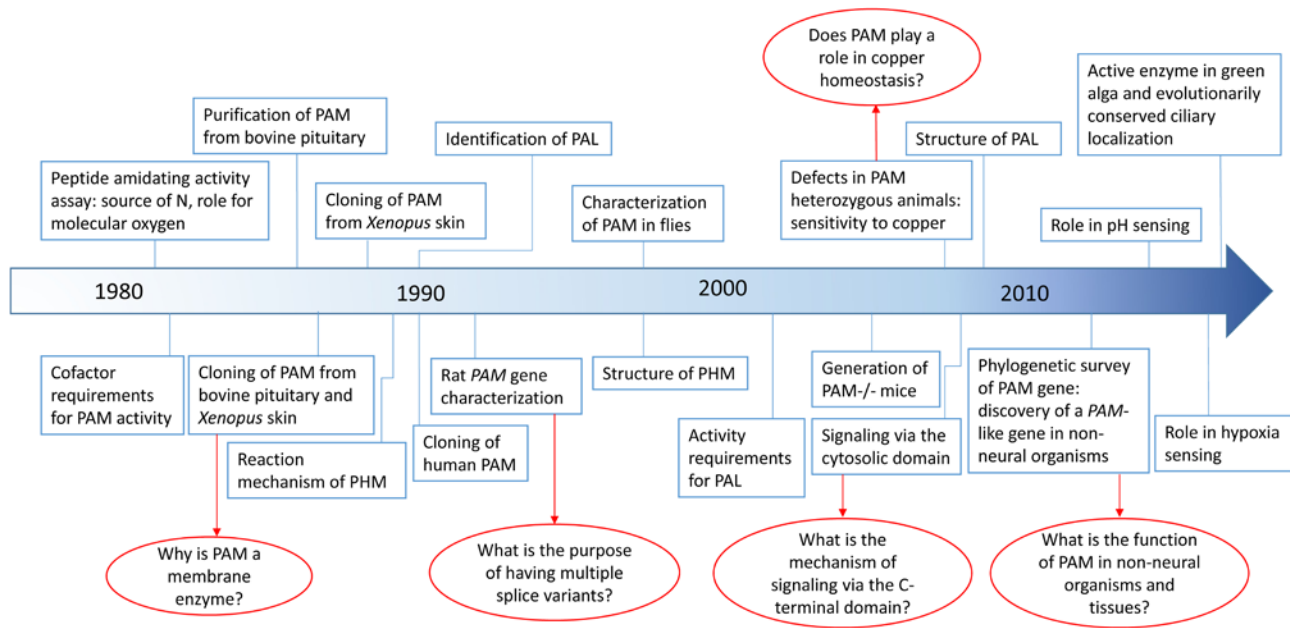
- ▶ amidation
- ▶ peptides
- ▶ cilia
- ▶ copper
- ▶ monooxygenase
- ▶ sensory
- ▶ signaling
- ▶ obesity
- ▶ energy homeostasis

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## POMC and PAM: where it all began

Over the last three decades, we have amassed a great deal of information on the function, trafficking, and biochemical properties of the only known peptide-amidating enzyme, peptidylglycine  $\alpha$ -amidating monooxygenase (PAM). Until its discovery in 1982, even the existence of such an enzyme was questioned (Fig. 1). Based on the first

biologically active peptides identified (vasopressin, oxytocin, and  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH)), it was clear that a C-terminal amide group was essential; however, there was no reason to suspect that a mechanism other than transamination (such as in glutamine synthesis) might be in place. The discovery of



**Figure 1**

Timeline highlighting key developments leading from POMC processing studies to PAM. The landmark study of Bradbury and coworkers provided a means of assaying peptide amidating activity in tissue lysates (Bradbury *et al.* 1982). Purification, cloning, and structural/mechanistic studies focused on PAM and then expanded to include cell biological studies on secretory granule biogenesis, retrograde signaling from the granule lumen to the nucleus, and the delivery of essential cofactors (ascorbate and copper) to the secretory pathway. Key unanswered questions are marked by red arrows.

glycine-extended precursors for amidated peptides such as  $\alpha$ -MSH, adipokinetic hormone, and melittin raised the possibility that an enzyme recognizing the terminal glycine was involved in generating the mature amidated peptide (Harris & Lerner 1957, Stone *et al.* 1976, Suchanek & Kreil 1977).

Using a synthetic radiolabeled peptidylglycine substrate (based on the last three amino acid residues of the  $\alpha$ -MSH precursor), Bradbury and coworkers demonstrated the presence of an activity catalyzing the amidation reaction in secretory granules of bovine pituitaries (Bradbury *et al.* 1982). In this landmark study, the amide group nitrogen was shown to be derived from the glycine residue, ruling out the possibility of a transaminase reaction; the formation of glyoxylate during the reaction pointed to a hydroxylation step in the reaction mechanism (Bradbury *et al.* 1982).

Around this time, our laboratory was focused on understanding the tissue-specific differential processing of POMC. The proteases that produced the longer peptides (such as pro- $\gamma$ -MSH, adrenocorticotrophic hormone (ACTH), joining peptide (JP), and  $\beta$ -lipotropin (LPH) in corticotropes) or the shorter peptides (such as  $\alpha$ -MSH,  $\gamma$ -MSH, and  $\beta$ -endorphin in melanotropes and hypothalamic pro-opiomelanocortin (POMC) neurons) were of special interest. Establishing primary rat

intermediate pituitary cultures seemed to be a convenient way to characterize the production of  $\alpha$ -MSH from what was then known as pro-ACTH. In order to study secretion, it was important to culture cells in a serum-free medium. However, it soon became obvious that serum contained a factor that was essential for the conversion of  $\alpha$ -MSH-Gly into amidated  $\alpha$ -MSH. Antibody specific for amidated  $\alpha$ -MSH was key in realizing that otherwise healthy pituitary cells maintained in a serum-free medium performed all of the processing steps required for generating active  $\alpha$ -MSH, including proteolytic cleavage and acetylation, except for amidation (Glembotski *et al.* 1983, Eipper *et al.* 1983a). In order to identify the serum factor(s) required for amidation, we turned to the newly developed enzyme assay to take a closer look at the amidating enzyme.

As a first step, secretory granules were purified from rat and bovine anterior, intermediate, and neural pituitary;  $\beta$ -endorphin immunoreactivity served as a convenient marker for granule fractions (Eipper *et al.* 1983b). Using the assay developed by Bradbury and coworkers, amidation activity could be detected in the secretory granules from all three regions (Bradbury *et al.* 1982). A simple experiment (determining the pH optimum) proved to be key in determining the cofactor requirements of the enzyme. Activity was greatly reduced in citrate and phosphate buffers, which can chelate metals, compared to

sulfonic acid buffers. Subsequent experiments confirmed an essential role for copper (Eipper *et al.* 1983b). Similar to other copper-dependent enzymes involved in redox reactions requiring molecular oxygen, the amidation reaction was dependent on oxygen availability. The remarkable similarity of the amidation reaction to that of dopamine  $\beta$ -monooxygenase (DBM), which converts dopamine to norepinephrine in a copper-, molecular oxygen-, and ascorbate (vitamin C)-dependent manner, suggested that ascorbate might provide the reducing equivalents essential for peptide amidation. As predicted, ascorbate was a potent stimulator of peptide amidation catalyzed by PAM (Eipper *et al.* 1983b). Knowing the cofactors required for its catalytic activity, we were not surprised that simply adding ascorbate to our serum-free medium allowed melanotropes to produce amidated  $\alpha$ -MSH. PAM (EC 1.14.17.3) and DBM (EC 1.14.17.1) were thought to form a family of related copper-dependent monooxygenases; this meant that the detailed structural and mechanistic studies carried out on DBM (Stewart & Klinman 1988) could guide studies of PAM.

## What have we learned about PAM since its identification?

### Structure of the PAM gene

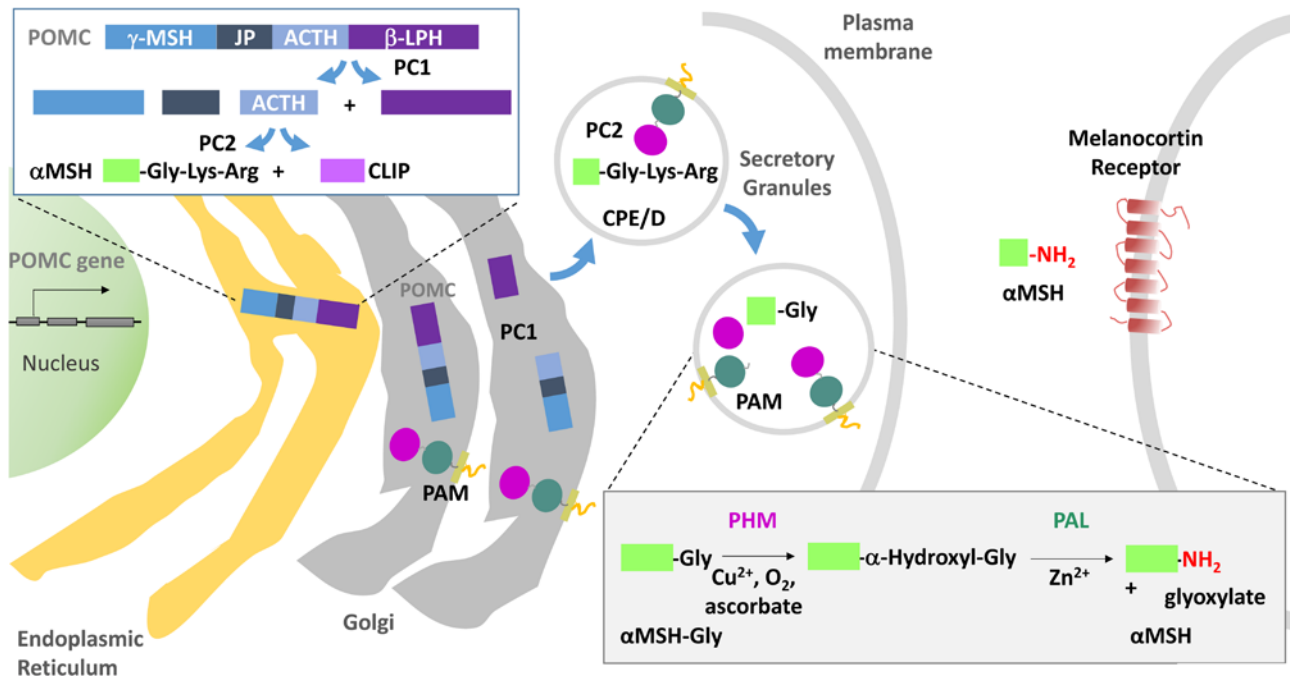
The flurry of activity that ensued centered on cloning the gene-encoding PAM (Fig. 1). The protein was purified from bovine pituitaries and used to generate antibodies (Murthy *et al.* 1986, Eipper *et al.* 1987). Having antibodies allowed identification of a cDNA-encoding PAM using a phage expression library generated from bovine intermediate pituitary RNA (Eipper *et al.* 1987). At the same time, a cDNA-encoding PAM was cloned from *Xenopus laevis* skin (Mizuno *et al.* 1987, Ohsuye *et al.* 1988). Analysis of the protein encoded by the PAM cDNA delivered a few surprises. As expected, a cleaved signal peptide was found, allowing the entry of PAM into the secretory pathway lumen. The cDNA encoded a protein more than twice the size expected. Although the enzyme purified from the pituitary was soluble, the cDNA encoded what was predicted to be a type 1 integral membrane protein; its single membrane-spanning domain was followed by a short stretch of hydrophilic residues predicted to reside in the cytoplasm. Several pairs of basic amino acids (recognition sites for prohormone convertase-like endoproteases) were also present in the intraluminal part of the PAM protein. Several questions arose: Why would an enzyme catalyzing amidation of bioactive peptides include a transmembrane

domain? Why did it include endoproteolytic cleavage sites and how did they affect its processing and activity? Efforts spanning over two decades have unraveled the answers to some of these puzzling questions.

### A PAL for PAM

It was soon discovered that the peptidyl- $\alpha$ -hydroxyglycine  $\alpha$ -amidating lyase (PAM) cDNA encoded two enzymatic domains, both of which were necessary to yield an amidated peptide (Fig. 2). Formation of an  $\alpha$ -hydroxyglycine intermediate by the stereo-specific hydroxylation of the glycine-extended peptide precursor was proposed as the first step in the reaction mediated by PAM (Young & Tamburini 1989). Although the second step of this reaction, cleavage of the N–C bond to yield amidated product is spontaneous in alkaline pH, it is impeded in the acidic environment of secretory granules. The stability of synthetic peptides terminating with a COOH-terminal  $\alpha$ -hydroxyglycine residue was shown to decline at pH values above 6, with half-lives of 8 h at pH 7.4 (Bundgaard & Kahns 1991). An enzyme catalyzing N–C bond cleavage was identified in bovine neurointermediate pituitaries; it was found that the bovine PAM precursor also contained this enzymatic activity. Thus, the PAM gene encodes two enzymatic domains that function sequentially to generate amidated peptides: peptidylglycine  $\alpha$ -hydroxylating monooxygenase (PHM; EC 1.14.17.3) and peptidyl- $\alpha$ -hydroxyglycine  $\alpha$ -amidating lyase (PAL; 4.3.2.5) (Katopodis *et al.* 1990, Perkins *et al.* 1990). Studies with purified PAL protein revealed its pH optimum to be in the acidic range and its dependence on zinc (Eipper *et al.* 1991).

Apart from endoproteolytic processing, functionally different forms of PAM can also be generated by alternative splicing. The longest isoform (PAM-1) (Fig. 3A) contains the two enzymatic domains, a transmembrane domain and a cytosolic domain, with an endoprotease-sensitive linker region between PHM and PAL. This endoprotease-sensitive region is not included in the PAM-2 isoform; as a result, PHM and PAL are rarely separated by cleavage. A third major isoform (PAM-3) lacks both the endoproteolytic cleavage site and the transmembrane domain, allowing soluble, bifunctional PAM to be secreted. PAM expression is not limited to neuroendocrine tissues; PAM is expressed at widely varying levels in almost all mammalian cell types, with significant expression in airway epithelium, ependymal cells in the brain, endothelial cells, and adult atrium as well as the brain and pituitary (Eipper *et al.* 1988, Oldham *et al.* 1992, Schafer *et al.* 1992). PAM expression is



**Figure 2**

POMC processing: amidation of  $\alpha$ -MSH. Following the cotranslational removal of its N-terminal signal sequence, POMC moves through the Golgi complex. As luminal pH begins to fall and prohormone convertase 1 (PC1) is activated, the first POMC cleavage produces ACTH biosynthetic intermediate and  $\beta$ -LPH. Subsequent cleavages (upper left box), which occur largely in maturing secretory granules, separate JP from ACTH; the C-terminus of JP can be amidated. Melanotropes, which express both PC1 and PC2, cleave ACTH(1–39) to produce an N-terminal fragment (precursor to  $\alpha$ -MSH) and CLIP (corticotropin-like intermediate lobe peptide). The production of  $\alpha$ -MSH requires a carboxypeptidase, PAM, and an *N*-acetyltransferase (not shown). The sequential actions of PHM and PAL on  $\alpha$ -MSH-Gly are illustrated (lower right box).

developmentally regulated; isoform-specific regulation is especially apparent in heart and neural tissues, suggesting that different isoforms have distinct functions (Braas *et al.* 1989, Stoffers *et al.* 1989, 1991, Eipper *et al.* 1992). Both in neurons and in endocrine cells, soluble PHM and PAL are secreted along with neuropeptides and peptide hormones upon stimulation; PHM and PAL can be detected in serum and cerebrospinal fluid (Mains *et al.* 1985, Wand *et al.* 1985).

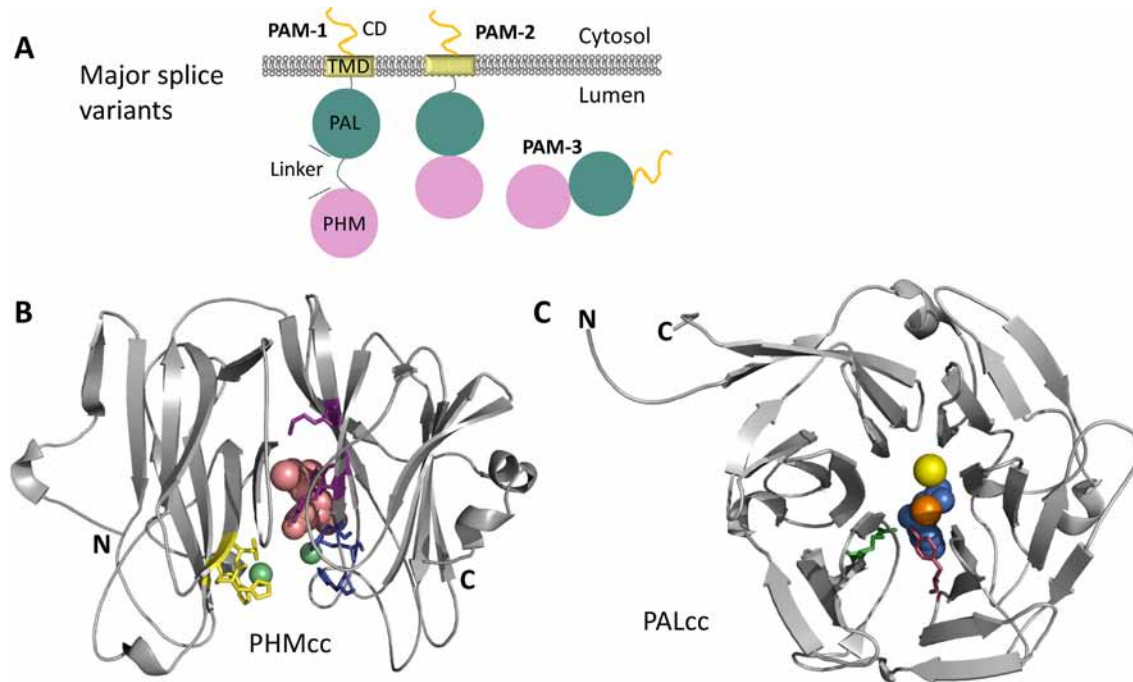
### Cellular requirements for PAM activity

PAM activity requires the delivery of copper and ascorbate to the lumen of the secretory pathway. Cells devote considerable energy to compartmentalizing and tightly regulating the flow of copper, a transition metal that can participate in free radical formation. Copper transporters deliver copper to intracellular copper chaperones such as COX17 and Atox1, which deliver copper to specific acceptor proteins. For the secretory pathway, two P-type ATPases, ATP7A and ATP7B, receive copper from Atox1 and transport it into the lumen (Prohaska 2008). No copper chaperones have been identified in the secretory pathway

lumen, and it has been suggested that PHM is metallated directly by the P-type ATPases (El Meskini *et al.* 2001, Otoikhian *et al.* 2012). Although most organisms, including rodents, synthesize ascorbate from glucose, humans lack this ability and must acquire it from their diet. Two Na<sup>+</sup>-dependent ascorbate transporters (SVCT1 and SVCT2) expressed in intestinal epithelial cells and neuroendocrine cells, respectively, import ascorbate into cells (Buzle & Hediger 2012); neuroendocrine cells lack gulonolactone oxidase and must use SVCT2 to obtain ascorbate from the circulation. It is not known how ascorbate enters the secretory pathway lumen, but it is maintained in the reduced state by the action of cytochrome *b*<sub>561</sub>, which shuttles electrons across the secretory pathway membrane (Asada *et al.* 2005, Iliadi *et al.* 2008).

Another important factor necessary for amidation is the low pH of the secretory pathway lumen. Both PHM and PAL exhibit optimal activity under acidic conditions. Intraluminal pH is maintained by the proton-pumping activity of the multisubunit vacuolar or V-ATPase. In addition to its role in acidification, the V-ATPase plays a role in secretory granule biogenesis through its role in sorting of vesicular cargo (Sobota *et al.* 2009). This



**Figure 3**

Major PAM splice variants and PHM and PAL catalytic core structures. (A) The major splice variants (isoforms) of PAM are shown. Although PAM-1 and PAM-2 are type 1 integral membrane proteins, PAM-3 is a soluble, secreted protein. (B) Crystal structure of rat PHM (PDB identifier: 1OPM) in the oxidized state bound to a substrate, *N*-acetyl-3,5-diiodotyrosylglycine (shown in pink), rendered here using PyMOL; bound copper, green spheres. The copper-binding site in the N-terminal domain of PHM ( $\text{Cu}_H$ , in yellow) is separated from the copper-binding site in the C-terminal domain ( $\text{Cu}_M$ , in blue) by an 11 Å solvent-filled cleft; the peptidylglycine substrate and molecular oxygen bind near  $\text{Cu}_M$ . Other essential catalytic residues involved in substrate binding ( $\text{R}^{240}$ ,  $\text{Y}^{318}$ , and  $\text{M}^{320}$ ) are shown in purple. After considering many mechanisms, quantum mechanical tunneling is thought to facilitate electron transfer from the  $\text{Cu}_H$  site, through solvent, to the  $\text{Cu}_M$  site (Francisco *et al.* 2004, Klinman 2006, McIntyre *et al.* 2010). (C) The structure of PAL (PDB identifier: 3FW0) crystallized in the presence of mercury ion (orange) instead of zinc to capture the binding of a nonpeptide substrate ( $\alpha$ -hydroxyhippuric acid, in blue) is shown. The six-bladed  $\beta$ -propeller structure of PAL positions Zn near a key Tyr residue (shown in purple) and a key Arg residue (shown in green). The structurally important calcium ion is depicted as a yellow sphere (Chufan *et al.* 2009).

proton pump also relays information about amino acid levels in the interior of lysosomes to mammalian target of rapamycin (mTOR), a cytosolic Ser/Thr kinase that serves as a master energy regulator (Jewell *et al.* 2013). It is intriguing to consider a role for PAM in signaling nutritional status information from distal parts of the secretory pathway (glycine levels, for example) to mTOR through the V-ATPase.

### Crystal structures and insights into the mechanism of peptide amidation

As mentioned previously, the cofactor requirements for the PHM domain of PAM are strikingly similar to those of DBM. The similarity extends to their amino acid sequences, with about 30% identity in the catalytic cores of the two molecules (Prigge *et al.* 1997). Another member of this small family of copper-dependent monooxygenases is the endoplasmic reticulum-localized monooxygenase X,

predicted to hydroxylate an unidentified hydrophobic substrate (Xin *et al.* 2004). Of these three monooxygenases, PHM is the only one with a solved crystal structure (Fig. 3B and C), providing key insights into reaction mechanism (Prigge *et al.* 1997, 1999, 2004). Only small structural differences were observed when the protein was crystallized in the reduced, oxidized, or substrate-bound form. The catalytic core of PHM has two domains, each composed primarily of eight antiparallel  $\beta$ -strands (Fig. 3B). Resembling a jelly roll motif, each domain has a hydrophobic interior held together by disulfide linkages; the two domains are linked by a single polypeptide chain. Each domain has one copper binding site: the three His residues of the  $\text{Cu}_H$  site are in the N-terminal domain; the two His and one Met of the  $\text{Cu}_M$  site are in the C-terminal domain. An 11 Å solvent-exposed hydrophilic cleft separates  $\text{Cu}_H$  from  $\text{Cu}_M$ ; in two single-electron steps, both sites are reduced. Molecular oxygen binds to the  $\text{Cu}_M$  site, with the peptidylglycine substrate bound close by;

glycine and D-alanine are the only amino acids that can be accommodated, and there is no indication that the distance between Cu<sub>H</sub> and Cu<sub>M</sub> varies during the reaction. The active site of PHM can accommodate large substrates (e.g., ubiquitin and selected immunoglobulin heavy chains) as well as fatty acyl glycines and other nonpeptide substrates (Wilcox *et al.* 1999, Chew *et al.* 2005, Skulj *et al.* 2014).

The crystal structure of the catalytic core of PAL was solved about 10 years later (Fig. 3C): it is a six-bladed β-propeller, with long loops extending from the propeller surface (Chufan *et al.* 2009). The central cavity houses a calcium ion required for structural integrity and a zinc ion required for catalytic activity. The peptidyl-α-hydroxyglycine substrate binds close to the zinc, which is coordinated by three His residues. Well-conserved tyrosine (Tyr<sup>654</sup>) and arginine (Arg<sup>706</sup>) residues play a critical role in catalysis (De *et al.* 2006, Chufan *et al.* 2009). As in the catalytic core of PHM (PHMcc), disulfide linkages play an essential role in the structural integrity of PAL. The N- and C-termini of PAL are positioned close to each other, and its C-terminus is tethered to the membrane; the unique geometry of the β-propeller structure positions PHM close to the membrane to receive copper and ascorbate from transmembrane P-type ATPases and cytochrome *b*<sub>561</sub>.

With the ability to produce active PHMcc and site-directed mutants in mammalian cells, the unique properties of the two essential copper-binding sites and the effects of pH on PHM were determined (Siebert *et al.* 2005, Evans *et al.* 2006, Jaron *et al.* 2002, Kline *et al.* 2013). The detailed mechanistic studies carried out previously on purified dopamine β-monooxygenase guided similar studies on PHMcc (Stewart & Klinman 1988). The initial focus was on the 11 Å solvent-filled gap separating the two essential copper-binding sites: Cu<sub>H</sub>, which has the properties expected of an electron transfer site, and Cu<sub>M</sub>, which binds O<sub>2</sub> and is adjacent to the peptidylglycine substrate, are both essential. Current evidence indicates that the two copper domains are directly coupled through a solvent bridge that facilitates redox and catalysis, a remarkable level of precision for a solvent-accessible active site (Francisco *et al.* 2002, Jaron *et al.* 2002, Bauman *et al.* 2006). The effects of temperature on intrinsic isotope effects and modeling studies have led to the consensus that PHM-dependent C(α)–H bond activation is dominated by quantum mechanical tunneling and tightly coupled with oxygen activation (Francisco *et al.* 2002, 2004, Klinman 2006, McIntyre *et al.* 2010). Efforts to develop inhibitors of PHM and PAL continue (Merkler *et al.* 2008, Langella *et al.* 2010). Disulfiram, a copper chelator, reduces levels

of amidated peptides in rat pituitary and cerebral cortex, presumably by inhibiting PHM activity (Mueller *et al.* 1993). A mechanism-based suicide inhibitor, 4-phenyl-3-butenic acid, has been shown to inhibit PHM activity *in vivo* (Mueller *et al.* 1999). Other potent inhibitors include hippurate analogs and N-substituted homocysteine analogs (Erion *et al.* 1994, Merkler *et al.* 2008).

## More than just an enzyme: a multitasking protein

### Evolutionary distribution of PAM

Amidated peptides are widespread, with roles in organisms with simple nervous systems, such as *Hydra* and *Aplysia* (Fujisawa *et al.* 1999, Grunder & Assmann 2015). Furthermore, PAM has been characterized in sea anemone, fly, and flatworm (Kolhekar *et al.* 1997, Williamson *et al.* 2000, Han *et al.* 2004, Mair *et al.* 2004). Although it was assumed that PAM co-evolved with the nervous system, recent studies suggest otherwise. A phylogenetic study identified PAM-like sequences in non-neural organisms such as *Amphimedon queenslandica*, a sponge, and *Trichoplax adhaerens*, a placozoan. Neuropeptide-like sequences have been found in the *Trichoplax* genome, and immunoreactivity to amidated peptides has been demonstrated in cells of *Trichoplax* (Jekely 2013, Smith *et al.* 2014). Perhaps more surprising was finding PAM-like sequences in several green algal genomes; the presence of PAM in *Chlamydomonas reinhardtii*, *Volvox carteri*, and *Ostreococcus tauri* raised the possibility that PAM evolved before the divergence of plants and animals (Attenborough *et al.* 2012). Active PAM enzyme has been demonstrated in the unicellular green alga, *C. reinhardtii*, raising the question of ancestral function (Kumar *et al.* 2015). Although amidated peptides have not been identified in green algae, the evolutionary conservation of components essential for PAM activity (copper homeostatic machinery, ascorbate synthesis, the V-ATPase) in eukaryotes certainly supports the possibility of amidation reactions occurring in these nonneural organisms. It is also possible that PAM amidates nonpeptide substrates in these organisms or has a completely novel role distinct from those discovered in mammals.

### The cytosolic domain of PAM

Although a cytosolic domain is not essential for PHM or PAL activity, the PAM gene in *C. reinhardtii* encodes a protein of identical topology (Kumar *et al.* 2015). Unlike soluble granule content proteins, granule membrane

proteins are not secreted; after exocytosis and insertion into the plasma membrane, membrane PAM can be retrieved, traveling through the endocytic pathway in a regulated manner. In *Drosophila*, gene duplication is thought to have produced separate genes encoding soluble PHM, soluble PAL, and membrane PAL.

Unlike the catalytic domains of PAM, its cytosolic domain is protease sensitive; the specific activity of PHM is increased following removal of the cytosolic domain. The cytosolic domain of PAM plays an essential role in its trafficking in mammalian cells; although soluble PHM and soluble PAL accumulate in secretory granules, PAM lacking its cytosolic domain accumulates on the plasma membrane (Tausk *et al.* 1992, Milgram *et al.* 1993). Furthermore, an epitope-tagged protein consisting of the transmembrane and cytosolic domains of PAM localizes to the *trans*-Golgi network and secretory granules, indicating that it contains key trafficking information (El Meskini *et al.* 2001).

The cytosolic domain (86 residues in mammalian PAM) is unstructured and contains no characteristic motifs. It is sensitive to proteolytic degradation, a feature characteristic of intrinsically disordered proteins involved in signaling, where the signal must be turned off just as rapidly as it is turned on (Rajagopal *et al.* 2009). Unstructured domains are often hubs of protein/protein interactions, and multiple PAM cytosolic domain interactors have been identified. Kalirin and Trio, members of the Rho GDP/GTP exchange factor (Rho-GEF) family, play roles in cytoskeletal control and the maintenance of synapses. Uhmk1, a Ser/Thr kinase, phosphorylates the C-terminus of PAM, and Rassf9, a member of the Ras-association domain family, plays a role in epidermal homeostasis (Alam *et al.* 1996, Lee *et al.* 2011). More recently, PAM has been shown to interact with the adaptor protein-1 (AP-1) complex (Bonnemaison *et al.* 2015). The AP-1 complex belongs to a family of endosomal and secretory granule coat proteins that link cargo proteins to clathrin; both PAM and Atp7a, the P-type ATPase that transports copper into the *trans*-Golgi network, interact with AP-1. Diminished AP-1 function affects PAM and Atp7a trafficking through the endocytic pathway. Antibodies to amidated (18 kDa fragment) and nonamidated POMC products allowed development of an assay to measure PAM activity in cells following manipulation of copper levels; cells with reduced AP-1 levels were more sensitive to copper restriction than control cells (Bonnemaison *et al.* 2014, 2015).

Mass spectrometry and two-dimensional gel electrophoresis revealed multiple phosphorylation sites

(Ser and Thr) in the cytosolic domain of PAM (Rajagopal *et al.* 2009). Phosphomimetic mutants revealed a role for phosphorylation in PAM trafficking through various biosynthetic and endocytic compartments. Development of an antibody specific to the C-terminal domain of PAM allowed identification of the various proteolytic fragments generated. Prohormone convertase-mediated cleavage of PAM in the secretory granules generates a slightly larger transmembrane/cytosolic domain fragment than  $\alpha$ -secretase-mediated cleavage of PAM. Both transmembrane and cytosolic domain fragments can be cleaved by  $\gamma$ -secretase; this intramembrane cleavage produces a short-lived, soluble 16-kDa fragment (soluble fragment of PAM cytosolic domain (sf-CD)) that accumulates in the nucleus (Rajagopal *et al.* 2009, 2010). Upregulation of PAM-1 levels in a corticotrope cell line caused alterations in cell morphology and a reduction in secretagogue-responsiveness, and altered the expression of a subset of genes, including copper chaperones, aquaporin 1 and Slpi, a protease inhibitor (Francone *et al.* 2010). In addition to its enzymatic role, these studies indicate that PAM relays signals from the lumen of the secretory/endocytic pathway to the nucleus, in a manner similar to other type 1 integral membrane proteins in the endoplasmic reticulum such as sterol regulatory element binding protein (SREBP) and activating transcription factor 6 (ATF6) (Rajagopal *et al.* 2012).

#### Lessons learned from knockout and heterozygous mice

Mice lacking both copies of *Pam* show no detectable peptide amidation activity and do not survive past mid-gestation, dying at e14.5–e15.5 (Czyzyk *et al.* 2005). The *Pam* null embryos display severe edema, cardiac malformations, and poor vasculature compared with wild-type littermates. However, the heterozygous animals have half the wild-type level of PAM activity and are viable. They showed only minor changes in the levels of the amidated peptides tested, suggesting that the reduced levels of PAM in PAM<sup>+/-</sup> mice were sufficient to catalyze the amidation reaction (Czyzyk *et al.* 2005).

As they age, PAM<sup>+/-</sup> mice display increased adiposity compared to age-matched wild-type littermates. Glucose metabolism, which is regulated in part by amidated peptides, is impaired in older PAM heterozygous mice compared to wild-type controls (Czyzyk *et al.* 2005). The PAM<sup>+/-</sup> animals demonstrate other behavioral deficits: they are unable to regulate body temperature, show increased anxiety-like behavior, and are more susceptible

to seizures compared with wild-type mice. Moreover, wild-type mice maintained on a copper restricted diet mimic many of the behavioral deficits observed in PAM<sup>+/-</sup> mice, and supplemental dietary copper reverses some of the behavioral defects observed in PAM<sup>+/-</sup> animals (Bousquet-Moore *et al.* 2009, 2010). These data suggest that altered copper homeostasis contributes to the behavioral defects observed in PAM heterozygous mice. A role for PAM in copper homeostasis had not previously been suspected.

### Sensory roles of PAM

Comparative genomic analyses suggest that cuproproteins evolved following oxygenation of the earth; thus, PAM belongs to a small set of cuproenzymes with primitive roles predating multicellularity. As one of the few proteins dependent on a steady supply of copper and molecular oxygen for activity, PAM is well positioned to function as a sensor for these critical factors. As mentioned previously, studies with mice lacking one copy of *Pam* and signaling mediated by PAM sf-CD suggest an involvement in copper sensing. Additional support for this hypothesis comes from studies using corticotrope cell lines. Depleting or overloading these cells with copper alters the trafficking of PAM through the secretory and endocytic pathways but does not lead to its degradation, as in the case of other cuproproteins (De *et al.* 2007).

The absolute dependence of the amidation reaction on molecular oxygen has been known for a long time; however, whether physiologically relevant changes in oxygen levels affected PHM activity was not known. A recent study shows that the ability of PAM to produce amidated products is as sensitive to changes in oxygen level as the prolyl hydroxylases that control the stability of hypoxia-inducible factor (HIF), a key oxygen-signaling protein. The speed and sensitivity with which PAM activity responds to hypoxia suggest a novel, paracrine signaling mechanism that could operate during oxygen homeostasis *in vivo* (Simpson *et al.* 2015).

The pH gradient in the lumen of the secretory pathway plays an essential role in the biosynthesis and processing of proteins. Enzymes like PAM, which operate late in the secretory pathway, display acidic pH optima; the pH gradient in the luminal compartment often dictates which biosynthetic enzymes are active and which processing steps can occur. The protease-sensitive linker region that connects the catalytic core of PHM to the catalytic core of PAL contains a cluster of histidine residues (His–Gly–His–His) that confer pH sensitivity (Vishwanatha *et al.* 2014).

PAM lacking these histidine residues (Ala–Gly–Ala–Ala) is handled differently in corticotrope cell lines; instead of being recycled, endocytosed PAM lacking this His cluster is rapidly degraded. The  $\gamma$ -secretase-mediated production of PAM sf-CD is largely eliminated, and the morphological changes associated with PAM expression no longer occur.

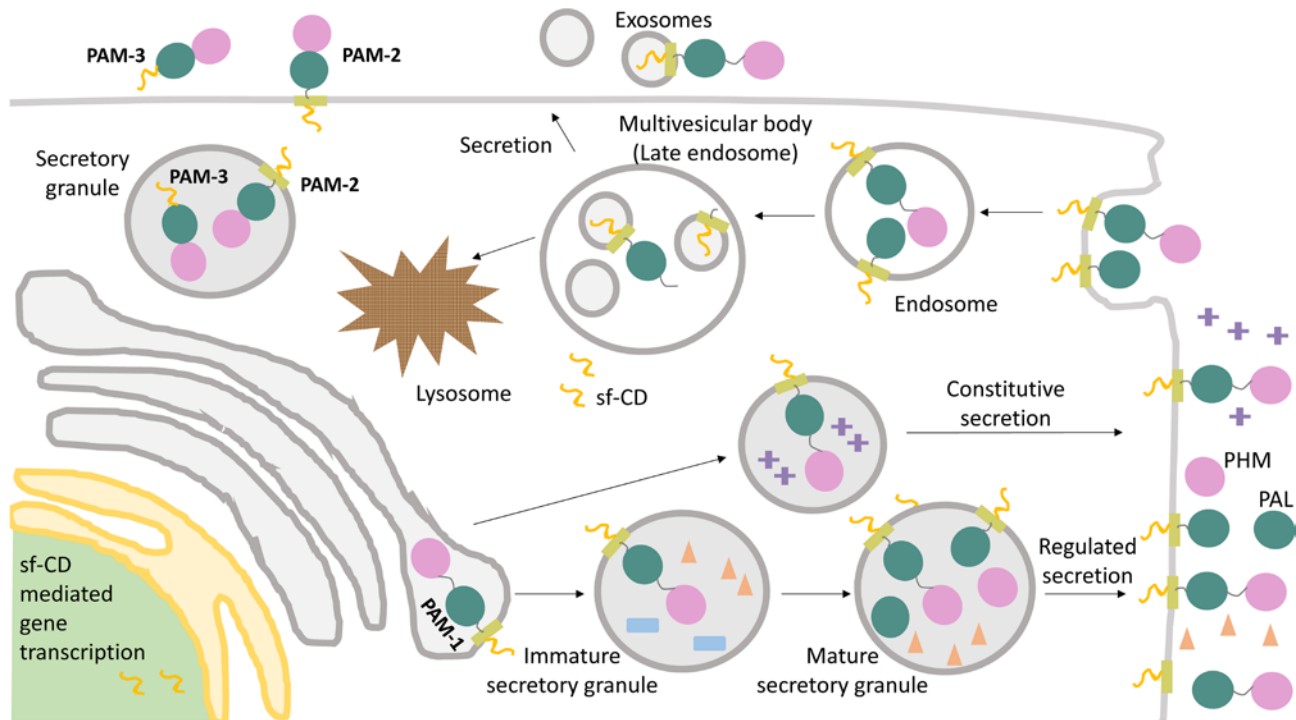
### What the future holds for PAM biology

The involvement of PAM in sensing environmental signals and generating factors involved in transcriptional regulation led us to expand our studies beyond the role of PAM as an amidating enzyme. Thus, there has been a paradigm shift in our understanding of this multitasking protein. We see understanding the role of PAM in non-neural tissues and organisms as key to uncovering all of the functions of this ancient, evolutionarily conserved protein. For instance, it will be interesting to determine the significance of abundant levels of PAM in the atrium, where it comprises about 1% of the total proteome (O'Donnell *et al.* 2003).

Mutations in the luminal copper-transporting P-type ATPase, ATP7A, cause Menkes disease, a copper metabolism disorder characterized by neurodegeneration, mental retardation, connective tissue abnormalities, and early childhood lethality. Several cuproenzymes, including PAM, are affected by the loss of functional ATP7A. Using the mottled-brindled mouse, an animal model for Menkes disease, normal levels of PAM expression but reduced levels of amidated peptides such as joining peptide,  $\alpha$ -MSH, and cholecystokinin were shown, suggesting that alterations in PAM activity might contribute to some of the phenotypes observed in these patients (Stevenson *et al.* 2003). Altered PAM levels and activity have also been reported in the cerebrospinal fluid of multiple sclerosis and post-polio syndrome patients (Tsukamoto *et al.* 1995, Gonzalez *et al.* 2009). The ability to assay PAM activity in serum makes it an attractive biomarker candidate (Gaier *et al.* 2014).

During its endocytic trafficking, PAM enters the intraluminal vesicles that characterize multivesicular bodies (Fig. 4); the fusion of multivesicular bodies with the plasma membrane results in release of these intraluminal vesicles, which are then known as exosomes (Rajagopal *et al.* 2010). As they can be isolated from a variety of sources such as saliva, serum, and urine, exosomes are being explored as a diagnostic tool (Simpson *et al.* 2009). PAM was identified in exosomes derived from human saliva and prostate cancer cells (Gonzalez-Begne





**Figure 4**

PAM trafficking. Membrane PAM (PAM-1, PAM-2) travels through both the biosynthetic and endocytic pathways; soluble PAM (PAM-3) is efficiently packaged into secretory granules and secreted. Cleavage of PAM in the biosynthetic pathway involves the prohormone convertases and cleavages at pairs of basic residues in the linker region between PHM and PAL and immediately following PAL. Cleavages on the cell surface and in the endocytic pathway involve  $\alpha$ -secretase and  $\gamma$ -secretase. Phosphorylation of its cytosolic domain affects the ability of PAM to move from the limiting membrane of multivesicular bodies (MVBs) into the intraluminal vesicles. Endocytosed PAM can be returned to the trans-Golgi network, for re-entry into secretory granules, or degraded in lysosomes.

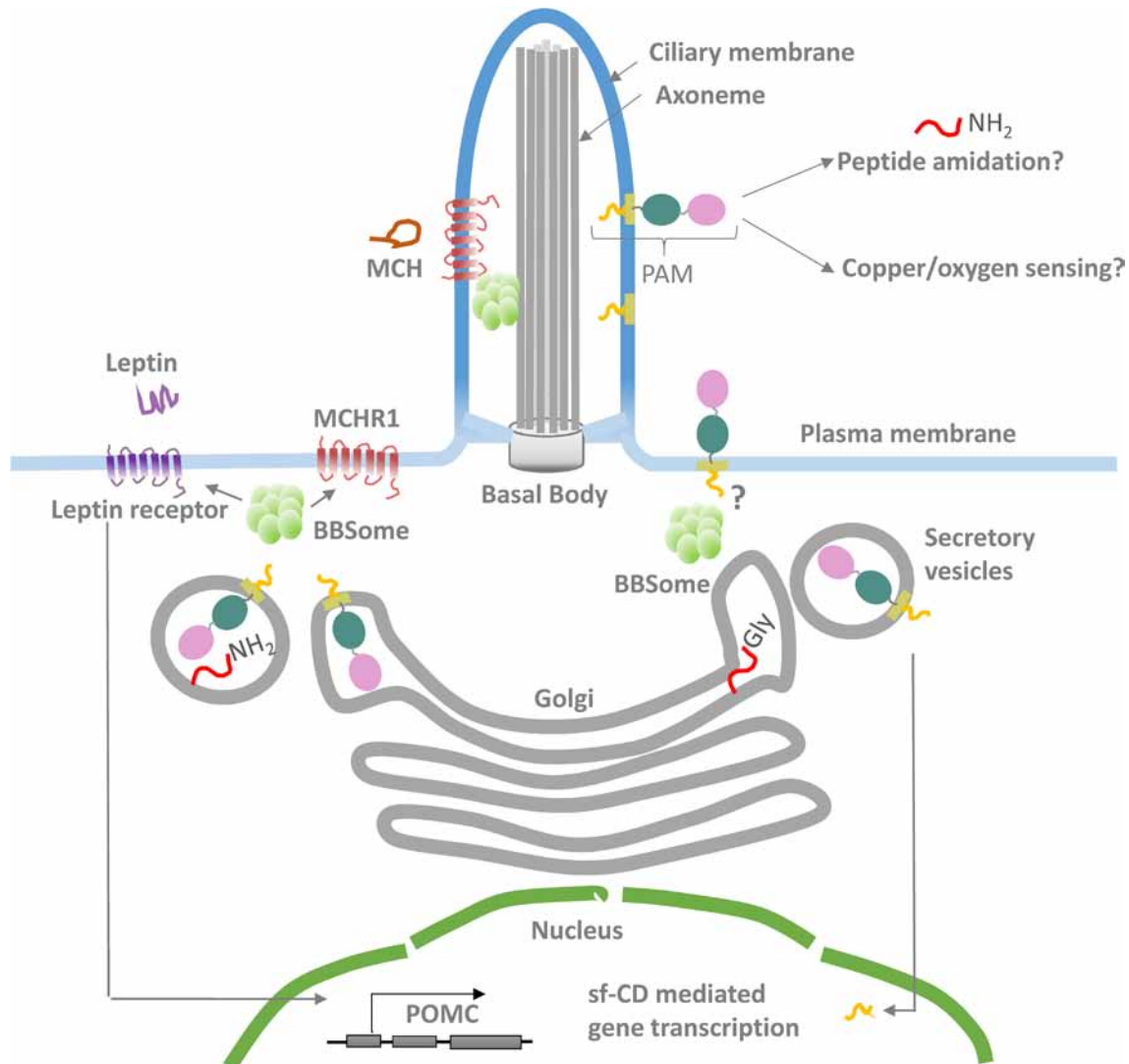
*et al.* 2009, Minciocchi *et al.* 2015). It will be interesting to determine if PAM-containing exosome release is differentially regulated, especially during genetic or metabolic alterations.

#### PAM, cilia, and POMC in obesity

A close look at the phylogenetic distribution of PAM suggested a strong correlation with the presence of cilia, leading us to explore the presence of PAM in this signaling organelle (Kumar *et al.* 2015). PAM localizes to primary and motile cilia in mammalian cells and to the motile cilium of *C. reinhardtii*, a unicellular eukaryote, suggesting an important, evolutionarily conserved role for PAM in this organelle (Fig. 5). Cilia are tiny hairlike, microtubule-based organelles that extend from the cell surface of almost all mammalian cells. Primary cilia are critical sensory and signaling structures, important for sensing and responding to environmental stimuli. Multiple motile cilia in unicellular eukaryotes and tracheal and ependymal cells play additional roles in cell motility and fluid propulsion. Acting as tiny reaction

chambers, cilia compartmentalize signaling pathways and proteins. Ciliary protein entry and exit is tightly regulated by specialized trafficking pathways. Only proteins destined for the cilium are recognized by the intraflagellar trafficking machinery. Ciliary dysfunction affects a number of different tissues, including the kidney, heart, and eyes, resulting in disorders collectively known as ciliopathies (Fliegauf *et al.* 2007).

The significance of POMC in energy homeostasis is clear: loss of POMC or melanocortin receptors, MC3R and MC4R, causes an obesity phenotype in mice, and mutations in *POMC* result in obesity in humans (Hinney *et al.* 1998, Yaswen *et al.* 1999, Challis *et al.* 2004). The leptin/melanocortin system plays a central role in regulating feeding behavior and obesity. Leptin, a satiety hormone, is secreted by adipocytes in proportion to body fat and acts on POMC and neuropeptide Y (NPY) neurons in the hypothalamus to reduce food intake and promote thermogenesis. Increased POMC production as a result of leptin signaling leads to a rise in  $\alpha$ -MSH and  $\beta$ -MSH levels; their binding to melanocortin receptors regulates energy balance (Sen Gupta *et al.* 2009).

**Figure 5**

Model of PAM function in cilia. PAM localizes to cilia, where it may play roles in peptide amidation and sensing of copper or oxygen. Ciliary PAM may also be subjected to cleavages leading to the release of sf-CD and changes in gene expression. It is not known if the BBSome is essential for PAM trafficking into the ciliary compartment. However, trafficking of the MCHR1 into the cilium requires the BBSome, and leptin receptor localization is affected by loss of BBS components. The leptin/melanocortin system is critical for establishing energy homeostasis. Leptin binding to its receptor leads to upregulation of POMC and satiety responses. It is not known if PAM also plays a role in energy balance in POMC neurons.

A number of recent studies have shown that cilia play an important role in energy homeostasis. Several G protein coupled receptors (GPCRs), including melanin-concentrating hormone receptor 1 (MCHR1), localize to cilia (Fig. 5), suggesting that G-protein signaling might occur in cilia (Berbari *et al.* 2008a). Furthermore, this localization is perturbed in mice lacking components of the BBSome (Berbari *et al.* 2008b). The BBSome, a multiprotein complex composed of seven Bardet-Biedl syndrome proteins, is implicated in membrane protein trafficking and ciliary biogenesis (Jin & Nachury 2009). Mutations in these *BBS* genes result in a ciliopathy (BBS)

characterized by mental retardation, retinopathy, and renal defects. Obesity is also observed in these patients and mouse models of the disease, further strengthening the link between energy homeostasis and cilia (Sen Gupta *et al.* 2009).

Leptin signaling has recently been linked to the cilium: hyperphagic BBS mutant mice have increased levels of circulating leptin and are resistant to exogenous leptin treatment, even when weight matched with control animals (Rahmouni *et al.* 2008, Seo *et al.* 2009). Although the leptin receptor has not been localized to cilia, it interacts with components of the BBSome and loss of BBS

components causes mislocalization of the leptin receptor in large vesicles (Seo *et al.* 2009). Further support for the importance of cilia in energy regulation comes from the conditional knockout of intraflagellar transport proteins, which leads to the loss of primary cilia in adult mice. Ablation of cilia only on POMC neurons causes mice to become hyperphagic and obese; a model proposing the disruption of a feedback mechanism between leptin and somatostatin signaling that regulates satiety responses has been proposed (Davenport *et al.* 2007, Satir 2007). These data are interesting, especially in light of the localization of PAM to cilia. It is currently not known if PAM localizes to cilia in POMC neurons, or if this localization is BBSome mediated (Fig. 5). It is also unclear if PAM performs a catalytic or signaling role in the cilium; however, there is certainly a link between PAM and energy metabolism, as seen in the increased adiposity phenotype in older PAM<sup>+/-</sup> mice. Two genome-wide association studies linked PAM to altered insulinogenic index and susceptibility to type 2 diabetes in Finnish and Icelandic populations, warranting a closer look at altered energy homeostasis in the context of PAM (Huyghe *et al.* 2013, Steinthorsdottir *et al.* 2014).

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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