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PMEL: A PIGMENT CELL-SPECIFIC MODEL FOR FUNCTIONAL AMYLOID FORMATION

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Abstract

PMEL is a pigment cell-specific protein responsible for the formation of fibrillar sheets within the pigment organelle, the melanosome. The fibrillar sheets serve as a template upon which melanins polymerize as they are synthesized. The PMEL fibrils are required for optimal pigment cell function, as animals that either lack PMEL expression or express mutant PMEL variants show varying degrees of hypopigmentation and pigment cell inviability. The PMEL fibrils have biophysical properties of amyloid, a protein fold that is frequently associated with neurodegenerative and other diseases. However, PMEL is one of a growing number of non-pathogenic amyloid proteins that contribute to the function of the cell and/or organism that produces them. Understanding how PMEL generates amyloid in a non-pathogenic manner might provide insights into how to avoid toxicity due to pathological amyloid formation. In this review we summarize and reconcile data concerning the fate of PMEL from its site of synthesis in the endoplasmic reticulum to newly formed melanosomes and the role of distinct PMEL subdomains in trafficking and amyloid fibril formation. We then discuss how its progression through the secretory pathway into the endosomal system might allow for the regulated and non-toxic conversion of PMEL to an ordered amyloid polymer.

Introduction

Melanosomes are membrane-bound organelles in pigment cells in which melanin pigments are synthesized and stored (Delevoye *et al.*, 2011; Sitaram and Marks, 2012). In vertebrate pigment cells that make predominantly black and brown eumelanins, melanins polymerize within ellipsoidal melanosomes upon intralumenal fibrils or fibrillar sheets that run the length of the organelle (Birbeck, 1963; Moyer, 1966; Seiji *et al.*, 1963). Often referred to as “the melanosome matrix”, it is now clear that these proteinaceous fibrils are composed predominantly – if not entirely – of proteolytic fragments of a single pigment cell-specific protein, PMEL. This remarkable protein – which has been referred to in the literature by many names, including Pmel17, Silver, SILV, gp100, and ME20, or by reference to the apparent molecular mass of its full-length or prominent proteolytic products (p100, p85, p95, p115, etc.) – is initially synthesized as an integral membrane glycoprotein in the endoplasmic reticulum (ER). Through post-translational modifications, proteolytic processing steps and precisely timed oligomerization, the glycoprotein becomes transformed

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into fibrillar structures that laterally assemble into sheets. The fibrils have biophysical features of amyloid, making PMEL a prime model for a class of proteins called functional amyloids (Blanco *et al.*, 2012; Fowler *et al.*, 2007). In this review, we detail the experimental evidence that PMEL underlies the melanosome fibrils and update (relative to an earlier review on the same topic (Theos *et al.*, 2005b)) our current understanding of PMEL processing and trafficking to melanosomes in pigment cells. We focus on the amyloid properties of PMEL and its new role as a model for distinguishing functional from pathological amyloid, discussing those characteristics of PMEL amyloid formation that might underlie its lack of toxicity and how alterations in this pathway might result in pathology.

What is amyloid?

The amyloid fold is a cross β-pleated sheet structure in which the β sheets stack upon themselves in a plane perpendicular to that of the sheets themselves (Dobson, 2003; Greenwald and Riek, 2010). Amyloid is characterized by the formation of stable fibrils that are insoluble in detergents, can be detected by electron microscopy, bind to specific dyes such as thioflavins and Congo Red, and are highly resistant to protease digestion (Chiti and Dobson, 2006). Historically, amyloid formation has been associated with disease, particularly neurodegeneration as observed in Alzheimer Disease (AD) and Parkinson Disease (PD) and the prion-associated spongiform encephalopathies, in which specific proteins misfold into an amyloid conformation (Buxbaum and Linke, 2012); indeed, many proteins can adopt an amyloid fold “by mistake” (Goldschmidt *et al.*, 2010). However, there is a growing body of evidence pointing to an evolutionarily conserved use of the amyloid fold in functional processes such as adaptation to and protection from environmental stresses (Blanco *et al.*, 2012; Iconomidou *et al.*, 2000; Uptain and Lindquist, 2002; Wickner *et al.*, 2004), concentration of biologically active peptides (Maji *et al.*, 2009), organization of extracellular environments (Chapman *et al.*, 2002; Kenney *et al.*, 2002), establishment and maintenance of long-term memory in the central nervous system (Majumdar *et al.*, 2012), and others. It is therefore important to understand (1) what makes some proteins acquire amyloidogenic properties and how this might be associated with disease, (2) the similarities and differences among pathological and functional amyloids, and (3) the conformational changes that direct amyloidogenic proteins towards either a benign or pathological role in cells and organisms.

Fibrils within melanosomes

Melanosomes are generated in the skin and hair within epidermal melanocytes and in the eye within choroidal melanocytes and retinal, iris and ciliary body pigment epithelia. Eye pigment cells retain their melanosomes, and a body of evidence indicates that melanosome formation in the retinal pigment epithelia is largely limited to pre- and early post-natal development (Lopes *et al.*, 2007). By contrast, melanosomes in skin melanocytes are constantly generated and transferred to neighboring keratinocytes in a process that is poorly understood (Van Den Bossche *et al.*, 2006). Melanosomes in skin melanocytes that generate predominantly red and yellow pigments, or pheomelanins, are fundamentally distinct in content and morphology from those that make predominantly black and brown eumelanins, and harbor neither PMEL nor intralumenal fibrils (Furumura *et al.*, 1998; Moyer, 1966; Simon *et al.*, 2009). This review will thus focus only on melanosomes that generate predominantly eumelanins in the eye and skin.

Melanosome formation can be divided into four stages of development (Figure 1). Stage I and II melanosomes, or premelanosomes, lack pigment. The onset of melanin synthesis allows for the conversion of stage II melanosomes to pigmented stage III and ultimately

stage IV melanosomes (Seiji *et al.*, 1963). This onset correlates with the delivery of melanogenic enzymes, such as Tyrosinase (TYR) (Hirobe, 1982; Huizing *et al.*, 2001; Novikoff *et al.*, 1968; Theos *et al.*, 2005a), the Tyrosinase-related protein 1 (TYRP1) (Raposo *et al.*, 2001; Vijayasaradhi *et al.*, 1991; Vijayasaradhi *et al.*, 1995) and likely DOPAchrome tautomerase (DCT) (Kushimoto *et al.*, 2001), and of transporters such as OCA2/ *pink-eyed dilute* protein (Puri *et al.*, 2000; Sitaram *et al.*, 2009) and the copper transporter ATP7A (Setty *et al.*, 2008), that modify the lumenal environment of the melanosome to favor melanin synthesis. These melanosome components are delivered to fully formed stage II and III melanosomes from early endosomes by membrane transport mechanisms that are just beginning to be understood (reviewed in (Sitaram and Marks, 2012)). The resulting melanin precursors then polymerize on the fibrillar matrix, resulting in the thickening and blackening of the fibrillar sheets in stage III and their ultimate masking in stage IV (Seiji *et al.*, 1963). The deposition of melanin onto fibrillar sheets allows for the concentration and retention of pigment into a single functional unit.

The fibrils begin to form in stage I melanosomes, which are vacuolar structures with few intralumenal membrane vesicles (ILVs; Figure 1) and patches of flat, electron dense clathrin-containing coats on the cytoplasmic leaflet of the limiting membrane (Hurbain *et al.*, 2008; Raposo *et al.*, 2001; Seiji *et al.*, 1963). Unlike later stage melanosomes which are segregated from the endosomal system in melanocytes, stage I melanosomes are accessible to internalized cargoes and correspond to sorting endosomes within the classical endocytic pathway (Raposo *et al.*, 2001). ILVs form by invagination of the endosomal limiting membrane. In vacuolar endosomes of conventional cell types, ubiquitylated growth factor receptors and other cargoes become sequestered on ILVs, which accumulate in late endosomal multivesicular bodies (MVBs) and are eventually degraded upon fusion of the MVBs with lysosomes (Babst, 2011). However, PMEL accumulates on the ILVs in stage I melanosomes (Berson *et al.*, 2001; Raposo *et al.*, 2001) and is destined for fibril formation instead of degradation. The melanosome fibrils begin to form and elongate in association with the ILVs, and as the fibrils grow, the ILVs become “pushed” to the periphery of the organelle (Hurbain *et al.*, 2008). Concomitantly, the fibrils assemble into sheets, generating the classic striated melanosome morphology as observed by electron microscopy and distending the organelle into its ellipsoid shape (Figure 1) (Hurbain *et al.*, 2008). Ellipsoidal melanosomes that are enriched in sheet-like fibrils but lack pigment are referred to as stage II melanosomes, and are no longer accessible to endocytic cargoes – marking the first clear separation between the late endosomal/ lysosomal pathway from the melanosomal pathway (Raposo *et al.*, 2001).

PMEL as the biogenetic component of the fibrils

Orlow and colleagues were the first to characterize components of the “melanosome matrix”. They isolated melanosome-enriched subcellular fractions from melanocytes, and used differential detergent extraction and antibody screening to identify proteolytic fragments of PMEL that were enriched in the insoluble fibrillar fraction (Orlow *et al.*, 1993; Zhou *et al.*, 1994). Later experiments using well-characterized antibodies identified the predominant PMEL species within stage II melanosome-enriched subcellular fractions as corresponding to proteolytic fragments of the lumenal domain (Berson *et al.*, 2003; Harper *et al.*, 2008; Hoashi *et al.*, 2006; Kushimoto *et al.*, 2001; Watt *et al.*, 2009). Using immunoelectron microscopy, Raposo and colleagues showed that PMEL was indeed detected on stage I and II melanosomes and was associated with the fibrils (Raposo *et al.*, 2001), supporting earlier qualitative results (Lee *et al.*, 1996). In addition, PMEL immunodetection was reduced as melanization increased (Raposo *et al.*, 2001), suggesting that PMEL becomes buried in melanins, as had been previously inferred from biochemical analyses (Donatien and Orlow, 1995). Numerous studies have now shown that PMEL

expression is both necessary and sufficient for fibril formation. Ectopic expression of PMEL in non-pigment cell types that do not normally express fibrils results in the production of fibrillar arrays that are morphologically similar to those observed in melanocytes (Figure 2a, b) (Berson *et al.*, 2001; Berson *et al.*, 2003), whereas naturally occurring mutations that prevent PMEL delivery to melanosomes (Theos *et al.*, 2006a; Zhou *et al.*, 1994) or loss of PMEL expression by gene ablation (Hellström *et al.*, 2011) result in a loss of fibrils from melanocytes (see Figure 3). Finally, a purified recombinant fragment of PMEL – corresponding to the largest fragment associated with melanosome fibrils (see below) – is capable of very efficiently forming fibrillar structures when allowed to refold in native buffers (Figure 2c) (Fowler *et al.*, 2006; Watt *et al.*, 2009); other fragments of PMEL do so less effectively (McGlinchey *et al.*, 2011; McGlinchey *et al.*, 2009) as discussed later in more detail. Together, these data convincingly show that PMEL is necessary and sufficient to generate, and likely the sole protein component of, melanosome fibrils.

PMEL fibrils are amyloid

The melanosome fibrils are insoluble in non-ionic detergents and are highly stable (Berson *et al.*, 2003; Hoashi *et al.*, 2006; Orlow *et al.*, 1993). Moreover, as will be discussed later, PMEL maturation to the fibrillar form requires a number of proteolytic processing steps from an integral membrane precursor (Berson *et al.*, 2001; Berson *et al.*, 2003; Kummer *et al.*, 2009; Kushimoto *et al.*, 2001; van Niel *et al.*, 2011). The similarity of these characteristics with those of the formation of Aβ amyloid from the amyloid precursor protein (APP) in Alzheimer disease was noted early on (Berson *et al.*, 2003; Kelly and Balch, 2003). Accordingly, Fowler et al (Fowler *et al.*, 2006) showed that isolated bovine retinal melanosomes bound to amyloidogenic dyes in a pattern that overlapped with that of labeling for PMEL. Moreover, they showed that fibrils consisting of recombinant PMEL fragments isolated from bacteria were amyloid based on binding to amyloidogenic dyes, characteristic X-ray diffraction, circular dichroism and attenuated total reflectance Fourier transform infrared spectra, and electron microscopy analyses (Fowler *et al.*, 2006). These data strongly support the notion that PMEL is a functional amyloid protein.

What function do the fibrils serve?

PMEL is an extraordinarily well conserved protein; for example, human and teleost fish orthologues share >40% amino acid identity throughout most domains (Theos *et al.*, 2005b). What is the evolutionary advantage for the melanocyte to promote the formation of a potentially hazardous protein aggregate?

One possible function for the fibrils is to sequester highly reactive oxidative intermediates that are generated during melanin synthesis (Simon *et al.*, 2009). Such intermediates could potentially oxidize and thus damage melanosome protein contents, melanosome membrane integrity, and/or cytosolic contents within melanocytes. In support of a role in melanin sequestration, PMEL has been shown to accelerate the polymerization of the intermediates 5,6-dihydroxyindole-2-carboxylic acid and 5,6-dihydroxyindole into melanins (Chakraborty *et al.*, 1996; Fowler *et al.*, 2006; Lee *et al.*, 1996). Intriguingly, Fowler and colleagues noted the chemical similarity of these melanin intermediates with the amyloid dye thioflavin T, and found that several amyloid fibrils, but not non-amyloid collagen fibrils, were similarly capable of accelerating the conversion of 5,6-dihydroxyindole to insoluble melanin (Fowler *et al.*, 2006). These studies suggest that the PMEL amyloid fold might have evolved to detoxify oxidative intermediates and thereby protect melanocytes from oxidative damage. Consistent with this notion, the hypomorphic PMEL mutant *silver* mouse, which expresses a truncated form of PMEL that is unable to form fibrils due to protein mislocalization (Martínez-Esparza *et al.*, 1999; Theos *et al.*, 2006a), shows premature graying of coat color

on some backgrounds (Dunn and Thigpen, 1930), correlating with reduced survival of hair bulb melanocytes (Quevedo *et al.*, 1981). A strain on cell viability upon loss of fibrils is further supported by delayed growth in culture of immortalized melanocytes derived from *silver* mice relative to wild-type immortalized melanocytes (Spanakis *et al.*, 1992), although it is not clear whether this phenotype is a direct result of the *silver* mutation, a consequence of an undefined stress induced by cell culture, or whether it is replicated in *Pmel*$^{-/-}$ melanocytes (Hellström *et al.*, 2011)). Complete loss of PMEL expression in *Pmel*$^{-/-}$ mice also causes a modest dilution of coat color pigmentation and impaired integrity of the melanosomal membrane in electron microscopy analyses (Figure 3) (Hellström *et al.*, 2011). In both *silver* and *Pmel*$^{-/-}$ mice, the loss of coat color is more pronounced in the background of mutations at the *Tyrp1* locus, encoding the melanogenic enzyme TYRP1 (Dunn and Thigpen, 1930; Hellström *et al.*, 2011; Lamoreux *et al.*, 2010), perhaps suggesting that PMEL is most effective in detoxifying intermediates that accumulate in the absence of TYRP1. Finally, a recent study has shown that polymorphisms in the PMEL promoter region are associated with vitiligo in Chinese populations and with reduced PMEL expression in vitiligous lesions (Tang *et al.*, 2012). Together, these data support the notion that PMEL fibrils protect melanocytes from toxicity.

A second potential function for the PMEL fibrils is to concentrate melanins to facilitate intracellular and intercellular transport. For example, aggregation of melanin polymers onto a network of PMEL fibrillar sheets might facilitate efficient melanin transfer from epidermal melanocytes to neighboring keratinocytes by at least one potential mechanism. The mechanism by which melanin transfer occurs is debated (Van Den Bossche *et al.*, 2006), but one model proposes that melanins are secreted – by fusion of the melanosome limiting membrane with the melanocyte plasma membrane – into the tightly enclosed space between the melanocyte dendrite and the keratinocyte; subsequent engulfment of free melanin by the keratinocyte would then complete the transfer. By this mechanism, aggregation of melanins into a single large particle would provide a more efficient package for phagocytic uptake than would endocytosis of many small, irregular-sized aggregates (Watt *et al.*, 2010). Moreover, the presence of a protease-resistant amyloid core (Schraermeyer and Dohms, 1996) might make this structure more stable within keratinocytes. In the retinal pigment epithelia, melanosomes temporarily fuse with phagosomes that harbor internalized outer photoreceptor segments, likely providing a means to scavenge free radicals from oxidized photoreceptor membranes (Schraermeyer, 1995; Schraermeyer *et al.*, 1999). In these cells, aggregation of melanins into a large particle with a sheet-like matrix would allow for rapid reformation of melanosomes following fusion with phagolysosomes. Consistently, melanosomes in the retinal pigment epithelia are more severely compromised than those in skin melanocytes of *Pmel*$^{-/-}$ mice (Hellström *et al.*, 2011).

One might have imagined PMEL fibrils playing a similar role in regulating organelle segregation during transient interactions between endosomes and maturing melanosomes within melanocytes (Delevoye *et al.*, 2009). However, the effective segregation of melanosomal and lysosomal cargoes in melanocytes from *silver* (Theos *et al.*, 2006a) and *Pmel*$^{-/-}$ (Hellström *et al.*, 2011) mice suggests that this is not the case, and that PMEL is not required in general to segregate the melanosomal and endosomal systems.

PMEL structure/ function relationships

PMEL is a type I transmembrane glycoprotein, meaning that it is anchored to membranes by a single membrane spanning domain with its bulky, glycosylated N-terminal region facing the extracellular space/ lumen of organelles and its short C-terminus facing the cytoplasm. In humans, alternative splicing leads to the generation of four distinct primary PMEL protein products, the largest of which consists of 645 residues (reviewed in (Theos *et al.*,

2005b)). For the purpose of this review we will focus on the long form. The large lumenal domain can be divided into a short signal peptide, which is excised from the mature protein, and four major subdomains based on primary sequence and homology to known structural domains (Figure 4a). Other groups have subdivided the lumenal domain into additional subdomains (Hoashi *et al.*, 2006), but we will use the simpler architecture that we originally proposed (Theos *et al.*, 2005b).

The N-terminal region (NTR) immediately follows the signal peptide (SP) – which is removed cotranslationally by signal peptidase – and spans amino acid residues 25–216. The NTR lacks homology to known protein domains, except for a similarly placed region in a homologous protein, GPNMB (Theos *et al.*, 2005b). The NTR contains three highly conserved consensus N-glycosylation sites and three cysteine residues that might participate in disulfide bonding. C-terminal to the NTR is a domain of ~90 amino acids with homology to a repeated domain in the Polycystic Kidney Disease associated protein, polycystin 1 (PKD1), and hence referred to as the PKD domain. This domain lacks glycosylation sites and is predicted to adopt a β-sheet conformation like other characterized PKD domains (Figure 4b) (Bycroft *et al.*, 1999). A single highly conserved cysteine residue is present on the last predicted β-sheet strand. A short linker region between the NTR and the PKD domain is required for efficient PMEL folding, trafficking and amyloid formation (Leonhardt *et al.*, 2010). Following and partially overlapping with the PKD domain is the repeat (RPT) domain, which consists of a varying number (10 in the most prominent human PMEL isoforms) of imperfect direct repeats of a 13-residue sequence rich in glutamic acid, proline, and serine/ threonine residues. This domain is highly modified by O-glycosylation in the mature protein (Harper *et al.*, 2008; Valencia *et al.*, 2007).

The major fibrillogenic proteolytic fragment of PMEL, Mα (see next section), comprises the NTR, PKD and RPT domains (Figure 4a, c). The remaining region of the lumenal domain contains a cysteine-rich region referred to as the Kringle-like domain (KLD) because the spacing of the cysteine residues resembles that of the Kringle cysteine knot structure that often functions in protein-protein interactions (Cao *et al.*, 2002). The KLD also harbors an N-linked glycosylation site that is essential for effective folding and secretion (Hoashi *et al.*, 2010) and additional cysteine residues that might form disulfide bonds with the NTR or PKD cysteines. The KLD is flanked by linker regions that have been referred to as GAP2 and GAP3 by Hearing and colleagues (Hoashi *et al.*, 2006). GAP3 links the KLD with the 26-residue transmembrane domain, which is followed by a 45-residue cytoplasmic domain. The NTR, PKD and KLD domains are highly conserved throughout vertebrate evolution (Theos *et al.*, 2005b), and although the sequence of the RPT, transmembrane, and cytoplasmic domains is not as conserved, the general features of these domains are (Theos *et al.*, 2005b).

How does each of these domains contribute to PMEL function? Their role in PMEL trafficking and fibril formation *in vivo* has been addressed by a number of studies in which PMEL variants lacking distinct domains have been expressed in HeLa cells or other non-pigment cell types. In such cells, the trafficking features of PMEL (described more below) are largely conserved and PMEL is capable of forming fibrils (albeit with less efficiency than in melanocytic cells and not appropriately compartmentalized). By this approach, the PKD domain is essential for proper PMEL processing and intracellular trafficking to ILVs of premelanosome-like multivesicular endosomes, both prerequisites for fibril formation (Hoashi *et al.*, 2006; Theos *et al.*, 2006b). Moreover, the RPT domain is dispensable for localization to multivesicular endosomes but is required in HeLa cells – and likely other non-pigment cell types such as CHO – for the generation of amyloid fibrils within these compartments (Hoashi *et al.*, 2006; Theos *et al.*, 2006b). O-glycosylation of the RPT domain has also been suggested to be required for fibril formation (Valencia *et al.*, 2007), supported

by the lysosomal degradation of PMEL expressed in CHO cell variants that fail to extend O-linked oligosaccharides (Harper *et al.*, 2008). The functions of the NTR and KLD in trafficking and fibril formation are more controversial, likely due to the use of distinct domain boundaries in different studies that might impact protein folding (e.g. see (Leonhardt *et al.*, 2010)). The NTR is clearly important for ultimate fibril formation, and at least part of the KLD is required for protein folding (Hoashi *et al.*, 2006; Theos *et al.*, 2006b). One caveat of these studies is that fibrillar melanosomes do not segregate from the endocytic pathway in non-pigment cells, and so fibrils form and accumulate aberrantly within multivesicular late endosomes (Berson *et al.*, 2001) (see Figure 2b). This might influence the ability of protofibrils to resist proteolysis or to assemble into sheets. The use of a PMEL-negative human melanoma cell line for such studies (Leonhardt *et al.*, 2011; Leonhardt *et al.*, 2010) will likely circumvent this problem in future analyses.

Two groups have used bacterially expressed PMEL fragments to define the subdomains that are capable of forming amyloid. Consistent with the requirement for the RPT domain in amyloid formation *in vivo*, McGlinchey *et al.* showed that prolonged incubation of a recombinant peptide corresponding to a His_6-tagged human PMEL RPT domain resulted in amyloid fibril formation under acidic conditions (McGlinchey *et al.*, 2009); at neutral pH, RPT fibrils failed to form and preformed fibrils dissolved (McGlinchey *et al.*, 2009; Pfefferkorn *et al.*, 2010). Similar results were obtained with PMEL RPT domains from other species, despite a lack of primary sequence conservation (McGlinchey *et al.*, 2011), and NMR spectroscopy revealed a parallel β-sheet structure, similar to that of Aβ amyloid, composed of non-uniform RPT segments (Hu *et al.*, 2011; McGlinchey *et al.*, 2011). While these authors argued that pH-dependent formation of RPT domain amyloid was consistent with the acid pH of melanosomes, the data fail to account for a number of features of melanosome biology that raise concerns about the significance of the RPT as the PMEL amyloid core *in vivo*. First, the recombinant form of the RPT domain isolated from bacteria lacks the extensive O-linked glycosylation that modifies this domain *in vivo* and that seems to be required for amyloid fibril accumulation in mammalian cells (Harper *et al.*, 2008; Valencia *et al.*, 2007). The modification of serine and threonine residues by these highly charged oligosaccharide side chains would likely interfere with the assembly of a closely packed cross-beta sheet that forms the structural basis for amyloid. Second, pH increases as melanosomes mature (Raposo *et al.*, 2001), and TYR is largely inactive at acid pH (Wang and Hebert, 2006) suggesting that mature melanosomes might be near neutral pH; hence, the dissolution of RPT fibrils at neutral pH would likely destroy the fibrils before they were significantly bound to melanins. Third, fibrils can be isolated from subcellular fractions of early stage melanosomes at neutral pH (Berson *et al.*, 2003; Kushimoto *et al.*, 2001; Watt *et al.*, 2009), indicating that they do not dissolve like the RPT fibrils do *in vitro*. Finally, the kinetics of recombinant RPT fibril formation are seemingly incompatible with the rapid kinetics of melanosome fibril formation *in vivo*; it takes several weeks to form fibrils initially, and several days to form fibrils if "seeded" with preformed fibrils (McGlinchey *et al.*, 2009). Thus, while an interesting *in vitro* experimental observation, it seems unlikely that the RPT domain forms the core of the PMEL amyloid.

By contrast, Watt et al found that His_6-tagged recombinant fragments corresponding to the NTR and PKD domain both had amyloid properties *in vitro* that were more consistent with experimental observations *in vivo* (Watt *et al.*, 2009). Like full-length recombinant Mα (Fowler *et al.*, 2006; Watt *et al.*, 2009), these fragments isolated under denaturing conditions from bacteria formed amyloid within seconds to minutes of dilution into non-denaturing buffers of varying pH, as evaluated by amyloid dye binding, detergent insolubility, X-ray diffraction and electron microscopy analyses. Under similar conditions, the RPT domain did not form amyloid (Watt *et al.*, 2009). Moreover, whereas deletion of the PKD or NTR domains from Mα diminished amyloid dye binding, deletion of the RPT domain did not.

Protease-resistant fragments obtained from Mα fibrils contained regions corresponding to the NTR and/or PKD domain, whereas the RPT domain was completely digested by limited protease treatment. Unlike the RPT domain fibrils characterized by McGlinchey and colleagues, the NTR and PKD fibrils were stable at neutral pH, and the morphology of the PKD fibrils resembled those of amyloid protofibrils observed in stage I premelanosomes (Watt *et al.*, 2009). Finally, a fragment of the PKD domain was identified in detergent-insoluble fibril fractions isolated from melanocytic cells (van Niel *et al.*, 2011; Watt *et al.*, 2009). These data support the notion that the PKD and/or NTR domains form the PMEL amyloid core *in vivo*. Because the PKD domain is unmodified by glycosylation *in vivo* and is predicted to fold into a beta sheet-rich conformation that might be primed for incorporation into the cross β-sheet amyloid conformation (Figure 4b) (Greenwald and Riek, 2010), we favor the model that the PKD is the physiological core of PMEL and that interactions with regions of the NTR might facilitate a conformational shift to the amyloid form. Rather than functioning in amyloid assembly per se, acidification of early stage melanosomes is needed for the proteolytic processing events that generate the amyloidogenic Mα precursor (Berson *et al.*, 2001) (see below) and we speculate might be additionally required for local PKD domain unfolding to initiate amyloid formation – a function that is not needed for the bacterial recombinant proteins that are isolated under denaturing conditions. We further speculate that the RPT domain functions in regulating the timing of this unfolding event and might additionally participate in regulating the assembly of fibrils into sheets and protecting them from lysosomal proteases within mature melanosomes.

PMEL synthesis, glycosylation and proteolytic processing

To avert toxicity, functional amyloid must be formed under tightly controlled conditions. In order to gain insight into these circumstances and when and how PMEL undergoes its transformation from a typical transmembrane glycoprotein to an insoluble amyloid, it is essential to understand the mechanisms controlling PMEL biosynthesis, maturation and trafficking.

PMEL is co-translationally translocated into the endoplasmic reticulum (ER) and modified by signal peptide cleavage and by addition of four N-linked core oligosaccharides (Berson *et al.*, 2001; Kwon *et al.*, 1987; Maresh *et al.*, 1994a; Maresh *et al.*, 1994b), generating a "precursor 1" (P1) form (Figure 4c). PMEL is slowly exported from the ER, likely due to slow folding of the large lumenal domain (Berson *et al.*, 2001); consequently, the unmodified P1 form is the most prominent form detected by immunoblotting using antibodies to the N- or C-termini (Berson *et al.*, 2001; Harper *et al.*, 2008; Kushimoto *et al.*, 2001). PMEL exit is facilitated by a C-terminal valine residue (Theos *et al.*, 2006a) that likely engages the COPII coat machinery for formation of Golgi-bound vesicles (Nufer *et al.*, 2002). In the Golgi, the core N-linked oligosaccharides are further modified, such that the oligosaccharide on N81 is maintained in high mannose form and the others are modified to the complex type (Berson *et al.*, 2001; Hoashi *et al.*, 2010; Maresh *et al.*, 1994b). In addition, the central "RPT" region of PMEL (see below) is modified extensively by terminally sialylated O-linked oligosaccharides (Figure 4a, c) (Harper *et al.*, 2008; Valencia *et al.*, 2007). Depending on the sialic acid linkages, these latter modifications make mammalian forms of PMEL detectable by the widely used monoclonal antibody HMB45 (Chiamenti *et al.*, 1996; Harper *et al.*, 2008; Valencia *et al.*, 2007). The fully modified full-length form of PMEL has been referred to as precursor 2 (P2; Figure 4c).

P2 is short-lived, and is subject to a number of proteolytic cleavages that generate the "mature" forms of PMEL (Figure 4c–e). P2 is proteolytically cleaved within an acidic compartment – either the trans Golgi network (TGN) (Leonhardt *et al.*, 2011) or early

endosomes (Theos *et al.*, 2006b), perhaps depending on the cell line analyzed. The cleavage occurs at a dibasic Lys-Arg sequence within the PMEL lumenal domain by furin or a related protease of the proprotein convertase (PC) family of enzymes (Berson *et al.*, 2001; Berson *et al.*, 2003). This produces two fragments, a large lumenal mature α (Mα) fragment and a smaller integral membrane mature β (Mβ fragment), that remain at least temporarily tethered by disulfide bonds (Figure 4a, c) (Berson *et al.*, 2001; Berson *et al.*, 2003). The dibasic residues on Mα are removed, likely by carboxypeptidase E (Fricker, 1988), to generate the Mα form that has been sequenced (Maresh *et al.*, 1994b). Because Mα remains covalently bound to the integral membrane Mβ subunit, PC cleavage is not sufficient to release the fibrillogenic Mα fragment from the membrane to allow for fibril formation. A second proteolytic cleavage in the lumenal juxtamembrane region liberates Mα and the bound lumenal region of Mβ (MβN) from the membrane (Kummer *et al.*, 2009). This cleavage is mediated by a “site 2 protease” (S2P; Figure 4c), occurs independently of but subsequent to PC cleavage (Kummer *et al.*, 2009) (and our unpublished data), and in transfected HeLa cells requires a metalloproteinase such as a disintegrin and metalloproteinase (ADAM) 10 or ADAM17 (Kummer *et al.*, 2009). It is not yet clear whether the ADAMs are also required for site 2 cleavage in melanocytic cells or whether they mediate site 2 cleavage directly. Cleavage of PMEL by both the PC and S2P is required for Mα release and fibril formation; inhibition of either cleavage results in the formation of non-fibrillar aggregates and loss of amyloid fibrils (Berson *et al.*, 2003; Kummer *et al.*, 2009). Interestingly, juxtamembrane cleavage is also required for the secretion of a small fraction of PMEL (Berson *et al.*, 2001; Esclamado *et al.*, 1986; Maresh *et al.*, 1994a; Maresh *et al.*, 1994b; Vennegoor *et al.*, 1988), most of which is cleaved by the PC and includes Mα bonded to MβN (Hoashi *et al.*, 2010). The sequence requirements within the PMEL juxtamembrane region for ectodomain shedding/ secretion and S2P cleavage/ fibril formation are distinct (Hoashi *et al.*, 2010; Kummer *et al.*, 2009), suggesting that they are mediated by distinct proteases and/or occur in distinct compartments.

While S2P cleavage liberates Mα-MβN fragments into the lumen of premelanosomes to initiate fibril formation, it also generates a membrane-bound C-terminal fragment or CTF (Figure 4c) (Kummer *et al.*, 2009). The CTF is a substrate for yet another protease, γ-secretase (Figure 4c) (Kummer *et al.*, 2009; van Niel *et al.*, 2011). γ-secretase is a multi-subunit intramembrane protease that cleaves transmembrane domain-containing substrates with a short lumenal extension – such as those produced by sheddases – at a site within their membrane-spanning segments (Prox *et al.*, 2012). This liberates short intracellular fragments, many of which are involved in intracellular signaling pathways (Fortini, 2002). In the case of PMEL, cleavage by γ-secretase is required for CTF degradation (Kummer *et al.*, 2009; van Niel *et al.*, 2011) and its segregation from the melanosomal pathway into the degradative late endosomal/ lysosomal system (van Niel *et al.*, 2011). While the cytoplasmic fragments of other γ-secretase substrates have been shown to initiate signal transduction cascades (Fortini, 2002), there is no current evidence to suggest a role for the PMEL intracellular fragment in cell signaling. Other melanosomal proteins including TYR, TYRP1 and DCT are also targets of γ-secretase, and loss of γ-secretase function results in TYR mistrafficking and loss of pigmentation (Wang *et al.*, 2006).

The progressive cleavage of PMEL by a PC, S2P and γ-secretase is reminiscent of the series of proteolytic cleavages of APP by BACE1 and γ-secretase that generate the amyloidogenic Aβ fragment in Alzheimer Disease (Prox *et al.*, 2012). Indeed, proteolysis-mediated amyloidogenic conversion is observed in many pathologic amyloidogenic proteins, such as for gelsolin in familial amyloidosis of Finnish type (Chen *et al.*, 2001) and perhaps for α-synucleins in Parkinson Disease (Choi *et al.*, 2011; Kim *et al.*, 2003; Levin *et al.*, 2009), and in functional amyloids such as polypeptide pro-hormones in mammals (Maji *et al.*, 2009). Proteolysis by similar classes of proteases can also interfere with amyloid formation, as

exemplified by the cleavage of APP (Prox *et al.*, 2012) and PrPc (Vincent *et al.*, 2001) to non-amyloidogenic fragments by ADAM10. Thus, proteolysis to fibrillogenic fragments – or proteolytic degradation of such fragments – is a commonly used theme in regulating amyloid formation. Nonetheless, given that appropriately cleaved secreted PMEL Mα fragments are isolated from soluble fractions of cell supernatants (Hoashi *et al.*, 2010; Maresh *et al.*, 1994b), proteolytic processing is necessary but not sufficient to drive PMEL amyloid conversion.

While intact Mα fragments are fibrillogenic and can be detected within fibril-enriched subcellular fractions (Berson *et al.*, 2003; Harper *et al.*, 2008; Kummer *et al.*, 2009), the major PMEL fragments detected in these fractions from pigment cells are products of further proteolytic maturation (Figure 4d–e) (Chiamenti *et al.*, 1996; Harper *et al.*, 2008; Hoashi *et al.*, 2006; Kushimoto *et al.*, 2001; Watt *et al.*, 2009). These fragments are also observed when PMEL is ectopically expressed in non-pigment cells (Harper *et al.*, 2008; Hoashi *et al.*, 2006) and likely arise by Mα proteolysis by lysosomal proteases which are present within melanosomes (Diment *et al.*, 1995; Novikoff *et al.*, 1968; Raposo *et al.*, 2001). While this proteolytic maturation does not seem to be required to form amyloid protofibrils *in vitro* (Watt *et al.*, 2009), it might be required for the formation of fully mature fibrils and the proper lateral association of fibrils into sheets (Figure 4d–e) (Hurbain *et al.*, 2008).

PMEL trafficking to premelanosomes

The sequence of PMEL processing described above reflects the segregation of processing enzymes and ultimate ordered oligomerization of PMEL into fibrils within sequential compartments of the secretory and endocytic pathways (Figure 5). PMEL synthesis, core N-linked oligosaccharide addition and trimming, and disulfide bond formation occur in the ER, and O-linked oligosaccharide addition and processing of N- and O-linked oligosaccharides occur in the Golgi and TGN, as for all known glycoproteins of the secretory and endocytic pathways. The site of PC cleavage is debated. Our group showed that PC cleavage of PMEL in MNT-1 human melanoma cells or transfected non-pigment HeLa cells is inhibited by agents that disrupt acidification (Berson *et al.*, 2001) and can be mediated by furin only when it is massively overexpressed (Theos *et al.*, 2006b). Furin is normally restricted to the TGN, but when overexpressed a substantial cohort is found in endosomes (Bosshart *et al.*, 1994). Moreover, surface expressed PMEL in MNT-1 or HeLa cells was not PC modified (our unpublished results), and PMEL mutants that accumulate at the plasma membrane of HeLa cells (see below) are largely uncleaved (Theos *et al.*, 2006a). By contrast, Leonhardt and colleagues reported conflicting results, suggesting that secretory forms or surface-exposed PMEL were PC cleaved in a different human melanoma cell line (Leonhardt *et al.*, 2011). It is not yet clear whether these conflicting results reflect differing experimental approaches or cell-type specific differences. Nonetheless, PC cleavage clearly precedes further maturation in premelanosomes.

From the TGN, PMEL accumulates in early endosomes/ stage I premelanosomes (Raposo *et al.*, 2001), but likely does so by clathrin-mediated endocytosis after first traversing the plasma membrane (Figure 5). PMEL is robustly internalized from the plasma membrane (Chen *et al.*, 2012; Theos *et al.*, 2006a) by virtue of a di-leucine-based consensus internalization signal in the cytoplasmic domain (Theos *et al.*, 2006a). This signal likely mediates internalization by binding to the AP-2 plasma membrane clathrin adaptor, as AP-2 depletion results in PMEL accumulation at the plasma membrane and interferes with its localization to melanosomes (Robila *et al.*, 2008). Similarly, a natural truncation of the PMEL cytoplasmic domain in the *silver* mouse (Martínez-Esparza *et al.*, 1999) blocks internalization (Theos *et al.*, 2006a) and a similar truncation introduced into human PMEL blocks localization to endosomes (Lepage and Lapointe, 2006). Whereas ectopically

expressed PMEL in HeLa cells accesses early endosomes effectively by an independent pathway that does not require cytoplasmic domain targeting sequences (Theos *et al.*, 2006b), such a pathway is likely not prominent in melanocytes, as melanosomes in *silver* melanocytes (Theos *et al.*, 2006a) or AP-2-depleted melanoma cells (Robila *et al.*, 2008) are severely depleted of PMEL. In wild-type melanocytes, the subsequent accumulation of PMEL in vacuolar endosomes/ stage I melanosomes (Raposo *et al.*, 2001) likely explains the cofractionation of PMEL with AP-1 adaptors (Valencia *et al.*, 2006), which themselves are localized primarily to early endosomes in melanocytes; depletion of AP-1 had no effect on PMEL maturation or fibril formation (Delevoye *et al.*, 2009).

Within vacuolar domains of early endosomes, PMEL is preferentially sorted to and becomes enriched in ILVs (Figure 5) (Berson *et al.*, 2001; Raposo *et al.*, 2001; Theos *et al.*, 2006b; van Niel *et al.*, 2011). Only the Mα fragment that has been segregated from CTF fragments preferentially accumulates on the ILVs, as is evident from the paucity of labeling for the PMEL C-terminus on ILVs (Raposo *et al.*, 2001; van Niel *et al.*, 2011). Whereas ILV sorting for cargoes such as activated epidermal growth factor receptors has been extensively studied and shown to be dependent on cargo ubiquitylation and sequential cargo hand-off by a series of ESCRT complexes (Babst, 2011; Hanson and Cashikar, 2012), selective accumulation of PMEL on ILVs is independent of both ubiquitylation and the ESCRT machinery (Theos *et al.*, 2006b; Truschel *et al.*, 2009). Rather, Mα associates with ILVs using a mechanism that is dependent on its PKD and NTR domains (Theos *et al.*, 2006b) and the tetraspanin protein, CD63 (Figure 5) (van Niel *et al.*, 2011). Interestingly, whereas Mα associates with ILVs in an ESCRT-independent manner, CTF fragments are degraded in a lysosomal pathway that requires the ESCRT machinery (van Niel *et al.*, 2011). CD63 seems to be critical to segregate Mα and CTF into distinct sorting pathways since depletion of CD63 from MNT-1 melanoma cells results in the ESCRT-dependent degradation of both domains (van Niel *et al.*, 2011). CD63 likely functions to generate microdomains on the limiting membrane of vacuolar endosomes that favor PMEL-Mα sequestration and subsequent ILV formation.

As discussed earlier, fibrils begin to emanate and elongate from the ILVs within stage I melanosomes (Figures 4d, 5) (Hurbain *et al.*, 2008). ILVs are essential for fibril formation, as the impaired ILV formation induced by CD63 depletion completely abrogates amyloidogenesis (van Niel *et al.*, 2011) and PMEL deletion mutants that fail to associate with ILVs also cannot form fibrils (Theos *et al.*, 2006b) (although interpretation of this latter result is complicated by their inefficient proteolytic maturation). How association with ILVs favors amyloid conversion of Mα is not clear. It is possible that interactions of lumenal subdomains with lipid headgroups that are enriched on ILVs induces local unfolding that promotes amyloid conversion, as has been previously shown *in vitro* for amyloid conversion of the prion protein (Wang *et al.*, 2007) and islet amyloid polypeptide (Jean *et al.*, 2010). Another possibility is that separation of PMEL-Mα from the MβN fragment is sufficient to drive the amyloid conversion, and that the ILVs merely serve as a mechanism to enhance this separation. Indeed, either the continued association of Mα with MβN (Hoashi *et al.*, 2010) or the absence of association with ILVs (our unpublished data) might explain the solubility of secreted PMEL-Mα. Finally, it remains possible that an as yet undiscovered conversion factor associates constitutively with the ILVs. Resolution of these mechanisms will require *in vitro* reconstitution with purified components in their native state. The maturation of the fibrils to sheets within premelanosomes correlates with a loss of ILVs, likely by their fusion with the limiting membrane, and with maturation to stage II melanosomes (Hurbain *et al.*, 2008). Protofibrils obtained with recombinant PMEL lumenal domain do not form the fibrillar sheets, suggesting that factors associated with melanosome maturation, including low lumenal pH, ILV association or proteolytic maturation, are necessary for lateral assembly of the fibrils. Intriguingly, this maturation step also correlates with the segregation of the melanosome pathway from the endosomal pathway, as stage II

melanosomes are no longer accessible to endocytosed cargoes (Raposo *et al.*, 2001). However, PMEL is not required for the segregation of these two pathways, as melanocytes derived from *Pmel*$^{-/-}$ or *silver* mice that lack melanosomal PMEL or fibrils altogether nevertheless segregate melanosomal cargoes from late endosomal/ lysosomal cargoes (Hellström *et al.*, 2011; Theos *et al.*, 2006a). How this segregation is established and how both stage II melanosomes and late endosomes derive from functionally and morphologically similar compartments is still not clear. It is possible that the small melanosomal protein, MART-1, and the G protein-coupled receptor, OA1, play roles in this segregation (Aydin *et al.*, 2012; Giordano *et al.*, 2009; Giordano *et al.*, 2011; Hoashi *et al.*, 2005).

Averting amyloid toxicity

How does PMEL amyloid avoid the toxicity associated with pathological amyloid? Several features of the PMEL life cycle might contribute to its lack of toxicity. One likely important feature is the sequestration of PMEL amyloid formation within stage I melanosomes. This compartmentalization might prevent the exposure of potentially harmful amyloidogenic intermediates from cellular proteins that might otherwise be subject to denaturation by exposed hydrophobic interactions (Klein *et al.*, 2001). A related mechanism might be the sequestration of amyloidogenic PMEL-Mα to the ILVs; this would allow specific concentration of the amyloidogenic species to favor fibril elongation, and exclude the majority of other cellular proteins that do not specifically concentrate on ILVs. Besides compartmentation, PMEL might be non-toxic due to the kinetics of amyloid conversion. Denatured recombinant PMEL-Mα, NTR and PKD fragments form amyloid almost immediately upon dilution into native buffers, and much more rapidly than conventional pathological amyloids (Fowler *et al.*, 2006; Watt *et al.*, 2009). If transient denaturation and rapid amyloid formation similarly occurs *in vivo*, as suggested by the rapid incorporation of the Mα fragment into detergent insoluble fractions in melanocytes, it would minimize exposure of potentially toxic oligomeric intermediates (perhaps amyloid seeds) and sequester amyloidogenic species into non-toxic fibrils. A third mechanism to avert toxicity might be the “protection” of a PKD/NTR domain amyloid core by binding to the highly hydrophilic RPT domain region (Figure 4d, e). The RPT clearly associates with fibrils in cells (Kushimoto *et al.*, 2001; Raposo *et al.*, 2001), and might prevent association of other lumenal contents with the growing fibrils. Lastly, lateral association of the fibrils into sheets (Hurbain *et al.*, 2008) might also protect the amyloid core from association with other lumenal contents and avert toxicity. These potential detoxification mechanisms are not mutually exclusive, and might cooperate with each other *in vivo* to minimize potential damage of PMEL amyloid.

If mechanisms are in place to avert PMEL-associated toxicity, one would predict that mutations that interfere with these mechanisms might result in pathology. Indeed, studies of naturally occurring PMEL mutations in animals such as chicken (Kerje *et al.*, 2004), horse (Brunberg *et al.*, 2006), cattle (Jolly *et al.*, 2008; Kühn and Weikard, 2007; Schmutz and Dreger, 2012), dog (Clark *et al.*, 2006), and perhaps zebrafish (Schonthaler *et al.*, 2005) are associated with more severe hypopigmentation than that observed in *Pmel*$^{-/-}$ mice and suggest that aberrant fibril formation might be deleterious. These mutations vary in nature from point mutations and small deletions/insertions to large truncations, and their phenotypic consequences range in severity from mild hypopigmentation, such as in *smoky* chickens (Kerje *et al.*, 2004), to a complete loss of eumelanin in *Dominant White* chickens (Keeling *et al.*, 2004; Kerje *et al.*, 2004) and *Silver* horses (Brunberg *et al.*, 2006). The latter two severe phenotypes are inherited as dominant mutations, suggesting a gain-of-function loss of pigmentation, and at least the *Dominant White* mutation (found in White Leghorn chickens in the United States) is associated with a loss of pigmented melanocytes in feathers

(Hamilton, 1940). Interestingly, both *Dominant White* chickens and *Silver* horses have mutations in or near the PMEL transmembrane domain (Brunberg *et al.*, 2006; Kerje *et al.*, 2004), far from the amyloidogenic domains. Analyses of epitope-tagged *Dominant White* chicken PMEL (Kuliawat and Santambrogio, 2009) or of human PMEL engineered with an orthologous mutation (hPMEL^{insWAP}; (Watt *et al.*, 2011) expressed in non-pigment cells shows that the *Dominant White* mutation does not influence PMEL maturation, processing or trafficking, and surprisingly has at most a minor effect on association with distinct membrane microdomains. Moreover, hPMEL^{insWAP} is capable of forming fibrils when expressed in HeLa cells (Watt *et al.*, 2011). However, both hPMEL^{insWAP} and human PMEL with the *Silver horse* mutation induce aberrantly assembled fibrillar sheets and a loss of pigmentation when expressed in melanocytes (Watt *et al.*, 2011) (Figure 6). This phenotype correlates with altered biophysical properties of the PMEL transmembrane domain (Watt *et al.*, 2011). Interestingly, the mildly hypopigmenting *smoky* allele arose as a revertant of the severely hypopigmented *Dominant White* allele and disrupts the PKD domain (Figure 4b; deleted amino acids in *smoky* are highlighted) (Kerje *et al.*, 2004); engineering this mutation into hPMEL^{insWAP} blocks trafficking to fibril-forming compartments and renders PMEL incapable of forming fibrils (Watt *et al.*, 2011). This indicates that the negative consequences of the *Dominant White* mutation are blocked by interfering with fibril formation, and support the notion that this and other dominant mutations generate fibrils or fibril intermediates with toxic properties.

Perspectives

Our extensive understanding of PMEL trafficking and processing and the availability of both loss-of-function and gain-of-function mutants makes PMEL an outstanding model to understand the generation of functional amyloid in mammals and to differentiate this process from the generation of pathological amyloid. While we have learned a great deal over the last 12 years, a number of key questions remain. The most fundamental questions revolve around the conformational change that initiates amyloid formation within stage I melanosomes. How is this conformational change initiated? Is protein unfolding a key step, and if so, what induces unfolding? What is the role of the ILVs – do they template amyloid conversion, provide a source of PMEL monomers for amyloid fibril elongation, or merely serve as a convenient site for fibril accumulation? What are the mechanisms leading to the formation of the ILVs and the accumulation of PMEL-Mα on them, and how do these mechanisms rely on CD63? How do the tertiary and quaternary structures of PMEL on precursor membranes contribute to the conformational change, and what are the respective roles of the different subdomains? Other questions revolve around how the amyloid "switch" is prevented in earlier secretory compartments prior to arrival at the stage I melanosome. Is PMEL in some way "tethered" to block the exposure of the amyloidogenic domain, and is the tether released by proteolytic processing? Does the KLD contribute to tethering the amyloidogenic domain until its removal in stage I melanosomes? What is the role of the RPT domain and its sialylated O-linked glycans? Finally, the new finding that several PMEL mutants might generate pathological amyloid warrants further investigation. Why are these mutants deleterious to pigment cells or pigmentation – is there a change in the kinetics of amyloid formation or in the lateral assembly of fibrils into sheets? Does it affect melanin binding to the matrix? What is the toxic species – the pre-amyloid oligomers or the mature amyloid fibrils? Are they actually toxic to the survival of melanocytes, and if so, why? Rather, do they merely disturb the integrity of the melanosome and interfere with melanization, and if so, how? Resolution of these issues will require a number of new experimental systems, including (1) better *in vitro* reconstitution systems with correctly modified and folded PMEL isoforms, (2) live cell imaging modalities, such as FRET and FLIM, to measure changes in inter-domain contacts during the progression of PMEL from the Golgi to stage I/ II melanosomes, (3) imaging methods such as RUSH (Boncompain *et*

al., 2012) to follow PMEL from its site of synthesis to stage II melanosomes or to monitor the segregation of stage I/ II melanosomes from the endocytic pathway, and (4) transgenic animal models to study the impact of natural PMEL mutants. Further analyses of this fascinating pigment cell-specific protein are sure to provide new avenues of research and new insights into amyloidoses.

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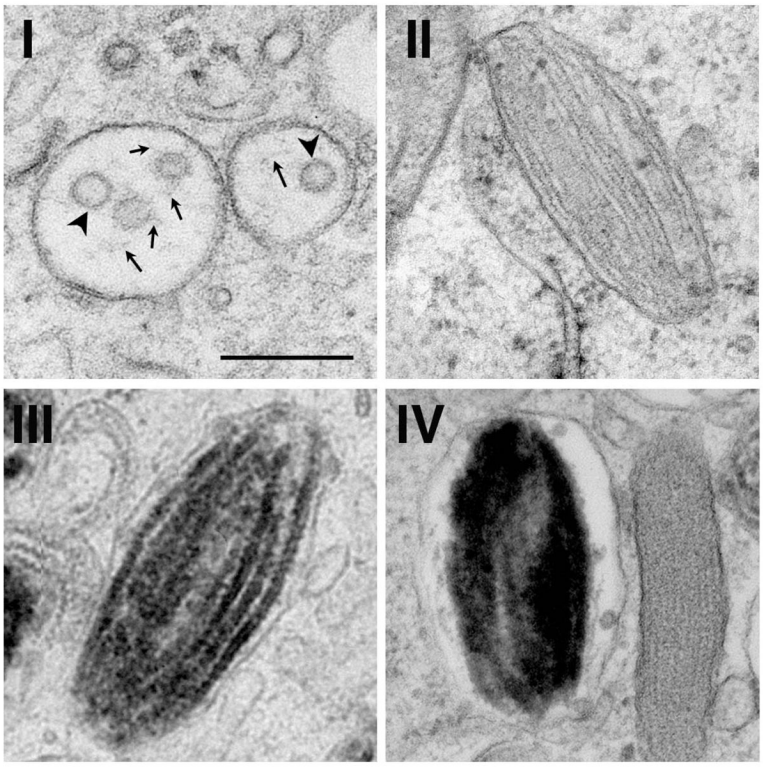

Figure 1. Morphology of different stage melanosomes

Shown are representative electron micrographs of melanosomes of stages I, II, III and IV from thin sections of human MNT-1 melanoma cells preserved by high pressure freezing and freeze substitution. Note the short fibrils (arrows) emerging from intralumenal vesicles (ILVs; arrowheads) in stage I, the parallel arrays of non-pigmented fibrillar sheets in stage II, the melanized fibrils in stage III, and the complete masking of intralumenal contents in stage IV. Scale bar, 200 nm. The figure is adapted from Figures 1 and 3 in (Hurbain *et al.*, 2008), copyright 2008, National Academy of Sciences, U.S.A. A modified form of this figure was also shown in (Watt *et al.*, 2010).

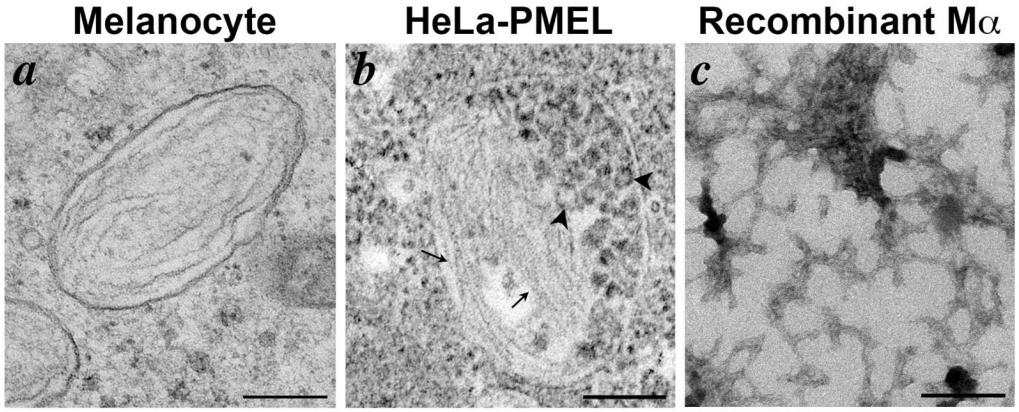

Figure 2. PMEL generates fibrils in melanocytic cells, transfected non-melanocytic cells, and in recombinant form

Shown are electron micrographs of fibrils generated by PMEL in three settings. **a**, thin section of human MNT-1 melanoma cells preserved by high pressure freezing and freeze substitution, highlighting a fibrillar stage II melanosome. **b**, thin section of transiently transfected non-melanocytic HeLa cells expressing a human PMEL transgene and fixed chemically with glutaraldehyde and formaldehyde. Note the fibrillar arrays (arrows) within a multivesicular body containing numerous intralumenal vesicles (ILVs; arrowheads). **c**, whole mount analysis of fibrils generated by dilution of purified recombinant PMEL-Mα fragment (tagged at the C-terminus with hexahistidine and purified from bacterial inclusion bodies by affinity chromatography in 6M guanidine-HCl) into a physiological buffer at neutral pH. Note the lattice-like fibrillar network. Scale bars, 200 nm. Panel a is from Figure 3 in (Hurbain *et al.*, 2008), copyright 2008, National Academy of Sciences, U.S.A. Panel b is from (Berson *et al.*, 2003), copyright 2003, The Rockefeller University Press. A modified form of this figure was shown in (Watt *et al.*, 2010).

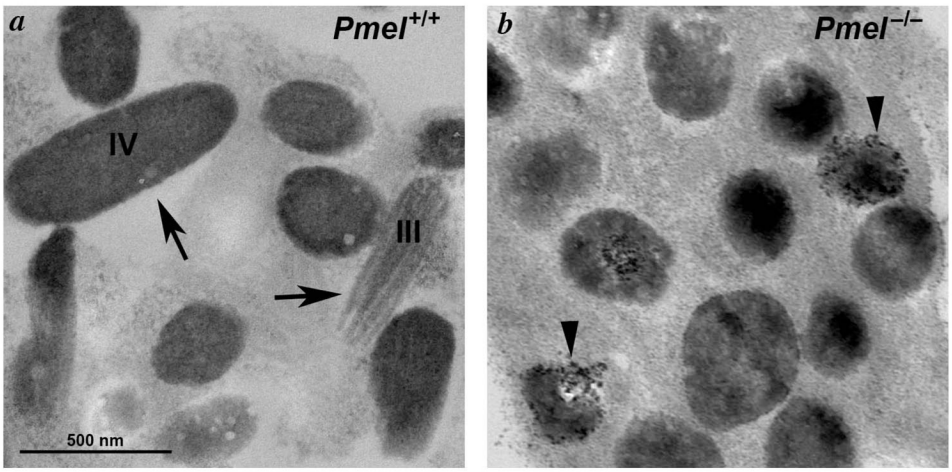

Figure 3. PMEL is necessary for fibril formation and melanosome shape

Shown are electron microscopy analyses of primary melanocytes from C57BL/6 wild-type mice (**a**, *Pmel*$^{+/+}$) and *Pmel*$^{-/-}$ mice (**b**), showing the morphology of the melanosomes in the presence and absence of PMEL. Note the loss of the oblong shape and the fibrillar morphology, and the irregular deposition of melanin deposits in melanosomes from *Pmel*$^{-/-}$ melanocytes. Similar data were shown in (Hellström *et al.*, 2011).

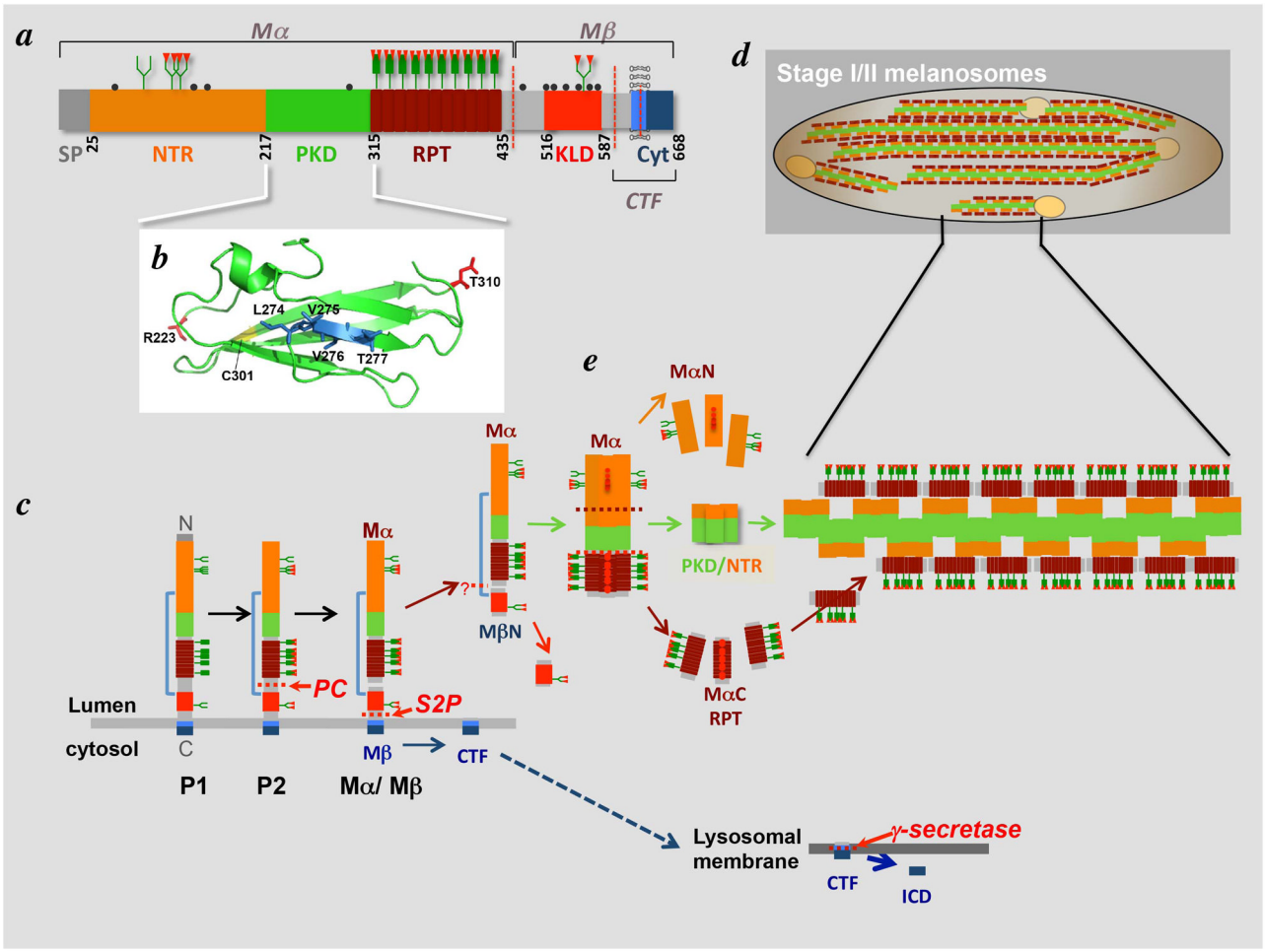

Figure 4. PMEL Structure and proteolytic processing during fibril formation

a, schematic structure of PMEL showing the individual domain structure, glycosylation of the "mature" form, and major cleavage products. NTR, PKD, RPT and KLD domains are defined in the text; SP, signal peptide; Cyt., cytoplasmic domain. The transmembrane domain (light blue) is indicated by association with the lipid bilayer. The two products of PC cleavage, Mα and Mβ, are indicated, as is the CTF; cleavage sites are indicated by dotted red lines. N-linked oligosaccharides are indicated as green branched structures; O-linked oligosaccharides in the RPT domain are indicated by green unbranched structures; sialic acid residues are indicated in red. Amino acid residue numbers are shown and correspond to those of the longest human PMEL isoform. **b**, model of the PMEL PKD domain, threaded onto the structure of the PKD domain of the *Saccharophagus degradans* cellulose-binding protein using the Phyre2 server (Kelley and Sternberg, 2009). The image was generated using MacPyMOL (http://www.pymol.org/). The N- and C-terminal residues within the model are highlighted in red, the single conserved cysteine is highlighted in yellow, and the four residues corresponding to those deleted in the *Smoky* chicken PMEL allele are highlighted in blue. **c**, proteolytic processing and maturation of PMEL prior to fibril formation, showing the P1, P2, Mα-Mβ, isoforms and subsequent cleavage products CTF and MβN. Colors of domains are as in panel **a**. Cleavage by PC and S2P are indicated by red arrows. **d**, model for PMEL domain composition within fibrils and fibrillar sheets in stage I and II melanosomes. Colors of domains are as in panel **a**. Note the emergence of fibrils from small ILVs (pale yellow). **e**, cleavages and model for assembly of PMEL fragments into

fibrils in stage I melanosomes. Mα fragments separate from MβN, and oligomerize. The oligomers are further processed into MαN, PKD/NTR and MαC fragments; note that the precise sites of cleavage have not yet been determined, and it is not yet clear whether NTR-derived fragments are present within the fibrils. In this hypothetical model, PKD-NTR fragments form the core of the fibril and MαC fragments assemble onto them. These fibrils would then assemble laterally into sheets in stage II melanosomes.

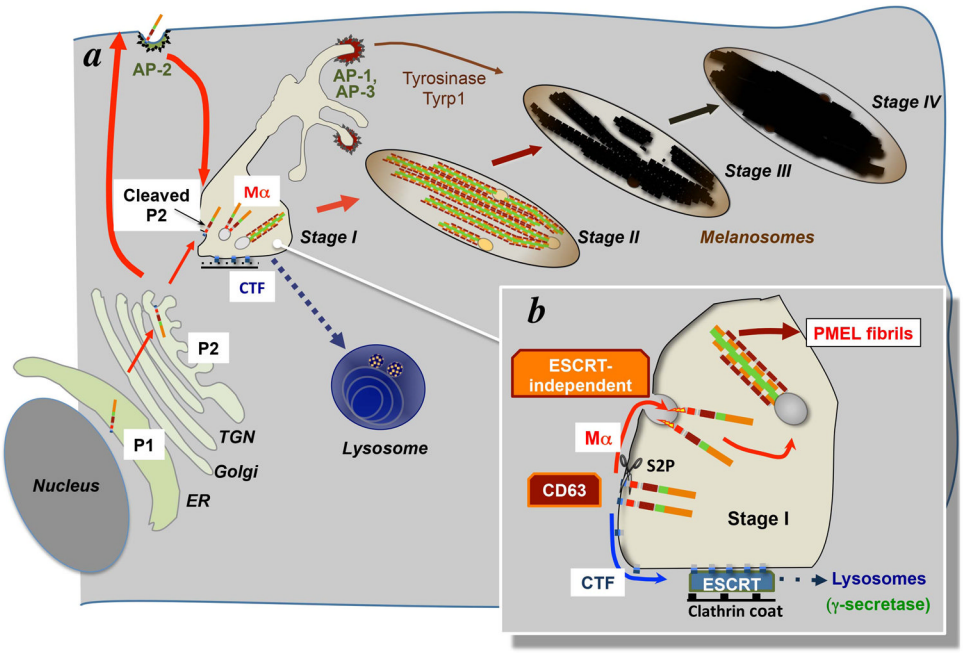

Figure 5. PMEL trafficking to stage I and II melanosomes

a, schematic of the trafficking pathway for PMEL from its site of synthesis in the endoplasmic reticulum (ER) through the Golgi and trans Golgi network (TGN) and the plasma membrane, ultimately to stage I and II melanosomes. Red arrows depict trafficking steps, and PMEL domains are colored as in Figure 4. The predominant processed PMEL isoform is indicated within each organelle. The blue arrow indicated PMEL CTF trafficking to lysosomes. Also shown are the clathrin-associated adaptors AP-2 – which facilitates PMEL endocytosis from the plasma membrane – and AP-1 and AP-3 – which associate with separate domains of early endosomes/ stage I melanosomes. Tyrosinase and Tyrp1 are delivered to maturing stage III and IV melanosomes via transport carriers that emerge from these domains. **b**, enlarged schematic of stage I melanosomes emphasizing processing of PMEL by S2P and subsequent ESCRT-independent sorting of (i) PMEL-Mα to ILVs, from which fibrils emanate, and (ii) CTF to an ESCRT- and γ-secretase dependent degradation pathway in lysosomes.

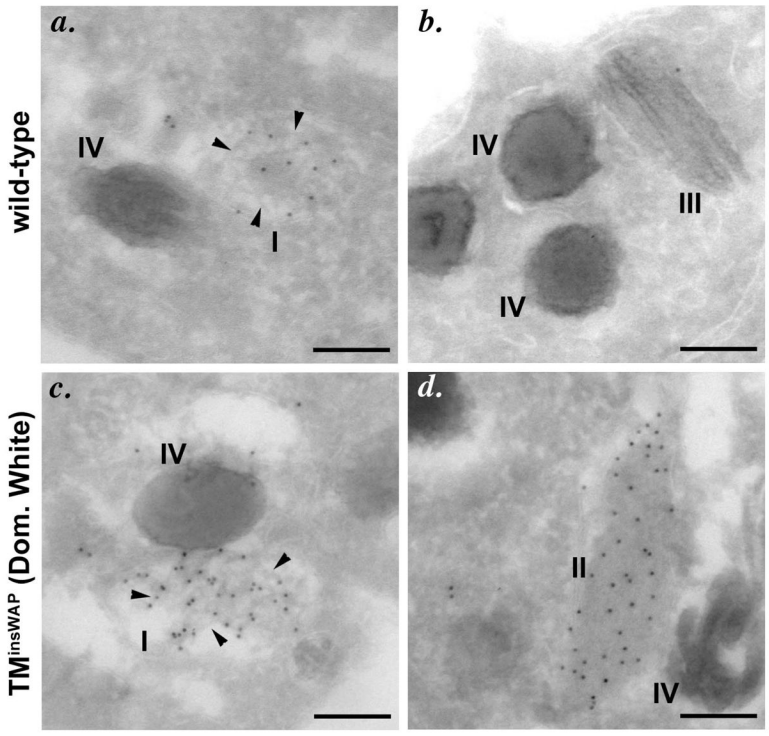

Figure 6. The *Dominant White* PMEL variant generates aberrant fibrils

Immunoelectron microscopy analysis of mouse melanocytes expressing wild-type human PMEL (wild-type; **a**, **b**) or PMEL-TM^{insWAP}, harboring an insertion in the transmembrane domain identical to that of the chicken *Dominant White* allele [TM^{insWAP} (Dom. White); **c**, **d**]. Ultrathin cryosections were immunogold labeled for human PMEL. Note the effective formation of fibrils within stage I melanosomes by both isoforms (**a**, **c**). Whereas wild-type human PMEL becomes incorporated into mature fibrillar sheets that are quickly coated with melanins and inaccessible to antibodies in stage II/III melanosomes (**b**), the TM^{insWAP} variant forms sheets in stage II-like melanosomes that do not become melanized and that remain accessible to antibody labeling (**d**). Adapted from Figure 6 of (Watt *et al.*, 2011).