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Why Study Functional Amyloids? Lessons from the Repeat Domain of Pmel17

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Abstract

One of the current challenges facing biomedical researchers is the need to develop new approaches in preventing amyloid formation that is associated with disease. While amyloid is generally considered detrimental to the cell, examples exist of amyloids that maintain a benign nature and serve a specific function. Here, we review our work on the repeat domain (RPT) of the functional amyloid Pmel17. Specifically, the RPT domain contributes in generating amyloid fibrils in melanosomes upon which melanin biosynthesis occurs. Amyloid formation of RPT was shown to be pH sensitive, aggregating only under acidic conditions associated with melanosomal pH. Furthermore, preformed fibrils rapidly dissolved at neutral pH to generate benign monomeric species. From a biological perspective, this unique reversible aggregation/disaggregation is a safeguard against an event of releasing RPT fibrils in the cytosol, resulting in rapid fibril unfolding and circumventing cytotoxicity. Understanding how melanosomes preserve a safe environment will address vital questions that remain unanswered with pathological amyloids.

Keywords

melanosomes; melanin; aggregation; pH; disassembly

Introduction

Amyloids are traditionally associated in the context of disease, however; the emerging concept of ‘functional amyloids’ has redefined this historic interpretation of amyloid [1]. Many examples of so called ‘functional amyloids’ have been discovered that serve a beneficial role where the amyloid carries out a specific function. Examples have been identified in bacteria [2], fungi [3], plants [4] and humans [5,6,7], offering molecular and cellular insights of using amyloids to carry out a function. Some of these functions are associated with bacterial physiology [8], hormone storage [6] and human reproduction [9]. With new functional amyloids constantly being discovered, a better understanding of what differentiates them from its pathological counterpart is emerging.

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One thought is that oligomers are the true pathogenic agents that are longer lived during pathological amyloid formation [10,11]. For example, soluble oligomers of A β correlate better with Alzheimer's disease severity than the insoluble fibrillar deposits that are present in amyloid plaques, suggesting that oligomeric forms are the toxic species [12]. In the case of functional amyloids, strict control would circumvent the buildup of toxic oligomers. This has been demonstrated with the rapid aggregation kinetics of the Orb2 protein [13], thereby bypassing oligomeric intermediate buildup. Tight kinetic control from the functional bacterial amyloid 'Curli' shows cell regulation at several steps by other proteins which facilitate localization and nucleate rapid polymerization of the protein CsgA [8]. Thus, *E. coli* has evolved ways to safely assemble amyloid and transport it to the extracellular matrix to carry out its specific function.

Sequestration of amyloids within membrane compartments is another possible mechanism to alleviate the detrimental effect caused by forming amyloid. For example, peptide hormones [6] and acrosomal matrix proteins [14] are stored in endocrine granules and acrosomes, respectively. These organelles provide an acidic environment optimal for the assembly of amyloid fibrils and prevent interaction with unwanted cellular components.

In this review, we highlight our work on the repeat (RPT) domain of Pmel17 that forms amyloid fibrils only at the biologically relevant pH of melanosomes [15,16]. Through a controlled series of proteolytic events of Pmel17, the RPT domain is released and self-assembles at acidic pH to form a fibrillar scaffold upon which melanin is deposited. This pH sensitive aggregation process was also shown to be reversible, with fibril disassembly occurring at near neutral pH. The ability to control fibril formation has strong biological implications. Unlike pathological amyloids, RPT fibrils rapidly dissolving at neutral pH would ensure that in the event of escaping from their melanosomal environment, they would disassemble and maintain a soluble benign form. This sensitive pH dependent mechanism sequesters fibril formation only in the melanosome where it aids in melanin synthesis.

Pmel17 and Its Structural Role in Melanin Synthesis

Pmel17 is a transmembrane protein that is proteolytically processed to generate intralumenal fibrils in melanosomes, acidic organelles that synthesize and store melanin [17]. The direct involvement of Pmel17 in melanin synthesis was shown using a Pmel17 knockout mutation in mice, where a 40–50% reduction in melanin content in hair was demonstrated [18]. This decline in melanin content suggests the fibrils might be responsible for enhancing melanin synthesis. To generate melanin, a multitude of oxidation steps are performed using tyrosinase on the substrate tyrosine. Specifically, L-tyrosine is oxidized by the action of tyrosinase to generate L-DOPA (L-3,4-dihydroxyphenylalanine), which is further oxidized to indole-5,6-quinone. This intermediate is then polymerized to produce either eumelanin or pheomelanin. For this review, we will focus on the formation of the brown-black pigment eumelanin, also referred here as melanin. Since melanin precursors are cytotoxic, it is suggested that intralumenal fibrils sequester these intermediates and subsequently polymerize along the fibril axis to form melanin [19]. These events permit a detoxifying environment to safely permit successful synthesis of melanin.

Melanosomes are tissue specific ‘lysosomal-like’ organelles that mature through four morphological stages, assigned based on fibril formation and melanin synthesis. Stages I and II are defined by formation of fibrils that initiate in association with intraluminal vesicles (ILV’s) during stage I and are fully matured by stage II [19]. Spherical shaped stage I melanosomes deform into ellipsoidal stage II structures that originate from mature fibrils (6–10 nm in diameter), forming parallel arrays that span the length of the organelle. Melanin synthesis begins at stage III where fibrils are coated with melanin that grow 2-fold in diameter. By stage IV, fibrils are no longer visible and the organelle is completely enriched with melanin [19].

Pmel17 was first identified genetically as the ‘silver gene’ in mice, whose mutation resulted in hypopigmentation [20]. It was not until 60 years later in 1991 that the gene was identified and cloned [21]. Transient expression in nonmelanocytic cell lines generated fibril structures that were reminiscent to stage II melanosomes [22]. Pmel17 is synthesized in the endoplasmic reticulum as a 668-residue protein containing a single transmembrane domain, a small C-terminal cytoplasmic domain and a large luminal domain. Posttranslational modification in the Golgi apparatus introduces N- and O-linked oligosaccharides [23,24] that are attached mainly in the large luminal domain [25]. The resulting protein is then delivered to endosomes where it internally associates with ILV’s [19]. A proprotein convertase first cleaves Pmel17 between residues 467 and 468 to produce a C-terminal fragment M β (residues 468–668) and an N-terminal fragment M α (residues 25–467) [17,26] that remains disulfide linked by cysteine residues within the M α and M β regions. The M β transmembrane fragment is then cleaved by BACE2 [27,28], a transmembrane aspartic acid protease that liberates the luminal M α fragment associated to a portion of the M β fragment called M β N (Figure 1A). The remaining M β fragment, termed C-terminal fragment (CTF) generated by BACE2 is further proteolyzed by a γ -secretase [29].

Following release of M α , a series of unknown proteolytic events occur on M α to generate smaller fragments that then proceed to form amyloid fibrils. Several of these regions have been tentatively assigned based on sequence homology. An N-terminal domain (~200 amino acids) is thought to share sequence similarity to the closely related glycoprotein NMB (nonmetastatic melanoma B) [30]. Immediately following this region is the polycystic kidney disease (PKD)-like domain (~90 amino acids) despite its weak sequence identity to a repeat region found in the protein polycystin-1 [31]. PKD-like domain is proposed to contain a β -sandwich domain like PKD but no structural information for this region is currently available. Between the PKD-like region and the proprotein convertase cleavage site lies a region called the repeat domain (RPT) (~130 amino acids) due to a series of imperfect sequence repeats rich in proline, serine, threonine and glutamic acid residues [22] (Figure 1B). Deletion of M α or individual regions affected fibril formation in both melanocytic and non-melanocytic cell lines [22,32,33].

Defining the Fibril-Forming Region of Pmel17

Melanosomes were first shown to contain amyloid fibrils using the amyloidogenic dyes thioflavin S (ThS) and Congo Red [5]. To associate Pmel17 to these structures, a Triton X-100 insoluble melanosome fraction showed colocalization between ThS stained particles

and Pmel17 immunofluorescence. Direct involvement of Pmel17 in amyloid formation was demonstrated using a recombinant M α (residues 25–467) fragment expressed in *E. coli* which resulted in rapid aggregation upon dilution out of guanidinium-HCl [5,31]. These aggregates had all the hallmarks of amyloid and its rapid aggregation having biological relevance for a functional amyloid.

Our attempts to replicate fibril formation using M α (residues 25–467) did not yield the characteristic amyloid fibrils typically observed by TEM [34]. While rapid aggregation and a moderate ThT fluorescence response occurred, these structures looked more amorphous than filamentous. Supporting this observation, the human melanoma cell line Mel220 stably expressing Pmel17 showed by antibody epitope mapping to contain M α region (residues 25–467) in a Triton X-100 soluble fraction [33]. Since amyloid is generally associated in a detergent-insoluble fraction, it can be assumed M α remains non-amyloidogenic prior to further proteolytic processing. In fact, many studies based on antibody epitope mapping conclude smaller peptide fragments that contain regions of the RPT [22,23] and PKD domain [31,35] are found in detergent insoluble fractions.

To identify the likely amyloid-forming region of Pmel17, an array of peptide fragments that cover regions within M α (residues 25–467) were cloned and expressed in *E. coli*. Under a variety of conditions, only the protein fragment corresponding to the RPT domain (residues 315–444) formed amyloid fibrils [34] (Figure 1B). Interestingly, this recombinant protein was purified in an unstructured state and only aggregated when subjected to acidic pH (~4.5–5.5). Aggregation kinetics at pH 5 showed a typical sigmoidal kinetic behavior consisting of a lag, growth and stationary phase (Figure 1C). TEM images tracking aggregation over time revealed a transition from irregular fibril-like aggregates to long unbranched filamentous structures (Figure 1C). Amyloid presence was also confirmed by Congo Red birefringence, proteinase-K (PK) resistance, while β -sheet presence validated using circular dichroism (CD) spectroscopy, electron diffraction and solid-state NMR spectroscopy [34]. Corroborating this observation, deletion of RPT from Pmel17 *in vivo* showed a lack of fibrillar structures when using HeLa cells [22] and melanoma cell line Mel220 [33]. Furthermore, the antibody HMB-45 which reacts with the only O-glycosylation site in RPT (between residues 328–344) decorates *ex vivo* fibrils [22,23].

While it is universally agreed that the RPT domain is a feature of melanosome fibrils, others pinpoint the PKD domain (residues 201–314) [31] and core amyloid fragment (CAF) (residues 148–223) [36] as the amyloid-forming region, suggesting that RPT likely serves a regulatory rather than a structural role. While we cannot preclude the involvement of these regions in amyloid formation, further *in vitro* work is needed, especially confirming the CAF (residues 148–223) is amyloidogenic under melanosomal-like conditions. With that said, we do not believe the prevailing reasons against RPT forming amyloid *in vivo* are sufficient and conclusive. These reasons are discussed at a later section of this review.

Mechanistic Insights into pH-dependent RPT Amyloid Formation

During melanosome maturation, the pH environment changes from a starting pH ~4.0 in stages I and II to near neutral pH (~pH 6.0) in melanized stage IV melanosomes. Knowing RPT aggregates into amyloid at pH 5 prompted a more detailed study of aggregation as a

function of pH. Using an array of techniques that include ThT (an amyloidogenic dye) and intrinsic Trp (W423) fluorescence (Figure 1D), light scattering and TEM were used to track amyloid formation [37]. At pH 4.0–4.5, aggregation occurred rapidly with moderate ThT response and TEM images depicting small prefibrillar species that looked more amorphous than filamentous. Fluorescence measurements of the only tryptophan (W423) in RPT, previously shown as an excellent structural probe [37], revealed a high intensity and blue shifted spectral change that was completed within 5 h (Figure 1D). This spectral blue shift reflects a more hydrophobic environment for W423 and the increased emission intensity likely due to changes in the nearby carboxylate protonation states.

At pH 5, W423 fluorescence showed a moderate decrease in emission intensity and increased blue shift by ~20–30 h. The spectral differences between pH 5 and pH 4–4.5 is reflected in fibril morphology, where pH 5 aggregates show more homogeneous fibrillar structures. Increasing the pH initially showed W423 emission at pH 5.5 is nearly identical to that observed for pH 6.0; however, after 40–50 h growth intensity drops dramatically, and a spectral blue shift is observed at pH 5.5 (Figure 1D). At pH 6.0, W423 emission is unchanged and no aggregates were visible by TEM and secondary structural analysis by CD spectroscopy, all indicative of RPT being unstructured [37].

To mimic RPT amyloid formation that parallels the pH change during melanosome maturation, preformed aggregates were first generated at pH 4.0 (Stage I) and then titrated to pH 5.0 (Stage II). Rapid (on the order of minutes) structural changes by TEM were observed with small prefibrillar aggregates forming long un-branched fibrils. This transition is reminiscent to morphological changes observed between stage I and II melanosome maturation [19]. Metabolic pulse/chase assays infer proteolytic processing of M α into insoluble fibrils also occurs on the order of minutes, making the *in vitro* data biologically relevant [17,26].

The sensitive pH dependence of RPT fibril formation would suggest that protonation sites of carboxylic acids influences aggregation kinetics. The amino acid content of RPT has 15 Glu and 1 Asp scattered throughout the sequence with a bias towards the C-terminus region (Figure 1B). NMR and limited PK proteolysis experiments suggest the amyloid forming region is located more towards the C-terminus of the RPT sequence, limiting the number of acidic residues that could contribute to this process. Specifically, limited PK experiments on RPT fibrils showed resistant peptide fragments corresponding to 390–444, 393–444, and 400–444 [38]. These fragments also contain the longest proline-free stretch (403–431) and a predicted amyloidogenic sequence (403–413). Solid state NMR experiments indicate the presence of protonated Glu side chains in RPT fibrils, especially those in the C-terminus [39]. Thus, Glu residues: E404, E422, E425 and E430 were investigated, assessing the importance of hydrogen bonding and protonation at these sites with Ala and Gln mutations, respectively [38]. Combinations of single, double, and quadruple mutations were generated to assess possible allosteric effects.

Aggregation kinetics with Ala and Gln mutants revealed E422 had the greatest sensitivity towards a pH change. A shift of more than one pH unit was observed with E422A and E422Q (Figure 2A) mutants, with aggregation now occurring at pH 6.5. The lag phase

lengthened and a lower ThT fluorescence was observed as the pH increased. At pH 6.5, fibrils could be observed by TEM (Figure 2A) that are reminiscent to those formed at pH 5. Other single mutations (E425 and E430) had no effect except for E404, which occasionally aggregated and formed fibrils at pH 6. A double mutation neutralizing E404/E422 with either Ala and Gln (Figure 2B), caused aggregation to now occur at pH 7.0, while additional double mutations at E425 and E430 had no effect. These data suggest that protonation of E422 is essential at defining this strict pH-dependence in RPT fibril formation, and that E404 has an ancillary role in this process.

From a structural perspective, E404 and E422 likely reside in the amyloid core region of RPT. Their involvement is further supported by deletion mutants ⁴⁰⁵VSIVVL⁴¹⁰ which ablates amyloid formation and ⁴¹¹SGTTA⁴¹⁵ causes morphological changes in fibril structure (Figure 2C). A schematic representation for one possible structure is shown in Figure 2D where two β -strands are connected *via* a β -turn to form a hairpin-like conformation, representing one monomeric unit in the fibril structure. Glu side chains are oriented with E404 outside and E422 inside the filament (Figure 2D). The position of these residues would imply that upon protonation, both intra- and inter- β -sheet contact are important in maintaining fibril structure.

Melanosomal Membrane Lipids Modulate RPT Fibril Formation

It has been suggested that Pmel17 processing and liberation of the amyloid forming region (RPT domain) occurs in conjunction with ILVs [40,41] (Figure 3A). This association is most evident during stage I melanosome development where prefibrillar aggregates formed from the proteolytic processing of Pmel17 appear to emanate from ILVs [19]. Recently, Apolipoprotein E was shown to be associated with ILVs and regulate the formation of Pmel17 amyloid fibrils in endosomes [42]. As the melanosome matures and fibrils elongate (stage I to II), ILV sizes decrease (Figure 3A). In stage II melanosomes most of the ILVs are near the limiting membrane, a result of mature fibrils perhaps forcing them to the poles of the now formed ellipsoidal membrane. Interestingly, melanosome membranes contain a high content (>10% total lipid) of lysophospholipids (lysolipids), cone-shaped structures that contribute to positive spontaneous curvature [43]. Their presence would support formation of highly curved small ILVs and stabilize the ellipsoidal-shaped stage II melanosomes.

To directly evaluate the effects of lysolipids on RPT fibril formation, small unilamellar vesicles containing either negatively charged lysophosphatidylglycerol (LPG) or zwitterionic lysophosphatidylcholine (LPC) were added to RPT under aggregation conditions at pH 5 [44]. Vesicles composed of 90% phosphatidylcholine [1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-choline (POPC)] and 10% either lysolipid accelerated RPT aggregation kinetics, with LPG having the greatest influence. TEM images taken post aggregation show fibrils formed with no obvious morphological difference from using either lipid condition [44].

To specifically test a direct effect of lysolipids on RPT aggregation, both monomeric and micellar (above critical micelle conc. (CMC)) forms of lysolipids were used and monitored by ThT fluorescence. In the presence of LPG monomer (<1 mM), a shortened lag time and faster growth phase was observed when compared to RPT alone. Contrastingly, LPG micelles (CMC ~0.6 mM) significantly reduced RPT fibril formation. For LPC, both

monomer and micellar forms stimulated RPT aggregation, with micelles exerting the greatest influence. TEM images reveal fibrils formed in the presence of LPG and LPC monomer, that are reminiscent to those formed in the absence of lipids. For micelles, fewer fibrils are observed with LPG, consistent with a reduced ThT response, while in the presence of LPC micelles, RPT fibrils are abundant yet appear thinner in diameter [44].

From a mechanistic view, the influence lysolipids have on RPT aggregation is depicted in Figure 3B. Here, LPG monomers binds to RPT monomer, forming a protein-lipid complex through hydrogen bonding of the LPG glycerol head group and the amyloid forming C-terminal region of RPT. This in turn reduces protein-protein electrostatic repulsion, promoting intramolecular interaction in the C-terminus to initiate nucleation events that ultimately lead to faster aggregation (Figure 3B). The reduced aggregation propensity of RPT in the presence of LPG micelles can be rationalized by an increase in helical content. Here, an ~20% helical content is measured in the presence of LPG micelles. This increase fits nicely to a predicted helical region spanning a proline-free C-terminal stretch of 29 residues (403–431), the critical region in amyloid formation. As a result, the presence of the α -helix inside the amyloid core would arrest β -sheet structure formation, leading to fewer formed fibrils (Figure 3B).

For LPC, a different mechanism for stimulated RPT aggregation is proposed, since the choline moiety of LPC cannot form intermolecular hydrogen bond. To account for an increase in fibril formation, the non-specific binding of LPC monomers to RPT causes greater exposure of hydrophobic regions in the monomer, which in turn drives protein-protein interaction and faster aggregation kinetics (Figure 3B). A similar mechanism is proposed for LPC micelles, where exposed regions of RPT accumulate and concentrate on the surface of micelles, creating more nucleation sites that promote enhanced aggregation kinetics (Figure 3B).

Probing Disassembly of RPT Fibrils as a Function of pH

The strict pH dependence of RPT fibril formation was determined to be reversible, with preformed fibrils at pH 5 rapidly disassembling when exposed to neutral pH [34,37,38,45]. This unique characteristic is in total contrast to pathological amyloids that generally resist the harshest of treatments. However, in the case of functional amyloids this observation has been shown from many peptide hormones [6] which have a reversible aggregation/disaggregation process. In the acidic environment of secretory granules, these peptide hormones are densely packaged and stored in an inert amyloid-like state. Upon release from these membrane-enclosed organelles, fibrillar aggregates disassemble to their biologically active monomeric state due to neutral pH exposure.

Using solution-state NMR spectroscopy, the fibril disassembly of RPT was monitored as a function of time [45]. ^{15}N HSQC spectrum was first measured using soluble isotopically-labeled RPT at pH 6.5 to obtain backbone amide hydrogen and nitrogen assignments. Under these conditions, spectroscopic features of all assigned residues were consistent with a random coil configuration. In the fibrillar state at pH 5, no backbone assignments could be obtained for residues 378–444 likely due to a large rotational correlation time in the amyloid core. In contrast, residues 316–377 are visible with broadened line widths that increase as

the residues approach the C-terminus. No significant chemical shift changes were observed compared to the soluble form, supporting a flexible N-terminus in the fibrillar state.

To obtain structural changes during disassembly, pre-formed isotopically-labeled fibrils were resuspended in pH 6.5 buffer and ^{15}N HSQC spectrum recorded every 30 minutes at 22 °C [45]. Figure 4A shows a comparison of the intensity ratios of the initial (time zero) and 30-minute spectra of fibrils exposed to pH 6.5 (cyan) to those of the fibril and monomer spectra (purple). Overall, the disassembly event followed a trend consistent of fibrils unfolding directly to monomer with no evidence of intermediates during this time frame. Special attention to the Glu backbone amides revealed no significant differences among the 15 different glutamates, suggesting fibril disassembly is a global effect involving multiple deprotonation events. A comparison of backbone amide kinetics for E318 (outside amyloid core) and E422 (within amyloid core) (Figure 4A inset) exemplifies this observed trend.

Using atomic force microscopy, fibril disassembly was probed at the ultrastructural level [45]. Upon washing pre-formed fibrils at pH 6.5, a gradual (order of minutes) morphological change was observed where larger fibrils fragment before completely disappearing (Figure 4B). This fragmentation pattern appeared random along the fibril axis, resulting in smaller fibrils varying in length (Figure 4B). A technique known as scanning force kymography was used to examine the kinetics of fibril disassembly as a function of time. A total of 18 individual events were recorded and revealed asymmetric dissolution rates at both fibril ends (Figure 4C). The observed variation in dissociation rates could be due to structural differences in the RPT fibril.

RPT Amyloid Enhances Melanin Synthesis at Acidic pH

To recapitulate the involvement of RPT in melanin synthesis, an *in vitro* assay was performed using RPT fibrils, tyrosinase and L-3,4-dihydroxyphenylalanine [34,39]. Under acidic pH, tyrosinase activity is known to be significantly diminished, with optimal activity at pH 6.8 [46]. However, in the presence of RPT fibrils at pH 5, a six-fold increase in melanin content was observed when compared to a reaction containing only soluble RPT [34]. This enhancement offers support for the direct involvement of RPT fibrils in catalyzing the polymerization step during melanin synthesis. Also, since RPT fibrils are likely more unstable in mature melanosomes where the pH is less acidic, deposition of polymerized melanin would likely protect fibrils from disassembling. While RPT fibrils enhanced melanin synthesis, other amyloid fibrils formed by the yeast prion Sup35 and the fungal protein Het-s also showed enhanced melanin rates [5,34]. This implies that a fibrillar structure is the minimum requirement to boost melanin production.

RPT Fibrils Have an In-register Parallel β -Sheet Structure

Using solid state NMR spectroscopy, preformed RPT fibrils formed at pH 5 were shown by selective ^{13}C -labeled amino acids to adopt the typical in-register parallel β -sheet architecture observed for pathological amyloids [39]. By labeling with ^{13}C -labelled amino acid (Ala, Val, or Met) in the carbonyl position, the closest intra/intermolecular ^{13}C distance between β -stands in a β -sheet was approximately 0.5 nm, suggestive of in-register parallel structure. ssNMR measurements of three independently prepared $^{13}\text{C}^{15}\text{N}$ -labeled RPT fibril samples

showed dramatic differences in the ^{13}C - ^{13}C NMR spectra as revealed by chemical shift, cross-peak positions and intensity patterns [39]. Close inspections of the three samples revealed residues 406–422 were always shown to have back bone assignments and hence part of the amyloid core. Corroborating these data, a peptide fragment $^{405}\text{VSIVVLSGT}^{413}$ was shown to aggregate and form amyloid fibrils, supporting this region as the amyloidogenic core [47]. Outside of this region, the three samples have protein segments with unique backbone assignments that are not conserved between the samples. A term ‘segmental polymorphism’ [48] was coined to describe these polymorphic fibrils that differ in protein segments that contribute to the amyloid structure. Interestingly, TEM images showed similar morphology and PK experiments gave an identical resistant core of the three fibril samples. While these data support a structural architecture similar to pathological amyloids, a separate study using structural and computational analysis of the RPT domain (residues 315–444) proposed a β -solenoid model [49].

Pmel17 Orthologs Contain Amyloid Forming RPT Domains

Pmel17 is conserved across many species with the RPT domain being the most varied in sequence identity [25]. This may suggest that the diversity in the number and sequence of repeats in RPT makes it an improbable candidate for the amyloid core for an evolutionarily conserved functional amyloid. To observe if fibrils are formed from these regions, the repeat sequences from mouse (mRPT), zebrafish (zRPT) and a known splice variant of the human Pmel17 (sRPT) sequence where 42 amino acids is deleted from repeats 5 to 8 were studied [50] (Figure 5). While mRPT has ~50% sequence identity to the human form (Figure 5B), zRPT shares no sequence identity to either ortholog (Figure 5C).

Under acidic pH with gentle agitation, all three RPT orthologs formed amyloid fibrils with differing structural features (Figure 5A–C). The sRPT fibrils formed bundles of twisted protofibrils that contrasted the single fibril morphology observed for full-length RPT (Figure 5A). In the case of mRPT, a twisted fibril morphology with an average diameter of 14 nm was observed (Figure 5B). Mass per unit length measurements of unstained mRPT fibrils gave a mean mass/length 2.12 ± 0.3 monomers/4.7 Å, suggesting paired protofibrils. In the case of zRPT, TEM images showed these fibrils to be thinner and have a tendency to bundle (Figure 5C). Solid-state NMR experiments of all three orthologs revealed a conserved in-register parallel structure [50].

While amyloid formation is conserved at acidic pH across species, it is also interesting to note the high number of Glu residues present from the different sequences. In human RPT there are fourteen, while mRPT has six and zRPT having twenty-seven Glu residues. The conservation of the critical residue E422 in human RPT appears to be conserved in mRPT, yet due to its highly repetitive sequence, zRPT is harder to interpret. However, a sequence alignment involving ten putative RPT sequences from other Pmel17 orthologs revealed many of these species could have a conserved critical Glu residue that defines a pH dependence in amyloid formation [50].

Controversy Surrounding a Structural Role for RPT

While our work highlights RPT as highly amyloidogenic under melanosomal conditions, there is controversy surrounding whether RPT forms amyloid *in vivo*. Here, we discuss reasons supporting our *in vitro* work and addressing prior concerns. First, the statement that the RPT domain is heavily O-glycosylated and for that reason cannot be considered to participate in amyloid formation *in vivo* is misinterpreted. Yes, the RPT domain is O-glycosylated, but the only known glycosylation sites are in the region between residues 328–344. No O-glycosylation sites have been identified outside of this region. Our data show residues 328–344 are located far away from the C-terminal amyloid core that we have extensively characterized *in vitro* and therefore, should not be hindered by O-glycosylation. Specifically, we found that a small C-terminal peptide region (residues 405–410), when deleted from RPT ablated fibril formation. In addition, a short C-terminal segment of RPT composed of ⁴⁰⁵VSIVVLSGT⁴¹³ alone was capable of self-assembling into amyloid fibrils.

Second, the idea that recombinant RPT aggregation is slow and not biologically relevant is misleading. In our hands, aggregation at pH 4 (stage I melanosomal pH) occurs instantaneously, while titration to pH 5 results in structural changes to generate long filamentous material on the order of minutes. This time frame is highly supportive of Pmel17 processing to generate fibrils. Additionally, lipid vesicle compositions containing melanosomal lipids showed enhanced aggregation of recombinant RPT, adding further biological relevance to our *in vitro* data.

Third, the sequence diversity in the RPT sequences from various Pmel17 orthologs had inferred that this makes RPT amyloid formation even more improbable. However, both mouse and zebrafish RPT domains, which share low to no sequence identity to human RPT form amyloid in a pH dependent manner. Differences in amino acid sequences should not discount its ability to be amyloidogenic.

Fourth, prior work showed Mel220 cells stably expressing RPT (residues 315–431) formed ellipsoidal melanosomes harboring fibril-like aggregates [33], inferring this domain was not essential in forming the melanosomal matrix. While this would discount a structural role for RPT, it was noted by the authors that there were morphological changes in aggregate structures upon removing RPT. Paradoxically, an absence of fibrils was observed upon expression of Pmel17 lacking the RPT domain (residues 315–444) in non-melanocytic cells [22]. The former points to other Pmel17 regions having structural roles, while the latter would suggest a structural role for RPT in amyloid formation.

Lastly, dissolution of RPT fibrils at neural pH would not align with fibril structures present in mature melanosomes where the pH is thought to be near neutral [51]. While we cannot ignore this observation, we hypothesize that RPT fibrils are dispensable at this point since melanin is already formed. This in turn could be an elegant way to recycle amyloid fibrils within melanosomes once melanin synthesis is complete. Alternatively, the coating of melanin to these fibrillar structures could offer protection to fibril disassembly. Assessing fibril integrity in mature melanosomes needs to be established to address the fate of RPT.

Conclusion and Future Direction

In this review, we focus on our work on the RPT domain of Pmel17 that forms amyloid fibrils only at the physiological pH (pH 4.0–5.5) of early stage melanosomes. Our work provides an elegant mechanism that shows a reversible aggregation/disaggregation process that is governed by pH. The strict pH dependence was largely controlled by the protonation of residue E422. Strikingly, removal of a single negative charge at E422 out of 16 carboxylic acids shifted the pH dependence by a full pH unit. The ability to disassemble pre-formed RPT fibrils at neutral pH was shown by AFM and solution-state NMR spectroscopy to occur in the absence of stable oligomeric intermediates. We have shown that LPG and LPC, abundant melanosomal lysolipids strongly influences RPT aggregation. This observation supports an important role for membranes in modulating fibril formation. It also motivates future studies of other abundant lipids found in late endosomes such as lysobisphosphatidic acid [52]. Knowledge gained from this will also be pertinent in comparing the role lipids play in pathological amyloids.

In a broader context, this strict pH dependence in fibril structure modulation has biological implications, where amyloids can only form at the acidic conditions associated with melanosomal pH. Furthermore, the dissolution of fibrils at neutral pH suggests that in an event of releasing RPT fibrils in the cytosol, rapid fibril disassembly to benign monomeric species would provide an essential response to circumvent cytotoxicity. The striking reversible pH dependent aggregation/disaggregation process has parallels to functional amyloids formed from peptide hormones [6]. Such mechanisms are clearly lacking in pathological amyloids that generally resist the harshest of treatments. However, it has been shown that phosphorylation in the fibril forming core of the protein fused in sarcoma (FUS) inhibits hydrogel binding and droplet formation. Such posttranslational modifications could be a way to control pathological amyloid formation [53].

While we have a better understanding of RPT amyloid formation *in vitro*, questions surrounding how Pmel17 is proteolytically processed to initiate fibril formation *in vivo* remain unanswered. Interestingly, Pmel17 processing towards amyloid formation is reminiscent to the pathway associated with the Alzheimer's Disease-associated amyloid precursor protein [54], where a set of proteases generate amyloidogenic fragments. Here, fibril formation involves proprotein convertases and α -, β -, and γ -secretases that result in liberating amyloid- β peptides. In the case of Pmel17, tighter control and regulation exists to coordinate the release of the luminal M α fragment prior to fibril formation. This in turn could explain why fibrils remain benign when sequestered in melanosomes. As for A β processing, such latter steps are likely lacking, further differentiating pathological from functional amyloid formation.

Once fibril formation occurs, deposition of polymerized melanin ensues; however, mechanistic insight is also lacking as to how melanin precursors bind and remain strongly associated with fibrils. The function of melanosomal fibrils is to sequester melanin, and hence defined as a beneficial amyloid, yet we only have a basic understanding of how it carries out its function. Resolution of these mechanisms will require *in vitro* reconstitution

with purified components in their native state. Only then would the complete mechanism of Pmel17 amyloid formation be ultimately understood.

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TEM images taken during aggregation at 14 h (red) and 61 h (blue) are shown. Inset scale bar is 100 nm. **(D)** W423 emission intensity surfaces as a function of pH (4.0–6.0) (top to bottom). RPT emission surfaces are plotted as a function of aggregation time (0–90 h) and wavelength (300–400 nm). Intensity units for W423 emission are arbitrary units (blue to red) normalized to pH 4.0 intensity.

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Figure 2.

(A) Effect of pH on aggregation kinetics of E422Q monitored by ThT fluorescence at pH 5.5 (black circles), pH 6.0 (open triangles), pH 6.5 (open squares) and pH 7.0 (black lines). Representative TEM image taken post-aggregation at pH 6.5 (B) Aggregation kinetics of E404Q/E422Q monitored at pH 5.0 (black lines), pH 6.0 (dark grey lines) and pH 7.0 (light grey lines). Representative TEM image taken post-aggregation at pH 7.0. (C) Representative TEM images of VSIVVL (cyan) and SGTTA (green) RPT mutants aggregated at pH 5.0. (D) Schematic representation showing one possible conformation of the amyloid forming region of RPT. Two β -strands (403–411) and (415–423) connected by a β -turn is depicted where side chain orientation for E404 (red) and E422 (pink) as being outside and inside of

the β -strands, respectively. Position of residues VSIVVL (cyan) and SGTTA (green) are shown and highlighted in the structure. Cross section of fibril axis is coming out of the page.

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Figure 3.

(**A**) Schematic representation showing morphological changes of melanosome maturation. Stage I melanosomes contain ILVs varying in size that are closely associated with prefibrillar aggregates that are generated from proteolytic processing of Pmel17. Stage II consist of long fibrillar striations that generally span the length of the melanosome. ILVs are shown to be more closely associated to the limiting membrane. (**B**) Schematic depicting the influence of lysolipids on RPT aggregation. (*top left*) LPG monomers bind to RPT monomers via hydrogen bonding between the LPG headgroup and C-terminal region of RPT. The resulting lipid-protein complex drives faster aggregation by increasing the intermolecular interactions between RPT monomers. (*bottom left*) LPG micelles interact with RPT monomers generating an increase in helical content, possibly in the proline-free

region (residues 403–431) that includes the critical amyloid-forming residues VSIVVL. This in turn reduces β -sheet formation and inhibits aggregation. (*top right*) RPT monomer in the presence of LPC monomer. The non-specific interaction between lysolipid and protein exposes hydrophobic regions of RPT, promoting protein-protein interaction and aggregation. (*bottom right*) LPC micelles form a scaffold for RPT monomers to accumulate on the surface, concentrating the protein and enhancing aggregation propensity.

Figure 4.

(A) Comparison of NMR amide resonance intensities for RPT fibril dissolution (right axis) and fibrillar vs monomer RPT (left axis). Intensity ratios [$I_{\text{fibril}}/I_{\text{monomer}}$] (purple circles) and [$I_{\text{initial}}/I_{\text{final}}$] (blue squares) are plotted as a function of residue number for the fibril (I_{fibril} , pH 5), monomer (I_{monomer} , pH 6.5), initial (I_{initial}), and final (I_{final}) time points of the disassembly process at pH 6.5. The inset shows disassembly kinetics monitored by E318 (green triangles) and E422 (red diamonds) with corresponding resonance peaks at selected times shown above. (B) AFM measurement of RPT fibril disassembly where pre-formed fibrils at pH 5.0 are washed with pH 6.5 buffer and recorded after 128 seconds. (C) Scanning force kymogram showing a single fibril scanned repetitively along its long axis as

a function of scanning time. Ends have been labeled for relative rates (an asterisk denotes fragmentation). Grayscale denotes fibril height. Time scale 0–900 s.

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Figure 5.

Sequences of (A) human short RPT (hsRPT), (B) mouse RPT (mRPT) and (C) zebrafish RPT (zRPT) (C) are shown. The hsRPT domain lacks the 42 amino acids and is shown with a strike through the residues. Glutamate residues are shown in red. Representative TEM images for each RPT ortholog formed at pH 5. Tobacco mosaic virus (TMV) is shown in mRPT sample for size comparison.