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# The Role of Glycosylation in the Control of Processing and Cellular Transport of the Functional Amyloid PMEL17

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## 1. Introduction

In this chapter, we focus on the effects of glycosylation in the cell biology of an amyloid, specifically in melanocytic cells. The actual operational definition of amyloid is that amyloid fibers display the cross- $\beta$  fiber diffraction pattern, which is the basis of fiber formation, that leads to aggregation of misfolded proteins [1]. Under this concept, classical disease-associated amyloids such as amyloid  $\beta$ -peptide (Ab) with Alzheimer's disease join other non-disease associated or functional amyloids such as PMEL/Pmel17/gp100/silver (herein referred to as PMEL), which is involved in the deposition of melanin [2-4]. Early reports suggested that two domains inside PMEL are contributors to fibril formation [5]. Today, unequivocal evidence recognizes that the so called repeat (RPT) domain is the only region capable to form amyloid fibrils *in vivo* and *in vitro* [6-8]. Unlike disease-associated amyloids, functional amyloids are the product of coordinated and regular cellular processes that ensure that amyloidogenesis does not result in cell damage or death [2;9]. Several reports have indicated the role of post-translational modifications, specifically glycosylation, in the protein aggregation that leads either to neurodegeneration [10;11] or physiological amyloid deposition [12]. In Alzheimer's disease, a cytosolic phosphoprotein called Tau, is abnormally phosphorylated [13]. The reason for this was attributed to an overall decrease in O-GlcNAcylation, a novel type of O-glycosylation by which the monosaccharide  $\beta$ -N-acetylglucosamine (GlcNAc) attaches to serine /threonine residues via an O-linked glycosidic bond. O-glycosylated tau protein aggregates which leads to the formation of neurofibrillary tangles leading to toxicity and neuron death [13;14]. The aberrant deposition of proteins as cellular inclusions or plaques in the form of amyloid fibrils is a characteristic hallmark of all amyloid diseases (or amyloidosis) and of the so-called conformational diseases. Additionally, the  $\beta$ -amyloid precursor protein ( $\beta$ APP) has also been found to be

modified in its cytoplasmic domain by both phosphorylation and glycosylation [15]. Large glycosylated secreted ( $\beta$ APP) derivatives are present in human cerebrospinal fluid, in brain, and in conditioned cell culture medium.

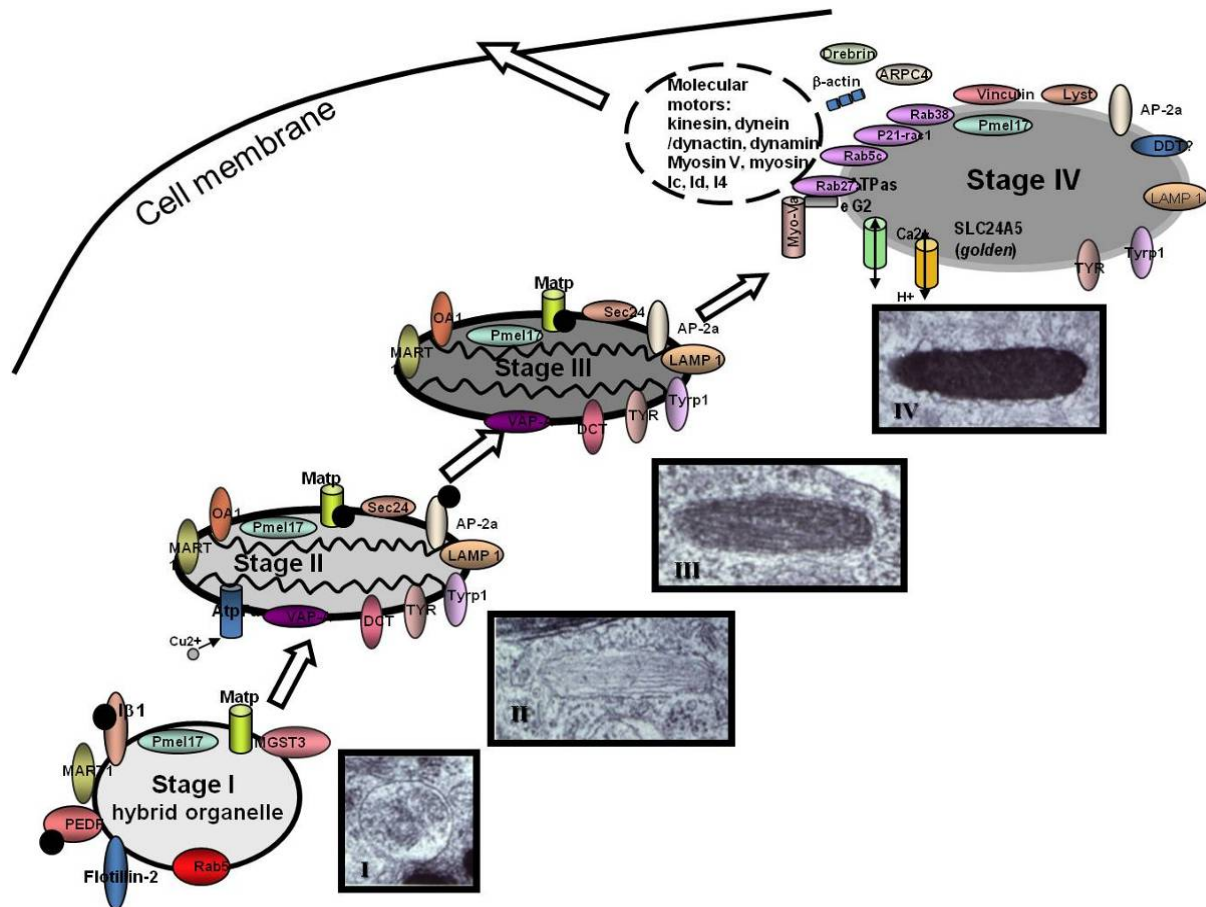
Despite the vast number of reports and clinical studies conducted to date, neurodegeneration and amyloid formation are still one of the least understood processes, which is mostly due to the complex nature of molecular interactions as well as the challenging properties of naturally aggregating systems. In particular, the low solubility of the proteins involved, their tendency to aggregate and their difficult synthesis/isolation *in vivo*. In this chapter, we detail the influence of glycosylation in the formation of functional amyloid, specifically for PMEL. The study of this type of amyloid raises the question on why these amyloids are non-toxic and whether there are lessons to be learned related with the cellular management of these amyloids that could be used to understand and possibly alter the detrimental effects of disease-associated amyloids.

## 2. Melanosomes and the functional amyloid PMEL

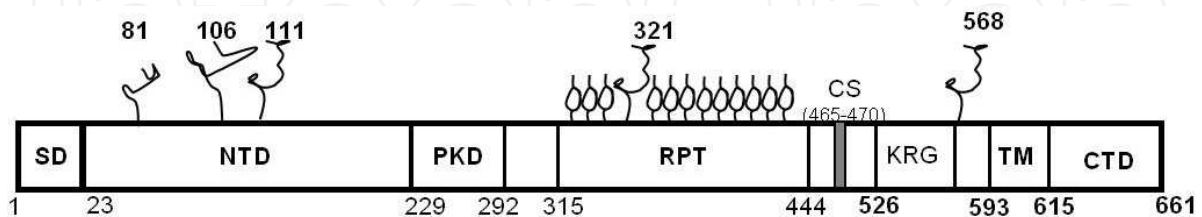
The ability to form pigment or melanin requires that the melanocyte handle the biochemical steps it takes in a safe and controlled environment. In many organisms, melanocytes build a specialized organelle called the melanosome. Melanosomes are membrane-bound organelles, specialized in the production and distribution of melanin pigment, that are conserved in structure from primitive organisms to mammals. However, the nature of this organelle has recently been studied in detail using novel techniques for isolation and global proteome analysis [16-18]. Those studies shed light on the complex nature of this organelle. As shown in Figure 1, melanosomes mature from undifferentiated vesicles (stage I) to elongated vesicles containing internal fibrils (stage II). In the presence of specific enzymes such as tyrosinase, melanin is synthesized and deposited on the internal fibrils (stage III) of melanosome until they become uniformly dense (stage IV) in pigmented cells. Previously, we detailed that this maturation process involves the acquisition of several specific proteins that we will call the maturation package that stabilizes the organelle for the subsequent maturation steps. As any structure, melanosomes build an internal matrix to store melanin in an orderly and efficient manner. The internal fibrils identified in stage II melanosomes have been unequivocally identified as amyloid aggregates [5;7]. Amyloid fibers derive from a class I transmembrane glycoprotein called PMEL that undergoes a series of post-translational modifications that involve processing by pro-protein convertases (PC) [5;19;19;20] and glycosylation [12;21] (Figure 2). These steps are highly regulated and follow an exact succession of processing steps which prevents the formation of amyloids during assembly or intracellular trafficking of PMEL.

Despite evidence that PMEL is a good substrate for PC in melanoma cells, Leonhard and coworkers confirmed that PC does not trigger immediate amyloid formation [19]. Our evidence suggests that glycosylation is responsible for such protection [12]. Glycosylation of proteins involves the addition of N- and/or O-linked oligosaccharide chains to acceptor asparagine residues or serine/threonine residues, respectively. N-linked core glycosylation begins in the ER, while O-linked glycosylation is a post-translational event which starts in the *cis*-Golgi [22]. PMEL has 5 potential N-glycosylation sites distributed at the N-terminal

(3), RPT (1) and C-terminal (1) domains [12;23]; while O-glycosylation sites have been predicted at the repeat sequences of proline, serine, and threonine in the RPT domain [24].



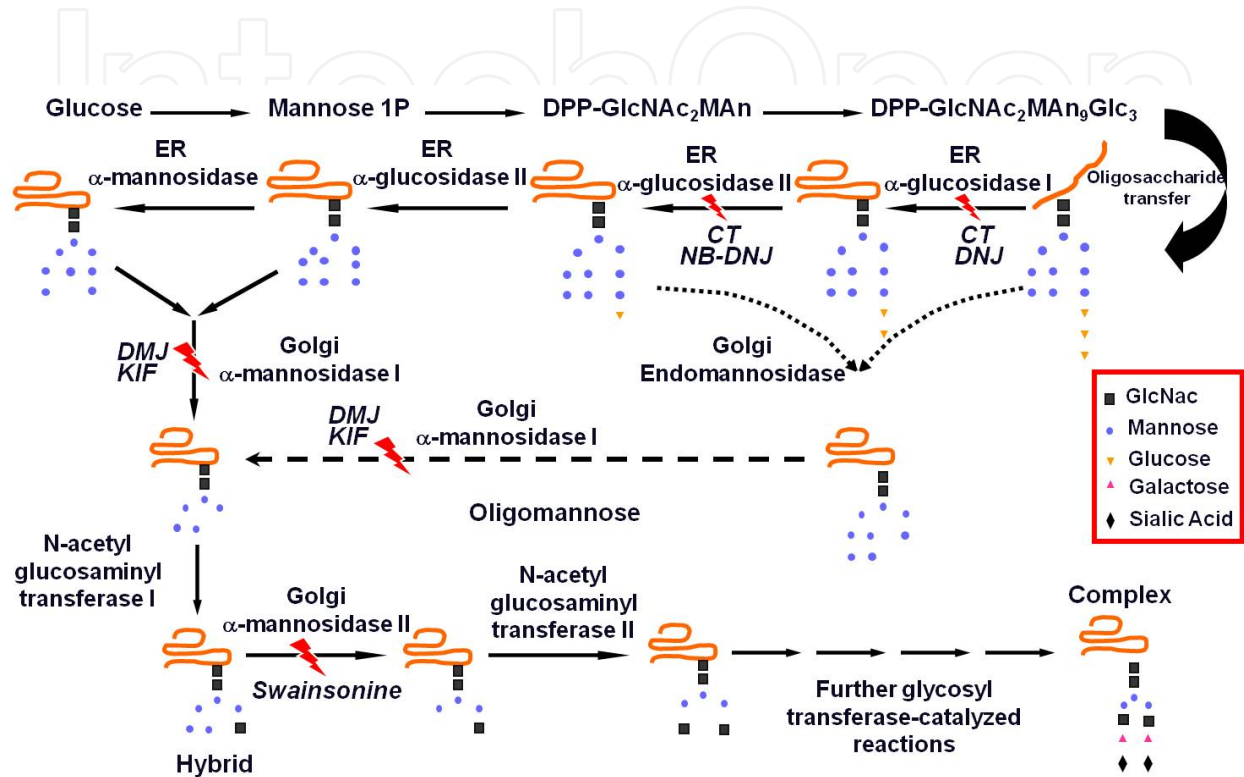
**Figure 1. Melanosome maturation at molecular and morphological levels.** The scheme details the process of melanosome maturation including variations at the molecular level (drawings) and morphological level (images). Note that every stage is characterized by a group of melanosome-specific and non-specific proteins. Electron microscopy images show the transformation of this organelle from an amorphous, non-pigmented and non-differentiated organelle (stage I) to a well-defined, differentiated and pigmented organelle (stage IV). Scheme adapted from [16].



**Figure 2. Scheme of PMEL domains and modification sites.** PMEL has only one conserved domain called polycystic Kidney disease (PKD) that is located near the center of the protein. During processing, the signal domain (SD) is removed early and then PMEL is cleaved (CS) by PC. The N-terminal (NTD) and C-terminal (CTD) domains undergo N-glycosylation, while the repeat domain (RPT) undergoes O-glycosylation. Numbers above the structures indicate the predicted aminoacid position for the N-glycan sites; while numbers below the protein graph indicate the aminoacids where each domain approximately starts and ends. Scheme adapted from [12].

## a. N-Glycosylation in PMEL

The  $\beta$ -glycosylamine linkage of GlcNAc to Asn represents the most widely distributed carbohydrate-peptide bond and is the site of attachment for a large variety of complex and poly-mannose oligosaccharides in proteins with demonstrated biological importance (See [25;26]). A summary of this process is presented in Figure 3.



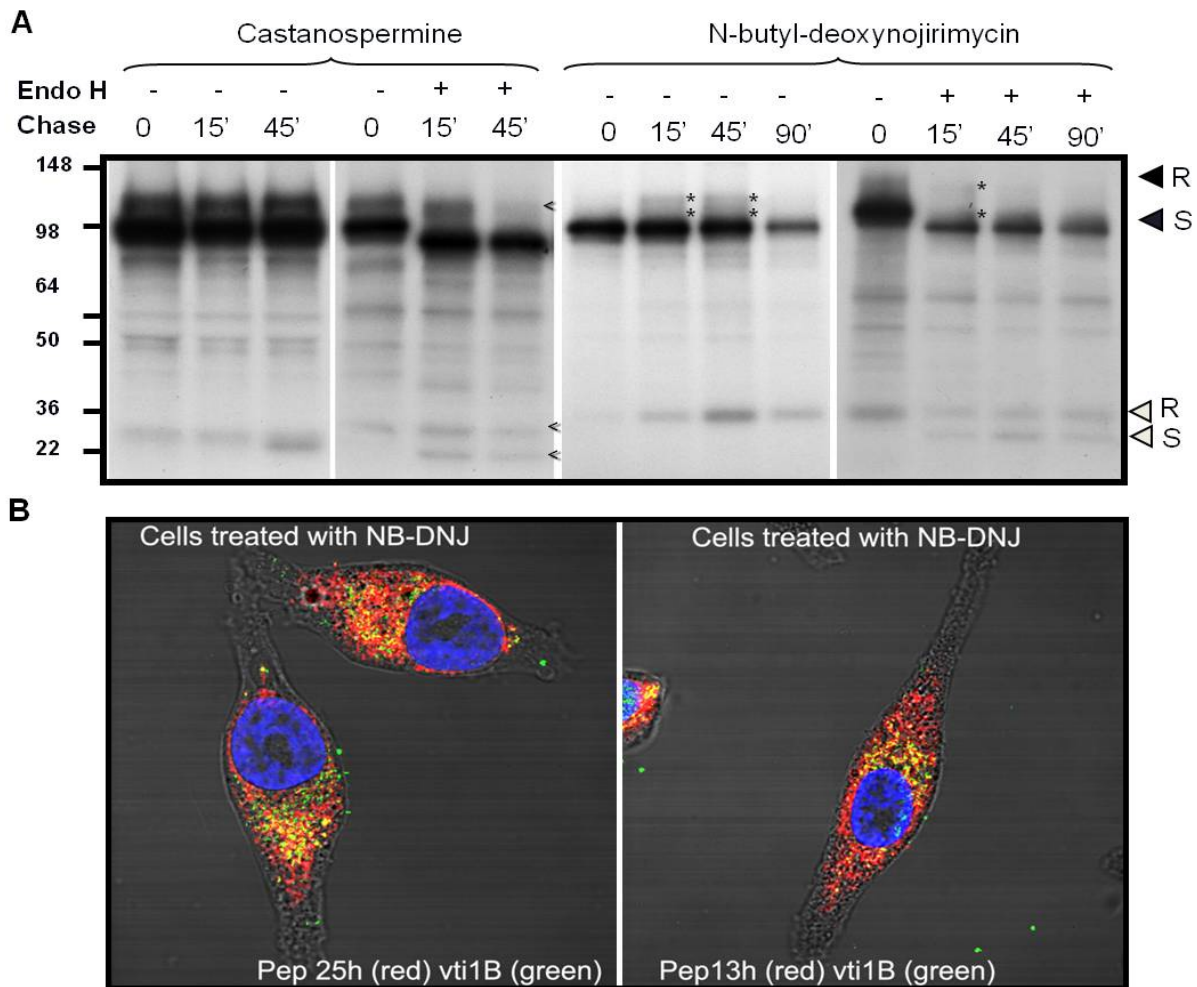
**Figure 3. N-glycosylation processing in mammalian cells.** The scheme details the place and different enzymes involved in the post-translational modification of glycoproteins with N-linked oligosaccharides. It also includes the different enzymes or steps targeted by different glucosidase inhibitors such as castanospermine (CT), deoxynojirimycin (DNJ), deoxymannojirimycin (DMJ), and kifunesine (KIF). Note that a series of sialyltransferases (not shown) are in charge of the addition of sialic acid at the end of the glycan chains late in the process. Peptide sequence is represented as an orange line. Scheme has been modified and adapted from [27].

In the ER, N-glycosylation starts with the attachment of a core unit of 14 monosaccharide residues ( $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ ), which is then processed by the removal of the 3 glucose residues attached to the terminal mannose D1 on the  $\alpha$  1-3 arm of the oligosaccharide by  $\alpha$ -glucosidases I and II [27]. In the Golgi, N-glycan processing starts when  $\text{Man}_6\text{GlcNAc}_2$  is trimmed to  $\text{Man}_5\text{GlcNAc}_2$  by Golgi mannosidase I (Figure 3). The extent of N-glycan processing towards triantennary and tetraantennary structures is limited by glycosidase availability and their accessibility to the processing sites. Noticeably, the enzyme endo- $\alpha$ -D-mannosidase provides an alternate route for de-glucosylation during a glucosidase blockade [27]. To determine the role of N-glycosylation in the processing and the trafficking of PMEL, we used several inhibitors that block the modification reactions either in the ER or Golgi

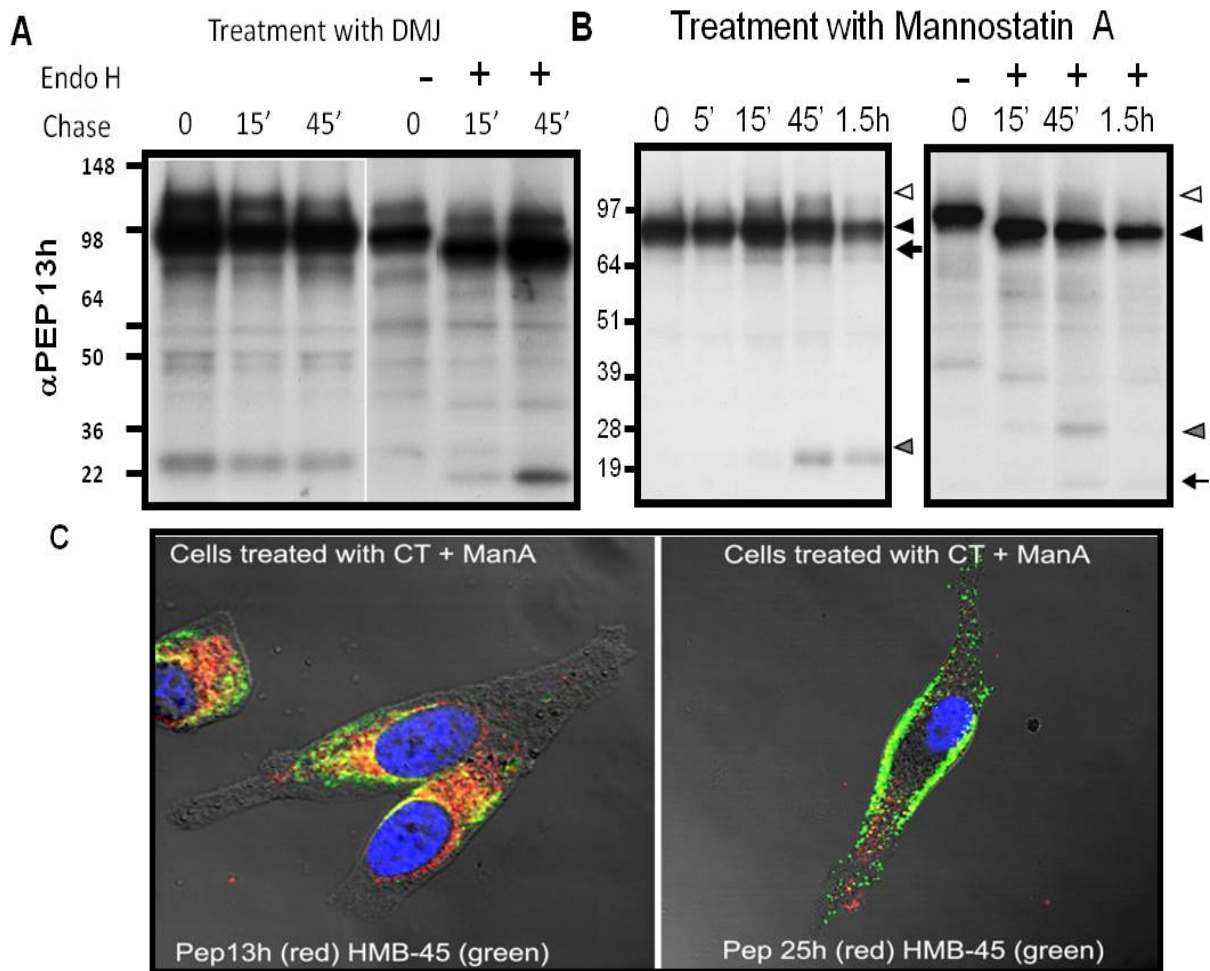


followed by metabolic labeling with [<sup>35</sup>S] Met/Cys in MNT-1 melanoma cells. We first targeted ER glucosidases with either Castanospermine (CT), a competitive inhibitor of  $\alpha$ - and  $\beta$ -glucosidases, or N-butyl-deoxynojirimycin (NBDNJ), an inhibitor of both glucosidases with preference towards glucosidase II [28]. These inhibitors prevent the processing of complex glycoproteins and cause the production of immature N-linked glycoproteins with Glc<sub>3</sub>Man<sub>7-9</sub>GlcNAc<sub>2</sub> structure. As detected by  $\alpha$ PEP13h (an antibody targeting the C-terminal domain of PMEL), treatment with either CT or NBDNJ did not affect processing or stability of PMEL. Similar results have been obtained for tyrosinase, a key melanin producing enzyme, although it was inactive [28;29]. To confirm the effect of this intervention, cells were treated with Endo-H, which removes high mannose/hybrid N-glycans. Endo H treatment revealed that all forms of PMEL contained immature N-glycans sensitive to Endo H (Figure 4A). These results indicate that PMEL, similar to tyrosinase [27;30], is not a substrate for Golgi endo- $\alpha$ -mannosidase which is capable of by passing ER glucosidases that remove end glucose residues and proceed with the glycosylation process in the Golgi. Confocal microscopy analysis revealed that PMEL (red) reaches intracellular targets but does not localize to the plasma membrane (Figure 4B). Thus, these results suggest that N-glycans may have a limited impact on PMEL processing but may play a role in its trafficking to the plasma membrane, possibly for secretion.

To further verify our results, we blocked the synthesis of complex type N-glycans using a fairly potent and specific inhibitors of mannosidase I and  $\alpha$ -mannosidase, called deoxymannojirimycin (DMJ) and Mannostatin A (ManA), respectively. These inhibitors have been extensively studied and have proven to be useful for studying the processing pathway and for comparing processing enzymes (For review see [28]). Pre-treatment of melanoma cells was followed by metabolic labeling with [<sup>35</sup>S] Met/Cys for at 3 hrs at 37°C. Consistently these inhibitors affected the formation of the mature form of PMEL without affecting cleavage of the C-terminal domain. As expected, digestion with Endo H revealed that the resulting "mature" PMEL contains non-complex glycans and as such that band was sensitive to this enzyme and suggest that this glycoprotein is getting additional modifications independent of N-glycosylation. These results further suggest that the mature PMEL could be distinguished by SDS-PAGE despite it has non-complex modified N-glycans (Figure 5, A and B). We then assessed whether PMEL intracellular localization was altered after these conditions in the same melanoma cells. To explore this possibility, cells were cultured in the presence of CT and ManA for 3 hrs previous to fixation. Confocal microscopy analysis revealed that PMEL (red) had a limited distribution in the cytoplasm, mostly perinuclear, compared to HMB-45 (green) which is already processed and localized in stage II melanosomes (Figure 5C). Superimposed confocal images with DIC showed that treatment did not compromised cell morphology or adhesion. Noticeably, PMEL staining was absent nearby the plasma membrane further supporting that N-glycosylation plays a role in the distribution of PMEL at the plasma membrane. Toxicity and viability assays were performed using different combinations of inhibitors and incubation times to determine optimal treatment conditions in culture for pigmented and non-pigmented melanoma cells (data not shown).



**Figure 4. Inhibition of N-Glycan maturation does not alter PMEL processing.** A. MNT-1 melanoma cells were treated for 3hrs with 5  $\mu\text{g}/\text{ml}$  of CT, which is known to produce minimal toxicity as reported by Takahashi et al.[31], were [ $^{35}\text{S}$ ]-labeled for 30 minutes and chased for the indicated times. Collected samples were left undigested or digested with Endo H where noted for 3h at 37 C, after which samples were immunoprecipitated with the  $\alpha\text{PEP13h}$  antibody, which recognize the C-terminal domain of PMEL. Immunoprecipitated bands were visualized by autoradiography. Faint bands are indicated with either an open arrow (<) or an asterix (\*). B. MNT-1 cells were cultured in the presence of 5 mM of NBDNJ for 3 hrs prior to fixation with 4% p-formaldehyde. Cells were dual stained either with  $\alpha\text{PEP13h}$  or  $\alpha\text{PEP25h}$  (red), anti-PMEL antibodies, and vti1b, a Golgi marker (green). Images are representative of the staining pattern after three repeated experiments in melanoma cells. Note the granular distribution patterns of PMEL. Images are merged with DIC to visualize the unstained parts of the melanoma cells. Colocalization of the red and green signals is shown in yellow. Nuclear counterstaining was done with DAPI (blue).



**Figure 5. Complete inhibition of N-Glycan maturation alters the intracellular localization of PMEL.** A and B. MNT-1 melanoma cells were treated for 3hrs with 5  $\mu\text{g/ml}$  of DMJ or overnight with 50  $\mu\text{g/ml}$  of mannostatin A (ManA). Samples were [ $^{35}\text{S}$ ]-labeled for 30 minutes and were chased for the indicated times. Collected samples were left undigested or were digested with Endo H where noted for 3h at 37  $^{\circ}\text{C}$ , after which samples were immunoprecipitated with the  $\alpha\text{PEP13h}$  antibody. Immunoprecipitated bands were visualized by autoradiography. C. MNT-1 cells were cultured in the presence of both CT and ManA for 3 hrs previous to fixation with 4% p-formaldehyde. Cells were dual stained either with  $\alpha\text{PEP13h}$  or  $\alpha\text{PEP25h}$  (red) and HMB-45 (green). Images are representative of the staining pattern after three repeated experiments in melanoma cells. Note limited granular distribution patterns of PMEL antibodies, while HMB-45 reactivity seems to be conserved. Images are merged with DIC to visualize the unstained parts of the melanoma cells. Colocalization of the red and green signals is shown in yellow. Nuclear counterstaining was done with DAPI (blue).

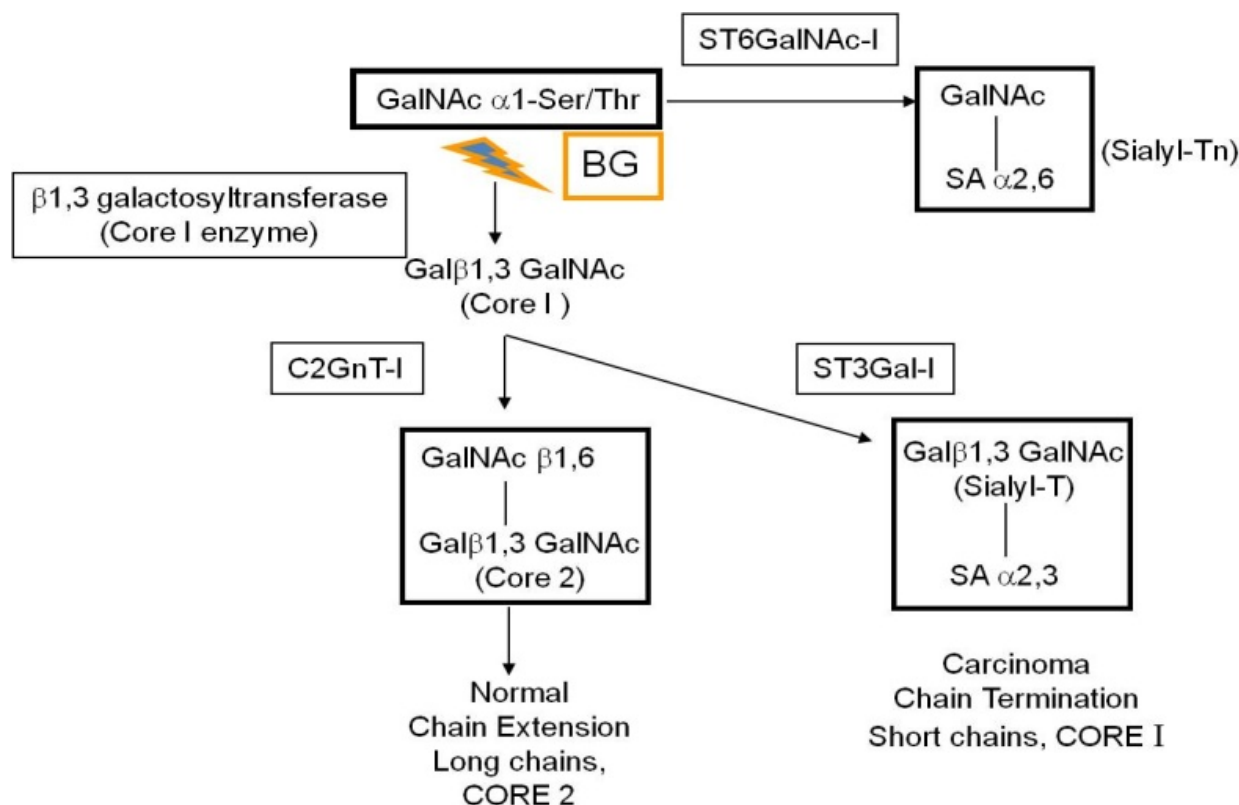


To establish a specific role for individual N-glycan sites in PMEL, Hoashi et al., performed mutational analysis using several PMEL mutants at the predicted N-glycosylation sites [23]. In that study, it was determined that 2 out of 4 N-glycosylation sites were not fully glycosylated in HeLa cells, which were derived from an adenocarcinoma of the cervix. Consistent with our data, the authors concluded that this does not affect PMEL processing but pointed that the mutation of the site at S570A altered secretion of PMEL. It is worthwhile to note that a glycoform of PMEL is known to be secreted to the extracellular space and in the medium of cells in culture [23;32]. However, there is no evidence of amyloid accumulation either in skin samples or in skin keratinocytes when co-cultured with normal human melanocytes (JVC, unpublished data). One possible reason for this is that PMEL amyloid formation is sensitive to pH [7]. Thus, the neutral pH in the extracellular compartment most likely undermines PMEL amyloid fiber ability or if any amyloid is formed it is dissolved; while the intra organelle acidic pH (5.0) will favor amyloid formation. Interestingly, the secretion of amyloid generating proteins has been reported previously for other functional (hormones) and disease-associated amyloids [9]. In the case of secreted PMEL, the effects of this activity are still unknown both in the context of human skin physiology and melanoma disease progression. Taken all together, we conclude that the N-glycosylation of PMEL might influence trafficking to and from the plasma membrane while playing a limited role in the protein shedding process and direct trafficking to melanosomes in melanocytic cells.

#### b. O-glycosylation in PMEL maturation

Prediction of O-glycosylation sites is not standardized but is known in several proteins as linkages in which the sugar is attached to an amino acid containing a hydroxyl group. However, every amino acid with a hydroxyl functional group (i.e. Serine (Ser), Threonine (Thr), Tyrosine (tyr), Hydroxyproline, and Hydroxylysine) has been implicated in that process (See details in [25;26]). As summarized in Figure 6, O-glycan structures are generated following the action of polypeptide N-acetylgalactosaminyltransferase (GalNAcT) enzyme and include 4 common subtypes based on differential monosaccharide linkage reactions to the unsubstituted GalNAc (GalNAc $\alpha$ -Ser/Thr). All O-glycans may be modified by one of several enzymes to generate different core structures known as core 1, core 2, core 3, etc. The core 1 O-glycan is generated by the core 1  $\beta$ 3 galactosyltransferase (Gal-T), which adds galactose to generate Gal $\beta$ 1-3GalNAc $\alpha$ 1-Ser/Thr. This is a key precursor for all core 1 and 2 mucin-type O-glycans in vertebrates and invertebrates. The biosynthesis of O-glycans can be modified and terminated with the addition of sialic acid residues relatively early in biosynthesis. For example, certain sialyltransferase enzymes are capable of acting on GalNAc $\alpha$ -Ser/Thr, or early O-glycan core subtypes after Core-1-GalT action. These sialic acid additions give rise to a series of O-glycan structures that generally restrict further biosynthetic steps and have been commonly referred to as tumor-associated (T) antigens.

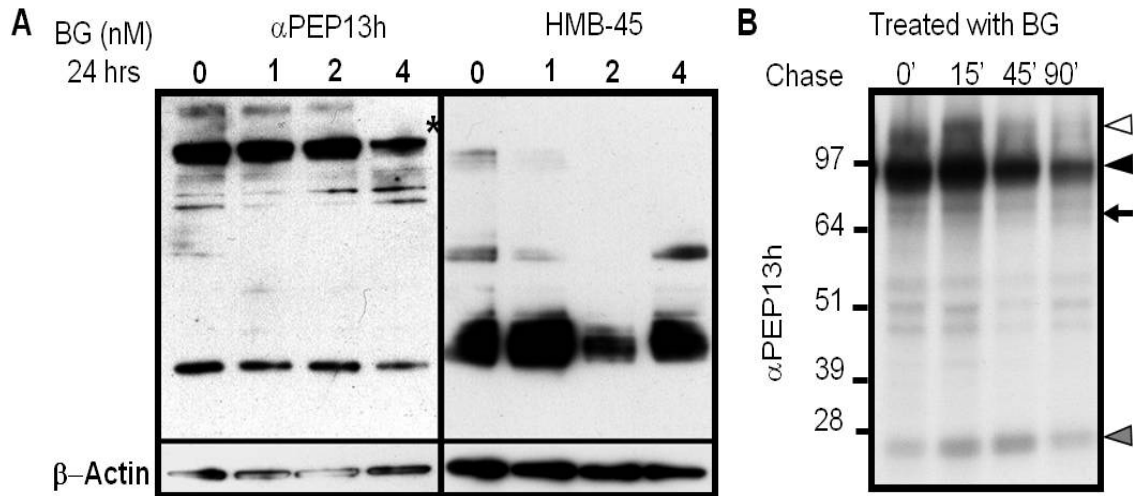
In PMEL, the RPT domain has 10 imperfect repeats rich in proline, serine, threonine and glutamic acid amino acids [5;33]. Recognition of this domain in amyloid fibers inside stage II melanosomes by the HMB-45 antibody, which recognizes an unknown sialic acid structure, suggests that glycan structures in the RPT domain are modified with sialic acid.



**Figure 6. O-glycosylation process in mammalian cells.** The scheme details the different enzymes involved in the post-translational modification of glycoproteins with O-linked oligosaccharides. It includes the step targeted by the inhibitor benzyl-N-acetyl- $\alpha$ -D-galactosaminide (BG). Note that the conformation of O-glycan chains varies in cancer cells compared to normal cells due an early addition of sialic acid. Adapted from [37].

Previously, we have confirmed that PMEL is modified with sialylated core-1 type O-glycans in MNT-1 melanoma cells [12]. To assess the role of O-glycosylation in PMEL function, we used the sugar analog benzyl-N-acetyl- $\alpha$ -D-galactosaminide (BG), known to inhibit O-glycosidation [34-37]. MNT-1 melanoma cells were cultured overnight in the presence of 1 to 4nM BG. Immunoblotting analysis revealed that maturation of PMEL (upper band) was completely inhibited in a dose dependent manner reaching a maximum inhibition at 4 nM (Figure 7A). As expected, HMB-45 staining decreased substantially at the doses of 2 nM and 4 nM of BG (Figure XB). Because HMB-45 reactivity depends on sialic acid, we then infer that O-glycan structures in PMEL were modified with sialic acid. To further confirm this and assess the impact on PMEL stability, MNT-1 cells were metabolically labeled with [ $^{35}$ S] met/cys and chased at the indicated times. As shown in Figure 7B, the mature form of PMEL (white arrow) was inhibited as early as 45 min and the stability of PMEL at 90 min. The observed shift in electrophoretic mobility following synthesis reflects mainly the loss of negatively charged sialic acid residues to O-linked sugars, with the maturation of N-linked chains to hybrid or complex forms contributing only slightly to the change in molecular weight. Noticeably, the observation of the cleaved 26 kDa band at all time points further suggest that PMEL processing by PC is also independent of O-glycan modification. At this point, there is

enough evidence to conclude that protein cleavage is independent of post-translational glycosylation.



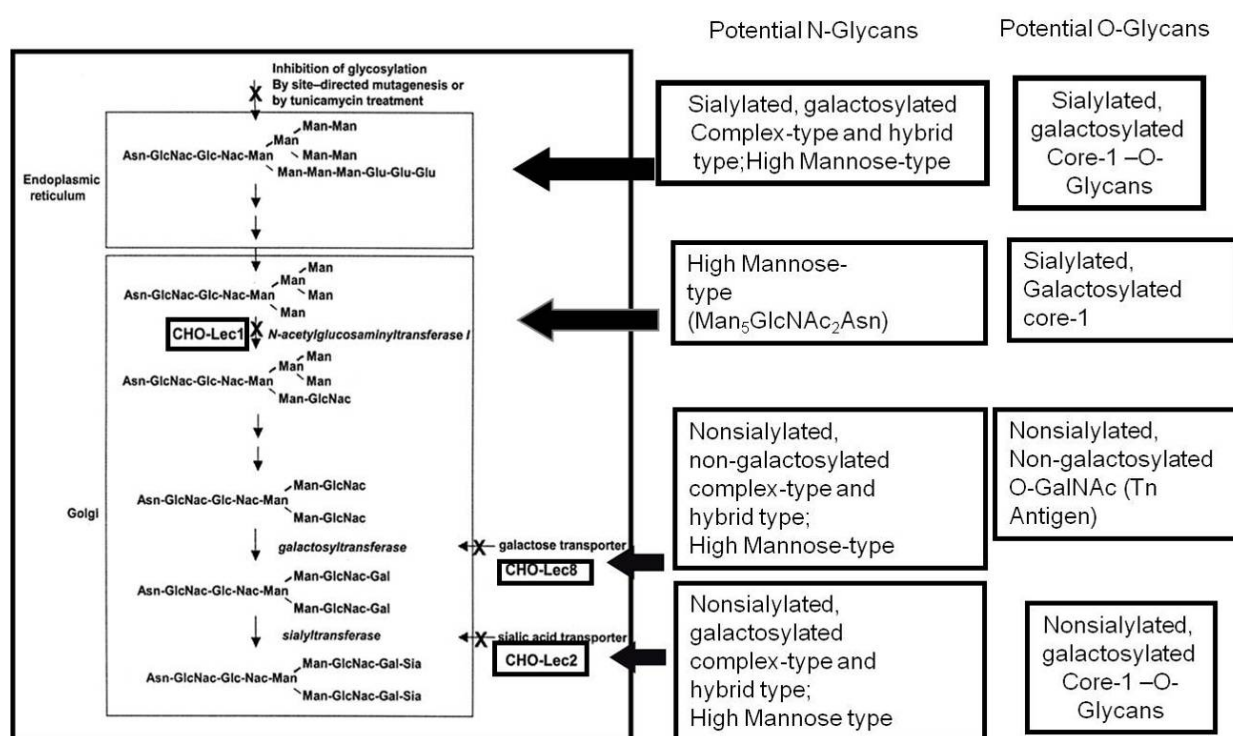
**Figure 7. O-glycosylation is important for proper PMEL maturation.** A. MNT-1 melanoma cells were cultured in the absence or in the presence of 4 nM BG overnight. Cells were then harvested and cell lysates were analyzed using SDS-PAGE. Detection of PMEL was done with  $\alpha$ PEP13h or HMB-45 as indicated. Actin was used as a loading control. B. After treatment with BG overnight, samples were [ $^{35}$ S]-labeled for 30 min and then chased for the indicated times. Collected samples were immunoprecipitated with the  $\alpha$ PEP13h antibody. Immunoprecipitated bands were visualized by autoradiography. Arrows next to PMEL bands represent: mature PMEL (white), cleaved (grey), intermediate (black).

### c. Sialylation in PMEL

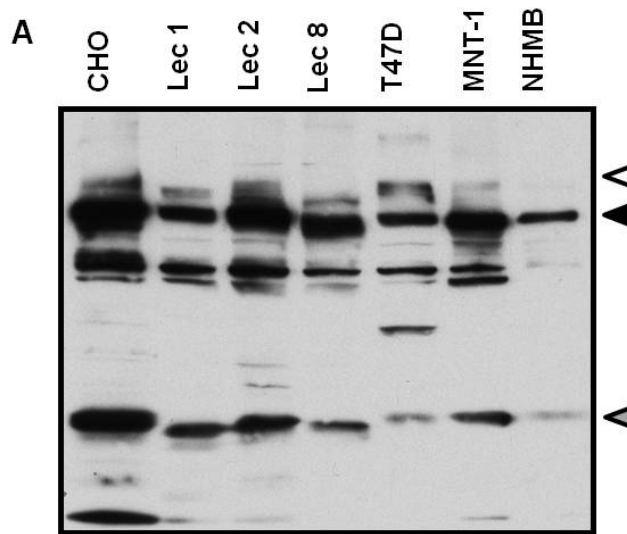
The negatively charged residues of sialic acid exert a particular influence on the 3 dimensional structure of the protein backbone. Sialic acid can be added to complex N- and O- linked glycans preferentially in  $\alpha$ 2,3 and  $\alpha$ 2,6 linkages to a penultimate galactose residue [38;39]. The transfer of these residues is catalyzed by several enzymes known as sialyltransferases (ST). To examine the impact of sialic acid addition to N- and O-glycan chains in PMEL, we transfected full length PMEL into wild-type and mutant Chinese hamster ovary (CHO) cells that exhibit a specific glycosylation defect. These cells provide an excellent tool to study the role of individual sugar residues in protein processing. Thus, the outcome of the glycoproteins could be predicted and it is summarized in Figure 8.

To test whether sialic acid addition affects the PMEL amyloid formation ability, we transfected full length PMEL into wild type- and mutant- (Lec1, Lec2 and Lec8) CHO cells. The Lec1 mutant is a leuco-phytohemagglutinin resistant cell line unable to synthesize complex and hybrid N-glycans due to the lack of N-acetylglucosaminyltransferase I (GnTI) activity [40]. The Lec2 mutant has a deletion mutation in the CMP-sialic acid transporter resulting in N- and O-glycans with a greater than 90% decrease in sialic acid

content [41]. The Lec8 mutant has a deletion mutation in the UDP-galactose transporter resulting in a truncated protein with a greatly reduced ability to translocate UDP-galactose (UDP-Gal) inside the Golgi [42]. Thus, Lec8 cells generate non-galactosylated and non-sialylated N- and O-glycans. Immunoblotting analysis showed PMEL was successfully expressed in these cell lines. Noticeably, PMEL was correctly processed in all mutant cells showing a band pattern that was no different than those in melanoma cells, except for minor differences in the molecular weight of the mature PMEL (white arrow) which reflects the different glycosylation background of the host cells (Figure 9). We noted that the mature PMEL (white arrow) band intensity is stronger in wild-type CHO cells than that observed in melanoma cells and in melanocytes. On the contrary, this same band in the absence of sialic acid modification is undefined despite correct modification with core 1-O-glycans in Lec2 cells.



**Figure 8.** Scheme detailing the specific mutation and the affected step in glycosylation processing. Summary of the outcomes for N- and O-glycans expected from the different CHO cell mutants (Lec1, Lec2 and Lec8) Adapted and modified from [41].



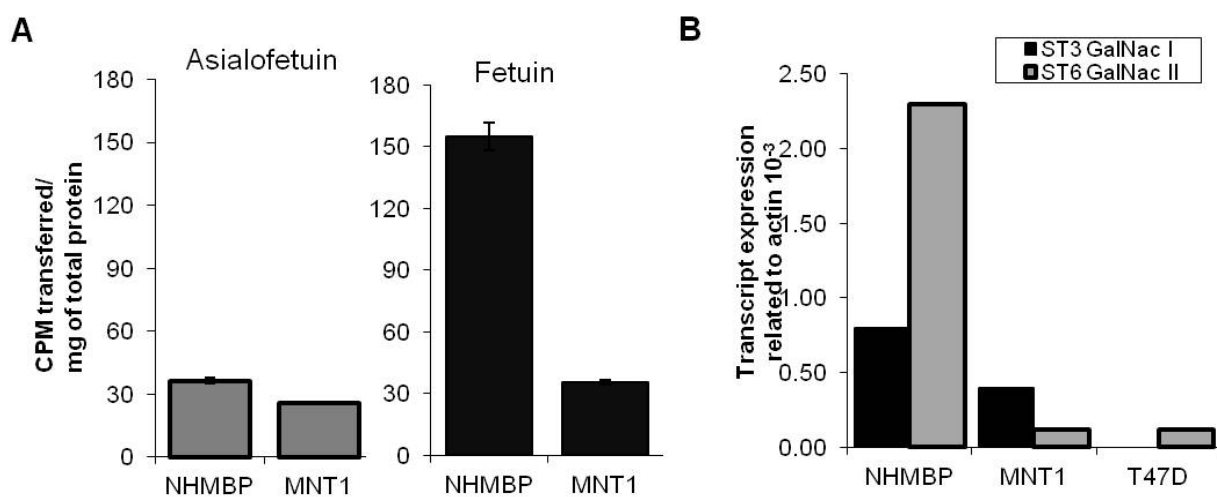
**Figure 9. PMEL expression in CHO cells results in different glycoforms.** A V5-His 6 tag full length PMEL was transiently transfected into the indicated cells lines. At 48 hr post-transfection, cells were harvested and lysates were analyzed by immunoblotting. PMEL was detected using the  $\alpha$ PEP13h antibody. Lysates from normal human melanocytes from black donors (NHMB) and from MNT-1 melanoma cells were used as controls for the PMEL band pattern. Arrows next to PMEL bands represent: mature PMEL (white), cleaved (grey), intermediate (black).

These results suggest that the band shifts in the mature PMEL band mainly reflect either the addition or loss of sialic acid residues. Previous reports have focused on the band size and location to infer about the maturation state of PMEL [20;23]. However, our results clearly indicate that PMEL shares a similar band pattern in all transfected cell lines despite the different N- and O-glycan composition. In other words these band patterns represent glycoforms of PMEL. Glycoform is a term that indicates differences in the composition of the glycan chains derived from an identical backbone protein [25;26;43]. This phenomenon is also known as microheterogeneity. From the practical point of view, microheterogeneity explains the anomalous behavior of glycoproteins in various forms of chromatography (such as the diffuse bands observed on SDS-PAGE gels) and makes the complete structural analysis of most 'glycoproteins a difficult task. From a functional point of view, the meaning of this heterogeneity remains unclear. It is possible that this is a type of "diversity generator" intended either to diversify endogenous recognition functions and/or to evade detection after malignant transformation. Interestingly, we reported that the amyloid formation abilities of PMEL were altered *in vivo* at different levels depending on the glycosylation defect [12]. Thus, lack of sialic acid resulted in the accumulation of thick amyloid fibers (Lec 2 cells), while the lack of the end terminal galactose modified with sialic acid at core-1-O-glycans resulted in complete loss of amyloid fibrils at target organelles (Lec 8 cells). Thus, these results indicate that mature PMEL is mainly formed by O-glycan derived-structures and the loss of this modification decreased PMEL stability in melanoma cells.

There have been several reports indicating that changes in sialylation patterns correlate with changes in cell motility and invasiveness [44-47]. In melanoma, it has been shown that



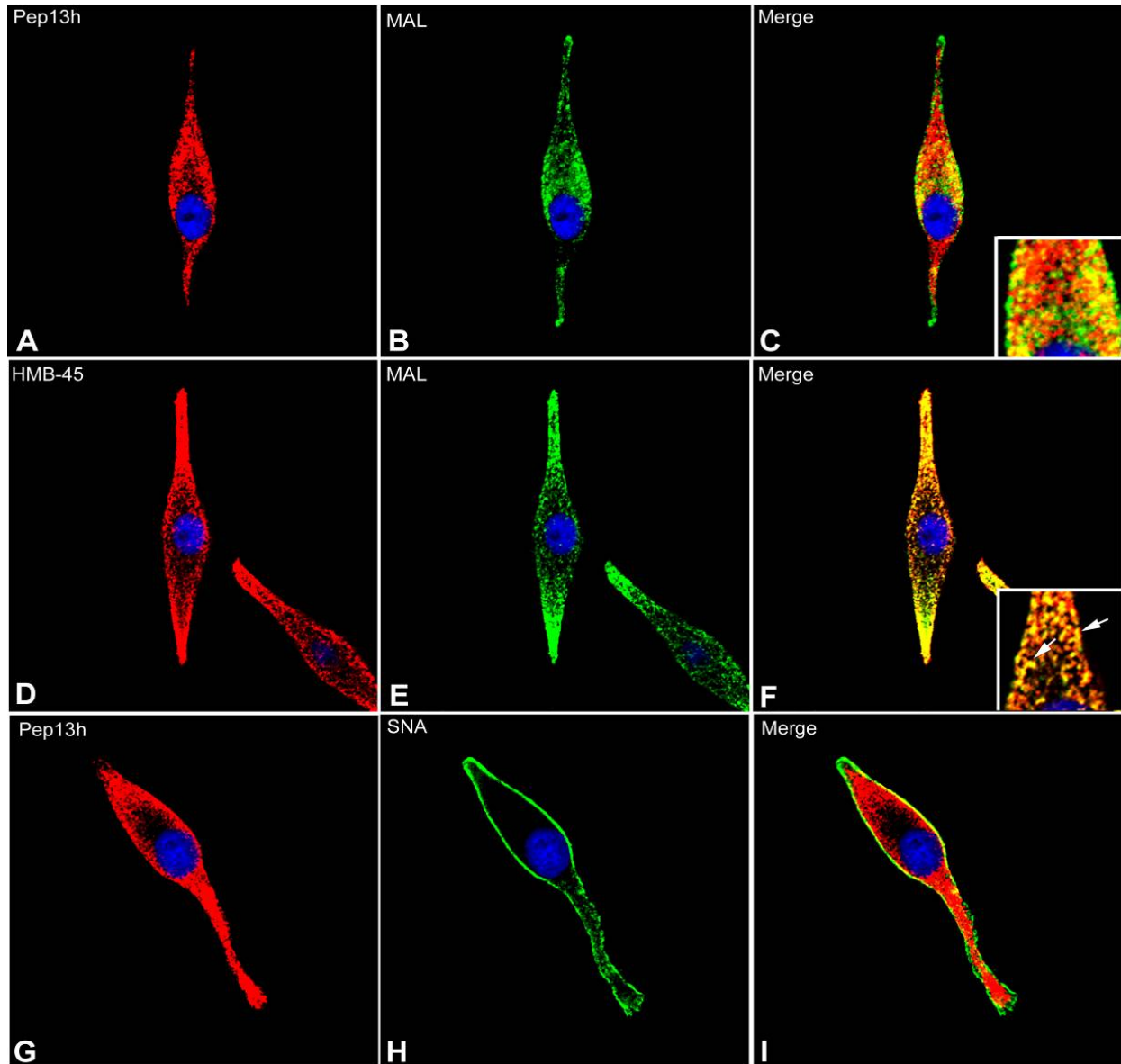
adhesion molecules such as N-cadherin and integrins undergo altered glycosylation and sialic acid modification [48-51]. Thus, we hypothesize that melanoma cells may exhibit altered sialyltransferase (ST) activities compared to either melanocytes or other cell lines that affects the glycosylation pattern of most proteins, including PMEL. Analysis of sialyltransferase activity in MNT-1 melanoma cells compared to normal human melanocytes (NHM) using different glycoproteins as acceptor substrates confirmed differences between these cell lines. As shown in Figure 10, both cell lines exhibited sialyltransferase activity towards asialofetuin, which bear unsialylated N-glycans and unsialylated core 1 O-glycans, although MNT-1 cells were less active than NHM. In contrast, very high levels of sialyltransferase activities were detected using fully sialylated fetuin as acceptor (Figure 10A), showing that these cells can substitute sialic acid on already sialylated carbohydrates an ability known as oligo- or poly-sialylation [52]. Thus, NHM is 5 times more active than MNT-1 and 25 times more active than the breast cancer cell line T47-D, used as a control, in the addition of sialic acid to glycan chains. Using real-time PCR, we confirmed that the mRNA levels for  $\alpha$ 2,3-sialyltransferase (ST3 GalNac I) and  $\alpha$ 2,6-sialyltransferase (ST6 GalNac II) were higher in melanocytes, which correlated with the increased ST activity detected in these cells. Furthermore, we reported that melanocytes preferentially add sialic acid to O-glycans while melanoma cells do it without specific preference, although in a less active fashion [12]. Thus, our results prove that there are cell-specific differences related to addition of sialic acid to the ends of both N- and O-glycans.



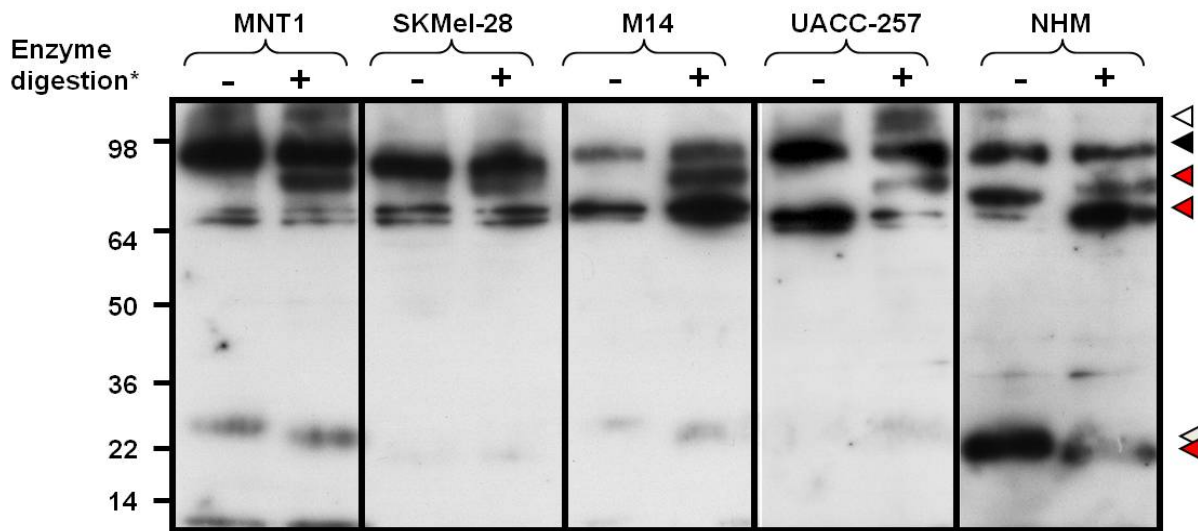
**Figure 10. Melanocytes exhibit increased ability to transfer sialic acid to glycans.** A. Sialyltransferase activity against the acceptor substrates asialofetuin or fetuin measured in lysates of normal human melanocytes from black donors (NHMB) or MNT-1 melanoma cells; values are expressed as total counts/min of <sup>14</sup>C against  $\mu$ g of protein. B. Real-time PCR mRNA expression of ST3 GalNac I and ST6 GalNac II enzymes in NHMB and the human cancer cell lines, MNT1 (melanoma) and T47D (breast). Values are expressed in transcript units related to actin. PCR primer sequences have been reported in Valencia et al., [12].

Lectins are sugar-binding proteins (not to be confused with glycoproteins, which are proteins containing sugar chains or residues) that are highly specific for their sugar moieties. They play a role in biological recognition phenomena involving cells and proteins. Lectins have a multimeric structure, which is responsible for the ability to agglutinate cells or form precipitates glycoconjugated in a manner similar to antigen-antibody interactions. During the past few years, lectins that discriminate between various types of sialylated sequences have been reported [53]. The lectin from elderberry (*Sambucus nigra*) bark (SNA I) has been shown to bind with high affinity to glycoconjugates containing the terminal sequence sialic acid  $\alpha$ 2,6-galactose/N-acetylgalactosamine. Similarly, the leucoagglutinin from *Maackia amurensis* (MAL) binds with high affinity to sialic acid  $\alpha$ 2,3 galactose  $\beta$ 1,4 N-acetylglucosamine but not to 2,6-linked isomers and, therefore, is a most interesting complementary probe to the SNA [53]. To more specifically determine the most frequent sialic addition related with PMEL, we performed a detail cytochemical analysis of the distribution of  $\alpha$ 2,6- and  $\alpha$ 2,3- linked sialic acid residues using MAL and SNA I in MNT-1 melanoma cells (Figure 12). Confocal microscopy dual immunofluorescence revealed that PMEL (red), as detected by  $\alpha$ PEP13h or HMB-45 antibodies, colocalized more frequently with MAL (green) in the cytoplasm of melanoma cells (Figure 12,A-C). Specifically, the staining pattern of HMB-45 antibody colocalized almost exclusively with MAL (Figure 12, D-F) suggesting that the  $\alpha$ 2,3 addition is more likely recognized by this antibody. These result also suggest that  $\alpha$ 2,3 is the most frequent modification in the glycan chains of PMEL. On the other hand, SNA staining was limited to the plasma membrane where it colocalized with PMEL (Figure 12, G-I) suggesting that plasma membrane forms of PMEL, including the secreted PMEL glycoform, are more likely to be modified with  $\alpha$ 2,6 instead of  $\alpha$ 2,3 terminal sialic acids ends.

In melanoma cells, immunopurified forms of PMEL show that N-glycosylation sites located at the N- and C-terminal domains were modified with sialic acid [12]. Detection of unsialylated N-glycan chains is an indication of the predominantly hybrid nature of the structures in PMEL. This type of variability opens the possibility that at least two glycoforms of PMEL are produced in one cell type or in cell lines from the same lineage. To effectively prove that PMEL glycan chains are differentially managed in a cell-specific manner, lysates from different melanoma cell lines and NHM were digested with a combination of the following enzymes:  $\alpha$ 2,3,6,8,9 neuroaminidase,  $\beta$ 1,4 galactosidase,  $\beta$ -N-acetylglucosaminidase known to remove complex and hybrid structures (Figure 13). As detected by the anti-PMEL antibody  $\alpha$ PEP13h, enzyme digestion rendered new bands that migrated different between cell lines, specially between melanoma cells and melanocytes. Thus, all cells exhibited bands of around 85 kDa and 20 kDa after the combined enzyme digestion (red arrows). These new bands are composed by non-complex unsialylated N- and O-glycans. Interestingly, the band patterns of pigmented cells (NHM, MNT-1 and UACC-257) were somewhat different than those in non-pigmented cells (SK-Mel-28 and M14). The only consistency was the minor molecular shift of the cleaved band at 26 kDa suggesting that post-translational modification of this domain in PMEL was similarly modified independent of the cell type. These results confirm that the majority of N-glycans on this band are hybrid not complex type.



**Figure 11. Immunohistochemical distribution of  $\alpha 2,3$  and  $\alpha 2,6$  sialic acid additions in MNT-1 melanoma cells.** MNT-1 cells were grown in Lab TeK II chamber slides with cover at a density of  $1 \times 10^4$  cells per chamber. After 48hrs, cells were fixed with 4% p-formaldehyde and dual stained either with  $\alpha$ PEP13h or HMB-45 (red) and the lectins MAL or SNA (green). Images are representative of the staining pattern after three repeated experiments in melanoma cells. Note the granular distribution patterns of PMEL antibodies and MAL that differ from the predominantly plasma membrane distribution of the lectin SNA. Colocalization of the red and green signals is shown in yellow. Nuclear counterstaining was done with DAPI (blue).



\* Combined treatment with Neuraminidase  $\alpha$  2-3,6,8,9;  $\beta$  1-4 Galactosidase; and  $\beta$ -N- Acetyl glucosaminidase

**Figure 12. PMEL is differently glycosylated in human melanoma cells compared to melanocytes.** Lysates from indicated cell lines were digested in the presence or absence of the enzymes noted and were analyzed by SDS-PAGE. Samples were separated on a 4-20% Tris glycine gels for 2 hr. Arrows next to PMEL bands represent: mature PMEL (white), cleaved (grey), intermediate (black), digested bands (red).

#### d. Glycosylation enzymes in melanosomes

Previously, we made possible global melanosome proteome characterization by using LC/MS to analyze both non-pigmented (pre-melanosomes) and pigmented melanosomes in solution digest after removal of melanin by immobilized metal affinity chromatography (IMAC) [16;17]. Briefly, we took advantage of the heavy metal ion sequestering property of melanin by loading the resulting peptides onto an IMAC column activated with an excessive volume of  $\text{FeCl}_3$ , assembled back-to-back with a reverse-phase (RP) precolumn. The melanin was retained on the IMAC column due to the high affinity of Fe (III) for the o-diOH groups of melanin, while the peptides passed through and were subsequently caught on the C18 RP-precursor. This assembly allowed the efficient and concurrent removal of melanin and the loading of the sample onto the column in a single step prior to the LC/MS analysis. Thus, proteomic analysis of melanosomes required an effective approach for purifying and solubilizing them, and removing the melanin.

Arrival of PMEL to melanosomes initiates a well coordinated sequence of events to incorporate and shred the protein into smaller pieces to expose the RPT domain and start the formation of amyloid fibers. Several reports agree that this process is initiated by a series of PC [19;54]. After that process, we hypothesized that the sialylated O-glycans covering the RPT domain of PMEL must be removed before amyloid formation. This is what we propose as the protective role these glycans exert in the amyloid processing. To perform such task, melanosomes will have to be equipped with enzymes specialized in the removal of carbohydrate modifications, such as sialyltransferases or hexosaminidases. To evaluate whether or not this is possible, we analyzed our proteome database for the presence of

enzymes involved in the modification of glycan chains. As shown in Table 1, there were several enzymes involved in either the transfer of sialic acid (like  $\alpha$ 2,6 ST), removal of fucose (another terminal modification of N- and O-glycan products), or removal of carbohydrates such as glucose ( $\alpha$ -glucosidase II, glucosidase I), mannose (mannosidase) and galactose (GalNAc-T2). Many of these enzymes have been identified to localize throughout the Golgi cisternae like GalNAc-T2 or T3. Note that most of these enzymes were identified in early maturation stages of melanosomes (stage I and II), not the fully mature late stage, of the pigmented MNT-1 and non-pigmented SK-Mel-28 cells. Interestingly, there is evidence that the localization of these enzymes is highly variable not only with respect to their normal localization inside the Golgi stack [55;56], but also in ectopic Golgi sites including the plasma membrane (see review [57]).

Protein AC	Protein ID	Group	Protein Name
Q9H553	ALG2	MNT1_stage2 MNT1_stage2	Alpha-1, 3-mannosyltransferase ALG2 (EC 2.4.1.-) (GDP- Man:Man(1)GlcNAc(2)-PP-dolichol mannosyltransferase
Q9BTY2	FUCO2	MNT1_stage1	alpha-L-fucosidase precursor (EC 3.2.1.51) (Alpha-L-fucosidase 2) (Alpha-L-fucoside fucohydrolase 2)
Q96L58	B3GT6	Skmel28_stage1	Beta-1, 3-galactosyltransferase 6 (EC 2.4.1.134) (beta 3GalT6) (Galactosylxylosylprotein 3-beta-galactosyltransferase) (Galactosyltransferase II) (GAG GalTII)
Q92685	ALG3	Skmel28_stage1; Skmel28_stage2; uniqueSkmel28	Dolichyl-P-Man:Man(5)GlcNAc(2)-PP-dolichyl mannosyltransferase (EC 2.4.1.-) (Dol-P-Man-dependent alpha(1-3)-mannosyltransferase)
Q16739	CEGT	MNT1_stage 1 and 2; Skmel28_stage1; Skmel28_stage2; common_earlystage	Ceramide glucosyltransferase (EC 2.4.1.80) (Glucosylceramide synthase) (GCS) (UDP-glucose:N-acylsphingosine D-glucosyltransferase) (UDP- glucose ceramide glucosyltransferase) (GLCT-1)
Q14697	GANAB	MNT1_stage1,2, and 4; Skmel28_ stage1; Skmel28_stage2; common_early_ stage	Neutral alpha-glucosidase AB precursor (EC 3.2.1.84) (Glucosidase II subunit alpha)
Q14435	GALT3	Skmel28_stage1	Polypeptide N-acetylgalactosaminyltransferase 3 (EC 2.4.1.41) (Protein-UDP acetylgalactosaminyltransferase 3) (UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase 3) (Polypeptide GalNAc transferase 3) (GalNAc-T3) (pp-GaNTase 3)



Protein AC	Protein ID	Group	Protein Name
Q13724	GCS1	MNT1_stage1; Skmel28_stage2	Mannosyl-oligosaccharide glucosidase (EC 3.2.1.106) (Processing A- glucosidase I)
Q10471	GALT2	Skmel28_stage1	Polypeptide N-acetylgalactosaminyltransferase 2 (EC 2.4.1.41) (Protein-UDP acetylgalactosaminyltransferase 2) (UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase 2) (Polypeptide GalNAc transferase 2) (GalNAc-T2) (pp-GaNTase 2)
P34059	GA6S	NG; Skmel28_stage1	N-acetylgalactosamine-6-sulfatase precursor (EC 3.1.6.4) (N- acetylgalactosamine-6-sulfate sulfatase) (Galactose-6-sulfate sulfatase) (GalNAc6S sulfatase) (Chondroitinsulfatase) (Chondroitinase)
P15907	SIAT1	Skmel28_stage1	CMP-N-acetylneuraminate-beta-galactosamide-alpha-2,6-sialyltransferase (EC 2.4.99.1) (Beta-galactoside alpha-2,6-sialyltransferase) (Alpha 2,6-ST) (Sialyltransferase 1) (ST6Gal I)
P15586	GL6S	MNT1_stage 1,2,4; commonMNT1; unique MNT1	N-acetylglucosamine-6-sulfatase precursor (EC 3.1.6.14) (G6S) (Glucosamine-6-sulfatase)
P07686	HEXB	MNT1_stage4; NG; Skmel28_stage1; Skmel28_stage2;	Beta-hexosaminidase beta chain precursor (EC 3.2.1.52) (N-acetyl-beta- glucosaminidase) (Beta-N-acetylhexosaminidase) (Hexosaminidase B)
P06865	HEXA	MNT1_stage1; Skmel28_stage1;	(Cervical cancer proto-oncogene 7) (HCC-7) Beta-hexosaminidase alpha chain precursor (EC 3.2.1.52) (N-acetyl- beta-glucosaminidase) (Hexosaminidase A)
O94766	B3GA3	Skmel28_stage1	Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase 3 (EC 2.4.1.135) (Beta-1,3-glucuronyltransferase 3) (Glucuronosyltransferase-I) (GlcAT-I) (UDP-GlcUA:Gal beta-1,3-Gal-R glucuronyltransferase) (GlcUAT-I)
O60701	UGDH	Skmel28_stage1 Skmel28_stage1	UDP-glucose 6-dehydrogenase (EC 1.1.1.22) (UDP-Glc dehydrogenase) (UDP-GlcDH) (UDPGDH)
O00754	MA2B1	MNT1_stage1; MNT1_stage4; Skmel28_stage1;	Lysosomal alpha-mannosidase precursor (EC 3.2.1.24) (Mannosidase, alpha B) (Lysosomal acid alpha-mannosidase) (Laman) (Mannosidase alpha class 2B member 1)

Source: Full database access could be found at [http://pir.georgetown.edu/iproxpress/melanosome/suppl\\_data/](http://pir.georgetown.edu/iproxpress/melanosome/suppl_data/).  
For details of the database features please refer to [16;17]

**Table 1.** List of glycosylation enzymes identified in the proteome of melanosomes

Despite these initial data, we should consider that the identification of these enzymes in melanosomes might be due to contamination of the purified melanosome sample. A crucial issue in proteomic analysis of subcellular proteomes is the ability to distinguish between true components and contaminants coming from other cell compartments, especially when working with low abundance proteins. Interestingly, except for known melanosome proteins that give the organelle its unique structure and functions, a majority of proteins detected in the melanosome proteome are not organelle-specific. Some, such as ribosomal protein complexes, are obvious minor contaminants that were co-purified during sucrose gradient fractionation. Even though extra precaution was taken, sensitive mass spectrometers can always detect trace amounts of peptides that originate either from resident low abundance proteins or from low-level contaminants. By searching our data against known human mitochondrial proteins annotated in UniProtKB, we estimate that the melanosome fractions at various stages are of high purity (94 % at early stage, and 98-99% at late stage). On the other hand, many proteins identified in our study demonstrate that melanosomes are highly dynamic. They may be viewed as a microcosm of organelles, representing a dynamic balance of proteins as well as small molecules being transported in and out. Many of the “nonspecific” proteins might be associated with melanosomes only for a short period of time, or they may be proteins that reside in other subcellular compartments. In that sense, we considered the possibility that true permanent “resident” molecules for organelles may not exist. Because of the uniqueness of melanosomes our study has become the gold-standard for future analysis. Taken all together, it is feasible that certain enzymes relocate to early melanosomes and become involved in the removal of glycan chains from PMEL to start the amyloid formation process. Further studies will be necessary to convincingly answer this question.

### 3. Conclusions and future directions

We suggest that the dynamics of amyloid formation could be managed or restored by jointly manipulating protein synthesis, *N*- and *O*-glycosylation, and Golgi remodeling. In the case of PMEL, we believe that the main role of glycosidation is to avoid the early start of the amyloid formation process in non-suitable intra or extra cellular locations. Thus, the sialic acid modified core-1-Oglycans in the RTP domain protect this domain from early protein shredding. The influence of glycosylation can be attributed to both the steric demand of the sugar moiety and hydrogen bonding, respectively. First, the incorporation of a single or a series of sugars will increase the serine side-chain volume dramatically, which results in a tight packing interaction which disrupts protein self-assembly into fibrils and prevents the exposure of this domain. The reported finding that the *N*-glycan site at aminoacid 321, located in the RPT domain of PMEL, is minimally modified indirectly supports this notion [12;33]. However, carbohydrate hydroxyl groups can still be involved in hydrogen bonding and sugar competition for intermolecular hydrogen bonds and could have an effect on the overall integrity of the structure. To avoid this situation, sialic acids are added at the non-reducing ends of glycans, conferring strong negative charges on the protein. We predicted that sialic acid when added to *N*-glycans makes then hydrophilic and leads to exposure of

hydrophobic protein domains, as occurs during cleavage of PMEL, favoring the formation of polymers through hydrophobic interactions. In contrast, sialic acid added to O-glycans make them hydrophobic and protects them from early cleavage and degradation, a function that has been reported previously in other cells [12;33;58;58]. Thus, the extent and type of O-glycans modified with sialic acid in the RPT domain of PMEL play key roles to determine the proper PMEL glycoform destined to form fibrils [59], or to be targeted to the plasma membrane [60; 61]

In light of the presented evidence, it is critical to understand that cancer cells present altered glycosylation patterns compared to their normal counterparts and that it will impact all glycoproteins. This is the case in murine melanoma cells, the interaction of cell surface lectins (such as calreticulin [62]) that bind proteins modified with mannose type glycans are necessary to initiate the increases of murine melanoma [63]. Similarly, the expression of high-mannose type glycans increases the development of hepatic metastasis in B16 melanoma cells [64] or that high-mannose type glycans are also involved in the functionality and cell surface expression of the “mature” human transferrin receptor [65;66]. Furthermore, differences in the patterns of expression of sialyltransferases, such as ST3 Gal I, between normal and malignant cells had already been associated with a cancer-specific regulation of glyco-epitopes [67]. Therefore, it is not unusual that “mature” proteins contain unmodified mannose-rich/ hybrid type N-glycans and that such conformation plays an active role in cellular processes. On the contrary, these findings reveal a new factor to consider in the processing of cancer-specific proteins, especially in Melanomas. Future research should be aimed to understand in depth and consider these alternatives.

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