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Review

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The Ocular Albinism type 1 (OA1) G-Protein Coupled Receptor functions with MART-1 at early stages of melanogenesis to control melanosome identity and composition

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ABSTRACT

OA1 (GPCR143), the protein product of the ocular albinism type 1 gene, encodes a pigment-cell specific G Protein-Coupled Receptor (GPCR) that localizes intracellularly to melanosomes. OA1 mutations result in ocular albinism due to alterations in melanosome formation, suggesting that OA1 is a key player in the biogenesis of melanosomes. To address the function of OA1 in melanosome biogenesis we have used siRNA inactivation and combined morphological and biochemical methods to investigate melanosome ultrastructure, melanosomal protein localization and expression in human pigmented melanocytic cells. OA1 loss of function leads to decreased pigmentation and causes formation of enlarged aberrant premelanosomes harboring disorganized fibrillar structures and displaying proteins of mature melanosomes and lysosomes at their membrane. Moreover we show that OA1 interacts biochemically with the premelanosomal protein MART-1. Inactivation of MART-1 by siRNA leads to a decreased stability of OA1 and is accompanied by similar defects in premelanosome biogenesis and composition. These data show for the first time that melanosome composition and identity are regulated at early stages by OA1 and that MART-1 likely acts as an escort protein for this GPCR.

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Introduction

Albinism comprises a heterogeneous group of inherited genetic disorders characterized by a variable loss of pigmentation in the eye, hair or skin. Ocular albinism type 1 (OA1) is an X-linked disorder that, differently from other forms of albinisms, is not caused by impairment of melanin production but by reduced number of melanosomes (1), the organelles in which melanin is synthesized and stored in Retinal Pigment Epithelium (RPE) and skin melanocytes. Mutations in OA1 gene also induce formation of aberrant "giant" melanosomes, called macromelanosomes (2). Thus, this form of albinism appears to be caused by a defect in melanosome organellogenesis. The protein product of the OA1 gene is a pigment cell specific G protein-coupled receptor (GPCR) (3-6). Differently to canonical GPCR, OA1 preferentially localizes to intracellular compartments such as melanosomes and lysosomes where it can signal from the organelle lumen to the cytosol (4, 7). Although the genetic defect was reported more than 10 years ago (8), how OA1 functions to control melanosome biogenesis has remained enigmatic, frustrating efforts toward development of new therapeutic strategies for ocular albinism.

Melanosomes are lysosome-related organelles that are distinct from conventional lysosomes within pigment cells (9, 10). Melanosomes develop through sequential maturation stages that can be defined based on morphology and that are enriched in a specific set of protein components (9, 11). Stage I and II melanosomes are characterized by the presence of intraluminal fibrillar sheets (12-14), in which the major component is the fibrillogenic protein Pmel17 (13, 15-17), but lack melanogenic enzymes such as Tyrosinase and Tyrosinase-related proteins. Deposition of black melanin along the fibers is initiated in stage III melanosomes as a consequence of the delivery of melanogenic enzymes to preformed stage II melanosomes. Once formed, the mature stage IV melanosomes are transported to the cell periphery for transfer to neighboring keratinocytes or stored within ocular melanocytes in the retinal pigment epithelium (18, 19).

Stage I premelanosomes correspond to early endosomal vacuoles that serve as intermediates for cargo bound both for lysosomes and melanosomes at different stages. As a first step towards melanosome formation, the transmembrane protein Pmel17 is sorted to intraluminal vesicles (ILVs) of endosomes thereafter called multivesicular bodies (MVBs) (9, 15, 16). Concomitant with the sequestration in ILVs,

Pmel17 is cleaved by a prohormone convertase liberating a fibrillogenic fragment ($M\alpha$) in its luminal domain which leads to the generation of the non-membranous amyloid like fibrillar structures (14, 16, 20). Whereas sorting of Pmel17 appears to be independent of ubiquitylation and of the (Endosomal Sorting Complex Required for Transport) ESCRT-0/ESCRT-I machinery (21), sorting of another melanosomal protein, MART-1 that is similarly sequestered in ILVs of MVBs, appears to require ubiquitylation (22, 23) and ESCRT proteins (21, 24). MART-1 has been reported to interact with Pmel17 (25), but the function of MART-1 in melanogenesis is not well understood.

In this study, we investigated the role of OA1 in melanosome biogenesis addressing its intracellular localization and how loss of function may alter trafficking steps required for melanosome maturation. In addition we investigated how it may exert its role together with other melanosomal proteins. Using a combination of light and electron microscopy and biochemical methods we show that OA1 controls the generation of premelanosomes and that it cooperates with MART-1 for this function. Altogether our data suggest that OA1 functions together with MART-1 to maintain melanosome composition and identity within the endocytic system.

Results

OA1 localizes to immature and mature melanosomes

Previous studies have addressed the subcellular localization of endogenous OA1 or GFP-tagged recombinant OA1 in melanocytic cells (4, 7, 26), but its localization within the distinct melanosomal stages and/or endosomes and lysosomes was not completely resolved. To qualitatively and quantitatively evaluate the subcellular distribution of the endogeneous OA1 protein in the melanocytic cell line MNT-1 we have used immunogold labeling on ultrathin cryosections (IEM). MNT-1 cells resemble untransformed skin melanocytes by expression of mRNAs encoding melanogenic proteins (27) and by ultrastructure, presenting melanosomes at all four stages (9). We have also investigated the localization of recombinant OA1 protein (OA1-Flag or OA1-EGFP) in transfected MNT1 cells (see below, fig S1) and in mouse melan-a immortalized melanocytes (data not shown). Consistent with previous studies, OA1 was detected at the limiting membrane of a small cohort of pigmented melanosomes labeled for the melanosomal enzyme Tyrp1 (stage III-IV) (fig 1A, arrows). Within these mature pigmented melanosomes, OA1 was often associated

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3 with small intraluminal vesicles (arrowhead fig 1A) and was also present at the
4 membrane of cytoplasmic vesicles often located in close proximity to melanosomes
5 (fig 1B, arrowhead). OA1 was detected at the limiting membrane of lysosomes (fig
6 1E, arrows) and in intraluminal vesicles of late endosomes/Multivesicular Bodies
7 (MVBs) (fig 1E, arrowheads). In agreement with its association with ILVs of MVBs,
8 like other ILV associated membrane proteins such as CD63, OA1 was detected in
9 extracellular exosomes secreted from MNT-1 after 48h of culture (not shown).
10 Although OA1 appeared broadly distributed, our observations revealed that the bulk
11 of OA1 was localized to organelles highly labeled for Pmel17 (fig 1C, 1D, arrows; see
12 quantification in fig 1F) that correspond to stage I and stage II premelanosomes (9).
13 This is consistent with but extends quantitatively the observations reported
14 previously (3) and (7). Similarly to endogenous OA1, the recombinant transfected
15 proteins (OA1-Flag or OA1-EGFP) localize to the distinct melanosomal stages and to
16 lysosomes. However, as compared to the endogenous protein we observed a shift in
17 the localization of OA1 towards more mature melanosomes (fig S1A, S1B, arrows).
18 We also observed that cells expressing higher levels of OA1 were characterized by
19 the presence of large electron-lucent OA1 positive membrane vesicles (350 nm).
20 These vesicular structures were also labeled for Tyrp1, and often localized in close
21 proximity to melanosomes in both human (fig S1A, asterisks) and mouse cells (data
22 not shown). These vesicles were possibly induced by the overexpression of OA1
23 since they were not observed in Flag/EGFP empty vectors transfected cells (data not
24 shown). The observed phenotype indicates that the trafficking of recombinant OA1
25 may not fully reflect that of the endogenous protein. However, on the other hand it
26 also suggests a dominant negative effect of OA1 expression that maybe result in the
27 accumulation of transport intermediates whose trafficking may be controlled by OA1.
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46 Altogether these data show that OA1 is widely distributed throughout the
47 endo-melanosomal system but that most of endogenous protein is localized in
48 unpigmented Stage II melanosomes. This finding is in agreement with the suggested
49 function for OA1 at early stages of melanogenesis as we previously proposed (1).
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OA1 loss of function affects melanosomal protein trafficking and melanosome size at early stages of biogenesis

To get further insights on the function of OA1, we inactivated OA1 by RNAi in pigmented MNT1 cells and analyzed melanosome morphology and composition right after the loss of OA1 activity. This approach overcomes the limits and/or compensations of immortalized melanocytes from OA1 patients or mice that contain primarily mature organelles and preclude the study of the different sequential events leading to the formation of macromelanosomes.

We designed and tested three oligonucleotides targeting three different regions of the human OA1 mRNA. As a control we have used a non-targeting siRNA. One of the three siRNAs (siRNA #3) was very efficient in reducing the levels of OA1 transcripts (15% siRNA #1, 78% siRNA #2, 95% siRNA #3)(fig 2A) and was used for experiments presented here. Downregulation of endogenous OA1 protein was confirmed by WB and IF (fig 2B-D). We found that mature melanosomes and melanin content in siOA1 MNT1 cells were reduced as much as 50% compared to the cells treated with the control siRNA (fig 2E-G). Analysis at the ultrastructural level, by conventional EM, also showed a reduction of the total number of melanosomes in MNT1 cells depleted of OA1 (fig 2H-K), consistent with the phenotype characterized in ocular albinism mouse model and patients. Although we did not observe the "classical" pigmented macromelanosomes, we found that OA1 depleted MNT1 cells harbor abnormally enlarged organelles with a heterogeneous content that are mixed with normal melanosomes and could possibly correspond to the precursors of the macromelanosomes (fig 2I; fig S2A). These aberrant mixed compartments contains Pmel17 positive fibrils characteristic of immature melanosomes and an electron dense core of melanin (fig 2I, asterisk; fig S2B-C). Similar phenotypes were observed with a second siRNA sequence (siRNA #2) excluding that the phenotype derives from mistargeting. Of note, similar enlarged "mixed" organelles were also observed in OA1 in vitro cultured melanocytes from knockout mouse (melan-Oa1^{-/-}) (fig S2D-E). Cells depleted of OA1 also harbored enlarged immature unpigmented melanosomes that had lost the typical striated appearance and whose internal fibrils were clearly disorganized (fig 2I, arrowhead; fig S2A; fig 3B, 3D, 3F). In order to investigate if these morphological alterations were accompanied by a defect in the localization of melanosomal proteins, we analyzed the distribution of melanosomal proteins in OA1 depleted MNT1 cells by IEM. The fibrillar structures apposed to or contained within the abnormal melanosomal structures were highly labeled with an antibody directed

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3 against Pmel17, the major component of the fibrils (fig 3B, arrowhead; fig S2C,
4 arrows). Of note, Tyrp1 that is nearly absent from Pmel17 enriched Stage II
5 premelanosomes in control cells, is detected at high levels at the membrane of the
6 enlarged organelles (fig 3A-B, arrows, see also fig 3F) suggesting an alteration of its
7 trafficking upon OA1 depletion. Another protein that appeared to be mislocalized to
8 the enlarged melanosomal organelles is LAMP-1. LAMP-1 is a lysosomal protein that
9 is present only in small amounts at the membrane of Tyrp1 positive melanosomes
10 ((9) and fig 3C, 3E, arrows). In OA1 depleted cells LAMP1 was abundant in both
11 aberrant enlarged pigmented organelles and normal size melanosomes (fig 3D, 3F,
12 3G, arrows). Quantification of these observations is shown in Table 1. LAMP1 also
13 accumulated in Tyrp1 positive vacuolar structures (fig 3H, inset) that were
14 reminiscent of the vesicles observed upon OA1-Flag overexpression (fig S1)
15 suggesting that depletion of OA1 or a dominant negative effect may lead to similar
16 phenotypes. It also suggests that OA1 may function at a fusion/fission step
17 regulating Tyrp1 and LAMP1 content of melanosomes. Although the melanin content
18 was generally reduced in siOA1 MNT1 cells, western blot experiments performed in
19 these cells revealed a slight increase of the amount of Tyrp1 in the OA1 depleted
20 cells and no significant differences in the amount of LAMP1 (fig 3I), consistent with a
21 defect in their trafficking and not expression/degradation. To get further insight on
22 the trafficking defects and nature of the organelles affected upon OA1 loss of
23 function, we performed IEM on cells that were allowed to internalize Transferrin
24 (early-recycling endosomes), BSA-gold (endo-lysosomal compartments) or that were
25 incubated with the weak base DAMP, to evaluate the acidity of the organelles (9).
26 Transferrin (data not shown) and the endocytic tracer BSA-gold (BSAG) were not
27 detected in the enlarged organelles (fig S3A-B) and vacuoles (data not shown)
28 indicating that the defects in trafficking and organelle biogenesis caused by OA1 loss
29 of function were not due to aberrant mistargeting within the endosomal system. The
30 enlarged melanosomal organelles appeared to acidic as are premelanosomes in wild
31 type cells as assayed with DAMP (9) (fig S3C-D). However, and despite that DAMP
32 accumulation is not a quantitative assay for pH, these organelles show an increased
33 labeling for the radical DNP (fig S3D), suggesting that they may be slightly more
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3 **OA1 and MART-1 loss of function similarly affect Pmel17 final processing**
4 **and early melanosome biogenesis.**
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7 We next aimed at further investigating how premelanosome biogenesis could be
8 affected by the loss of OA1 function. The major biogenetic component of the stage II
9 fibrils is the pigment cell-specific protein Pmel17. In stage II melanosomes the
10 Pmel17 forms are detectable with HMB45 antibody, which recognizes the $M\alpha$
11 fragment and its proteolytic products (28-30) and with Pep13h antibody, recognizing
12 the processed full length (P1, 100kD) and the cleaved forms ($M\beta$, 26kD and CTF,
13 10kD) (31).
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19 Enlarged organelles displayed disorganized fibrils and this prompted us to evaluate
20 Pmel17 expression and processing. We did not detect significant changes of Pmel17
21 transcript levels upon OA1 loss of function (fig S4A). Next we analyzed the different
22 maturation forms of Pmel17 by using HMB45 and Pep13h antibodies. Although
23 Pep13h did not reveal significant differences in the levels of P1 and $M\beta$ (fig 4Ac), an
24 accumulation of $M\alpha$ fragment (80kD) and a reduction of one of its products ($M\alpha C$,
25 35kD) was observed with the HMB45 antibody in both the TX-soluble (fig 4Aa) and
26 TX-insoluble pool (fig 4Ab) of siOA1 cell lysates (fig 4A). The TX-insoluble pool is
27 enriched in amyloid fibers that are insoluble in non-denaturing detergent TX100 (16).
28 Since $M\alpha C$ is considered to be the major form of the HMB45-reactive species within
29 the fibers (29, 30) its reduction is likely to reflect the disorganization of amyloid-like
30 fibers observed in stage II melanosomes in siOA1 MNT1 cells (fig 2I, arrowhead; fig
31 3D, 3F). These results indicate that although Pmel17 appears to be cleaved in its
32 luminal domain, its final processing to form the $M\alpha C$ fragment is impaired consistent
33 with the altered morphology of the intraluminal contents of the premelanosomal
34 compartment.
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47 Such effects observed upon OA1 depletion were reminiscent of those described in
48 cells that do not express MART-1 (25). MART-1 is an integral membrane
49 melanosomal protein that has been shown to form complexes with Pmel17 and has
50 been suggested to control Pmel17 processing and stability (25). We therefore used
51 siRNA to deplete MART-1 from MNT-1 cells. MART-1 was effectively downregulated
52 (reduction of 90% of detectable MART-1 transcript and protein) (fig 6I and fig 4B).
53 We confirmed that Pmel17 maturation was affected in MART-1 depleted cells and in
54 particular, similarly to OA1 knockdown, we detected accumulation of the $M\alpha$
55 fibrillogenic fragment accompanied by a reduction of its subproduct $M\alpha C$ (fig 4B). To
56 better define the compartments affected by MART-1 depletion, we performed
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3 immunogold EM analysis on ultrathin cryosections of siMART-1 MNT1 cells. No
4 normal stage I premelanosomes were found, but we noted the presence of many
5 aberrant enlarged compartments that often appeared as a mixture of striations and
6 electron dense pigment, similar to those observed in siOA1 cells (fig 4D).
7 Interestingly, these compartments contained abnormally large intraluminal vesicles
8 and their Pmel17-positive fibers were disorganized and/or apposed to one or both
9 sides, indicating altered early melanogenesis. We also found that Tyrp1 (fig 4E, 4F,
10 arrows), and LAMP1 (data not shown) accumulated in early Pmel17 positive
11 premelanosomal compartments with concomitant reduction at the melanosomal
12 membrane as compared with the control cells.
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20 Overall these observations show that loss of function of either OA1 or MART-1 leads
21 to an impairment of melanosome biogenesis at its earliest steps and to mis-
22 trafficking of distinct melanosomal proteins likely en route to their final destination,
23 in this case lysosomes or melanosomes. Moreover these results show that MART-1,
24 similarly to OA1, regulates melanosome maturation and in particular the
25 maintenance of melanosomal identity and composition.
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33 **MART-1 interacts with OA1 and is required for OA1 stability in MNT1 cells**

34 Based on the data presented so far we hypothesize that either OA1 and MART-1
35 fulfill a similar redundant function in early melanogenesis or that one of the two
36 proteins affects the function of the other. We thereby evaluated possible interactions
37 between these proteins and investigated the consequences of MART-1 loss of
38 function on OA1 expression and localization.
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42 For this purpose, we immunoprecipitated endogenous OA1 from MNT1 extracts and
43 tested for the presence of MART-1 by immunoblotting. Consistent with our
44 hypothesis, the 18kDa form of MART-1, corresponding to the not-ubiquitylated form
45 (23), was detected in the anti-OA1 immunocomplexes (fig 5A). We confirmed the
46 specificity of this interaction by co-immunoprecipitation of OA1 with Galpha-i3 (data
47 not shown), as already published (4), but not with the melanogenic enzyme Tyrp1
48 (fig 5A). Although we detected a biochemical interaction between OA1 and MART-1,
49 co-localization, as analyzed by immunofluorescence followed by 3D deconvolution or
50 IEM, was limited to only a small subset of cellular compartments (fig 5B-L). OA1 was
51 distributed in vesicular structures that mainly correspond to immature and mature
52 melanosomes (see also Fig.1), but MART-1 was essentially localized in the
53 perinuclear area in a region that mainly corresponds to the Golgi/TGN and post-Golgi
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3 endosomal compartments (fig 5B-I and 5J). These data were confirmed by IEM, in
4 which OA1 and MART-1 were detected together only within the Golgi, vesicles close
5 to the Golgi apparatus and endosomal compartments (fig 5J, 5K,5L, arrows).
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8 Interestingly, when we analyzed cell lysates from cells in which MART-1 was
9 depleted with siRNA, we found that MART-1 depletion greatly affected OA1 protein
10 expression and distribution, reducing the levels of OA1 protein (fig 6E-H, 6J) but not
11 its transcript (fig 6I). By contrast, MART-1 RNA and protein levels were not affected
12 in the absence of OA1 (fig S4A-B). These observations suggested that MART-1 loss
13 of function induces an increased degradation of OA1. To further test this hypothesis
14 and to investigate the mechanisms involved in its decreased expression/stability,
15 MART-1 depleted cells were treated for 5 hrs with drugs known to inhibit lysosomal
16 (chloroquine) or proteasomal degradation (MG132 or epoxomycin) respectively.
17 Chloroquine is an inhibitor of intra-lysosome catabolism that raises intralysosomal pH
18 disrupting the activity of lysosomal acid hydrolases (32) (33). As analyzed by western
19 blot, in control cells treated with Chloroquine, the levels of OA1 increased after 5hrs
20 of treatment, showing that OA1 generally undergoes a lysosomal degradation. A
21 similar increase of OA1 protein levels was observed in Chloroquine-treated MART-1-
22 depleted cells (fig 6Ka), showing a partial rescue of OA1 protein levels after only 5
23 hrs of treatment. In order to address whether the proteasome was involved in OA1
24 degradation, MART-1 depleted cells were then treated with two proteasome
25 inhibitors MG132 or Epoxomycin. MG132 is a widely used potent synthetic inhibitor
26 of a number of proteasome-associated proteases (34) (35). Epoxomycin is a natural
27 proteasome inhibitor highly specific for the 20S proteasome (36). OA1 protein levels
28 did not significantly change in treated versus untreated control cells. On the other
29 hand, in siMART-1 MNT1 cells we observed significantly higher levels of OA1 protein
30 after 5 hrs of incubation with proteasome inhibitors as compared to the siMART1
31 untreated cells (fig 6Kb-c). These results suggest that, in absence of MART-1, OA1 is
32 likely to follow also a distinct degradation pathway and in particular proteasomal
33 degradation. The stronger effect of MG132 as compared to epoxomycin could be due
34 to the fact that this compound is not specific for proteasome and is also a potent
35 inhibitor of various cysteine proteases and cathepsins (37, 38). Overall, these data
36 show that OA1 stability/expression requires MART-1.
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Discussion

Melanosomes represent a class of lysosome-related organelles that coexist with late endosomes and lysosomes within melanocytes and retinal pigment epithelium. The pathways by which melanosomal proteins are diverted from the classical endocytic organelles toward melanosomes and/or differentially targeted to different melanosomal stages are incompletely defined, but are disrupted in several genetic diseases (39). These diseases, leading to defects in melanosome biogenesis and transfer, are due to mutations in genes encoding proteins involved in intracellular trafficking but also mutations in melanosome specific proteins whose functions remain so far unclear (10, 39). We show here that the OA1 functions early in melanosome biogenesis controlling not only melanosome size but also melanosome composition, and thereby appropriate maturation. In addition we show that OA1 stability and function is ensured by its interaction with the premelanosomal protein MART-1 assigning a significant role to this protein in melanosome biogenesis.

OA1 functions in early melanogenesis and melanosome composition

Our studies evidence that the bulk of intracellular OA1 localizes to Pmel17-positive unpigmented premelanosomes. The presence of endogenous OA1 in the precursors of premelanosomes that also display other melanosomal proteins such as Pmel17 and MART-1 (9, 22, 40), suggest a function for this GPCR early in melanogenesis as previously predicted based in our genetic studies (1).

The albinism phenotype caused by OA1 loss of function is characterized not only by the presence of macromelanosomes but also by a reduction in melanin content and the number of melanosomes at early stages of their maturation (1, 41). After 5 days of OA1 inactivation we were able to reproduce the defect in melanosome number but not the "classical" macromelanosome phenotype probably because a longer time is required to complete melanosome maturation. However even after short inactivation (3 to 5 days) we observed abnormal enlarged pigmented structures with a heterogeneous content. These structures display a size similar to macromelanosomes with an average diameter of 0,9-1 μm (1). In addition, several of the enlarged compartments observed in OA1 knocked-down cells, although not fully electron dense like macromelanosomes in melanocytes from OA1 mutant mice, display a mixed content with an "amorphous" melanin core and fibrillar sheets ((2) and fig S2B-E.). For all these reasons we believe that the enlarged

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3 structures observed in si-OA1 cells correspond to the precursors of
4 macromelanosomes. These aberrant compartments could result from fusion of
5 anomalous premelanosomes with mature melanosomes. Otherwise and not mutually
6 exclusive, these compartments could also arise from an improper
7 formation/composition of immature Stage II melanosomes resulting from abnormal
8 fusion of transport intermediates – or from lack of retrograde transport from
9 maturing melanosomes – to remove cargoes like LAMP1. This is supported by our
10 observations that OA1 depleted cells accumulate Tyrp1 containing vesicular
11 structures and that immature melanosomes (stage I-II) displayed consistent
12 amounts of Tyrp1 at their limiting and internal membranes. This melanogenic
13 enzyme is in general absent from Stage I/Stage II unpigmented melanosomes and
14 enriched in pigmented Stage III and Stage IV (9). These observations indicate
15 mistargeting of melanogenic enzymes to immature melanosomes and this event may
16 lead to abnormal pigment synthesis within premelanosomal compartments. These
17 observations can also explain the reduced number of early stage melanosomes
18 observed in OA1 mutant RPE (1). Abnormal protein targeting is not limited to
19 melanogenic enzymes but also to proteins of other cellular organelles such as the
20 lysosomal membrane protein LAMP1. LAMP1 is normally present in lysosomes, it is
21 also detectable at lower amounts in mature melanosomes but absent from immature
22 unpigmented melanosomes (9). The presence of Tyrp1 and LAMP1 in vesicular
23 structures and within Pmel17 positive early melanosomes favors the hypothesis of an
24 “aberrant” fusion of LAMP1 and Tyrp1 loaded vesicles with StageI/Stage II
25 premelanosomes. The nature of the Tyrp1/LAMP1 containing transport intermediates
26 is not totally clear but based on our recent studies they most likely correspond to
27 post Golgi endosomal compartments (24). These studies indicate that endosomes are
28 required intermediates for Tyrp1 transport from the Golgi to the limiting membrane
29 of melanosomes. These endosomal domains are distinct from EEA1 positive early
30 sorting endosomes and appear to be located just downstream of early recycling
31 endosomes as in the absence of functional Tsg101 there is a reduced flux of Tyrp1
32 via the plasma membrane and accumulation of Tyrp1 in endosomal vacuoles that are
33 also positive for LAMP1 (24) and CD63 (data not shown). The vacuolar structures
34 observed upon OA1 inactivation show similar characteristics, containing both Tyrp1
35 and LAMP1 but not internalized Transferrin (data not shown) or the endocytic tracer
36 BSAG, consistent with the notion that these are post-Golgi/endosomal intermediates
37 that are placed downstream of the sorting decision point to segregate endosomal
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3 from melanosomal cargo. Similar vesicular structures bearing OA1 at their membrane
4 were observed in OA1 overexpressing cells supporting our hypothesis that OA1 is
5 involved in regulating trafficking of such intermediates. One plausible hypothesis is
6 that OA1 controls their transport/fusion to/with melanosomes. Since we detected the
7 bulk of endogenous OA1 in immature melanosomes, OA1 possibly functions in
8 these compartments to negatively control a "too early" fusion of transport
9 intermediates before complete maturation of Stage II premelanosomes. In the
10 absence of functional OA1 these transport intermediates aberrantly fuse with
11 immature melanosomal compartments leading to mistargeting of these proteins to
12 their membranes before completion of maturation. Thereby it seems plausible that in
13 melanocytes issued from OA1 mutant mice, pigmented melanosomes rather
14 correspond to immature aberrant organelles, with an inappropriate composition
15 leading to synthesis of melanin pigments in the wrong organelle. This explains the
16 morphological features of melanosomes and macromelanosomes in OA1 mutant cells
17 and maybe underlies the transport defects of melanosomes recently reported in
18 these cells (42).
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32 **OA1 and MART-1 similarly influence Pmel17 processing and melanosome** 33 **maturation** 34

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36 The enlarged premelanosomal compartments harboring Tyrp1 and LAMP1 at
37 their limiting membrane display Pmel17 positive fibrils in their lumen although the
38 fibrils do not appear to be fully organized as in classical Stage II premelanosomes.
39 The premelanosomal protein Pmel17 is properly processed as judged by western
40 blotting and is able to generate fibrils indicating that its cleavage by prohormone
41 convertases is not affected in the absence of functional OA1. However we observed
42 an increased amount of the M α fragment over M α C indicating that one very last step
43 of its processing required for fibril formation is impaired resulting in abnormally
44 assembled fibrils. The cause of the observed alterations in fibril
45 formation/organization remains unclear. The intraluminal acidification of the
46 aberrant organelles, as assayed with the weak base DAMP, does not appear to be
47 significantly altered as compared to control unpigmented premelanosomes.
48 Although, we cannot exclude that the slightly higher labeling observed in the
49 inactivated cells could be due to a defective deacidification required to progress the
50 fibrils and consequently mature melanosomes. Otherwise, the inefficient processing
51 could also be the consequence of an altered trafficking of the enzymes required for
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3 these last steps of Pmel17 processing. Confirming this hypothesis is difficult at the
4 moment because, although some of the enzymes involved in early steps of Pmel17
5 processing have been at least partially identified (16, 31), those allowing these final
6 steps of cleavage are still unknown.
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10 Interestingly, a similar alteration of Pmel17 processing was observed in cells
11 in which the premelanosomal protein MART-1 was inactivated. MART-1 has been
12 suggested to control Pmel17 expression, stability, trafficking and processing (25). We
13 show here that in cells in which MART-1 expression was reduced by 90%, Pmel17
14 processing is affected but not its expression and trafficking. MNT1 cells show only a
15 partial phenotype of that observed in primary human melanocytes and SK-MEL-28
16 cells (25), however we confirmed MART1 functional relevance in the generation of
17 Pmel17 M α fragment. In this study we also unraveled a morphological phenotype in
18 MART-1 knocked-down cells: enlarged aberrant premelanosomes with disorganized
19 fibrils and mistargeting of Tyrp1 and LAMP1 to Pmel17 positive organelles. These
20 vesicles are similar to those observed in OA1 depleted cells. Moreover and despite
21 the higher enrichment in premelanosomes, OA1 such as the premelanosomal protein
22 MART-1 is broadly distributed throughout the biosynthetic pathway, mature
23 melanosomes and lysosomes. Interestingly, the ubiquitylation deficient mutant
24 MART-1 is mistargeted to mature melanosomes (23) in a manner similar to the
25 overexpressed OA1 in human and mouse melanocytes (our study). We therefore
26 hypothesized that OA1 and MART-1 could function at a similar step and/or
27 reciprocally regulate each other.
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43 **OA1 expression and function requires MART-1 as an escort protein.**

44 Our observations are consistent with the hypothesis that MART-1 is an
45 essential regulator of OA1 expression and function. These two proteins interact
46 biochemically, as shown by co-immunoprecipitation, and this interaction appears to
47 be independent of the reported MART-1/Pmel17 interaction (25) since we did not
48 observed co-immunoprecipitation of OA1 with Pmel17 (data not shown). MART-1
49 probably functions in the biosynthetic transport/stabilization of OA1 and this
50 indicates that the phenotypes observed in MART1 depleted cells could rather be the
51 consequence of a decreased expression/function of OA1. In the absence of OA1
52 MART-1 expression was not affected at the RNA and protein levels. In absence of
53 functional MART-1, OA1 expression was not affected at the RNA level but
54 significantly decreased at the protein level as assayed by western blot and
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3 immunofluorescence. We have also shown that in absence of MART-1 OA1 appears
4 to be degraded by a lysosomal pathway involving the ubiquitin/proteasome system.
5 Consistent with this hypothesis, OA1 expression is restored in cells depleted for both
6 MART-1 and Tsg101, a component of the ESCRT machinery involved in degradation
7 of ubiquitinated cargo proteins (data not shown). These observations show for the
8 first time that OA1 can be degraded by Lysosomal/proteasomal degradation similarly
9 to other GPCRs (43, 44). In addition we showed that MART-1 can interact with OA1
10 early during its biosynthetic pathway acting as an escort protein that helps its
11 stabilization to prevent degradation. Innamorati et al. showed that arrestin induces
12 an accelerated degradation of OA1 in transfected cells (5) and it is commonly
13 accepted that arrestins can directly induce GPCR degradation by a process distinct
14 from internalization i.e. by promoting receptor ubiquitination through recruitment of
15 E3 ligases and subsequent lysosomal and/or proteasomal-mediated destruction (45,
16 46). Future studies are needed to unravel the relationship of OA1 interaction with
17 MART-1, arrestin and the ESCRT machinery in the regulation of its degradation.
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29 In conclusion, we show that the GPCR OA1 functions in early melanogenesis
30 controlling melanosome size and composition that impacts on their identity and
31 consequent maturation. In addition we evidence that OA1 expression is ensured by
32 its interaction with MART-1 revealing a role for this protein in melanosome
33 biogenesis. Altogether our data sheds light on how OA1 regulates key steps of
34 melanosome formation also unraveling pathways by which melanosomal proteins are
35 diverted from the classical endocytic organelles and/or differentially targeted to
36 different melanosomal stages. These studies have important implications for
37 understanding the pathophysiology of OA1, the involvement of GPCRs in intracellular
38 trafficking and how highly specialized cells exploit intracellular trafficking pathways to
39 generate unique organelles.
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Materials and methods

Cell culture, transfection and siRNA depletion

The immortalized Oa1 null (melan-Oa1^{-/-}) and wild type melanocytic cell lines were derived from wild type or Oa1^{-/-} mouse skin (42) and maintained in RPMI-1640 supplemented with 10% FCS and 200nM phorbol 12-myristate 13-acetate at 37°C with 10% CO₂. MNT-1 human melanoma cells were maintained as previously described (9). MNT-1, melan-O and melan-a were transfected with plasmid constructs using Lipofectamine 2000 (Invitrogen) following manufacturer's recommendations. Cells were collected and analyzed after 48 h. Transfection in MNT-1 with siRNA duplex oligonucleotides proceeded as reported (21) and according to the manufacturer's instructions. Cells were subjected to two rounds of transfection (day 0 and day 2), were passaged as required, and processed at day 3 or day 5 for real time qPCR, immunofluorescence microscopy, electron microscopy, western blotting and melanin assay.

The sense strands for the indicated double stranded siRNAs (21-mers) were synthesized with the following sequences or derived from the following references. siRNA OA1 #1: 5'- GGA TAT GAA CCA CAC GGA A -3' (Hs_GPR143_6_HP siRNA from QIAGEN); siRNA OA1 #2: 5'- GGC TGT AAA GTA AGT GTA A -3' (Hs_GPR143_3_HP siRNA from QIAGEN); siRNA OA1 #3: 5'- GGT TGT CGA ATA TCA TCA A -3' (Hs_GPR143_1_HP siRNA from QIAGEN); siRNA MART-1 #1: 5'- AAG ACG AAA TGG ATA CAG AGC -3' (25); siRNA MART-1 #2: 5'- TCC GCT AGC AGT ACT AAT CAT -3' (Hs_MLANA_7_HP from QIAGEN); . siRNA non-targeting control: 5'- AAT TCT CCG AAC GTG TCA CGT -3'.

Reagents and Antibodies

Primary antibodies: polyclonal anti-human OA1, raised against the C-terminus of the human OA1, similar to the previously published (4); polyclonal anti EGFP (Molecular Probes); monoclonal anti flag M2 antibody (SIGMA-Aldrich); polyclonal anti flag (SIGMA-Aldrich); monoclonal anti β -tubulin antibody (SIGMA-Aldrich); TA99 mouse anti-TYRP11 mAb (American Type Culture Collection; (47)) for immunofluorescence and immunogold labelling and monoclonal anti-Typr1 75kDa (Abcam) for western blotting; melanoma Ab-2 anti-Pmel17 (clone HMB50) (Lab Vision Corporation); mouse monoclonal anti-Pmel17 (clone HMB45) (Novocasta); Pep13h antibody was a kind gift from M.S.Marks (University of Pennsylvania, Philadelphia); monoclonal anti-MART-1 7c10 (ABCAM); rabbit anti mouse IgG was purchased from Dakopatt; polyclonal anti biotin (Polysciences Biovalley). Monoclonal anti-LAMP1 (BD Biosciences). Chloroquine, MG132 and Epoxomycin (SIGMA Aldrich);

DAMP (3-(2,4-dinitroanilino)-3'-amino-*N*-methyldipropylamine) (Invitrogen). Bovine Serum Albumin coupled to 5 nm gold particles (BSAG) and Protein A Gold conjugates were purchased from Cell Microscopy Center, Utrecht The Netherlands.

Cloning of OA1-tagged constructs

The human OA1 coding sequence was amplified by polymerase chain reaction (PCR) by using *Pfu* high fidelity DNA polymerase (Promega) and the following primers containing EcoRI and XhoI restriction sites: OA1fw:CGGAATTCATGACCCAGGCAGGCCGG;OA1rw:CCGCTCGAGTCACACCTGGACACGGAAG. The PCR product was digested by EcoRI and XhoI and subsequently cloned into EcoRI/XhoI-digested pcDNA3-flag vector to express OA1 with a Flag-tag at the C-terminus. The same PCR product was also cloned into pEGFP-N1 vector (Invitrogen) to express OA1 with an EGFP-tag at the C-terminus.

Quantitative Real time-PCR

Total RNA was extracted from siRNAs and control transfected MNT-1 cells using RNeasy MiniKit (Qiagen) according to the manufacturer's instructions. The same amount of cDNA was synthesized using Superscript II (Invitrogen) and random primers. Real time PCR was carried out with the GeneAmp 7000 Sequence Detection System (Applied Biosystem). The RT-PCR reaction was performed using cDNA, 12.5 μ l SYBR green master mix (Applied Biosystem) and 400 nM primers for each gene (*see list below*). Water was added to a final reaction volume of 25 μ l. The PCR conditions were as follows: preheating at 50°C for 2 min and 95°C for 10 min; 40 cycles of 15 s at 95°C and 1 min at 60°C.

Quantification results were expressed in terms of the cycle threshold (Ct). All real-time qPCR reactions were run in triplicate and the Ct values were averaged from three independent samples. Data were normalized to the internal control, the reference gene S26. Differences between the mean Ct values of each gene and those of the reference gene were calculated as $\Delta Ct = Ct_{\text{gene}} - Ct_{\text{reference}}$ and represented as $2^{-\Delta Ct}$.

Sequences of the forward (fw) and reverse (rw) intron-spanning primers used for Real-time qPCRs:

Human S26 fw	CCG TGC CTC CAA GAT GAC AA
Human S26 rw	GCA ATG ACG AAT TTC TTA ATG GCC T
Human OA1 fw	CGG AGA TCG GCA GGA CTG AGC AC

Human OA1 rw	ATA GTG GGG GAT GGC GTG GT
Human MART-1 fw	GAAGGGTTTGATCATCGGGA
Human MART-1 rw	GCATTGGGAACACAGGTTC
Human Pmel17-fw	CAGCTCAGCCTTCACCATTA
Human Pmel17 rw	AAGTGCTTGTTCCCTCCATC

Immunoprecipitations and Western Blotting

siRNA treated MNT-1 cells at 80% of confluency and control MNT-1 cells were washed in cold PBS and lysed on ice in lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 10 mM EDTA, pH 7,2 and protease inhibitor cocktail (Roche 11836153001). Lysates were then centrifuged at 21,000g for 20 min at 4°C. Protein concentrations were determined by Micro BCA™ Protein Assay Reagent Kit (Pierce). The protein samples were mixed with an equal volume of loading buffer containing 2% SDS (or 14% SDS for OA1) with 5% β-mercaptoethanol and then boiled for 2 min (or warmed 30 min at 37° for OA1 protein). For immunoprecipitation protein samples were first precleared using protein G agarose beads for 2 hrs at 4°C under rotation. Supernatants were collected and incubated with protein G agarose beads with 1 μg of rabbit anti-human IgG for 5 hrs at 4°C. To immunoprecipitate OA1, supernatants were incubated with 1 μg of anti-OA1 polyclonal antibody O/N at 4°C under rotation. As controls, 1 μg of rabbit anti-human IgG was used. After 10 washes in cold lysis buffer, immunoprecipitated proteins bound to the beads were incubated in sample buffer (containing 14% SDS for OA1 protein) and then boiled for 2 min (warmed 30 min at 37° for OA1 protein). Immunoprecipitated proteins were loaded and separated in 8% SDS-PAGE gel to reveal OA1 and in Nupage (4-12%) Bis-Tris gels (Invitrogen) to reveal the other proteins.

The separated proteins were then transferred to polyvinylidene difluoride membrane (Millipore) in XCell II™ Blot Module (Invitrogen) and the membranes were blocked in PBS/0.1%Tween-20 (PBS/T) with 5% non-fat dried milk, incubated with primary antibody in PBS/T, washed four times in blocking solution, and incubated with horseradish peroxidase-conjugated secondary antibody (anti-rabbit or anti-mouse; 1:10000; Jackson ImmunoResearch Laboratories) followed by washing in PBS/T. Visualization of the antibody binding was carried out with ECL Plus Western blotting detection system (GE Healthcare) according to the manufacturer's instruction. Signal intensities were quantified with Image J software.

Melanin assay

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Cells were disrupted by sonication in 50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol and protease inhibitors. Pigment was pelleted at 20,000g for 15 min at 4°C, rinsed once in ethanol/ether (1:1), and dissolved in 2M NaOH/ 20% dimethylsulfoxide at 60°C. Melanin content was measured as optical density at 492 nm (48).

Immunofluorescence (IF)

Transfected MNT-1 cells on coverslips were rinsed in PBS and fixed for 15min in 4% paraformaldehyde/PBS at room temperature (RT). Fixed cells were washed in PBS and quenched 10 min in PBS/ 50 mM glycine, saturated in PBS/ 1 mg/ml BSA (blocking buffer) and permeabilized in PBS/ 0.05% saponin/ 1 mg/ml BSA (incubation buffer, IB). Cells were incubated 1 hr with the primary antibody diluted in IB, washed three times in IB and incubated with the corresponding secondary antibody Alexa 488- or Alexa 568-conjugated anti-rabbit or anti-mouse secondary antibodies (1:200; Molecular probes) for 45 min in IB and washed again. Finally, coverslips were mounted in DABCO medium and examined on a Leica Microsystemes (Nanterre, France) DM-RXA2 3D deconvolution microscope equipped with a piezo z-drive (Physik Instrument, Pantin, France) and a 100x1.4NA-PL-APO objective lens for optical sectioning. Images are maximum-intensity z projections of 3D image stacks acquired using Metamorph software (MDS Analytical Technologies, Sunnyvale, CA) through a Coolsnap HQ (Photometrics Coolsnap HQ) cooled CCD-camera except images shown in fig 5B-I where single stacks were deconvoluted.

Electron Microscopy (EM)

For conventional EM, cells grown on coverslips were fixed with 2.5% glutaraldehyde in 0.1M cacodylate buffer for 24 hrs. After several washes with 0.1M cacodylate buffer, the cells were postfixed with 2% OsO₄, dehydrated in ethanol, and embedded in Epon while on the coverslips. Ultrathin sections were prepared and counterstained with uranyl acetate and lead citrate before the observation. For quantification, melanosomes were counted on 10 randomly acquired micrographs at X5,000 of siOA1 and control MNT1 cells. The area of cell analyzed was determined by iTEM software and expressed in square micrometers (160μm²).

For immunogold labelling, cells were fixed with a mixture of 2%PFA and 0.2% glutaraldehyde in 0.1M phosphate buffer, pH 7.4 and processed for ultracryomicrotomy as previously described (49). Ultrathin cryosections were single- or double-immunogold labelled with antibodies and protein A coupled to 5 or 10 or 15-nm gold, as indicated in the legends to the figures. Sections of resin embedded

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3 cells and immunogold labeled cryosections were observed under a Philips CM120
4 electron microscope (FEI company), equipped with a KeenView camera (Soft
5 Imaging System; SIS, Germany). For quantification of OA1 labeling, 500 gold
6 particles (endogenous OA1) or 800 (transfected OA1) were counted in randomly
7 selected cells profiles of cells in each of two experiments. Tyrp1 and LAMP1 gold
8 particles were randomly counted in a total of 80 unpigmented (stage I/II) or
9 pigmented (stage III/IV) melanosomes, in two separate experiments. Data are
10 presented as mean \pm SD.

17 **Uptake of BSAG and DAMP**

18 Uptake of the endocytic tracer BSAG and the weak base DAMP (3-(2,4-
19 dinitroanilino)-3'-amino-*N*-methyldipropylamine) were performed on living cells
20 before fixation and processing as previously reported (9). *Uptake of BSAG:* cells were
21 washed with serum-free DMEM, pulsed for 10 min at 37° with BSAG ($OD_{520nm}=5$),
22 washed with ice-cold medium/2%FBS and the endocytic tracer was chased for
23 45min. After washing with ice-cold medium, cells were fixed. *Incubation with DAMP:*
24 cells were washed with serum-free DMEM and incubated with DAMP (30 μ M) for 15
25 min at 37°. Cells were washed with ice-cold medium and then fixed.

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58 **Conflict of Interest:**

59 The authors declare no conflict of interest
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Legends to Figures

Figure 1.

Localization of OA1 in MNT-1 cells. Ultrathin cryosections of MNT-1 cells were double immunogold labeled with anti-OA1 and anti-Tyrp1 (A and B) or anti-OA1 and anti-Pmel17 (C and D) or anti-OA1 and anti-LAMP1 (E). (A) OA1 (PAG 15) is detected in the limiting membrane (arrows) and in small intraluminal vesicles (arrowhead) of pigmented Tyrp1 (PAG10)- positive melanosomes. (B) Note the presence of OA1 in the membrane of vesicles close to melanosomes (arrowhead). (C and D) Pmel17 (PAG 5)-positive compartments (unpigmented fibrillar melanosomes) show high labeling for OA1 (PAG 10) (arrows fig 1C,D). (E) OA1 (PAG10) localizes to the limiting membrane of LAMP1 (PAG15) positive lysosomes (arrow) and to the membrane of internal vesicles of late endosomes (arrowheads). I, stage I. II, stage II. III, stage III. IV, stage IV. I/II, stage I/II. LE, late endosome. Lys, lysosome. Bars, 200 nm. (F) Quantification of gold particles labeling OA1 on ultrathin cryosections of MNT-1 cells. Results are presented as the percentage of the total number of gold particles for OA1 (PAG 10) in the distinct morphologically defined compartments. The highest labeling for OA1 is observed in stage II premelanosomes (24%).

Figure 2.

Inactivation of OA1 affects melanosome number, melanosome size and composition. MNT-1 cells were transfected with control (Ctrl) non-targeting siRNA or 3 different siRNAs targeting OA1 (siRNA#1,#2,#3) (A) Cell harvested five days after siRNA transfection were analyzed by real time qPCR. Real time qPCR reactions were performed in triplicate and averaged. Y-axis: $2^{-(dCT)}$ value represents differences between the mean Ct (cycle threshold) values of tested genes (OA1) and those of reference gene (S26). $2^{-(dCT)}$ values are significantly lower in MNT-1 cells interfered with oligo siOA1#3 and siOA1#2 (reduction of OA1 mRNA levels: 15% siRNA #1, 78% siRNA #2, 95% siRNA #3) than in the control. (B) Cells treated with OA1 or control siRNAs were analyzed by immunoblotting with the polyclonal anti-OA1 antibody and anti β -tubulin. OA1 appears as a doublet of 45-48kDa and a band at 60kDa corresponding to different patterns of glycosylation. The extra band at 65kDa (asterisk) is a non-specific. OA1 protein levels are significantly reduced in cells interfered with siOA1#3 and siOA1#2. (C,D) OA1 distribution was analyzed by

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3 immunofluorescence, using the polyclonal anti-OA1 antibody in control and in siOA1
4 treated cells (siOA1#2 or siOA1#3). OA1 expression is clearly reduced in most
5 siOA1 treated cells. (E,F) Bright field images show the reduction of pigmentation in
6 siOA1 treated cells, as compared to control cells. (G) Evaluation of melanin content
7 in OA1 depleted cells shows a 50% decrease and as shown in Inset the decrease in
8 pigmentation is visible in cell pellets (same number of OA1 depleted and control
9 cells diluted in the same volume). Y-axis: Percentage (%) of Melanin content
10 normalized to the control (assumed as 1%). (H,I) Cells treated with OA1 or control
11 siRNAs were analyzed by conventional EM. Note the different stages of melanosomes
12 in control cells (I,II,III,IV) (H) OA1 depleted cells display only few pigmented,
13 normal sized melanosomes (IV) and show several "enlarged" organelles with
14 different morphological features: melanosomal structures with disorganized fibrils
15 (arrowhead), with melanin deposits (arrows) and aberrant mixed compartments
16 (asterisk) containing fibrillar striations and melanin pigment (I). (L) Histogram
17 representing the number of melanosomes (stage II+III+IV) per area ($160\mu\text{m}^2$) in
18 control and siOA1 treated cells. Bars, 200 nm. (K) Number of different stage
19 melanosomes in control and siOA1 cells. In siOA1 cells melanosomes were
20 distinguished between normal (organized fibers, normal size), aberrant melanosomes
21 (fibrils disorganized, bigger size) and mixed melanosomes. Melanosomes were
22 counted on 10 randomly acquired micrographs at X5,000 of siOA1 and control MNT1
23 cells. The area of cell analyzed was determined by iTEM software and expressed in
24 square micrometers ($160\mu\text{m}^2$).

Figure 3.

44 **Localization of Tyrp1 and LAMP1 in OA1 depleted cells.** Ultrathin cryosections
45 of MNT-1 cells treated with control (Ctrl; A,C,E) or OA1 siRNA (siOA1; B,D,F,G,H)
46 were double immunogold labeled for Tyrp1 (PAG10) and Pmel17 (PAG5)(A,B) or
47 Tyrp1 (PAG10) and LAMP1 (PAG15)(C,D,E,F,G,H). Labeling for Tyrp1 is observed at
48 the limiting membrane of mature pigmented melanosomes (A, arrows) but not in
49 Pmel17 positive premelanosomes (arrowhead). Note a lysosome highly labeled for
50 LAMP1 (C, arrow). (B) OA1 depleted cells show aberrant premelanosomes with
51 disorganized fibrils and those are labeled for both Tyrp1 (arrows) and Pmel17
52 (arrowhead). (D-G) Note the labeling for LAMP1 on the membrane of melanosomes
53 and pigmented enlarged compartments in siOA1 treated cells as compared to control
54 (arrows). (H) LAMP1 was present also in Tyrp1 positive vacuolar structures
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3 generated in OA1 depleted cells (arrows inset). II, stage II. IV, stage IV. Bars
4 200nm. (I) Western blot analysis of lysates of cells treated with control (Ctrl) and
5 OA1 (siOA1) siRNAs using anti-Tyrp1 or anti-LAMP1 antibodies and anti- β -tubulin as
6 a loading control. In siOA1 treated cells the expression of Tyrp1 is slightly increased
7 whereas LAMP1 levels remain unchanged.
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13 **Table 1.**

14 Quantitative analysis of Tyrp1 and LAMP1 distribution in different melanosomal
15 stages in siOA1 and control cells.
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18 Tyrp1 and LAMP1 gold particles were randomly counted in a total of 80 unpigmented
19 (stage I/II) or pigmented (stage III/IV) melanosomes, in two separate experiments.
20 Data are presented as mean \pm SD. N, number of gold particles. % percentage of
21 gold particles. In siOA1 cells melanosomes were distinguished between normal
22 (organized fibers, normal size) and aberrant melanosomes (fibrils disorganized,
23 bigger size).
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31 **Figure 4.**

32 **MART-1 depletion affects Pmel17 processing and melanosome biogenesis.**

33 (A) Western blot analysis of TX-soluble fraction extracted from cells treated with
34 control (Ctrl) and OA1 (siO) siRNAs using anti-HMB45 (Aa) or pep13h (Ac) antibody
35 directed against the processed forms and the C-terminal of Pmel17 respectively and
36 anti- β -tubulin as a loading control. Note the accumulation of Malpha ($M\alpha$) fragment
37 and the reduction of MalphaC ($M\alpha C$) fragment in siOA1 lysates (Aa, arrows). TX-
38 insoluble pellets (enriched in $M\alpha$, the luminal domain fragment of Pmel17) from
39 siOA1 and control cells were re-extracted in TX lysis buffer containing 8M Urea at
40 60° and analysed by western blot using anti-HMB45 (Pmel17; $M\alpha$ and $M\alpha C$) antibody
41 (Ab). The accumulation of Malpha ($M\alpha$) fragment and the reduction of MalphaC
42 ($M\alpha C$) are maintained. (B) Western blot analysis of lysates from cells treated with
43 control (Ctrl) and MART-1 (siM) siRNAs using anti-MART-1 (Ba), anti-HMB45 (Bb) or
44 pep13h (Bc) antibody against the processed forms and the C-terminal of Pmel17
45 respectively and anti- β -tubulin as a loading control. Note the accumulation of
46 Malpha ($M\alpha$) fragment and the reduction of MalphaC ($M\alpha C$) fragment in siMART-1
47 lysates (Bb, arrows). (C,D,E,F) Ultrathin cryosections of control and siMART-1 treated
48 MNT-1 cells were single immunogold labeled for Pmel17 (HMB50; PAG 5) (C and D)
49 or double immunogold labeled for Tyrp1 (PAG 10) and Pmel17 (HMB50; PAG 5) (E)
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3 and F). MART-1 depleted cells display aberrant enlarged compartments with a mixed
4 content of Pmel17 positive fibrils and electron dense pigment (arrow in D). As shown
5 in (F) in MART-1 depleted cells, Tyrp1 localizes to Pmel17 positive unpigmented
6 melanosomal compartments. II, stage II. IV, stage IV. Bars 200 nm.
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10 11 **Figure 5.**

12 **OA1 and MART-1 co immunoprecipitate and co localize in Golgi and TGN**

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14 (A) MNT-1 cell lysates were immunoprecipitated with polyclonal anti-OA1 (IP OA1) or
15 anti rabbit IgG (IP IgG rabbit) antibody. Immunoprecipitates were fractionated by
16 SDS-PAGE and immunoblotted with anti-MART-1, Tyrp1 or OA1 antibody as indicated
17 on the right of each panel. The arrow indicates a specific band detected by anti-
18 MART-1 antibody in OA1-immunoprecipitates.
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23 (B,C,D) MNT-1 cells were analyzed by immunofluorescence and deconvolution after
24 double labeling for OA1 (B; green) and MART-1 (C; red). Overlap of the two images
25 is shown in D and the phase (blue pseudocolored melanosomes) is shown in E.
26 Magnified insets are also shown (F,G,H,I). Arrows indicate colocalization between
27 OA1 and MART-1. (J,K,L) Ultrathin cryosections of MNT-1 cells were double
28 immunogold labeled for OA1 and MART-1 and analyzed by EM. OA1 and MART-1 are
29 detected in the Golgi and in associated vesicles (J, arrows), at the cytosolic side of
30 small endosomes (K, arrow) and in multivesicular compartments (L, arrow). TGN,
31 Trans Golgi Network. IV, stage IV. Bars 200 nm.
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40 41 **Figure 6**

42 **Depletion of MART-1 affects OA1 stability.** MNT-1 cells were treated with
43 control (Ctrl) siRNA or siRNA targeting MART-1 (siMART-1). (A-H)
44 Immunofluorescence analysis of control (A,B,C,D) and siMART-1 cells (E,F,G,H)
45 stained with anti-MART-1 (red) and anti-OA1 (green) antibodies. MART-1 depleted
46 cells (arrow) show a clear decrease in OA1 labeling. Phase is shown in D and H. (I)
47 Real Time quantitative PCR of MART-1 and OA1, in siMART-1 (siMART-1) and control
48 (Ctrl) MNT1 cells. Note that MART-1 RNA is significantly decreased (94%) and OA1
49 RNA does not significantly change in siMART1 cells. Y-axis: $2^{-(\Delta\Delta Ct)}$ value represents
50 differences between the mean Ct (cycle threshold) values of tested genes and those
51 of reference gene (S26). (J) Western blot analysis of lysates of cells treated with
52 control (Ctrl) and MART-1 (siM) siRNAs using anti-MART-1 or anti-OA1 antibody and
53 anti- β -tubulin as a loading control. Note the depletion of MART-1 (90%) and the
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3 reduction of OA1 protein (85%) in cells treated with MART-1 siRNA. Arrows point to
4 the specific forms of OA1 (60 kDa and doublet 45-48 kDa). (K) Western blot analysis
5 of control (Ctrl) and siMART-1 (siM) cells, treated with Chloroquine 200 μ M (Chlor,
6 lysosome inhibitor)(Ka) or MG132 50 μ M (MG132, proteasome inhibitor)(Kb) or
7 Epoxomycin 10 μ M (Epoxy, specific proteasome inhibitor)(Kc) for 5hrs, 5 days after
8 siRNAs transfection. Control cells treated with Chloroquine show an increase in OA1
9 as compared to untreated control cells. OA1 levels increase also in siMART-1 cells
10 treated with Chloroquine, as compared to untreated siMART-1. Note also the
11 increase of OA1 in siMART-1 cells treated with MG132 and Epoxomycin as compared
12 to the untreated inactivated cells. Arrows point to the specific forms of OA1 (60 kDa
13 and doublet 45-48 kDa). Graphs shows signal intensity of OA1 normalized to β -
14 tubulin in the different conditions of two separate experiments. Data are expressed
15 as arbitrary units and presented as mean \pm SD.
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Supplementary Figures

Figure S1.

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34 **Localization of OA1-Flag in transfected MNT-1 cells.** Ultrathin cryosections of
35 MNT-1 cells transfected with OA1-Flag construct were double immunogold labeled
36 with anti-Flag (OA1) and anti-Tyrop1 (A and B) or with anti-Flag (OA1) and anti-
37 Pmel17 (C). (A and B) OA1-Flag (PAG 15) is present at the limiting membrane of
38 Tyrop1-positive (PAG 10) pigmented melanosomes (arrows, A), in small intraluminal
39 vesicles that are also labeled for Tyrop1 (arrowhead, A, B) and in intracellular small
40 vesicles closed to melanosomes (asterisk, B). Note the presence of large electron
41 lucent OA1-Flag positive membrane vacuoles (350 nm) labeled for Tyrop1 that localize
42 at the proximity of melanosomes (asterisks, A). (C). OA1-Flag (PAG15) localizes to
43 stage IV pigmented mature melanosomes (arrows) while stage II unpigmented
44 premelanosomes do not show consistent labeling. Bars, 200 nm. (D) Quantitative
45 analysis of the distribution of OA1-Flag in MNT-1 transfected cells. Results are
46 presented as the percentage of the total number of counted gold particles for OA1
47 (PAG 15) in the distinct morphologically defined compartments in MNT-1 cells. The
48 highest labeling for OA1 is observed in stage IV premelanosomes (23%).
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Figure S2.

Aberrant melanosomes are generated in OA1 depleted MNT1 and in OA1 knock out mouse melanocytes. OA1 siRNA targeted or not targeted human MNT1 (siOA1; A,B,C) and OA1 Knock-out (melan-OA1^{-/-}) or wild type (melan-a) mouse melanocytes (D,E) were analyzed by conventional EM (A,B,D,E) or after double immunogold labeling for Tyrp1 (PAG 15) and Pmel17 (PAG 5) on ultrathin cryosections (C). (A) Electron micrograph showing a siOA1 targeted cell (defined on the base of the typical phenotype) close to a siOA1 not targeted cell. Melanosomes in the siOA1 targeted cell display disorganized luminal fibrils and are 3 times bigger than in the not targeted cell (arrows). I, stage I. II, stage II. III, stage III. IV, stage IV. Bar: 1 μ m. (B) OA1 depleted cells show aberrant melanosome displaying electron dense pigment surrounded by fibrils characteristic of immature premelanosomes. These fibrils are labeled for Pmel17 (arrows, C). Bars 200 nm. (D,E) Electron micrographs of regions of the cytoplasm of OA1 Knockout (melan-Oa1^{-/-}; E) and wild type (melan-a; D) melanocytes. Arrows indicate aberrant pigmented compartments with a mixed content in OA1 Knockout cells. Insets are 2,5X magnification of areas showing representative melanosomes. II, stage II. III, stage III. IV, stage IV. Bars 200nm.

Figure S3

(A,B) Control (Ctrl) or OA1 siRNA (siOA1) treated MNT1 cells were pulsed with BSAG (5nm) for 10 min and the endocytic tracer was chased at 37°C for 45 min. BSAG is present in MVBs (arrowheads, A-B) located close to melanosomes labeled for Tyrp1 (arrows, A-B). The enlarged melanosomal organelles in siOA1 cells were not accessed by the BSAG (B). (C,D) Control (Ctrl) and OA1 depleted (siOA1) MNT1 cells were incubated with DAMP for 15 min at 37°C and then fixed, processed and double immunogold labeled for Tyrp1 (PAG10) and DAMP, visualized with anti-DNP antibody (PAG 5). The weak base DAMP accumulates primarily in stage II melanosomes while stage IV melanosomes show less labeling. The enlarged melanosomal organelles (arrow, D) in siOA1 cells appear to be correctly acidified. III, stage III. II/III, stage II/III. IV, stage IV. Bars 200 nm.

Figure S4

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3 (A) Real Time quantitative PCR of OA1, Pmel17 and MART-1 and in siOA1 treated
4 (siOA1) and control (Ctrl) MNT1 cells. Y-axis: $2^{(-\Delta\text{CT})}$ value represents differences
5 between the mean Ct (cycle threshold) values of tested genes and those of reference
6 gene (S26). Pmel17 and MART-1 RNA levels do not change in siOA1 cells. (B)
7 Western blot analysis of lysates of cells treated with control (Ctrl) and OA1 (siOA1)
8 siRNAs using anti-MART-1 and anti- β -tubulin as a loading control. MART-1 does not
9 significantly change in siOA1 treated cells vs control.
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For Peer Review

Table 1

		Tyrp1			
		stage I/ II	stage I/ II	stage III/IV	stage III/IV
		n	%	n	%
	Ctrl	36 ±5	8%	448 ±19	92%
	siOA1 (normal melanosomes)	206 ±11	27%	554 ±22	73%
	siOA1 (aberrant melanosomes)	471 ±17	36%	833 ±30	64%

		LAMP1			
		stage I/ II	stage I/ II	stage III/IV	stage III/IV
		n	%	n	%
	Ctrl	16 ±4	25%	48 ± 7	75%
	siOA1 (normal melanosomes)	33 ±8	29%	79 ±12	71%
	siOA1 (aberrant melanosomes)	100 ±14	35%	186 ±20	65%

For Peer Review

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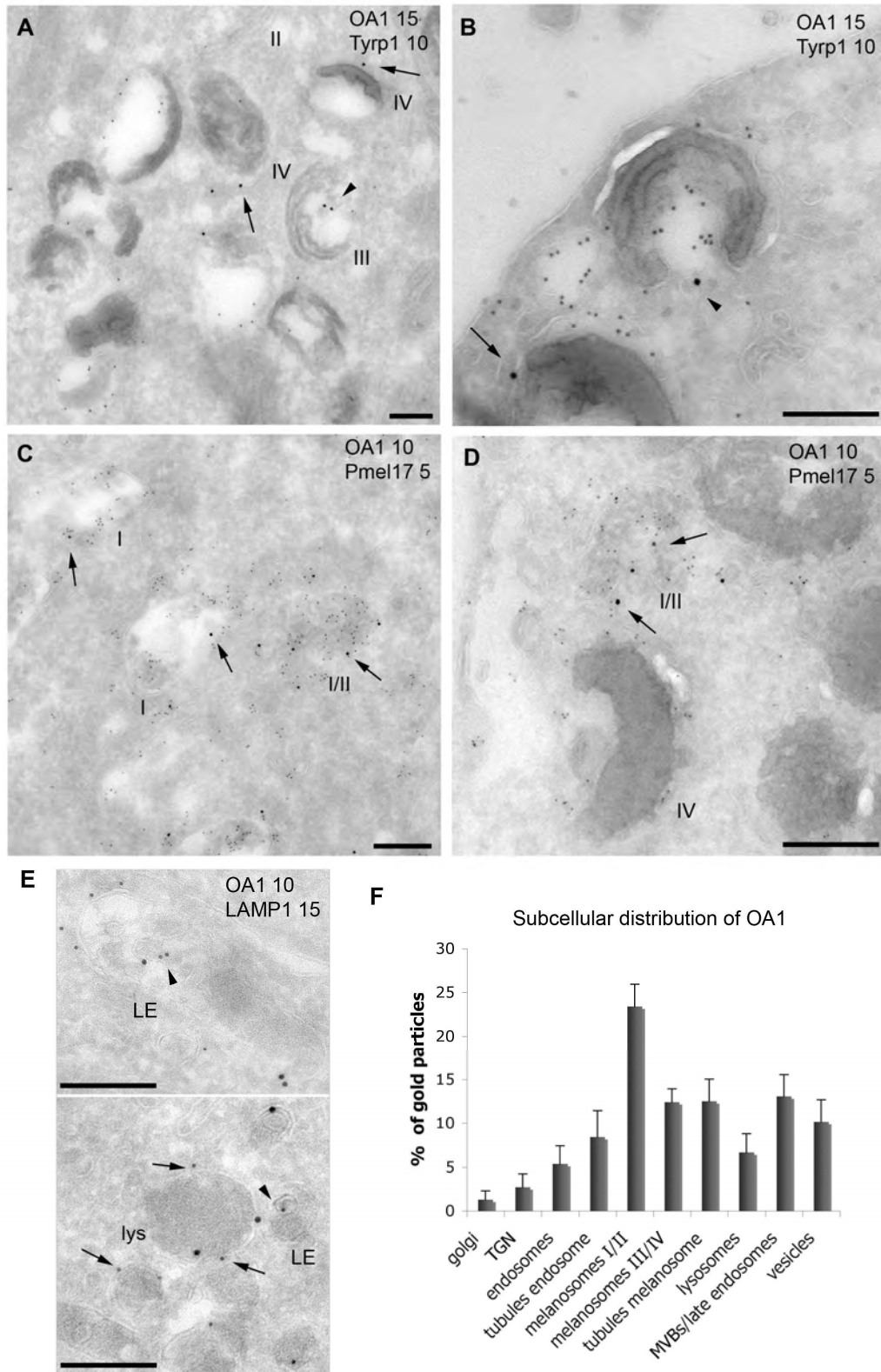


Figure 1

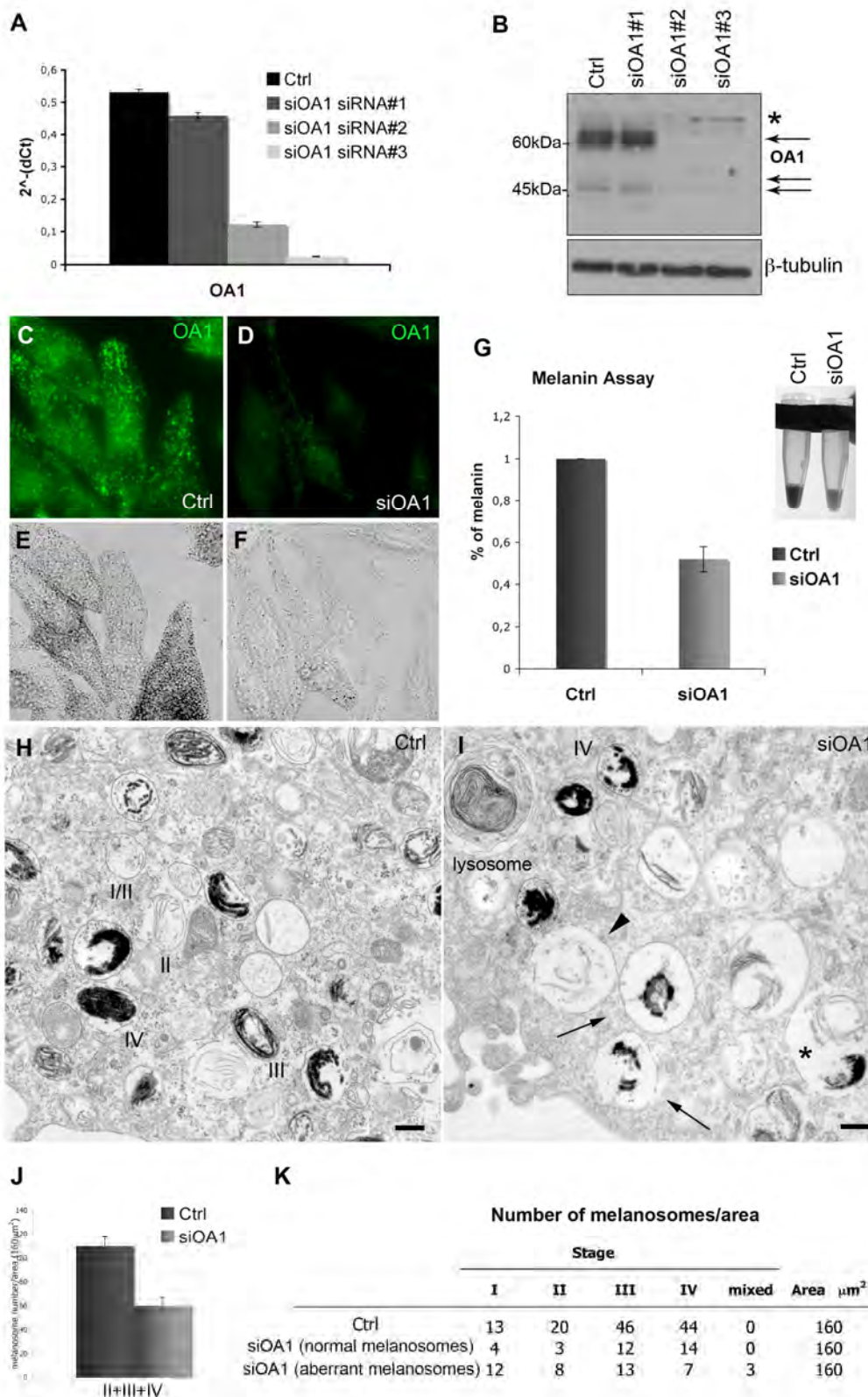


Figure 2

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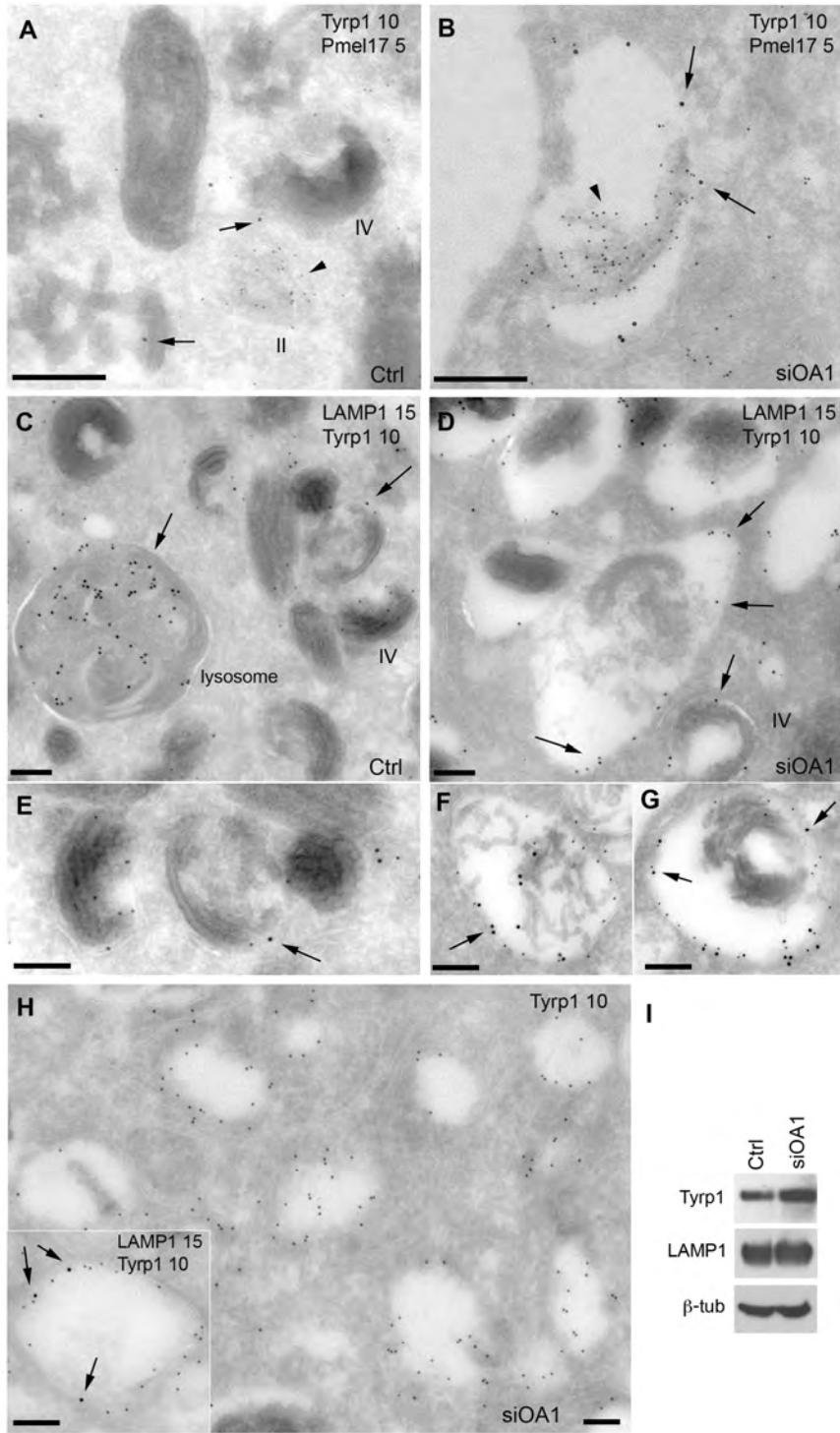


Figure 3

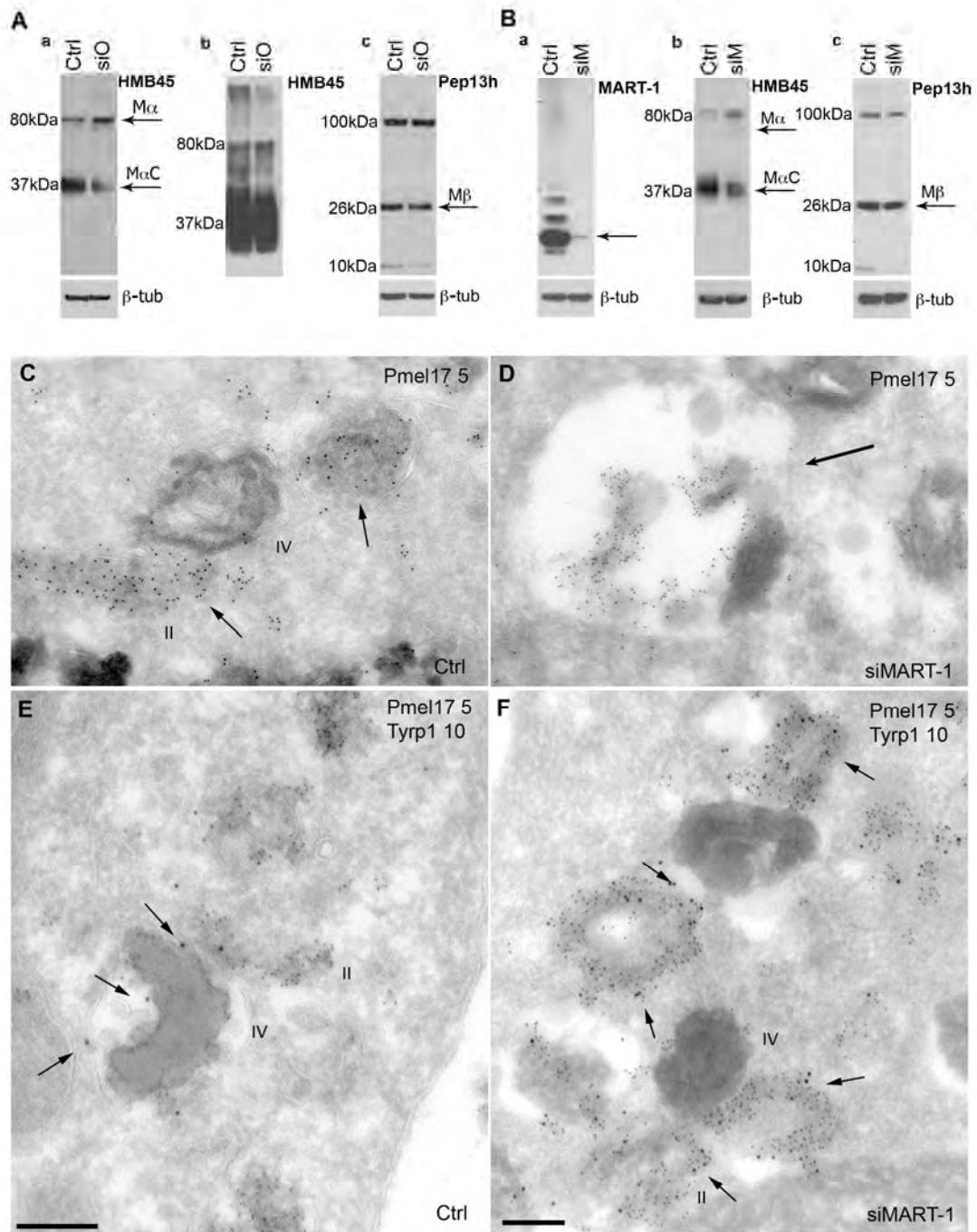


Figure 4

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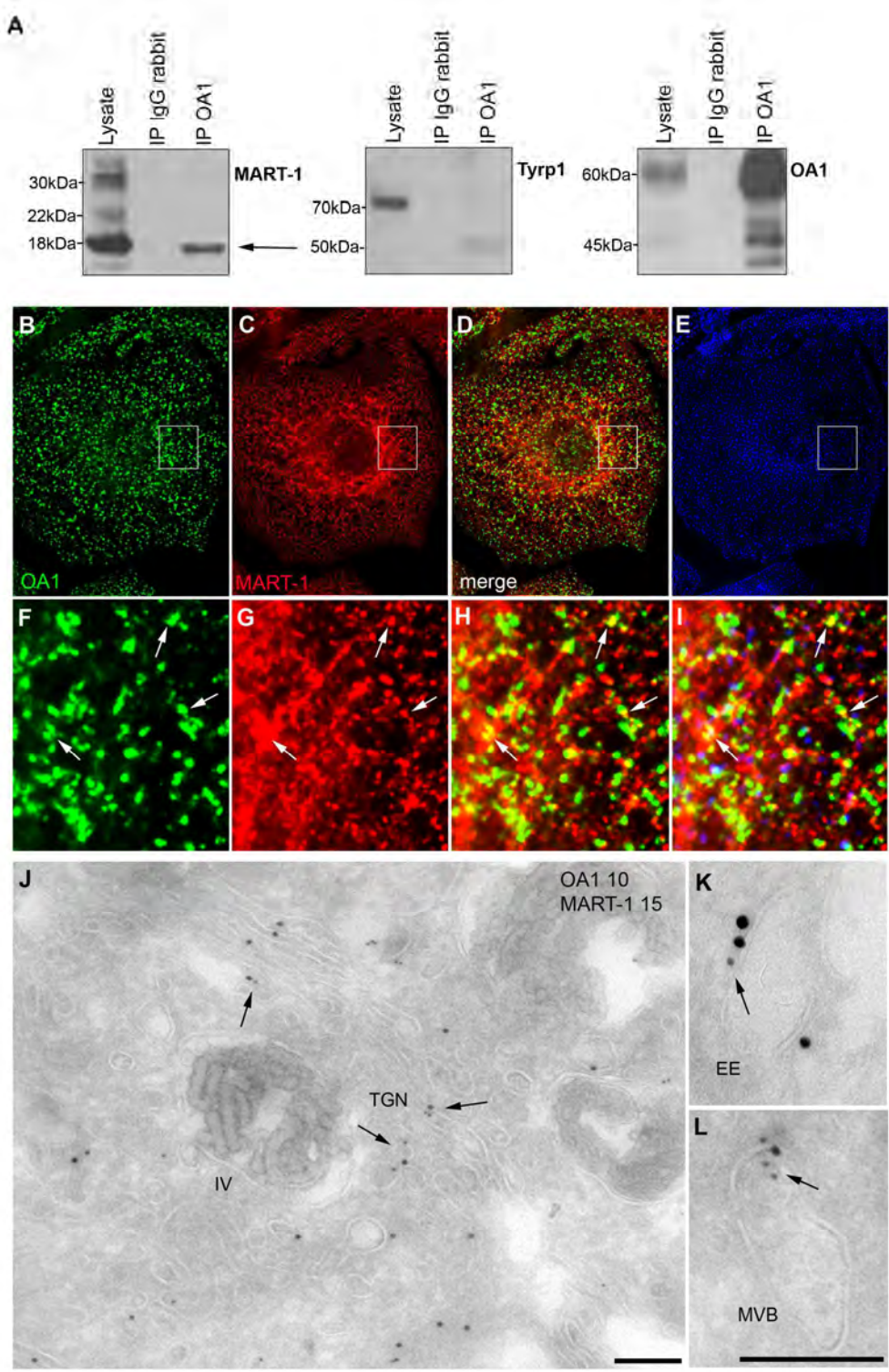


Figure 5

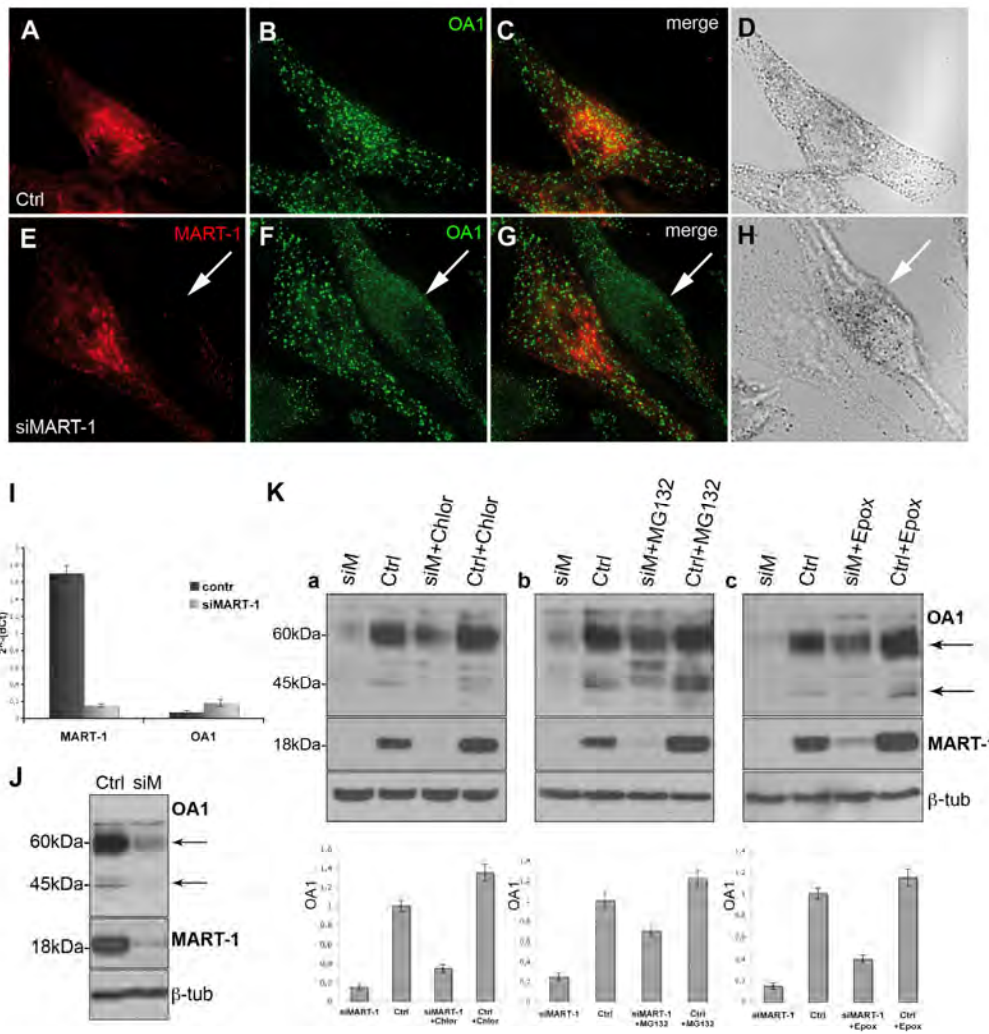
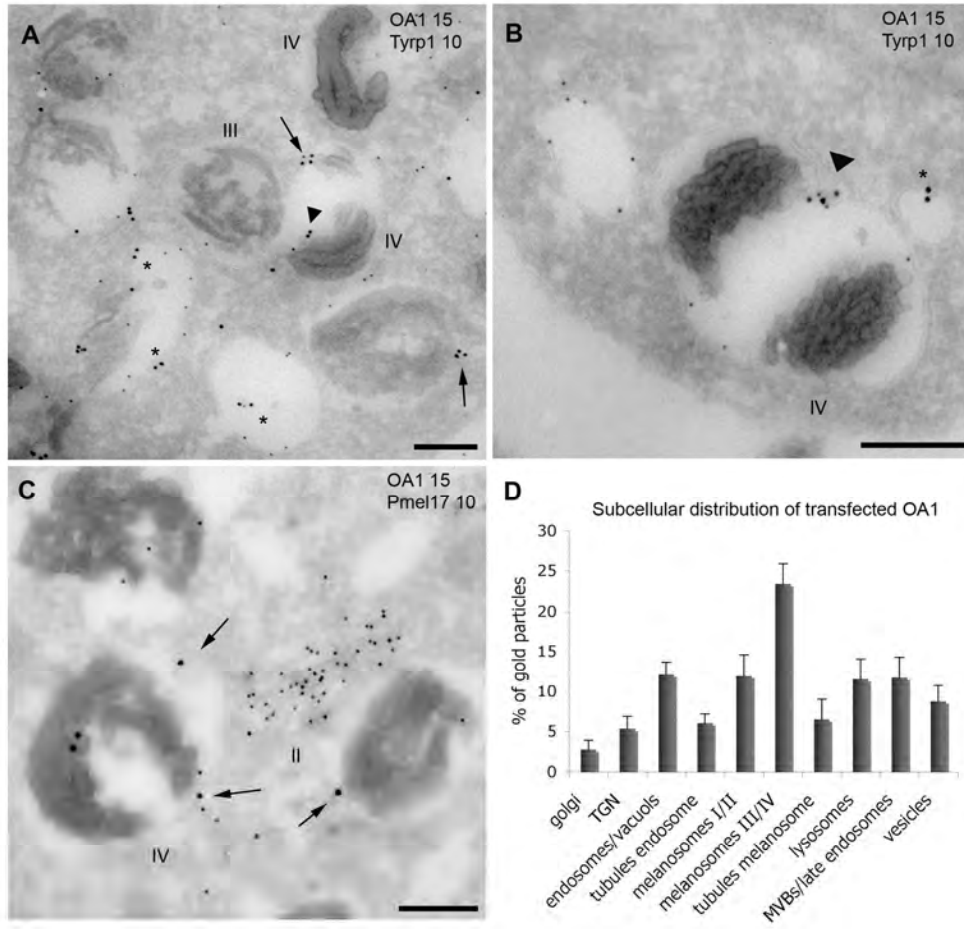


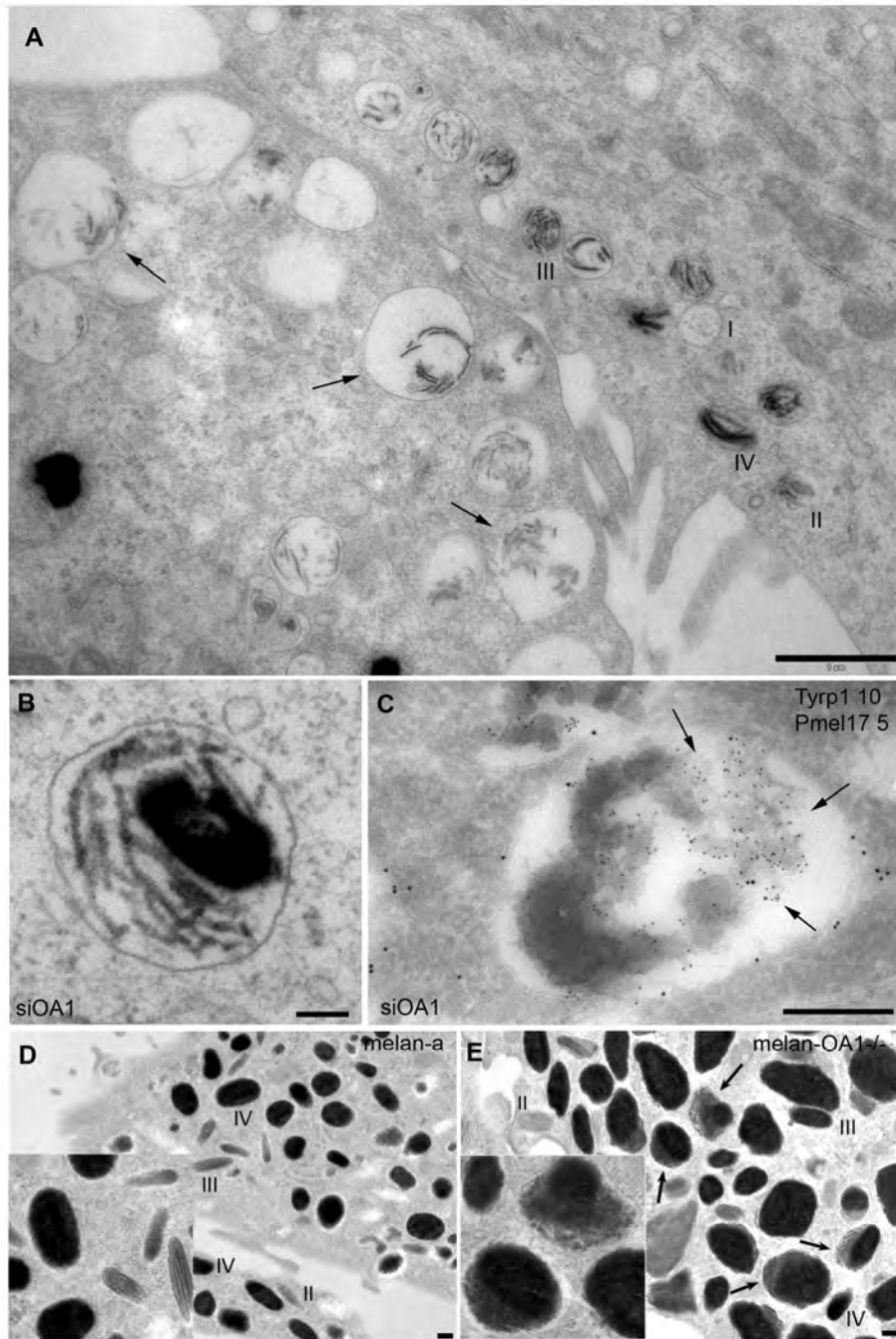
Figure 6

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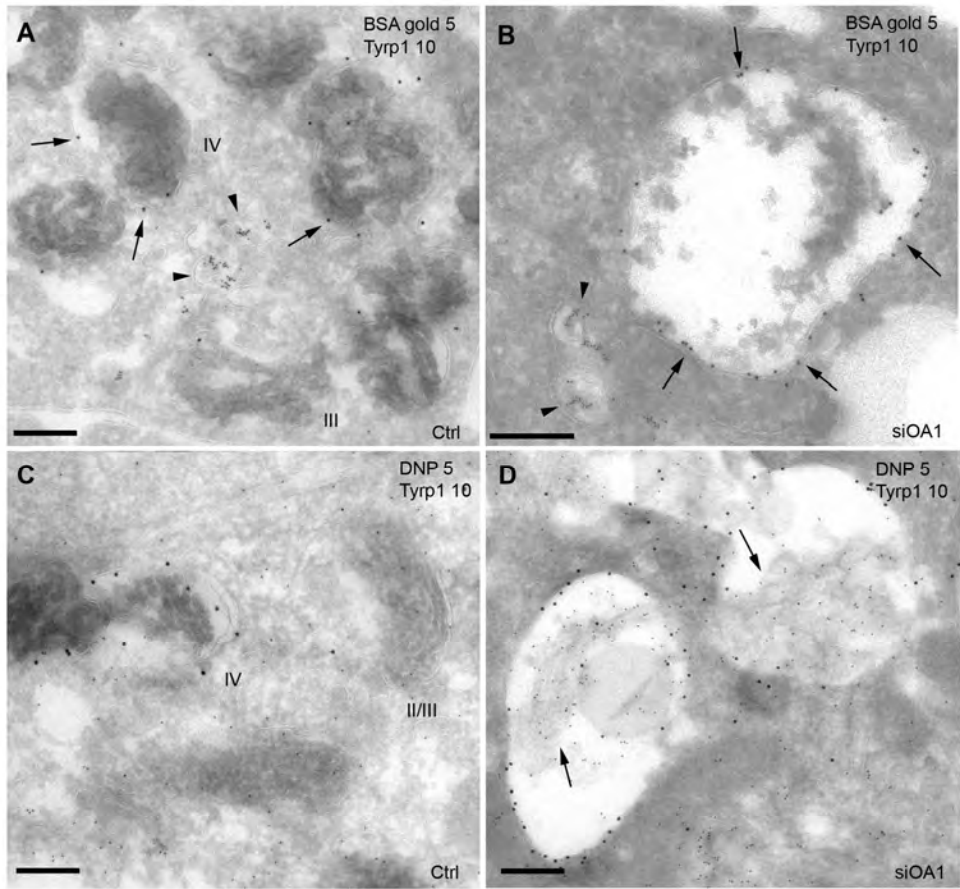
Supplementary Figure 1



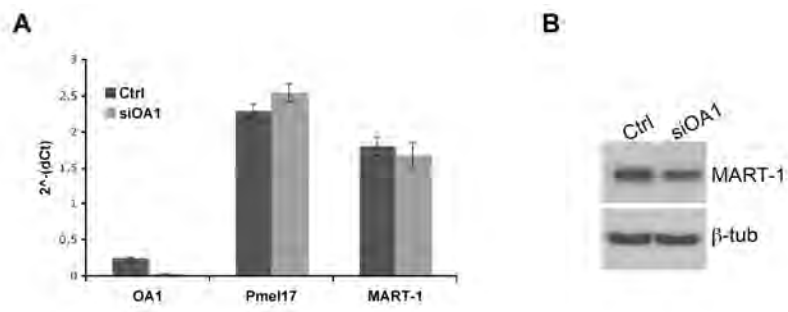
Supplementary Figure 2

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Supplementary Figure 3



Supplementary Figure 4