The Ocular Albinism Type 1 (OA1) Gene Controls Melanosome Maturation and Size

Katia Cortese,1,2 Francesca Giordano,2,3 Enrico M. Surace,3 Consuelo Venturi,1 Andrea Ballabio,1,4 Carlo Tacchetti,1 and Valeria Marigo3

PURPOSE. The authors took advantage of the Oa1 mutant mouse in combination with other albinism mouse models (i.e., Tyrosinase and Membrane-associated transporter protein [Matp]) to study the function of Oa1, the gene mutated in ocular albinism type 1, in the RPE during development and after birth.

METHODS. Enzyme activity and protein localization were analyzed by immunohistochemistry of tyrosinase (Tyr) in Oa1-null mice. Ultrastructural analysis and morphometry were performed by electron microscopy, of the RPE in Oa1-knockout mouse and double-mutant mice of Oa1 with either Tyr or Matp.

RESULTS. Differently from other albinism models, Tyr activity was not impaired in Oa11/1 eyes. Hyopigmentation of the RPE in Oa11/1 mice is due to a reduced number of melanosomes. Analysis of Oa11/1; Tyr<sup>−/−</sup>/Tyr<sup>−/−</sup> and Oa11/1;Matpuw<sup>−/−</sup>/Matpuw<sup>−/−</sup> double-knockout mice, which display a block at stages II and III of melanosome maturation, respectively, revealed that Oa1 controls the rate of melanosome biogenesis at early stages of the organellogenesis, whereas the control on the organelle size is exerted at the final stage of melanosome development (stage IV).

CONCLUSIONS. The findings indicate that Oa1 is involved in the regulation of melanosome maturation at two steps. Acting at early maturation stages, Oa1 controls the abundance of melanosomes in RPE cells. At later stages, Oa1 has a function in the maintenance of a correct melanosomal size. This study helps to define ocular albinism type 1 as a defect in melanosome organellogenesis and not in melanin production. (Invest Ophthalmol Vis Sci. 2005;46:4358 – 4364) DOI: 10.1167/iovs.05-0834

4358

The X-linked form of albinism (ocular albinism type 1) in humans is due to mutations in the ocular albinism type 1 (OA1, recently renamed GPR143) gene. Although skin pigmentation appears normal, patients manifest the characteristic defects of albinism in the eye, such as translucent iris, photophobia, foveal hypoplasia, nystagmus, and miswiring of the optic tract. Studies conducted in the mouse showed that Oa1 is specifically expressed in pigment cells of skin and eye. OA1 protein localizes on the membrane of mature melanosomes and in the endolysosome–premelanosome compartment. The protein has homology with members of the G protein-coupled receptor (GPCR) superfamily and binds heterotrimeric G proteins (Gi3); however, a ligand has not been identified yet. The Oa1-null mouse was generated by knocking out the first exon of the mouse gene. As observed in humans, Oa1 loss of function causes the formation of abnormally giant melanosomes (macromelanosomes). Macromelanosomes were detected in the retinal pigment epithelium (RPE) of these mice after birth. Electron microscopy demonstrated that macromelanosomes contain a central core region within the electron-dense melanin, which closely resembled the structure of a normal membrane-free melanosome. This result suggested a mechanism of macromelanosome formation based on overgrowth of a single melanosome rather than fusion of several melanosomes. Nevertheless, the function of Oa1 during melanosome maturation is still obscure. Furthermore, the correlation between macromelanosome and the hypopigmentation of the RPE that causes the albinism phenotype in patients is unclear.

Melanosomes are organelles specialized in the biosynthesis and storage of melanin, in melanocytes of skin and ear, and in RPE cells. Melanosome maturation occurs through sequential maturation stages (I–IV) that can be defined based on morphology. Stage I premelanosomes lack pigment and contain internal membranous vesicles. Stage II melanosomes are elongated in shape, lack pigmentation and contain intraluminal matrix fibers organized in a striated array. Deposition of black melanin along the matrix fibers begins in stage III melanosomes. Finally, stage IV melanosomes are characterized by a dense homogeneous deposit of melanin that covers all the internal structures of the matrix. Melanin is synthesized from the amino acid tyrosine. In the initial step, tyrosine is hydroxylated to DOPA, which is in turn oxidized to dopaquinone. Both steps are catalyzed by the enzyme tyrosinase (Tyr). Then dopaquinone is converted into melanin.

There is no evidence of differences in melanosome organellogenesis between RPE and skin melanocytes. However, in contrast to skin melanocytes that transfer their melanosomes to surrounding keratinocytes in the epidermis, ocular melanosomes are retained. Melanization in RPE cells apparently occurs only for a limited period after initial differentiation, and then they permanently halt melanin synthesis.

Mouse mutants have been of great value for the elucidation of the pigmentation process. The albino (c) mouse is a spon-
taneous mutant with a mutation in the Tyr gene, causing loss of tyrosinase enzyme activity and lack of pigmentation. Recently, the molecular basis of the underwhite (uw) mouse has been defined.12 The affected gene encodes the membrane-associated transporter protein (Matp). An in vitro study on Matp mutant melanocytes suggested that this mutation causes abnormal processing and intracellular trafficking of Tyr to the melanosome.13 These two spontaneous albino mutants show different phenotypes in the RPE. The Tyr mouse completely lacks melanin, and melanosomes do not develop beyond stage II. Ultrastructural studies of different Matp alleles suggested that the defect was at the level of the melanosome showing irregular shape, reduced size, and maturation impairment.14 Therefore, these two mouse models provided us with genetic tools to study Oa1 function, either in the absence of melanin production or in impaired melanosomal maturation conditions.

In this study, we addressed the function of Oa1 in pigment cells in vivo, to define better why mutations in Oa1 cause albinism. We generated double-mutant mice of Oa1 and either Tyr<sup>−/−</sup> or Matp<sup>−/−</sup> and analyzed melanosome phenotype in the absence of melanin synthesis and impaired melanosome maturation. The phenotype of the double-mutant mice compared with single mutants led us to the hypothesis that Oa1 performs two functions in melanogenesis. In particular, Oa1 loss of function causes a reduction in the number of melanosomes in the RPE, suggesting an Oa1 control on the rate of melanosome biogenesis. In addition, double mutant phenotype analysis unraveled a size-control function of Oa1 on the final maturation stage of melanosomes (stage IV).

**Material and Methods**

**Mouse Breeding**

The use of animals in this work was in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were bred and maintained in a 12-hour light–dark cycle. Oa1<sup>−/−</sup> mice were bred on a C57BL/6 genetic background. Tyr<sup>−/−</sup> and Matp<sup>−/−</sup> mice, both on a C57BL/6 background, were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained inbred in the animal house. The Tyr<sup>−/−</sup> mouse is due to a 7-hp deletion in exon 3 resulting in a 43 amino acid frameshift and premature stop codon, which predicts that the Matp protein lacks the C-terminal six-transmembrane domains.16 To generate Oa1<sup>−/−*/Tyr</sup><sup>−/−</sup>/Matp<sup>−/−</sup> and Oa1<sup>−/−*/Matp</sup><sup>−/−</sup>/Matp<sup>−/−</sup> mice, single homozygous mutant mice were crossed, and the resultant F<sub>1</sub> double-heterozygous progenies were intercrossed. F<sub>2</sub> progeny was scored for coat color (Tyr<sup>−/−</sup>/Tyr<sup>−/−</sup>, Matp<sup>−/−</sup>/Matp<sup>−/−</sup>) and by diagnostic PCR on genomic DNA obtained from the tail for Oa1<sup>−/−</sup>.14

**Tyrosinase Activity Assay**

Eyes were dissected from killed littersmates (P<sub>7</sub>) of different genotypes and frozen at −70°C. Each mouse was genotyped and at least four eyes for each genotype were used for the assay. Each pair of eyes was homogenized on ice in 10% glucose in PBS with 50 μg/mL leupeptin, 20 μg/mL aprotinin, and 5 mM benzamide. Protein concentration was determined using the Bradford method (Bio-Rad, Hercules, CA). Equal quantities of protein were used in each reaction. After the addition of tritiated tyrosine (GE Healthcare, Arlington Heights, IL) to the reaction, Tyr activity was assayed radiometrically by measuring production of tritiated water from hydrolysis of tyrosine, as reported in a published protocol.17 Activity was expressed as counts per minute per microgram of total protein.

**Quantitative RT-PCR**

Eyes were dissected from either wildtype or Oa1<sup>−/−</sup>null mice at birth (P0), and total RNA was extracted (TRIzol reagent; Invitrogen-Life Technologies, Gaithersburg, MD), according to the manufacturer’s instructions. cDNAs were synthesized (Superscript II; Invitrogen, Carlsbad, CA) and random primers and then amplified (SYBR Green PCR Master Mix; Applied Biosystems, Inc. [ABI], Foster City, CA) with the forward (f) and reverse (r) intron-spanning primers: GAPDHf (GTATGACCTCCTGACGGCAAA) and GAPDHz (TTCCCCATTCTGCGCCTTTG) for glyceraldehyde-3-phosphate; Tyri (CAAGGATCTGGATATGATGATGAC) and TyRr (TGGATATGTTAGATGATGATGATGAC) for Tyri; and Matpf (CCTCCTACATGCCCCTCCCT) and Matpr (CCTCCTATGCCCCTCCCT) for Matp. Quantitative PCR reactions were run in triplicate for each gene (model 7000; ABI). Cyclical reaction values were recorded and preprocessed with the accompanying software (ABI). Data were then normalized to the internal control and statistically analyzed (t-test).

**RPE Cell Culture**

Primary retinal pigment epithelium cells (RPE) and choroidal melanocytes were isolated from P1 eyes of wildtype, Oa1<sup>−/−</sup>, Tyr<sup>−/−</sup> and Matp<sup>−/−</sup> mice.

After a brief (30-minute) treatment with 2 mg/mL Dispase II (Roche, Indianapolis, IN) in Hanks’ balanced salt solution (Invitrogen) choroid and RPE were gently separated from neural retina, lens, and vitreous and incubated in 2 mL of 0.5 mg/mL trypsin (Sigma-Aldrich, St. Louis, MO) in Hanks’ at 37°C for 30 minutes.

After adding 1 volume of 0.2 mg/mL trypsin inhibitor (Roche) in DMEM (Invitrogen), the tissue was triturated 50 times with a borohole glass pipette. The single-cell suspension was resuspended in 1 mL of DMEM-Ham’s F-12 (1:1) (Invitrogen) containing 5% FBS and N2 hormone mix (Invitrogen) and plated on extracellular matrix (ECM) gel (Sigma) at 37°C in a 5% CO<sub>2</sub> atmosphere. Cells were cultured for 4 to 5 days and not propagated in culture. Cell identity was defined by pigmentation and by expression of Tyr, Trpl, and Trp<sub>2</sub> for RPE and choroidal melanocytes and by Crabl for RPE cells.21

**Immunofluorescence**

Cells were fixed in 4% paraformaldehyde/PBS, permeabilized with 0.1% saponin in 10% FBS/PBS and incubated with polyclonal antibodies anti-Oa1, raised against the C terminus (last 14 amino acids) of murine Oa1, anti-Tyr<sup>−/−</sup> anti-Crabl<sup>−/−</sup> in 0.1% saponin, 1% FBS/PBS. Alexa Fluor goat anti-rabbit was used as the secondary antibody (Molecular Probes, Eugene, OR). Immunofluorescence was visualized with a laser confocal microscope system (Leica, Heidelberg, Germany).

**Ultrastructural Analysis**

P7 and 3-month-old mice of different genotypes were anesthetized and perfused with 4% paraformaldehyde and 2% glutaraldehyde in PBS. Eyes were removed and placed in 0.1 M cacodylate buffer, containing 2.5% glutaraldehyde, for 4 hours at room temperature. Eyes were postfixed in osmium tetroxide for 1 hour and uranyl acetate another hour. Subsequently, samples were dehydrated through a graded ethanol series and propylene oxide and embedded in resin (Poly-Bed; Polysciences, Inc., Warrington, PA) overnight at 4°C and 2 days at 60°C. A dorsoventral orientation was maintained during embedding. Semithin sectioning (0.5 μm) was started at the frontal pole of the embedded eye (corresponding to the cornea) and ultrathin sections (50 nm) were collected, starting when the RPE was observed. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with an electron microscope (model CM10 or Model G2 Tecnai; Philips, Eindhoven, The Netherlands). We analyzed comparable regions of the different genotypes.

The number of melanosomes and their maturation stages in the RPE of single- and double-mutant mice were analyzed on 10 random micrographs at ×21,000, either at the apical zone or the basal zone of the
epithelium. The basal zone was defined by the presence of basal lamina, the apical zone by the presence of microvilli, and the limit between the two regions was drawn at half distance between the apical and basal surfaces. Diameters were measured on melanosomes at different stages of maturation (II, III and IV) in each micrograph (Photoshop 7.0 software; Adobe Systems, Mountain View, CA). For statistical analysis, we applied unpaired t-test assuming equal variances.

**RESULTS**

**Effect of Oa1 Loss of Function on Tyr activity and the Rate of Melanosome Biogenesis in the RPE**

The albino phenotype in patients with ocular albinism type 1 is often correlated with macromelanosome formation; however, hypopigmentation of the ocular fundus could not simply be explained by giant melanosomes. In fact, melanin appears to be synthesized in the absence of Oa1, as suggested by pigmentation of skin and hair in patients. To quantify this observation, we measured activity of Tyr in eye protein extracts from the Oa1 mutant, heterozygous, and wild-type mice 7 days after birth (P7) and found no significant difference among the different genotypes (Fig. 1). Therefore, in contrast to other albinism models,22 lack of Oa1 does not impair Tyr activity in the eye. It is otherwise possible that albinism in Oa1 is due to a decreased abundance of melanosomes in the RPE. In one of our previous studies, based on light microscopy analysis, we failed to detect differences in pigment granule number in the RPE of Oa1-knockout mice.23 We therefore chose a more sensitive technique, such as morphometric studies by electron microscopy. We evaluated density (number of organelles per square micrometer) and distribution of melanosomes in the RPE of wild-type and Oa1 mutant mice at different developmental and postnatal stages. At embryonic day (E)15.5, newborn (P0), and later postnatal stages (P7 and 3 months), the melanosome population in wild-type RPE comprised mostly mature stage-IV melanosomes (Table 1). However, a sizable fraction of the melanosomes was still at stage II at E15.5 (31%), and at stage III at P0 (14%). The total number of melanosomes increased progressively and reached a plateau at P7 (Table 1). The changes in the total number of melanosomes reflected almost exclusively the modifications in the stage-IV population, probably due to the relative paucity of stages II and III at P0, P7, and 3 months (Table 1).

![Figure 1. Tyrosinase activity assay on eyes at the age of 7 days after birth. ANOVA confirmed no significant difference in the enzymatic activity among wild-type (wt, n = 9), Oa1 heterozygous (Oa1+/−, n = 9), and homozygous (Oa1−/−, n = 6) mice. Tyrosinase activity in albino control CD1 mice was significantly different from that in wild-type (t-test P < 0.001). Data are shown as the mean divided by the blanc (CD1 mice that have no Tyr activity have a value close to 1, therefore activity equal to blanc).](https://iovs.arvojournals.org/doi/pdf/10.1167/iovs.05-2630)

**Table 1. Number of Melanosomes in the RPE of Wild-Type and Oa1−/− Mice at the Different Developmental Stages**

<table>
<thead>
<tr>
<th>Stage</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>Area μm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E15.5</td>
<td>9</td>
<td>1</td>
<td>18</td>
<td>186</td>
</tr>
<tr>
<td>P0</td>
<td>3</td>
<td>13</td>
<td>75</td>
<td>186</td>
</tr>
<tr>
<td>P7</td>
<td>0</td>
<td>4</td>
<td>132</td>
<td>186</td>
</tr>
<tr>
<td>3 months</td>
<td>9</td>
<td>2</td>
<td>136</td>
<td>186</td>
</tr>
<tr>
<td>Oa1−/−</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E15.5</td>
<td>2</td>
<td>2</td>
<td>17</td>
<td>186</td>
</tr>
<tr>
<td>P0</td>
<td>10</td>
<td>8</td>
<td>37</td>
<td>186</td>
</tr>
<tr>
<td>P7</td>
<td>0</td>
<td>4</td>
<td>63</td>
<td>186</td>
</tr>
<tr>
<td>3 months</td>
<td>0</td>
<td>5</td>
<td>58</td>
<td>186</td>
</tr>
</tbody>
</table>

The total area of the cell analyzed (area) was determined by the point-intersection method and expressed in square micrometers.

In Oa1 mutant RPE we found that the melanosome population, similar to wild-type RPE, comprised mostly stage IV melanosomes (Table 1), which included also macromelanosomes at P0, P7, and 3 months. Similar to the phenotype described in patients,25 only a fraction of melanosomes was enlarged, and we calculated that approximately 20% of melanosomes in the RPE displayed the macromelanosome phenotype (>1.5 μm; Table 2). Of note, when compared with wild-type RPE, the total number of melanosomes was lower, showing 25% reduction at E15.5 (P < 0.05), 40% at P0, 50% at P7, and 57% at 3 months (P = 0.01, Table 1). A more detailed analysis of the relative changes among different stages of melanosomes revealed that the reduction was mostly attributable to the stage-IV population at P0, P7, and 3 months, whereas at E15.5, it was due to changes in immature stage II melanosomes (Table 1). With respect to the cellular distribution of melanosomes, Oa1 mutants did not show differences when compared with wild-type RPE (data not shown).

The reduced number of stage-IV melanosomes was not accompanied by the appearance of autophagosomes, suggesting that the event was probably due to a reduced rate of melanosome biogenesis, rather than degradation of already formed organelles.

**Effect of Oa1−/−, Tyr−/−, and Matp−/− Mutations on Each Other and Cellular Distribution**

Our analysis of the Oa1 mutant RPE suggested that Oa1 is involved in controlling both the rate of melanosome biogenesis and the size of stage-IV melanosomes. To define which stage of melanosome maturation was the target of Oa1 activity, we generated double-mutant mice of Oa1 with two other albinism mouse models (i.e., Tyr−/− and Matp−/−) in which melanosomes are unable to undergo full maturation. Although in Tyr−/− mice melanin is not synthesized and melanosomes stop their maturation at stage II (Fig. 4B), in Matp−/− mice, the mutation causes a block of maturation at stage III (Ref. 24 and Fig. 4B). The double mutant strategy would have only been successful if each pair of selected gene mutations did not interfere with their reciprocal expression and/or distribution. Therefore, before generating double-mutant mice we analyzed expression and distribution of Oa1 protein in Tyr−/− and Matp−/− mutants. To this end, we generated primary cultures of pigment cells dissected from wild-type, Oa1−/−, Tyr−/−, and Matp−/− neonatal eyes. These cultures contained both RPE and choroidal cells as defined by immunodetection of pigment cell markers (i.e., Tyr, Tyrp1, and Tyrp2, data not shown). To discriminate between the two cell populations, we performed...
immunofluorescence studies using antibodies to Cralbp, a specific marker for RPE cells and confirmed that cells with elliptic big pigment granules were RPE cells (Fig. 2, inset). As expected, labeling with an Oa1-specific antibody demonstrated colocalization of Oa1 with pigmented granules in wild-type cell cultures, whereas no signal was detected in Oa1−/− cells. Oa1 was normally expressed and distributed in Tyrc-2J and Matpuw mutant RPE and choroidal melanocytes (Fig. 2).

We then analyzed expression of Tyr and Matp mRNA in Oa1 mutant eyes at birth by quantitative real-time PCR. Tyr and Matp expression was not impaired in the Oa1−/− genotype compared with the wild-type (Fig. 3A, 3B). Finally, we analyzed Tyr distribution in RPE and choroidal melanocyte primary cultures derived from Oa1−/− neonatal eyes. We found that Oa1 loss of function did not affect Tyr distribution (Figs. 3E, 3F).

**FIGURE 2.** Oa1 expression and distribution in Tyr−/− and Matpuw eyes. Pigmented cells dissociated from eyes of neonatal wild-type (wt), Oa1−/−, Tyr−/−, and Matpuw mice were stained with an anti-Oa1 antibody. The identity of RPE cells was assessed by staining with anti-Cralbp antibody (inset), which does not label choroidal pigmented cells (arrow). Top: interferential reflection micrographs of a typical RPE (left) and choroid (right) cell. Bottom: immunofluorescence detection of Oa1 in wild type, Oa1−/−, Tyr−/−, and Matpuw in RPE and choroidal cells. Expression of Oa1 in Tyr−/− and Matpuw RPE and choroidal cells was similar to wild-type, whereas Oa1 could not be detected, as expected, in Oa1−/− RPE, and choroid.

**FIGURE 3.** Tyrosinase and Matp expression in Oa1 mutant eyes. (A) Real-time quantitative RT-PCR of Tyr in neonatal (P0) wild-type (wt), and Oa1−/− (Oa1 mut) eyes. y-Axis: 2−ΔΔCt value represents differences between the mean Ct (cycle threshold) values of tested genes and those of reference gene. (B) Real-time quantitative RT-PCR of Matp in neonatal (P0) wild-type (wt) and Oa1−/− (Oa1 mut) eyes. (C–F) Immunofluorescence analysis of Tyr distribution in RPE (arrows) and choroid-dissociated cells. (C, E) Micrographs of cells derived from wild-type mice, (D, F) micrographs of cells derived from Oa1−/− mice, (C) and (D) are bright-field phase images of the cells in (E) and (F), respectively.
number of stage II melanosomes was reduced in the their maturation at stage II (Fig. 4, Table 3). Of interest, the Tyrc-2J

TABLE 2. Size Distribution of Melanosomes in the RPE of Different Genotypes

<table>
<thead>
<tr>
<th>Diameter</th>
<th>&lt;1 μm</th>
<th>1–1.5 μm</th>
<th>&gt;1.5 μm</th>
<th>Mean Diameter (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage IV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type P7</td>
<td>90</td>
<td>10</td>
<td>0</td>
<td>0.51</td>
</tr>
<tr>
<td>n</td>
<td>106</td>
<td>11</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3 months</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%</td>
<td>92</td>
<td>8</td>
<td>0</td>
<td>0.61</td>
</tr>
<tr>
<td>n</td>
<td>98</td>
<td>9</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Oa1&lt;sup&gt;+/−&lt;/sup&gt; P7</td>
<td>63</td>
<td>14</td>
<td>23</td>
<td>0.90</td>
</tr>
<tr>
<td>%</td>
<td>50</td>
<td>7</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>44</td>
<td>40</td>
<td>16</td>
<td>1.00</td>
</tr>
<tr>
<td>Stage II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyrc&lt;sup&gt;−/−&lt;/sup&gt;:Tyr&lt;sup&gt;−/−&lt;/sup&gt; P7</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0.56</td>
</tr>
<tr>
<td>%</td>
<td>118</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Oa1&lt;sup&gt;+/−&lt;/sup&gt;:Tyrc&lt;sup&gt;−/−&lt;/sup&gt;:Tyr&lt;sup&gt;−/−&lt;/sup&gt; P7</td>
<td>95</td>
<td>5</td>
<td>0</td>
<td>0.67</td>
</tr>
<tr>
<td>%</td>
<td>41</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Stage III</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Matpuw&lt;sup&gt;+/−&lt;/sup&gt;/Matpuw&lt;sup&gt;+/−&lt;/sup&gt; 3 months</td>
<td>87</td>
<td>13</td>
<td>0</td>
<td>0.54</td>
</tr>
<tr>
<td>%</td>
<td>14</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Oa1&lt;sup&gt;+/−&lt;/sup&gt;/Matpuw&lt;sup&gt;+/−&lt;/sup&gt;/Matpuw&lt;sup&gt;+/−&lt;/sup&gt; 3 months</td>
<td>97</td>
<td>3</td>
<td>0</td>
<td>0.56</td>
</tr>
<tr>
<td>%</td>
<td>40</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Data report the number (n) and the percentage (%) of the melanosomes counted in each condition and falling in each of the three ranges of diameter shown.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Thus), therefore, the absence of Oa1 does not interfere with the expression of Tyr and Matp.

Altogether, these results suggest that Oa1, Tyr, and Matp do not reciprocally influence each other’s expression and subcellular distribution.

**Effect of Oa1 on Rate of Melanosome Biogenesis and Organelle Size**

We generated Oa<sup>1<sup>+/−</sup>∶Tyr<sup>−/−</sup>:Tyr<sup>−/−</sup></sup> double-mutant mice and analyzed the melanosome phenotype in RPE at P7. At this time point, both the reduced number of melanosomes and the macromelanosome phenotype are easily detectable in Oa<sup>1<sup>−/−</sup></sup> mice (Tables 1, 2; Ref. 6). At P7, no melanin was deposited in the melanosomes of Tyrc<sup>−/−</sup>:Tyr<sup>−/−</sup>:stage II melanosomes. Bar, 0.47 μm.

![Figure 4](Downloaded From: https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932930/ on 09/14/2018)

The reduced number of stage-II melanosomes in double-mutant RPE demonstrates that Oa1 contributes to the rate of melanosome maturation at early stages and independently of melanin synthesis. However, we could not define yet whether melanin synthesis and/or melanosome maturation are necessary for the regulation of melanosomal size by Oa1.

**Effect of Melanosome Maturation**

To address whether melanin deposition and/or melanosome maturation are involved in the macromelanosome phenotype, we analyzed the RPE in P7 and 3 month old Oa<sup>1<sup>−/−</sup></sup>∶Matpuw<sup>+/−</sup>/Matpuw<sup>+/−</sup> double-mutant mice. In fact Matpuw<sup>+/−</sup>/Matpuw<sup>+/−</sup> adult single mutant RPE and of 0.67 μm in the double mutant RPE (Table 2). These values were similar to those obtained from wild-type stage-IV melanosomes (average value, 0.51 μm; Table 2). On the contrary, they were smaller compared with Oa<sup>1<sup>−/−</sup></sup> macromelanosomes (average value, 0.9 μm; Table 2).

![Figure 4](Downloaded From: https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932930/ on 09/14/2018)
RPE was previously reported to display smaller but pigmented melanosomes.14 Whereas at P7 we did not detect any melanosome in both Matpuw/Matpuw and double-mutant mice (data not shown), at 3 months of age, in both genotypes RPE melanosomes did not proceed beyond stage III (Table 3). The Matpuw/Matpuw single mutant showed a reduction in melanosome number compared with the Oa1+/−:Matpuw/Matpuw double mutant, though the difference is not significant. By morphometry, we measured similar diameters in the Matpuw/Matpuw single mutant (0.54 μm) and in the Oa1+/−:Matpuw/Matpuw double mutant (0.56 μm) RPE (Table 2C). However, when analyzed in detail, the relative distribution of the different stages of melanosome showed a reduction in the number of stage-III melanosomes in the double mutant compared with Matpuw/Matpuw single-mutant RPE (Table 3). This event was accompanied by an increased percentage of stage-III melanosomes (75% of total melanosomes) in Oa1+/−:Matpuw/Matpuw double-mutant RPE when compared with Matpuw/Matpuw single-mutant RPE (40% of total melanosomes; Table 3). Therefore melanosome maturation increased (P = 0.01) in the absence of both Oa1 and Matp, although the block of melanosome maturation at stage III characterizing Matpuw/Matpuw single-mutant RPE was retained.

Altogether these results suggested that Oa1 exerts a size control function only on fully developed stage-IV melanosomes, independent of melanin synthesis. Therefore, melanosome maturation and not melanin deposition has a direct influence on the formation of macromelanosomes.

### Discussion

Two main classes of albinism that affect pigmentation in the eye have been described: oculocutaneous albinism (OCA) and ocular albinism (OA). In OCA hypopigmentation is caused by inadequate melanization of an apparently normal number of melanosomes, whereas OA was suggested to result from reduced number of melanosomes.25 Patients with OA1 have a severe reduction of visual acuity, horizontal and rotary nystagmus, photophobia, and lack of stereoscopic vision due to misrouting of the optic tract. A peculiar melanosome phenotype in these patients was characterized by the presence of pigmented melanosomes with a giant size,25 suggesting that the absence of OA1 could interfere with the normal melanosome biogenesis. However, we still lack a comprehensive view of how this phenotype is acquired and how it relates with the albino phenotype observed in the RPE. In particular, it is unclear how the presence of pigmented melanosomes reconciles with the observed albino phenotype in the RPE, at which stage(s) of melanosome maturation OA1 exerts its function, and whether an impaired control of melanin synthesis and/or of rate of melanosome biogenesis is involved in giant melanosome formation. Our study addresses these important questions and allows defining the differences between OA (albinism isolated to the eye) and OCA. We focused our attention on two aspects of melanogenesis in the RPE: rate of melanosome formation and size control. We found that these events are both affected by lack of OA1 activity.

We first characterized Oa1 interaction with Tyr and showed that there is no impairment in Tyr activity and expression in Oa1 mutant mice. This aspect of the phenotype differentiates Oa1 from other albino mice in which melanin production is affected.22 However, the number of mature melanosomes in Oa1 mutant eyes was reduced by 50% in the RPE at birth. This reduction was in the number of stage IV melanosomes. It is possible that the effect on other melanosomal stages is masked by the lower number of stage-II and -III organelles after birth. In fact, a reduction in the number of stage-II melanosomes was detected both in mutants when melanosome maturation is impaired (Oa1+/−:Tyr−2/Tyr+/− and Oa1+/−:Matpuw/Matpuw double mutants) and at embryonic stages in Oa1+/− RPE. These results demonstrate, for the first time, that the reduced pigmentation of the RPE observed in patients with ocular albinism type I is caused by reduced number of pigmented melanosomes.

A second relevant aspect of Oa1 function highlighted by our results is the specificity of Oa1 size control activity on stage-IV melanosomes. This conclusion is based on the absence of melanosome enlargement in the RPE of Oa1+/−:Tyr−2/Tyr−2 and Oa1+/−:Matpuw/Matpuw double-mutant mice which lack stage-IV melanosomes and display either stage-II or -III, respectively. Therefore, in the absence of fully mature melanosomes Oa1 does not exert its size control function.

Whereas the Oa1 size control activity is specific for the most mature melanosomes, an additional Oa1 function appears to be exerted at earlier stages. In particular, in Oa1+/− single and double mutants, the number of stage-II melanosomes was reduced at the earliest stages of maturation evaluated: E15.5 for Oa1+/−, and P7 or 5-month postnatal stages for Oa1+/−; Tyr−2/Tyr−2 and Oa1+/−:Matpuw/Matpuw double mutants. In contrast, stage-III melanosomes were more abundant in Oa1+/−:Matpuw/Matpuw double mutants compared with Matpuw/Matpuw single-mutant mice. This suggests that Oa1 may at the same time control the maturation rate of melanosomes and inhibit the overgrowth of the most mature ones. In agreement with this interpretation is our previous report of OA1 acting as a negative regulator of melanosome maturation.26 We cannot exclude, however, that the phenotype observed in double-mutant mice could also be due to a specific interaction between the two genes.

Although we have not defined the mechanisms by which Oa1 may perform its double function, it is of note that heterotrimeric G-proteins associated with intracellular organelles have been described to participate in various steps of secretion and vesicular fusion27 and organelle maturation,28 even though no specific intracellular GPCR has yet been described for these functions. We suggest that Oa1 may act as a GPCR, either constitutively active, or activated by melanin or a byproduct of melanin synthesis that controls membrane delivery to the melanosomes. This interpretation could explain the reduction of early stage melanosomes and the macromelanosome phenotype.

Taken together, our findings indicate that Oa1 regulates melanosome maturation. Acting at early maturation stages, Oa1 controls the abundance of melanosomes in RPE cells, and, at later stages, Oa1 has a function in maintaining a correct melanosomal size. The dissection of these two Oa1 activities allows a better understanding of the albinism phenotype due to the
lack of this protein, and in the future it will be important in the design therapeutic approaches for this disease.

Acknowledgments
The authors thank Vittoria M. Schiaffino for collaborating in Oa1 antibody production, Salvatore Arbucci for confocal microscopy, Diego Di Bernardo, Graciana Diez-Roux, and Alberto Auricchio for discussion of the data; and Vincent J. Hearing and John Saari for the reagents.

References