Specific interaction of G\(\alpha_i3\) with the Oa1 G-protein coupled receptor controls the size and density of melanosomes in retinal pigment epithelium.

https://escholarship.org/uc/item/0z78n9bm

PloS one, 6(9)

1932-6203

Young, Alejandra
Jiang, Meisheng
Wang, Ying
et al.

2011

10.1371/journal.pone.0024376

Peer reviewed
Specific Interaction of Gαi3 with the Oa1 G-Protein Coupled Receptor Controls the Size and Density of Melanosomes in Retinal Pigment Epithelium

Alejandra Young1,2, Meisheng Jiang3, Ying Wang3, Novruz B. Ahmedli1, John Ramirez1, Benjamin E. Reese4, Lutz Birnbaumer5, Debora B. Farber1,2*

1 Jules Stein Eye Institute, University of California Los Angeles, Los Angeles, California, United States of America, 2Molecular Biology Institute, University of California Los Angeles, Los Angeles, California, United States of America, 3Department of Molecular and Medical Pharmacology, University of California Los Angeles, Los Angeles, California, United States of America, 4Department of Psychological and Brain Sciences, University of California Santa Barbara, Santa Barbara, California, United States of America, 5Laboratory of Neurobiology, Division of Intramural Research, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina, United States of America

Abstract

Background: Ocular albinism type 1, an X-linked disease characterized by the presence of enlarged melanosomes in the retinal pigment epithelium (RPE) and abnormal crossing of axons at the optic chiasm, is caused by mutations in the OA1 gene. The protein product of this gene is a G-protein-coupled receptor (GPCR) localized in RPE melanosomes. The Oa1-/- mouse model of ocular albinism reproduces the human disease. Oa1 has been shown to immunoprecipitate with the Gxi subunit of heterotrimeric G proteins from human skin melanocytes. However, the Gxi subfamily has three highly homologous members, Gαi1, Gαi2 and Gαi3 and it is possible that one or more of them partners with Oa1. We had previously shown by in-vivo studies that Gαi3-/- and Oa1-/- mice have similar RPE phenotype and decussation patterns. In this paper we analyze the specificity of the Oa1-Gαi interaction.

Methodology: By using the genetic mouse models Gαi1-/-, Gαi2-/-, Gαi3-/- and the double knockout Gαi1-/-, Gαi3-/- that lack functional Gαi1, Gαi2, Gαi3, or both Gαi1 and Gαi3 proteins, respectively, we show that Gαi3 is critical for the maintenance of a normal melanosomal phenotype and that its absence is associated with changes in melanosomal size and density. GST-pull-down and immunoprecipitation assays conclusively demonstrate that Gαi3 is the only Gαi that binds to Oa1. Western blots show that Gαi3 expression is barely detectable in the Oa1-/- RPE, strongly supporting a previously unsuspected role for Gαi3 in melanosomal biogenesis.

Conclusion: Our results identify the Oa1 transducer Gαi3 as the first downstream component in the Oa1 signaling pathway.

Introduction

Hypopigmentation mutations affecting melanin synthesis or melanosomal biogenesis in the retinal pigment epithelium (RPE) of mammals are known to have profound effects on the developing retina and visual pathways, including abnormal crossing of the optic axons, nystagmus, strabismus, loveal hypoplasia, and reduced visual acuity [1]. Two forms of albinism are commonly recognized: oculocutaneous albinism (OCA), in which neither the eye nor the skin or hair are pigmented and ocular albinism (OA), which affects primarily the eye pigmentation.

Ocular albinism type 1 (OA1, also called Nettleship-Falls type), is the most common form of ocular albinism. It has an estimated prevalence of 1/50,000 in the general population of the United States [2]. Although the cutaneous manifestations of OA1 are very mild, affected patients present abnormal macromelanosomes in both the RPE and skin [3]. In contrast to other forms of albinism, melanin is not dramatically reduced in OA1; in fact, this disease is characterized by the unusual coexistence of the typical albinopigmentary defects with a substantial amount of melanin in the eyes [4]. Different types of mutations in the OA1 gene have been associated with ocular albinism type 1 (http://albinismdb.med.umn.edu/oa1mut.html#mutations). OA1, the protein product of the OA1 gene, is a G protein-coupled receptor localized to RPE melanosomal membranes and the initiator of the observed abnormal visual phenotype in ocular albinism. The position of OA1 within these membranes, with its N-terminal towards the lumen of the melanosome and C-terminal towards the cytoplasm, suggests that it may function as a novel intracellular GPCR activated by the binding of a melanosomal ligand. This ligand could thus regulate melanosomal biogenesis through activation of specific G-proteins found in the RPE cytoplasm [5]. In addition,
this localization of OA1 is consistent with its proposed role as a stop signal for melanosome overgrowth during melanogenesis [6,7] and could explain the changes in RPE phenotype observed in ocular albinism: mutations or deletions in OA1 producing a non-functional OA1 protein would allow a continuous vesicular traffic of membrane proteins to melanosomes resulting in the formation of macromelanosomes [3].

It has been shown that the endogenous OA1 from human melanocyte extracts co-immunoprecipitates mainly with the alpha subunits of the Gα subfamily of heterotrimeric G-proteins [9]. The Gαi subfamily comprises three closely related members, Gαi1, Gαi2, and Gαi3, which share 85 to 95% amino acid sequence identity and partial overlapping expression patterns. In addition to their established function in signal transduction across the plasma membrane, these heterotrimeric G proteins localize to intracellular membranes and have been implicated in the regulation of membrane trafficking and fusion events along the secretory and endocytic pathways, such as vesicle formation by the endoplasmic reticulum, the Golgi/secretory pathway, and vesicle trafficking and fusion [9,10,11]. We have recently shown that mouse Oa1 and Gαi3 play an important role in the determination of melanosomal size and density and that both signal in the same pathway to regulate axonal guidance at the optic chiasm [12]. In the present study, we used Gαi knockout mouse models to investigate whether the other two members of the Gαi family of proteins, Gαi1 and Gαi2, are involved in the regulation of size and density of the RPE melanosomes. We also studied the effects of the loss of both Gαi1 and Gαi3 using the corresponding double knockout mouse (hereafter called DKO). In addition, we analyzed the specific interaction of each Gαi protein with Oa1 in in-vitro immunoprecipitation and GST-pull-down experiments. For the latter, two polypeptides corresponding to the Oa1 third intracellular loop (I3) and carboxy-terminal tail (CT), the two regions that have been shown to play a critical role as selectivity determinants in receptor–G-protein interactions [13,14], were tested. Furthermore, we investigated the expression and cellular response of Gαi proteins in the RPE of Oa1-/- mice using Western blots and ADP-ribosylation. Altogether, our in-vivo and in-vitro data demonstrate a previously unknown role of Gαi3 as the specific protein downstream of Oa1 that regulates the size and density of RPE melanosomes.

Results

Size, density and morphology of RPE melanosomes

To investigate the involvement of Gαi proteins in the regulation of size and density of RPE melanosomes, we analyzed electron-micrographs of RPE melanosomes from each Gαi1-/-, Gαi2-/-, Gαi3-/- and DKO mice, using 129 Sv wild-type mice as controls, as discussed in Materials and Methods. We had previously shown that Gαi3-/- mice, like Oa1-/- mice, have larger RPE melanosomes than those of control mice, and that they each have reduced density of melanosomes [12] (Figures 1A, B, C and D). Notably, the two background strains for these knock-out mice (129 Sv and C57Bl/NCrl, hereafter B6/NCrl) differ substantially in melanosome size and density, making clear the need for appropriate controls when considering these features of the RPE. Gαi1-/- macromelanosomes are conspicuously larger than Gαi3-/- macro-melanosomes, but the latter are still significantly larger than those found in the 129 Sv control mice [12]. Our current electron microscopy results compare the relative roles of Gαi1 and Gαi2 to that of Gαi3 upon melanosomal size, density and shape, and assess the effects of combining the loss of two of them in a double knockout mouse.

We first examined the size of melanosomes in the RPE of these different Gαi knockout mice. The RPE of Gαi1-/- mice shows melanosomes that appear slightly enlarged (Figure 1E), while melanosomes in Gαi2-/- mice appear no different (Figure 1F), when compared with the melanosomes of 129 Sv mice (Figure 1C). By contrast, the RPE in DKO mice appears to contain enlarged melanosomes relative to those in 129 Sv mice, being comparable in size to those in the RPE of Gαi3-/- mice (Figure 1D). In order to quantify these differences, we measured melanosomal size, and determined the relative frequency of larger, macro-melanosomes (>5000 nm²) within the RPE of the different Gαi-/- mice. A total of 2501 129 Sv, 1285 Gαi1-/-, 1609 Gαi2-/-, 1115 Gαi3-/- and 1997 DKO melanosomes were sampled.
Comparison of the area of melanosomes from each mouse line (Figure 2A) shows that the frequency of larger melanosomes significantly differs between the groups (ANOVA, p<0.05). Ga3-/ RPEs have the highest percentage of melanosomes larger than 5000 nm$^2$ (6.58%±1.47), and post-hoc Tukey tests confirmed that this group is significantly larger than the 129 Sv control retinas (p<0.05). Thus, loss of only Ga3 yielded a significant increase in the presence of larger melanosomes. Combining the loss of Ga1 with Ga3 proteins in the DKO mice certainly did not worsen the abnormal morphology of the Ga3-/ RPE melanosomes. Together, these results suggest that only Ga3 has a function in the determination of the size of these organelles.

Second, we evaluated melanosome density (number of melanosomes/RPE μm$^2$) in the RPE of all the Ga-/ and their 129 Sv control mice (Figure 2B). While melanosomal density need not be inversely related to melanosome size, we previously found that it was in both Ga3-/ and in Oa1-/ mice [12], and so extended these analyses to the present knock-out mice. Ga1-/ and Ga2-/ mice showed comparable densities to those of 129 Sv mice, while Ga3-/ mice had a large reduction (37.3%) in melanosomal density when compared to 129 Sv mice. DKO mice also showed a comparable effect (a 34.6% reduction). Statistical analysis confirmed an effect of group (ANOVA, p<0.05), and post-hoc Tukey tests confirmed that Ga3-/ and DKO are significantly different from both 129 Sv and Ga2-/ mice (p<0.05). These results and the fact that the loss of Ga1 alone had no significant effect on either melanosome size or density leads us to conclude that of the three heterotrimeric Ga proteins, Ga3 may be the endogenous downstream protein in the Oa1 signaling cascade that controls melanosomal size and density.

Third, we compared the morphology of the melanosomes in the RPE of the different Ga-/ mice with that of their control mice, 129 Sv, to determine the percentage of melanosomes with a round shape. We classified all melanosomes according to their sphericity level, as described in Materials and Methods. Comparisons of 129 Sv with each Ga-/ mouse studied showed that all animals except for Ga2-/ have a similar percentage of RPE melanosomes that are round: 129 Sv 8.75%±0.25, Ga1-/ 9.37%±0.77, Ga2-/ 5.39%±0.37, Ga3-/ 9.12%±1.85, and DKO 7.65%±0.31. One-way analysis of variance, however, failed to reveal any effect of group despite the appearance of fewer round melanosomes in Ga2-/ (Figure 2C).

Figure 2. RPE melanosomal size, density and morphology of all Ga-/ mice compared to control 129 Sv mice. (A) Percentage of melanosomes larger than 5000 nm$^2$ in each of the mouse lines analyzed. The Ga3-/ mice have the highest percentage of melanosomes larger than 5000 nm$^2$ in the RPE. (B) Mean density of melanosomes: The Ga3-/ group had the lowest mean number of melanosomes per RPE area (59±5.2), and the wild-type and Ga2-/ groups had the largest mean density of melanosomes (96.8±8.2 and 93.4±8.1, respectively). (C) Percentage of round melanosomes: The Ga1-/ and Ga3-/ groups have the larger number of round melanosomes (9.37±0.77 and 9.12±1.85 respectively), followed by the 129 Sv wild-type (8.75%±0.25), DKO (7.65%±0.31) and Ga2-/ (5.39%±0.37).

doi:10.1371/journal.pone.0024376.g002
Analysis of retinal function in all Gαi-/− mice by electroretinography

ERG responses obtained from 129 Sv, Gαi1-/−, Gαi3-/− and DKO mice are summarized in Figure 3A and from 129 Sv and Gαi2-/− mice in Figure 3B. In each panel, mean response amplitudes (±1sd) are plotted against stimulus intensity (left to right). The retinal function of Gαi1-/−, Gαi3-/− and DKO mice, as judged by the electroretinograms, appears to be not significantly different and within normal limits from control retinas (Figure 3A). Curiously, the ERGs of the Gαi2-/− mice show a reduced b-wave amplitude (Figure 3B), suggesting that the lack of Gαi2 had some other impact on rod-mediated, but not on cone-mediated, retinal function, but the reason for this difference is not yet understood.

The Gαi3 protein specifically interacts with the melanosomal GPCR Oa1

Seven transmembrane GPCRs are characterized by their ability to couple with and activate heterotrimeric G proteins in response to a ligand binding mainly through two well-known functional regions: the third intracellular loop (i3) and the carboxy-terminal tail (CT) [8,14]. Given that Oa1 shares all typical hallmarks of GPCRs, to characterize the potential interactions of Oa1 and Gαi3 we carried out in-vitro binding studies in which recombinant fusion polypeptides of glutathione S-transferase with i3 and CT regions of Oa1, immobilized on glutathione-agarose beads, were incubated with in-vitro-synthesized 35S-labeled Gα proteins. Figure 4A shows a diagram indicating how the GST fusion proteins were obtained using the PGEXT4-2 vector, as detailed in Material and Methods. Figure 4B corroborates that the apparent molecular masses of the fusion proteins, after SDS-PAGE electrophoresis, correspond to the expected 29 kDa for the GST::Oa1-i3 fusion protein and 37 kDa for the GST::Oa1–CT fusion protein; and Figure 4C shows the apparent molecular masses of the in-vitro synthesized 35S-labeled Gα proteins.

SDS-PAGE of the Oa1- Gαi interacting complexes eluted from the agarose beads and Coomassie blue staining of the gel showed that each lane contained the same amount of GST-fusion protein: lanes 2–7 and 8–13 correspond to GST::Oa1-CT and GST::Oa1-i3 reactions, respectively (Figure 4D). Interestingly, the autoradiograph of the same gel demonstrated that Gαi3 is the only one of all Gαi proteins tested that specifically binds to Oa1 (Figure 4E, lanes 4 and 10). No binding was detected when GST::Oa1-i3 and GST::Oa1-CT were incubated with 35S-labeled Gαi1, Gαi2, Gαs, or Gαq or when GST alone, used as control, was incubated with one of the 35S-labeled Gα proteins (we used Gαi3). Also, incubation of beads having only GST-fusion proteins (negative control) did not show any non-specific Oa1 binding with TNT rabbit retinoculocyte lysate or with the beads (Figure 4E, lanes 7 and 13).

Mass spectrometry also identifies Gαi3 as the specific OA1-interacting Gαi protein

To identify RPE proteins that interact with human OA1 and gain a better understanding of this GPCR’s function, we used a human antibody against OA1 in immunoprecipitation (IP) reactions followed by SDS-PAGE. For these experiments, we dissected the RPE from donor, adult human eyes, which allowed us to obtain sufficient amount of protein. Half of the gel was stained with Coomassie blue to visualize the protein bands. Bands in the appropriate molecular mass of Gαi proteins (39–41 kDa) and OA1 (45–48 kDa) were excised, destained, trypsinized and sent for mass spectrometry analysis to the Pasarow Mass Spectrometry core facility at UCLA. The other half of the gel was transferred to nitrocellulose membranes to confirm by Western blot analysis, using antibodies to the mass spectrometry-identified interacting protein, the specificity of the OA1 partner. As we anticipated, mass spectrometry of the 38–41 kDa excised band identified Gαi3 as one of the proteins immunoprecipitated together with OA1 (Table 1). We corroborated the...
specific interaction of these proteins by incubating the blot with anti-Gαi3 antibody and visualizing the Gαi3 band using the ECL detection system (Figure 5A). Given that the commercially available antibodies against Gαi3 recognize both, Gαi3 and Gβ, Gβ was another protein immunoprecipitated together with OA1 and identified by mass spectrometry (Table 1). RPE lysates not immunoprecipitated with antibodies against OA1 but incubated with pre-immune serum did not show any G-protein on the corresponding lane of the blot (Figure 5A). Further confirmation of the OA1 and Gαi3 interaction was obtained by performing a reciprocal IP experiment using lysates containing proteins of adult, human RPE and the anti-Gαi3 antibody. After separating the immunoprecipitated proteins by SDS-PAGE and transferring them to nitrocellulose membranes, the blot was incubated with the anti-OA1 antibody. Our results show the presence of two forms of OA1, previously described with apparent molecular weights of 45 and 48 kDa [15], in the lane corresponding to the RPE proteins immunoprecipitated with the anti-Gαi3 antibody and the absence of the OA1 protein in the lane corresponding to the RPE lysates incubated with pre-immune serum (Figure 5B).

Gαi3 compensates the lack of Gαi1 in Gαi1-/- mouse RPE

Compensatory increases elevating a Gαi subunit level in a tissue when another Gαi subunit is missing from it have been previously observed [16]. To test this possibility, we used Western blots incubated with Gαi common antibody to analyze the expression of

---

**Figure 4. Analysis of interactions between OA1 and heterotrimeric Gα proteins by in vitro pull-down assay.** A) Schematic representation of the vector used to make the GST-fusion polypeptides GST::Oa1-i3 and GST::Oa1-CT. Each amplified Oa1 sequence (i3 and CT) was cloned into the PGEXT4-2 vector containing GST between the selected restriction enzymes (BamHI and SalI). B) Coomassie-blue stained gel showing the SDS-PAGE-separated fusion proteins used in the pull-down experiment. GST by itself has an apparent molecular weight of 26 kDa, the GST::Oa1-i3 fusion protein of 29 kDa and the GST::Oa1-CT fusion protein of 37 kDa in agreement with their estimated molecular masses. C) Autoradiogram of in vitro transcribed and translated 35S-labeled Gα proteins that will be tested for OA1 binding activity below (D–E), after separation by SDS-PAGE on a 10% Tris-Glycine polyacrylamide gel (D–E). The apparent molecular weight of each Gα protein is in agreement with its predicted molecular mass, Gαi1: 41 kDa, Gαi2: 40 kDa, Gαi3: 41 kDa, Gαq: 42 kDa, Gαs: 46 kDa and luciferase, run as a control: 61 kDa. D) Coomassie blue staining of the gel used for SDS-PAGE of recombinant GST::OA1-CT and GST::OA1-i3 fusion proteins immobilized on glutathione-agarose beads and incubated with the indicated 35S-labeled Gα proteins. Lanes 1 and 14 had the GST protein incubated with one of the 35S-labeled Gα proteins in this experiment, 35S-Gαi3. Lanes 2–7: GST::OA1-CT incubated with the indicated 35S-labeled Gα. Lanes 8–13: GST::OA1-i3 incubated with the indicated 35S-Gα. Since the amount of 35S-Gα protein is minimal, only the fusion proteins are observed in this gel. Lanes 7 and 13: GST-fusion proteins immobilized on glutathione-agarose beads (Neg. C: negative control). All lanes with the same fusion protein have comparable amounts of protein. E) Autoradiograph of the same gel showing that of all 35S-labeled Gα proteins, Gαi3 is the only one that binds specifically to OA1 (lanes 4 and 10). Molecular weight markers are expressed in kDa.

doi:10.1371/journal.pone.0024376.g004
Table 1. Ga\(i\)3 is identified by LC-MS/MS as the specific Ga\(i\) protein interacting with OA1.

| Accession no. | Entry | Description | Mascot score | MW | Unique peptides matched |
|---------------|-------|-------------|--------------|----|-------------------------|
| NP_006487     | Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide3 (Ga\(i\)-3) [Homo sapiens] | Association with Golgi-derived vesicles and protein trafficking | 63 | 41076 | 4 |
| NP_0020661    | G protein beta subunit [Homo sapiens] | Proteins that cover a wide variety234 of functions in signal transduction, pre-mRNA processing and cytoskeleton assembly [32]. | | 38061 | | |

Mascot search (Matrix Science) identified both, Ga\(i\)3 and Ga\(i\)2 in the immunoprecipitate of adult human RPE with the OA1 antibody. The entry names and accession numbers (UniProtKB) in this Table are provided along with a brief description of the function of the proteins and the match score, as well as the number of unique peptides found by nLC–MSMS for each protein.
doi:10.1371/journal.pone.0024376.t001

the three Ga\(i\) proteins in membrane preparations from RPE of Ga\(i\)1-/-, Ga\(i\)2-/-, Ga\(i\)3-/-, DKO and 129 Sv mice. The relative amount of protein in each band was then measured by densitometry. On the Western blot, Ga\(i\)1 and Ga\(i\)3 run together as one band at 41 kDa. In the absence of Ga\(i\)1 (in Ga\(i\)1-/- mice), levels of Ga\(i\)3 much higher than those in wild-type RPE are observed (Figure 6, lanes 1 and 5, respectively) since the corresponding band from the 129 Sv RPE has both Ga\(i\)1 and Ga\(i\)3. Densitometry measurements (Figure 6B) confirm this, showing 3.8±0.7 optical density (OD) units for Ga\(i\)3 in lane 1 (Ga\(i\)1-/- RPE) and 3.6±0.3 for both Ga\(i\)1 and Ga\(i\)3 in lane 5 (129 Sv RPE). Ga\(i\)2 is also higher in Ga\(i\)1-/- RPE than in control RPE. In the case of Ga\(i\)3-/- mice, it is not possible to determine if a small increase in Ga\(i\)1 is present in its RPE, since we do not exactly know how much Ga\(i\)1 or Ga\(i\)3 is present in the corresponding band from the 129 Sv RPE; the levels of Ga\(i\)2 are comparable in these animals to those of control RPE. In contrast, absence of Ga\(i\)2 in the Ga\(i\)2-/- mice is accompanied by a reduced level of the 41 kDa band corresponding to the Ga\(i\)1 and Ga\(i\)3 proteins, and loss of both Ga\(i\)1 and Ga\(i\)3 in the DKO mice does not trigger an up-regulation of Ga\(i\)2 levels (Figures 6, A and B). These results suggest that at the protein level, Ga\(i\)3 may compensate completely for the loss of Ga\(i\)1 in the Ga\(i\)1-/- mice and that Ga\(i\)1 may compensate only partially, if it does so, for the loss of Ga\(i\)3 in Ga\(i\)3-/- mice.

Ga\(i\)3 may be absent or expressed at very low levels in the RPE of Oa1-/- mice

To get more insight into the Oa1 signaling cascade, we tested the functional expression of Ga\(i\)1, Ga\(i\)2 and Ga\(i\)3 in the RPE of Oa1-/- mice and their congenic controls, C57Bl/NCrl mice, by performing Pertussis toxin (PTX)-mediated \(^{32}\)P-ADP ribosylation
experiments. After incubation of the crude membrane preparations from RPE with PTX and 32P-nicotinamide adenine dinucleotide (32P-NAD), an autoradiography of the 32P-labeled proteins separated on 6 M Urea-SDS-PAGE was obtained. Two bands at 40 and 41 kDa corresponding to Gz2 and Gz1 plus Gz3, respectively, were observed on the lane of the gel containing the RPE from control mice (Figure 7A, lane 1). In contrast, the RPE from Oa1-/− mice only showed the band corresponding to the 32P-ADP ribosylated Gz2. No sign of Gz3 or Gz1 was present on the autoradiograph (Figure 7A, lane 2). To corroborate these results, a Western blot was prepared with the same samples, using the Gz3 antibodies. Figure 7B shows the Gz2 and Gz1 plus Gz3 bands in the control RPE (lane 1), but in Oa1-/− RPE, comparable levels of Gz2 but a very minimal amount of either or both Gz1 and Gz3 are seen (lane 2).

Discussion

We have previously demonstrated by in-vivo studies on mice that the heterotrimeric G protein Gz3 signals in the same transduction pathway controlled by Oa1 to regulate melanosomal biogenesis and axonal growth through the optic chiasm [12]. However, the interaction between Oa1 and the two other members of the Gz family of proteins, Gz1 and Gz2, remained to be explored. In this paper, taking advantage of the availability of knockout mice for each of the Gz genes, and of DKO (Gz1-/−, Gz3-/−) mice, we tested the hypothesis that Oa1 transmits its signal through one specific Gz protein and demonstrated with in-vitro and in-vivo experiments that this protein is indeed Gz3.

To investigate the involvement of all Gz proteins in the regulation of size, density and shape of RPE melanosomes, electron micrographs of the RPE from 3-month-old Gz1-/−, Gz2-/−, Gz3-/−, DKO and 129 Sv were analyzed. With regard to size, our results indicate that only the loss of function of Gz3 significantly increases the number of macromelanosomes in the RPE. Even with loss of function of both Gz1 and Gz3, no added effect in the size of DKO melanosomes was observed when compared to melanosomes of Gz3-/− RPE, suggesting that Gz1 is not involved in establishing the size of RPE melanosomes. We then looked at the number of melanosomes per unit area of RPE, and found that melanosomal density in Gz1-/− and Gz2-/− were not significantly reduced from that in 129 Sv wild-type RPE. Conversely, melanosomal density was reduced significantly and similarly in Gz3-/− and in DKO RPEs when compared to wild-type RPE. This again supports the notion that Gz1-/− does not contribute to the RPE melanosomal density decrease observed in DKO mice. We also established that despite the appearance of fewer round melanosomes in Gz2-/− RPEs, there is not a major change in the percent frequency of round melanosomes in all Gz1-/− RPEs when compared to those in the wild-type 129 Sv. In addition, as demonstrated by electrotetinography, the retinas of Gz3-/− as well as those of DKO mice are functional despite the phenotype of their RPEs (Figure 3A) similar to what was previously observed in the ERG of Oa1-/− mice [12]. Thus, these results together lead us to conclude that of the three heterotrimeric Gz proteins, Gz3 is the Gz1-associated protein involved in the regulation of RPE melanosomal size and density.

Potential binding sites for heterotrimeric G-proteins on GPCRs have been localized to the cytoplasmic loop 3 (i3) and carboxy-terminal tail (CT) of the seven-transmembrane receptors [14]. These intracellular segments are the ones that determine the interaction with specific G proteins, and as a result, which of several possible signaling pathways are activated [17,18,19,20]. Therefore, we generated GST-fusion proteins with the Oa1-i3 and Oa1-CT segments and used them in GST pull-down assays to determine which Gz protein bound in-vitro to Oa1. Results of these experiments showed that of the three heterotrimeric Gz proteins, Gz3 is the only one that binds specifically to Oa1. Most important, immunoprecipitation of RPE proteins using Oa1 antibodies, followed by SDS-PAGE and mass spectrometry analysis of the proteins, brought down Gz3 together with Oa1, conclusively identifying Gz3 as an Oa1-specific interacting protein. These results were further confirmed by immunoprecipitation experiments of RPE proteins using Gz3 antibodies, which also demonstrated that Oa1 co-immunoprecipitated with Gz3. Collectively, these data indicate that the GPCR Oa1 initiates in the RPE the signal transduction cascade that controls melanosomal size and density through activation of Gz3.

Compensatory increases elevating a Gz subunit level in a tissue when another Gz subunit is missing from it have been previously observed [16]. This raises the possibility that in the RPE of Gz knockout mice, at the protein level, compensatory expression of the other Gz subunits may reduce the effect that the loss of a particular Gz protein has on the control of melanosomal size and density. To test this, we measured the levels of the three Gz proteins in the RPEs of Gz1-/−, Gz2-/−, Gz3-/− and DKO mice using Western blotting with a Gz3 common antibody. Our results suggest that at the protein level, only the loss of Gz1 from the RPE may be compensated for by Gz3. However, with respect to functional compensation of the melanosomal phenotype, we cannot conclude that Gz3 functionally compensates for the loss of Gz1 since our results show that Gz1-/− melanosomes are not significantly different from those observed in control mice, even though they are a bit larger. Similarly, the fact that the DKO phenotype is no worse than that of Gz3-/− alone with respect to size, density or shape of RPE melanosomes, suggests that Gz1 is not critical for the regulation of these parameters. Furthermore, compensatory increases, if any, in Gz1 protein in the Gz3-/− retina seem to play no role in melanosome biogenesis. Although Gz2 levels are considerably increased in the Gz1-/− animals and to a quite lesser extent in Gz3 knockout mice, we have shown in the in-vivo experiments that Gz2 is not involved in the determination of melanosome size. Given the high levels of this protein in the RPE, it must play an important, though different, role in this tissue than Gz3, a role having quite discriminable effects, evidenced in the ERG itself (Figure 3B).

ADP-ribosylation of Gz subunits is a covalent modification catalyzed by PTX in which an ADP-ribose moiety is attached to the C-terminus of the protein. This prevents the Gi proteins to interact with their receptors and, therefore, it causes functional inactivation of all signal transduction pathways. ADP-ribosylation with NAD 32P-labeled on its ADP-ribose moiety allowed us to tag Gz subunits in membranes and to learn about their involvement in cellular responses. We carried out this reaction using RPE membranes of Oa1-/− and their congenic B6/NCr mice. Interestingly, our results showed that the three Gz subunits are present in the RPE of control mice, but that only Gz2 is in the Oa1-/− RPE. Similarly, when we used samples from the same RPE membranes to perform Western blotting with the Gz common antibody we found that Gz2 levels in −/− Oa1 RPE are comparable to those in the control RPE, but that there is very minimal, if any, amount of either or both Gz1 and Gz3 in the Oa1-/− RPE. These interesting results will lead to a whole series of studies in the future.

It is well established that in addition to their important roles in many pathways of transmembrane signaling, where they participate in processing and sorting of incoming signals as well as in adjusting the sensitivity of the signaling system, heterotrimeric G-
proteins are also localized to the Golgi complex [21], where they are involved in the formation of secretory vesicles from the trans-Golgi network (TGN) [9]. Gζ3, in particular, acts as an inhibitor of intra-Golgi and post-Golgi trafficking [22], and has been found, among other places, in the membranes of secretory vesicles in pancreatic acinar cells, from where it facilitates the fusion between zymogen granules and/or the expulsion of vesicular contents [23].

The specific function of Gζ3 in the RPE has not been identified. Our working hypothesis is that after the bulk of Oa1, together with tyrosinase, TRP1 and other compounds involved in melanin synthesis, has reached the stage II melanosomes, Oa1 starts to activate Gζ3, which in turn inhibits the traffic of vesicles carrying the membrane proteins required for melanization from the TGN. However, melanization occurs with no problem in stage III and IV melanosomes, as they use the proteins already present. This would explain why the levels of Oa1 decrease from those in stage II melanosomes to those in stages III and IV [24]. Oa1 is not being renewed in the melanosomes after its activation of Gζ3 because there are no longer vesicles bringing this protein to the melanosomes. Thus, the function of Gζ3 in the RPE would be to control the size of melanosomes through the inhibition of vesicle trafficking from the TGN to the melanosome, a function previously thought to be carried by Oa1 [25].

On the basis of our results and the accumulated information in the literature about GPCRs, Gζ3 functions and melanogenesis, we are proposing a hypothetical model for the beginning of the Oa1 signaling cascade (Figure 8A).

In this model, at the end of stage II melanogenesis, when melanosomes have acquired their melanoproteins and the bulk of Oa1, a signal must turn on an endogenous luminal ligand to activate Oa1 at the melanosomal surface membrane. Like in other GPCR cascades, activated Oa1 will cause the exchange of GDP for GTP on the Gζ3 subunit of the heterotrimeric G protein localized to the surface of the same membrane, activating Gζ3 (Gζ3*)—which then separates from the βγ subunits—and leading to the release of Gζ3* and Gβγ to the RPE cytoplasm. Gζ3* will at this stage inhibit the vesicular traffic of membrane proteins from the TGN to the melanosome. Prenylation of the Gγ subunit will target the Gβγ complex to the endoplasmic reticulum, where it will be processed fully before it is delivered to the Golgi. There, Gβγ will bind Gζ3 and after post-translational modification of Gζ3, the G protein heterotrimer may leave the TGN and get transported to the melanosome surface membrane using the classic secretory pathway.

This model could also explain the presence of macromelanosomes in the RPE of ocular albinism patients or Oa1 and Gζ3-/- mice (Figure 8B). Mutations in the OA1 gene in humans could render the OA1 protein incapable of activating the heterotrimeric Gζ3 on the surface membrane of the melanosome. The same effect would be observed in the absence of Oa1 or Gζ3 in knockout mice. Without the active form of Gζ3*, inhibition of the vesicular traffic of melanin-related proteins to stage II melanosomes cannot occur, and the continuous supply of this material to the melanosome would result in the formation of abnormally large organelles, the macromelanosomes.

In summary, while the precise function of Gζ3 in RPE melanosome biogenesis remains to be delineated, it is clear that this protein plays an important role in the control of the size of RPE melanosomes. As a consequence, Gζ3 is also controlling RPE pigmentation, which seems to be necessary during embryonic stages for the proper decussating pattern of the optic axons. Because Gζ3-/- mice, like Tyr-/- and Oa1-/- mice, all show abnormalities in the decussation of their optic axons [12], Gζ3 appears to be the common effector by which these three distinct RPE phenotypes affect the retinal ganglion cells as their axons navigate the optic chiasm.

Materials and Methods

Ethics Statement

All experiments involving mice were carried out using protocols approved by the UCLA Animal Research Committee, and in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research.

Animal and human tissues

C57BL/6NCrl (B6/NCrl) mice and congenic Oa1 knock-out mice (Oa1-/-) were obtained from The Charles River Labs, USA and Italy, respectively, and bred at UCLA. Gζ3-/-, Gζ2-/-, Gζ3-/- and the DKO mice were previously generated on the 129Sv background. The genotype of these mice was determined by Southern blot analysis of mouse tail genomic DNA as described by Jang et al. [26]. Mice were housed and bred in conventional cages and environmental conditions at the animal facilities of UCLA.
Healthy human donor eyes were obtained from the National Disease Research Interchange (Philadelphia, PA) and immediately frozen in liquid nitrogen. The donor eyes were handled in compliance with the Declaration of Helsinki.

Electron Microscopy

3 month-old 129 Sv, i1-/-, i2-/-, i3-/- and DKO mice were deeply anesthetized by an intraperitoneal injection of 120 mg/kg sodium pentobarbital, and perfused intracardially with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Eyes were enucleated, rinsed in 0.1 M phosphate buffer, post-fixed with 1% buffered osmium tetroxide, dehydrated in graded ethanol, and embedded in araldite 502. Sections for electron microscopy (60–70 nm) were cut on a Leica Ultracut UCT and collected on 200 mesh uncoated copper grids. For the ultrastructural analysis, stained sections (5% uranyl acetate and 0.4% lead citrate) were observed with a 910 Zeiss electron microscope. The RPE fields analyzed were photographed using a Keenview™ digital camera. For quantification of melanosomes and determination of their area in the RPE, we analyzed 50 micrographs for each 129 Sv and DKO mice and 30 micrographs for each i1-/-, i2-/-, and i3-/- mice at 16,000 x magnification using the analySIS™ software for LEO 900 TEM, version 3.2. (Soft Imaging System, Lakewood, CO). Images were cropped with Adobe Photoshop (Adobe Systems Inc., San Jose, CA). A total of 2501 129 Sv, 1285 i1-/-, 1609 i2-/-, 1115 i3-/-, and 1997 DKO melanosomes were studied, sampling 10 eyes for each 129 Sv and DKO mice and 6 eyes for each i1-/-, i2-/- and i3-/- mice. The data for the i3-/- mice were previously reported, but are included herein for direct comparison.

Melanosomal size and density.

We used the magic wand feature of the Soft Imaging System analySIS software to select and measure the area of every individual melanosome and of the total RPE area containing the melanosomes in each micrograph, as we have done previously in the analyses of i3-/- and the i1-/- mice and their controlling controls [12]. We analyzed the difference in the melanosomal size, the percent frequency of melanosomes in the >5000 nm² size range, and the number of melanosomes per RPE area among five mouse groups: 129 Sv, i1-/-, i2-/-, i3-/- and DKO. Comparisons among all groups were done using a one way analysis of variance (ANOVA) followed by post-hoc Tukey tests to identify significant differences between individual pairs of groups, conservatively using animal averages for each measure and thus an n of 5 for the 129 Sv and DKO groups and an n of 3 for the i1-/-, i2-/-, and i3-/- groups. A p-value of less than 0.05 was considered to be statistically significant.

Melanosomal Morphology.

To determine melanosomal shape we used the particle detection analysis of the Soft Imaging System analySIS software for LEO 900 TEM, version 3.2. The classification of the shape of the organelles is given by how round the organelle is. A melanosome with a shape factor of 1 is round, while elliptical melanosomes have a shape factor less than 1. We determined the organelle is. A melanosome with a shape factor of 1 is round, while elliptical melanosomes have a shape factor less than 1. We used the magic wand feature of the Soft Imaging System analySIS software for LEO 900 TEM, version 3.2. (Soft Imaging System, Lakewood, CO). Images were cropped with Adobe Photoshop (Adobe Systems Inc., San Jose, CA). A total of 2501 129 Sv, 1285 i1-/-, 1609 i2-/-, 1115 i3-/-, and 1997 DKO melanosomes were studied, sampling 10 eyes for each 129 Sv and DKO mice and 6 eyes for each i1-/-, i2-/- and i3-/- mice. The data for the i3-/- mice were previously reported, but are included herein for direct comparison.

Electroretinography

Mice were anesthetized with an intraperitoneal injection of xylazine (0.5 mg/ml) and ketamine (1 mg/ml) in normal saline. In adult mice, a dose of 0.1 ml was administered. Body temperature was maintained at 38°C with a heating pad. Pupils were dilated with Atropine (1%). A gold-wire electrode was placed on the corneal surface of the right eye and referenced to a gold wire in the mouth. A needle electrode in the tail served as the ground. Responses were amplified (Tektronix AM 502 Differential Amplifier, ×10,000) band pass filtered (0.1–300 Hz), digitized using an I/O board (PCI-6221, National Instruments, Austin, TX) in a personal computer, and averaged. A signal rejection window was used to eliminate electrical artifacts. All stimuli were presented in a large integrating sphere painted with a highly reflective white matte paint (#6080, Eastman Kodak Corporation, Rochester, NY). Rod mediated responses were obtained with blue flashes (Wrettan 47A; I max = 470 nm) varied over an intensity range of 3.5 log units. Cone-mediated responses were obtained with white flashes on a rod saturating background (32 cd/m²).

Production of GST::OA1-i3 and GST::OA1-CT recombinant proteins

Mouse OA1 cDNA was subcloned into the mammalian expression vector pCDNA3.1/V5-His-Topo using EcoRI and PsI sites in the poly linker. Using this construct, a 108 bp DNA fragment corresponding to the third cytosolic loop (i3) of OA1 (residues 213–248), with flanking fragments corresponding to the BamHI and Sall restriction enzymes, was amplified by PCR using the following primers: Forward, 5'-TGGATCCTTTTACAAAGCAATGACTTTCA-3'; and Reverse, 5'-AGTCGACTCTAGTTTAAAGGCGGTTGAT-3'. Similarly, a 275 bp DNA fragment corresponding to the C terminal (CT) of OA1 (residues 314–405), with flanking fragments corresponding to the BamHI and Sall restriction enzymes, was amplified by PCR using the following primers: Forward, 5'-TGATGTTCAACAGGAT-CAGCCTGGATGTC-3'; and Reverse, 5'-AGTCGACTCAGGTTCACCCGTGAGTTTCA-3'. The 25 μl reaction contained 2.5 μl of each of 5’ primer and 3’ primer, 100 ng/μl pCDNA3.1/V5-His-Topo-OA1, 0.6 Units of Taq DNA polymerase (Invitrogen Corporation, Carlsbad, CA), 1× PCR buffer, 2.5 mM MgCl₂ and 250 μM dNTP mix. After 30 cycles (melting for 3 min at 94°C; annealing for 1.5 min at 53°C; extension for 1 min at 72°C), the PCR reactions were subjected to an additional 5 min incubation at 72°C. Each PCR reaction yielded a single product that was subsequently isolated and gel purified, cut with BamHI and Sall, and subcloned into the pGEX-FT-2 vector (Amersham Bioscience, now GE Healthcare, Piscataway, NJ) using T4 ligase. Ligation reactions were used for transformation of BL21 (DE3). E. coli, and were selected on LB-ampicillin agar plates. Positive clones were screened by restriction analysis and DNA sequencing.

Preparation of GST-Fusion Proteins

E. coli cultures carrying the pGEX-FT-2 constructs were grown until they reached mid-logarithmic growth phase (0.4–0.5 A 600). Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.1 mM to induce GST protein expression, and the cultures were further incubated for 4 h at 30°C. GST was
purified from cultures incubated at 37°C for 3 h. The GST::Oa1-i3 fusion protein was obtained as previously described [27] with few modifications. Briefly, bacteria were harvested by centrifugation at 4,000 g for 15 min and the pellets were resuspended in 2 ml of STE Buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 25 μg/ml PMSF and protease inhibitors cocktail) containing 50 μg/ml of lysozyme (added immediately prior to resuspension), and incubated on ice for 15 min. Dithiothreitol (DTT) was then added to a final concentration of 2.5 mM. Bacteria were lysed by the addition of 1.0% N-lauroylsarcosine (Sarkosyl), disrupted on ice for 1 min using a MISONIX Sonicator 3000 (power level 3, 50% cycle) and centrifuged at 13,000 g for 10 min at 4°C to remove insoluble debris. To obtain the GST::Oa1-CT fusion protein, a similar procedure was followed, but no lysozyme or Sarkosyl was used.

Purification of GST-tagged proteins

GST-tagged proteins were purified from bacterial lysates by affinity chromatography using immobilized Glutathione Sepharose™ High Performance (GE Healthcare). 1 ml GSTTrap HP column was equilibrated with 5 ml of binding buffer (PBS, pH 7.3) and 1 ml of bacterial lysate was loaded onto it. The column was washed with 5 ml of binding buffer and the GST-tagged protein was eluted with 3 ml of elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0).

GST activity assay

The GST specific activity was measured spectrophotometrically using the GST assay kit (Sigma-Aldrich Corporation, St. Louis, MO) according to the manufacturers’ instructions, using 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 2 mM reduced-glutathione (GSH) as substrates at 340 nm and 25°C. Fractions with good GST activity (~600–800 μg/μl) were used for incubations with in-vitro transcribed/translated 35S-methionine-labeled Gα proteins (see below).

In-vitro synthesis and 35S-methionine-labeling of Gα Proteins

Gα1, Gα2, Gα3, Gαs and Gαq were in-vitro transcribed, translated and labeled with 35S-methionine using the pAGA-2 vector containing the cDNA of the corresponding Gα protein and the TNT Coupled Reticulocyte Lysate system (Promega, Madison, WI) with T7 RNA polymerase. The resulting 35S-labeled proteins (1.5 μg of each) were boiled in 2× SDS–PAGE sample buffer containing 3% β-mercaptoethanol, loaded onto a 10% Tris-Glycine gel. SDS-PAGE was carried out overnight at 44 V. Half of the gel was stained with Coomassie blue to visualize protein bands for mass spectrophotometry analysis and the other half was transferred to a nitrocellulose membrane (Hybond ECL, Amersham Biosciences) overnight at 33 V. The blot was then incubated with 1:10,000 anti-Gα1 (Abcam, Cambridge, MA) or with 1:7 μl anti-Gα3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) rabbit polyclonal primary antibodies, at 4°C overnight, and then with 50 μl of protein G-Sepharose for 2 h at RT. The complex-bound resin was washed 5 times with IP buffer (25 mM Tris–HCl, 150 mM NaCl, pH 7.2) and 3 times with water. Immunoprecipitated complexes were eluted with 2× SDS–PAGE sample buffer containing 3% β-mercaptoethanol and each of duplicate aliquots were loaded onto a different half of a 12% Tris-glycine gel. SDS-PAGE was carried out overnight at 44 V. Half of the gel was stained with Coomassie blue to visualize protein bands for mass spectrophotometry analysis and the other half was transferred to a nitrocellulose membrane (Hybond ECL, Amersham Biosciences) overnight at 33 V. The blot was then incubated with 1:10,000 anti-Gα3 or anti-OA1 rabbit primary antibodies, and with 1:10,000 goat anti-rabbit secondary antibodies. The Enhanced Chemiluminescence (ECL) detection reagent (Amersham Biosciences) was used to visualize the bands.

Tryptic Digestion. Protein bands of interest on the Coomassie blue-stained gel were excised and placed in microtubes. In-gel digestion with trypsin was performed according to standard procedures routinely used in the Pasarow Mass Spectrometry core facility at UCLA. Briefly, the gel pieces were destained with 10 μl acetonitrile (ACN) for 30 min at RT and treated with 10 mM DTT/100 mM NH4HCO3 (200 μl) for 1 hour at 37°C. Samples were then alkylated with 55 mM iodoacetamide (Sigma-Aldrich Corporation). The gel pieces were washed with 100 mM NH4HCO3 for 15 min at RT, dehydrated with ACN and digested with 1.25 μg trypsin (Promega) in 100 μl of 50 mM NH4HCO3 on ice, for 45 min. They were then incubated overnight in 10 μl of 50 mM NH4HCO3 without trypsin. The digests were extracted twice with 100 μl of 50% ACN/0.5% formic acid at RT for 60 min with constant mixing. The extracts were pooled and dried and each sample was then reconstituted with 8 μl of 2% formic acid and sent for
nano-liquid chromatography tandem mass spectrometry (nLC-MSMS) analysis at the Pasarow core facility. **Database Analysis.** The mass spectra were searched against a human trypsin indexed database, with variable modifications of carboxyamidomethylation, methionine oxidation, and deamination of asparagine residues using the BioWorks software (Thermo Fisher) based on the SEQUEST algorithm implemented in Discoverer software (Thermo Fisher). Spectra were also searched using Mascot software (Matrix Science, UK) and results with p<0.05 (95% confidence interval) were considered significant and indicating identity.

**Preparation of RPE Membranes**

The RPEs from Gzi1-/-, Gzi2-/-, Gzi3-/-, DKO, Oa1-/-, and their corresponding 129 Sv and B6/NCol control mice (12 eyes of each), were dissected, collected and frozen in liquid nitrogen. Each sample was homogenized in 250 μl of 27% (wt/wt) sucrose/1 mM EDTA/10 mM Tris-HCl, pH 7.5, in Dounce homogenizers. Homogenates were centrifuged for 5 min at 1,000 g and the supernatants were again centrifuged at 12,000 g for 20 min to obtain the RPE membranes. Quantification of protein from melanin-containing RPE membranes was carried out by the method of Sedmak and Grossberg, which depends on the conversion of Coomassie brilliant blue G-250, in diluted acid, from a brownish-orange to an intense blue color [25]. With this very sensitive method the melanin interference is negligible. The G-250 dye was prepared as a 0.06% solution in 19% perchloric acid (w/v) and was filtered through Whatman No. 1 filter paper to remove any undissolved material. The assay consisted of adding 0.5 ml of the G-250 dye to 5 μl of homogenized RPE membranes, mixing immediately, and determining absorbance at 620 nm.

**Western blot analysis**

The Gzi1, Gzi2, and Gzi3 protein levels in RPE membranes from 129 Sv, Gzi1-/-, Gzi2-/-, Gzi3-/- and DKO mice, were measured using an anti-Gzi_common antibody (Cell Signaling technology, Danvers, MA) that recognizes all the Gzi and Gzo proteins. Proteins (15 μg per lane) were separated by SDS-PAGE as above on 9% acrylamide/bis-acrylamide gels containing 6 M urea and blotted onto nitrocellulose. Blots were incubated with 1:2,000 anti-Gzi_common polyclonal anti-rabbit primary antibody, overnight, at 4°C and with 1:5,000 goat anti-rabbit secondary antibody for 3 hours at RT. The ECL detection reagents were used to visualize the bands.

**ADP ribosylation of Gzi proteins**

Using the membrane proteins from B6/NCol and Oa1-/- mice we performed ADP-ribosylation as described in Jiang et al. [29,30]. The reaction mixture (30 μl) contained 15 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 mM thymidine, 1 mM ATP, 1 mM guanosine 5’-o-(2-thiodiphosphate) (GDPβS), 2 mM dithiothreitol, 0.17% Lubrol PX, 0.02% bovine serum albumin, 10 μg/ml FITC activated by DTT (50 mM), 5×10^6 cpm ^32P-NAD (≈2×10^6 M) and 15 μg protein of crude RPE membranes. The mixture was incubated at 4°C overnight. The reaction was stopped by addition of 10 μl of complete 2× Laemmli’s sample buffer containing 9% mercaptoethanol and 4 mM unlabeled NAD. The ADP-ribosylated proteins were separated by high resolution urea-SDS-PAGE (9% acrylamide/bis-acrylamide gel containing 6 M urea), overnight, at 44 V. The gel was dried and autoradiographed. Expected bands were observed at 41 kDa for the Gzi1 and Gzi3 and at 40 kDa for the Gzi2 ribosylated proteins.

**Acknowledgments**

Special thanks to Drs. Vittoria Schiaffino and Rosella D’Ambrosio who kindly provided plasmid IC3AD8 (construct containing the i3 of OA1 in the pGEX2T vector) and the purification protocol to compare results with our construct. We would also like to acknowledge Dr. Fei Yu for his assistance with the statistical analysis; Scott Fish and Marcia Lloyd for their valuable initial help and guidance with electron microscopy; and Dr. Bo Wei who kindly helped us reach the number of Gzi2-/- eyes needed.

**Author Contributions**

Conceived and designed the experiments: AY, DBF. Performed the experiments: AY. Analyzed the data: AY BER DBF. Contributed reagents/materials/analysis tools: MJ DBF. Wrote the paper: AY BER DBF. Helped with the design of some experiments and discussion of results: MJ. Prepared Gzi vectors for binding experiments: YM. Contributed with the troubleshooting and discussion of molecular experiments: NBA. Carried out the electrotoretinographs of mice: JR. Conceived, with Gai knockout mice: LB.

**References**

1. King KC, Stanfield WD, Mulligan PK (2006) A Dictionary of Genetics, Seventh Edition Oxford University Press.
2. Oetting WS, Summers CG, King RA (1994) Albinism and the associatedocular defects. Meth Pediatr Syst Ophthalmol 17: 5–9.
3. Garner A, Jay BS (1980) Macromelanosomes in X-linked ocular albinism. Histopathology 4: 243–54.
4. O’Donnell FE, Jr., Hambrock GW, Jr., Green WR, Hill WJ, Stone DL (1976) X-Linked ocular albinism. Proc Natl Acad Sci U S A 73: 61–5.
5. Schiaffino MV, Tacchetti C (2005) The ocular albinism type 1 (OA1) protein, in melanosomal biogenesis and optic pathway formation. Invest Ophthalmol Vis Sci 49: 3245–52.
6. Weiss J (1997) G-protein-coupled receptors: molecular mechanisms involved in receptor activation and selectivity of G-protein recognition. FASEB J 11: 346–54.
7. Bourne HR (1993) How receptors talk to trimeric G proteins. Curr Opin Cell Biol 9: 134–42.
8. Schiaffino MV, Baschiratorio C, Pellegrini G, Montalini S, Tacchetti C, et al. (1996) The ocular albimism type 1 gene product is a membrane glycoprotein localized to melanosomes. Proc Natl Acad Sci U S A 93: 9055–60.
9. Gohla A, Klement K, Piekorz RP, Pexa K, vom Dahl S, et al. (2007) An obligatory requirement for the heterotrimeric G protein G33 in the antischlagic action of insulin in the liver. Proc Natl Acad Sci U S A 104: 3003–8.
10. Luttrell LM, Della Rocca GJ, van Biesen T, Luttrell DK, Lefkowitz RJ (1997) Gbetagamma subunits mediate Src-dependent phosphorylation of the epidermal growth factor receptor. A scaffold for G protein-coupled receptor-mediated Ras activation. J Biol Chem 272: 4637–44.
11. Namba T, Sugimoto Y, Negishi M, Irie A, Ushikubi F, et al. (1993) Alternative splicing of C-terminal tail of prostaglandin E receptor subtype EP3 determines G-protein specificity. Nature 363: 166–70.
12. Hayes RE, Luttrell LM, Exum ST, Lefkowitz RJ (1994) Inhibition of G protein-coupled receptor signaling by expression of cytoplasmic domains of the receptor. J Biol Chem 269: 15776–85.
13. Luttrell LM, Ostrowski J, Cotechia S, Kendall H, Lefkowitz RJ (1993) Antagonism of catecholamine receptor signaling by expression of cytoplasmic domains of the receptors. Science 259: 1453–7.
14. Ercolani L, Stosso JL, Boyle JR, Holtzman EJ, Lin H, et al. (1990) Membrane localization of the pertussis toxin-sensitive G-protein subunits alpha i-2 and alpha i-3.
alpha i-3 and expression of a metallothionein-alpha i-2 fusion gene in LLC-PK1 cells. Proc Natl Acad Sci U S A 87: 4635–9.

22. Stow JL, de Almeida JB, Narula N, Holtzman EJ, Ercolani L, et al. (1991) A heterotrimeric G protein, G alpha i-3, on Golgi membranes regulates the secretion of a heparan sulfate proteoglycan in LLC-PK1 epithelial cells. J Cell Biol 114: 1113–24.

23. Sattar AA, Boinpally R, Stromer MH, Jena BP (2002) G(alpha)(i3) in pancreatic zymogen granules participates in vesicular fusion. J Biochem 131: 815–20.

24. Giordano F, Bonetti C, Surace EM, Marigo V, Raposo G (2009) 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. Hum Mol Genet 18: 4530–45.

25. Cortese K, Giordano F, Surace EM, Venturi C, Ballabio A, et al. (2005) The ocular albinism type 1 (OA1) gene controls melanosome maturation and size. Invest Ophthalmol Vis Sci 46: 4358–64.

26. Jiang M, Spicher K, Boulay G, Martin-Requero A, Dye CA, et al. (2002) Mouse gene knockout and knockin strategies in application to alpha subunits of Gi/Go family of G proteins. Methods Enzymol 344: 277–98.

27. Frangioni JV, Neel BG (1993) Solubilization and purification of enzymatically active glutathione S-transferase (pGEX) fusion proteins. Anal Biochem 210: 179–87.

28. Sedmak JJ, Grossberg SE (1977) A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G250. Anal Biochem 79: 544–52.

29. Jiang M, Gold MS, Boulay G, Spicher K, Peyton M, et al. (1998) Multiple neurological abnormalities in mice deficient in the G protein Go. Proc Natl Acad Sci U S A 95: 3269–74.

30. Codina J, Gernet D, Chang KJ, Birnbaumer L (1991) Urea gradient/SDS-PAGE, a useful tool in the investigation of signal transducing G proteins. J Recept Res 11: 587–601.

31. Wylie F, Heimann K, Le TL, Brown D, Rabnott G, et al. (1999) GAIP, a Galphai-3-binding protein, is associated with Golgi-derived vesicles and protein trafficking. Am J Physiol 276: 497–506.

32. Gao B, Gilman AG, Robishaw JD (1987) A second form of the beta subunit of signal-transducing G proteins. Proc Natl Acad Sci U S A 84: 6122–5.