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# Identification of the key residues determining the product specificity of isomerohydrolase

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## Abstract

The efficient recycling of the chromophore of visual pigments, 11-*cis* retinal, through the retinoid visual cycle is an essential process for maintaining normal vision. RPE65 is the isomerohydrolase in retinal pigment epithelium and generates predominantly 11-*cis* retinol (11cROL) and a minor amount of 13-*cis* retinol (13cROL), from all-*trans* retinyl ester (atRE). We recently identified and characterized novel homologs of RPE65, RPE65c and 13-*cis* isomerohydrolase (13cIMH), which are expressed in the zebrafish inner retina and brain, respectively. Although these two homologs share 97% amino acid sequence identity, they exhibit distinct product specificities. Under the same assay conditions, RPE65c generated predominantly 11cROL, similar to RPE65, while 13cIMH generated exclusively 13cROL from atRE substrate. To study the impacts of the key residues determining isomerization product specificity of RPE65, we replaced candidate residues by site-directed mutagenesis in RPE65c and 13cIMH. Point mutations at residues Tyr58, Phe103 and Leu133 in RPE65c resulted in significantly altered isomerization product specificities. Particularly, our results showed that residue 58 is a primary determinant of isomerization specificity, since the Y58N mutation in RPE65c and its reciprocal N58Y mutation in 13cIMH completely reversed the respective enzyme isomerization product specificities. These findings will contribute to the elucidation of molecular mechanisms underlying the isomerization reaction catalyzed by RPE65.

### Keywords

isomerohydrolase; product specificity; visual cycle; 11-*cis* retinoid; 13-*cis* retinoid; site-directed mutagenesis

To maintain normal vision, an efficient recycling of the chromophore (11-*cis* retinal, 11cRAL) of visual pigments is essential. Chromophore recycling, referred to as the retinoid visual cycle, involves multiple enzymes and retinoid-binding proteins in the photoreceptors and retinal pigment epithelium (RPE) (1, 2). The key step of the retinoid visual cycle is the conversion of all-*trans* retinyl ester (atRE) into 11-*cis* retinol (11cROL), which is catalyzed by a single enzyme, isomerohydrolase, in the RPE (3–5). We and other groups independently showed that an RPE-specific protein with apparent molecular mass 65 kDa (RPE65) is the isomerohydrolase in the RPE and catalyzes the conversion of atRE to 11cROL, which is subsequently oxidized to 11cRAL, the chromophore of visual pigments (6–8). Furthermore, we successfully purified the active form of RPE65 and demonstrated

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that purified RPE65 has robust isomerohydrolase activity (9). This study provided solid evidence that RPE65 is the isomerohydrolase in the retinoid visual cycle. Finally, Kiser et al. recently reported the crystal structure of RPE65 (10), which confirmed our previous findings regarding the key residues for the enzymatic activity of RPE65 using structure modeling and site-directed mutagenesis (11–13).

On the other hand, the molecular mechanisms underlying the isomerization from all-*trans* to 11-*cis* retinoids and its isomerization specificity are not well understood. It was reported that an apocarotenoid-15, 15′-oxygenase (ACO), which belongs to the same enzyme family as RPE65, possesses a bent tunnel from the non-polar patch to active center, and that the actual isomerization may occur when the substrate passes through the bent tunnel to the catalytic domain (14). Moreover, Redmond et al. showed that RPE65 produces both 11cROL and 13-*cis* retinol (13cROL) from atRE (15). It was shown that several amino acid residues in the potential "substrate cleft" contribute to the isomerization specificity of RPE65. Specifically, the mutation F103L in canine RPE65 significantly increased 13cROL production, whereas the T147S mutation decreased 13cROL production, compared to wild-type RPE65 (15). These reports suggested that the structure of "bent tunnel" and "substrate cleft" may contribute to its product specificity. However, these two mutations did not completely reverse the product specificity from dominant 11cROL to 13cROL, suggesting that there are other residues contributing to the product specificity of RPE65.

We recently identified and characterized a novel homolog of RPE65, 13-*cis* specific isomerohydrolase (13cIMH), from the zebrafish brain (16). Although 13cIMH belongs to the same isomerohydrolase family as the RPE-specific RPE65 (RPE65a in zebrafish (17)), the gene encoding 13cIMH is located in a different chromosome than that encoding RPE65a, and it generates exclusively 13cROL without any detectable 11cROL (16). It is worth to mention that 13cIMH was previously named "RPE65b" based on its sequence homology to RPE65 (17). Later, we named it 13cIMH, since it generates exclusive 13cROL from atRE substrate in our enzymatic assay (16). In addition to 13cIMH, we have identified another homolog of RPE65, RPE65c, which is expressed in the inner retina of zebrafish, likely in retinal Müller cells and not in the RPE (18). RPE65c generates predominantly 11cROL (72.2 ± 3.0 %) and a minor amount of 13cROL (27.8 ± 3.0 %) from atRE substrate in our *in vitro* assay, similar to the RPE-specific RPE65. Zebrafish is a cone-dominant species with 79% cones and 21% rods based on immunohistochemistry analysis at 7 dpf (19). RPE65c expressed in the inner retina may serve as an alternative isomerohydrolase in the inner retinal visual cycle to meet the high demand for recycling of the chromophore in the cone-dominant retina.

It is known that a number of genes were duplicated due to fish-specific whole-genome duplication (20, 21). It is likely that the two novel homologs of RPE65 (13cIMH and RPE65c) are generated through gene duplication after the separation of fish RPE65 from the ancestral RPE65, since they showed extremely high sequence identity (97%) and located in the same chromosome 8 (18). However, they are encoded by distinct genes, show different tissue distributions and generate distinct isomerization products (16, 18). High sequence identity and distinct product specificities of zebrafish RPE65c and 13cIMH make them ideal molecular models to identify the key residues determining the product specificity of isomerohydrolase.

In the present study, we identified the key residues which determine the product specificity of these isomerohydrolases using site-directed mutagenesis and the *in vitro* isomerohydrolase assays.

## Materials and Methods

### Construction of expression vectors and site-directed mutagenesis

The wild-type (wt) zebrafish 13cIMH, RPE65c and chicken RPE65 were subcloned into cloning and expression vectors as described previously (16, 18, 22). It is noteworthy that the enzymatic activity of RPE65a in zebrafish has not been studied or characterized. At the present time, we know neither its enzymatic kinetics nor product specificity. In contrast, chicken is another cone-dominant species and chicken RPE65 has been cloned, expressed, and its enzymatic activity and product specificity well characterized (9, 22). Therefore, we used chicken RPE65 as a control for product specificity analysis in this study. The site-directed mutations of 13cIMH and RPE65c were generated using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the protocol recommended by the manufacture. The introduced mutations were confirmed by sequencing from both strands using ABI-3730 DNA sequencer (Applied Biosystems, Foster City, CA) and subcloned into an expression vector, pcDNA3.1 (−) (Invitrogen, Carlsbad, CA). Following the sequence confirmation, the expression constructs were purified by QIAfilter Maxi Prep kit (Qiagen, Valencia, CA).

### Plasmid transfection

The expression plasmids of RPE65 homologs and their mutants were transfected into 293A cells using the Fugene 6 transfection reagent (Roche, Indianapolis, IN) following manufacturer's instruction. Forty-eight hours following the transfection, the cells were harvested by cell scraper and rinsed twice with ice-cold PBS. The protein expression and its enzymatic activity were measured by Western blot analyses and *in vitro* activity assays.

### Western blot analysis

Total cellular protein concentrations were measured by Bradford assay (23). Equal amounts of protein (25 μg) were resolved by electrophoresis through 8% Tris-glycine SDS polyacrylamide gel and electrotransferred onto a nitrocellulose membrane. The membrane was blocked with 5% (wt/vol) non-fat dry milk in TBST (Tris-buffered saline with 0.1% Tween-20) for 30 min and subsequently incubated overnight at 4°C with 1:1000 dilution of an anti-RPE65 polyclonal antibody (24). After three washes with TBST, the membrane was incubated for 1.5 hr with 1:6700 dilutions of an HRP-conjugated anti-rabbit IgG antibody (Millipore, Billerica, MA) in TBST containing 1% non-fat dry milk. After four washes with TBST, the bands were detected using Super Signal West Dura Extended Duration Substrate (Pierce, Rockford, IL) or home-made ECL solution (25). As needed, the membrane was stripped in the stripping buffer (Pierce, Rockford, IL) and re-blotted with an antibody specific for β-actin (Sigma-Aldrich, St. Louis, MO) for loading control. The bands (intensity × area) were semi-quantified by densitometry using Fluorochem-Q software (AlphaInnotech, San Leandro, CA), averaged in at least 3 independent experiments.

### In vitro isomerohydrolase activity assay

The plasmids expressing red fluorescence protein (Rfp, as a negative control), wt chicken RPE65 (as a positive control of cone-dominant species), zebrafish 13cIMH, RPE65c and their mutants were separately transfected into 293A cells. The liposome-based isomerohydrolase activity assay was carried out as described previously (9, 16). The peak of each retinoid isomer was identified by high performance liquid chromatography (HPLC) based on its characteristic retention time and the absorption spectrum of each retinoid standard. The isomerohydrolase activity was calculated from the area of the 11cROL and 13cROL peaks. The ratio of the generated 11cROL to the sum of generated 11cROL and 13cROL was expressed as mean ± SD from 3 independent measurements.

### Statistical analysis

The product generated by RPE65 and its mutants were presented as mean ± SD (standard deviation) from 3 independent measurements and evaluated by student t-test. It is considered as "statistically significant" at p-value below 0.05 (*, $p< 0.05$; **, $p<0.01$; ***, $p<0.001$), but above 0.05 at p-value is "non-significant" (n.s, $p>0.05$).

## Results

### Prediction of key residues contributing to product specificity of isomerohydrolases

To predict the candidate residues determining product specificity of the isomerohydrolase, amino acid sequences of chicken RPE65, zebrafish RPE65a, RPE65c and 13cIMH were aligned using "Clustal-W" program in BioEdit (Ibis Therapeutics, Carlsbad, CA). We selected four candidate residues (Asn58, Leu103, Ser133 and Met222) which are specific in 13cIMH and were substituted by their counter parts in RPE65c and chicken RPE65 (Fig. 1).

### Isomerohydrolase activities of RPE65 and its homologs

HEK-293A cells were separately transfected with plasmids expressing chicken RPE65, zebrafish RPE65c and 13cIMH, and cultured for 48 hrs. The protein expression was confirmed by Western blot analysis (Fig. 2A), and the same batches of total cellular proteins were used for the *in vitro* isomerohydrolase activity assay. To avoid experimental variations of retention time in HPLC profile, we performed the isomerohydrolase assay for all of the enzymes at the same time. The HPLC profiles were presented side-by-side to compare the retinoid profiles produced by the three isomerohydrolases (Fig. 2D–F). Chicken RPE65 and zebrafish RPE65c generated predominantly 11cROL with a minor amount of 13cROL (actual values will be shown in Fig. 4, 5 and Table 2), whereas 13cIMH generated exclusively 13cROL. This result is consistent with our previous studies (16, 18).

### Structure model of RPE65 and the location of candidate residues

The structure of bovine RPE65 (PDB accession: 3FSN) was analyzed by a SwissPDB Viewer version 4.01 (http://www.expasy.org/spdbv/; (26)) and displayed the results by a POV-Ray version 3.61 (http://www.povray.org/) (Fig. 3). The candidate residues Tyr58, Phe103 and Leu133 are located in the random coils, which may be close to the "hydrophobic tunnel" of substrate or product (10), the "substrate cleft" that determines the isomerization product specificity of RPE65 (15), and Lys222 is located in α helix-4. The disordered segment (Phe109-Val126 (10); pink colored dotted-line in Fig. 3) may be responsible for its membrane association, since this segment contains palmitylated Cys112 residue (10, 27). The straight-line distances of the four residues to an iron in the predicted catalytic domain are Tyr58 (19.15Å), Phe103 (14.56 Å), Leu133 (10.79 Å) and Lys222 (25.91 Å), suggesting that these four residues are likely too far from the catalytic center to directly impact the hydrolysis and/or isomerization of substrate. These residues, however, can directly or indirectly alter the structure of "hydrophobic tunnel" and "substrate cleft" to affect its product specificity.

### The impacts of the candidate residues on the product specificity of RPE65c

To analyze the impacts of the candidate residues on product specificity of the isomerohydrolase, Tyr58, Phe103, Leu133 and Lys222 residues in zebrafish RPE65c were substituted by their respective counterparts of 13cIMH (Asn58, Leu103, Ser133 and Met222) using site-directed mutagenesis. Wt RPE65c and its mutants (Y58N, F103L, L133S and K222M) were expressed in 293A cells by plasmid transfection. The protein expression and its enzymatic activities were confirmed by Western blot analysis and *in vitro* isomerohydrolase activity assay. The isomerization product specificity and efficiency of

isomerization (pmol/hr) of all the tested wt enzymes and its mutants were summarized in Table 2. As a positive control, wt chicken RPE65 generated 87.3 ± 3.9 % of 11cROL and 12.7 ± 3.9 % of 13cROL. Similarly, zebrafish RPE65c generated 72.2 ± 3.0 % of 11cROL and 27.8 ± 3.0 % of 13cROL. As shown by the *in vitro* isomerohydrolase assay, the mutant Y58N of RPE65c generated exclusively 13cROL, same as wt 13cIMH. The F103L and L133S mutations substantially increased production of 13cROL (F103L, 95.7 ± 1.1 %; L133S, 71.3 ± 1.1 %), whereas the K222M mutation showed less of an effect on increasing 13cROL generation (55.1 ± 1.2 %), compared to the F103L and L133S mutations. Interestingly, the double mutant Y58N+F103L of RPE65c generated predominantly (74.1 ± 0.5 %) 11cROL, which is similar to that of wt RPE65 and RPE65c (Fig. 4B and Table 2).

### The reciprocal mutations in 13cIMH and their impacts on its isomerization specificity

To study the impacts of the selected residues on product specificity of RPE65, the reciprocal mutants of 13cIMH (N58Y, L103F, S133L and M222K) were generated. The expression plasmids of wt 13cIMH and these mutants were separately transfected into 293A cells. Their protein expression and enzymatic activities were measured by Western blot analysis (Fig. 5A) and the *in vitro* isomerohydrolase activity assay, respectively. As shown in Figures 2F and 5B, wt 13cIMH exclusively generated 13cROL, whereas point-mutations of 13cIMH, N58Y and L103F dramatically increased 11cROL (N58Y, 71.3 ± 2.4 %; L103F, 37.5 ± 2.6 %) while decreasing 13cROL generation. The S133L mutant showed a slight increase (5.6 ± 1.4 %) of 11cROL generation (Fig. 5B and Table 2). Particularly, the single mutation N58Y in 13cIMH completely reversed its product from exclusively 13cROL to a mixture of 11cROL and 13cROL (11cROL:13cROL=71.3:28.7), at an 11cROL to 13cROL ratio similar to that in wt chicken RPE65 (11cROL:13cROL=87.3:12.7) and RPE65c (11cROL: 13cROL=72.2:27.8). On the other hand, the single mutation M222K in 13cIMH, which is located far from the catalytic center in the RPE65 structure model showed no effect on product specificity, which generated exclusively 13cROL. Interestingly, a double mutant of N58Y and L103F showed an identical product specificity as wt 13cIMH (Fig. 5B and Table 2), although each of the single mutations N58Y and L103F in 13cIMH substantially increased 11cROL production.

## Discussion

RPE65 in the RPE was previously known as the only enzyme that generates 11cROL from all-*trans* retinoids (28). Recently, we have identified a homolog of RPE65, RPE65c, in the inner retina of zebrafish and showed that RPE65c is another isomerohydrolase that converts atRE to predominantly 11cROL with a minor amount of 13cROL, a product profile similar to that of the RPE-specific RPE65 (16). This is the first isomerohydrolase in the retina which generates 11cROL in vertebrates and thus, is suggested to participate in the generation of additional 11-*cis* retinoids to meet the high demand of chromophore in the cone-dominant retina in zebrafish. Interestingly, we have identified another isomerohydrolase, 13cIMH, which is encoded by a distinct gene in the same chromosome as that of RPE65c in zebrafish. Although 13cIMH shares 97% sequence identity with RPE65c, it generates exclusive 13cROL from the same substrate, atRE (16, 18). These features of these RPE65 homologs make them ideal models to study the structural basis for the product specificity of isomerohydrolase. Using site-directed mutagenesis and isomerohydrolase assay, the present study for the first time identified key residues responsible for the different isomerization products generated by these highly homologous enzymes.

In the present study, we predicted the key residues contributing to different product specificities of isomerization by amino acid sequence comparisons between these enzymes (see Fig. 1) and examined if the substitutions of the predicted four residues in RPE65c result in altered products. All of the four single mutations in RPE65c (Y58N, F103L, L133S and

K222M) significantly increased 13cROL production while decreasing 11cROL production (see Fig. 4B and Table 2), suggesting that these residues exert significant impacts on determining the isomerization specificity of products.

Among the four residues identified, our results showed that residue 58 plays a crucial role in determining the isomerization at the 11 or 13 bond of atRE. Substitution of Tyr58 in RPE65c with Asn residue, the counterpart of 13cIMH, completely reversed the isomerization product from predominantly 11cROL to exclusively 13cROL, a profile identical to that of wt 13cIMH. On the other hand, reciprocal mutation in 13cIMH, N58Y, also completely reversed the product to predominantly 11cROL, similar to that of RPE65c. These results indicate that the amino acid residue at 58 is the primary determinant of its isomerization specificity to generate either 11-*cis* or 13-*cis* retinol, at least in the zebrafish RPE65 homologs. Interestingly, an additional mutation at residue 103 in the Y58N mutant of RPE65c and in the N58Y mutant of 13cIMH (double mutants; Y58N+F103L in RPE65c and N58Y+L103F in 13cIMH) reversed these phenotypic changes induced by the single mutations at residue 58 to that of wt enzymes (Fig. 4 and 5). These results suggest that the additional mutation may reverse the tunnel structure in the mutant to that of wt.

Recently, Redmond et al. experimentally showed that a replacement of Phe103 by Leu residue (a F103L mutant) in canine RPE65 resulted in an approximately 2-fold increase in its 13cROL production compared to wt RPE65 (15). Actually Phe103 is conserved in all known RPE-specific RPE65 from different species, and is substituted by a Leu residue in zebrafish 13cIMH, suggesting that Leu103 in 13cIMH may contribute to its exclusive 13cROL production. Here, we examined the impact of residue 103, and the result showed that F103L in RPE65c greatly increased its 13cROL production compared to that of wt RPE65c (see Fig. 4B), consistent with the observation by Redmond's group (15). Likewise, a reciprocal mutant of 13cIMH at the same position, L103F, showed significantly increased 11cROL production, compared to that of wt 13cIMH (see Fig. 5B). Unlike the N58Y mutant of 13cIMH, L103F did not completely change the products of 13cIMH to those of RPE65c and RPE65 (Fig. 5B). This result suggests that the residue at 103 exhibits significant impacts on isomerization specificity, but it is not the primary residue determining isomerization specificity. Similarly, our results showed that residues at 133 and 222 play minor roles in determining isomerization specificity, since L133S and K222M mutants of RPE65c both showed significant increases of 13cROL production, whereas the reciprocal mutants of 13cIMH at the same positions, S133L and M222K, exhibited minor or no effects on 11cROL production.

Based on the 3-D structure model of RPE65, all of the four residues identified here are not in close proximity to the catalytic domain or the iron-binding site. Instead, they are located relatively close to the bent hydrophobic tunnel and substrate cleft, through which the substrate reaches the catalytic center. Our results suggest that the structures of the bent tunnel and substrate cleft are crucial for determining its product specificity. It is likely that isomerization of the substrate (atRE) in RPE65 occurs when substrate reaches the catalytic domain, similar to that in apocarotenoid-15, 15′-oxygenase (ACO) (14).

An efficient visual cycle to regenerate chromophore (11cRAL) of visual pigments is crucial for maintaining normal vision. It has been shown that depletion of 11cRAL in the retina causes vision loss as well as cone opsin mislocalization and accelerates photoreceptor death, whereas supplementation of 11cRAL recovered vision and normalizes opsin localization (29, 30). RPE65 is a key enzyme in the visual cycle and is essential for regenerating 11cRAL. Numbers of non-sense and missense mutations in RPE65 have been reported to impair vision by disrupting the visual cycle (31–33). Despite the significance of the efficient regeneration of 11cRAL, the molecular mechanism by which RPE65 generates 11cROL

from atRE has not been well understood. The present study has identified the key residues contributing to the generation of 11cROL, a precursor for 11cRAL. These findings will contribute to the understanding of the structure and function of RPE65. Furthermore, identification of key residues determining the product specificity will also contribute to the elucidation of the mechanism for isomerization of atRE to 11cROL catalyzed by RPE65.

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## Abbreviations

| | |
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| **11cRAL** | 11-*cis* retinal |
| **RPE** | retinal pigment epithelium |
| **atRE** | all-*trans* retinyl ester |
| **11cROL** | 11-*cis* retinol |
| **RPE65** | RPE-specific 65 kDa protein |
| **13cROL** | 13-*cis* retinol |
| **ACO** | apocarotenoid-15, 15′-oxygenase |
| **13cIMH** | 13-*cis* isomerohydrolase |
| **Wt** | wild-type |
| **RFP** | red fluorescent protein |
| **HPLC** | high performance liquid chromatography |

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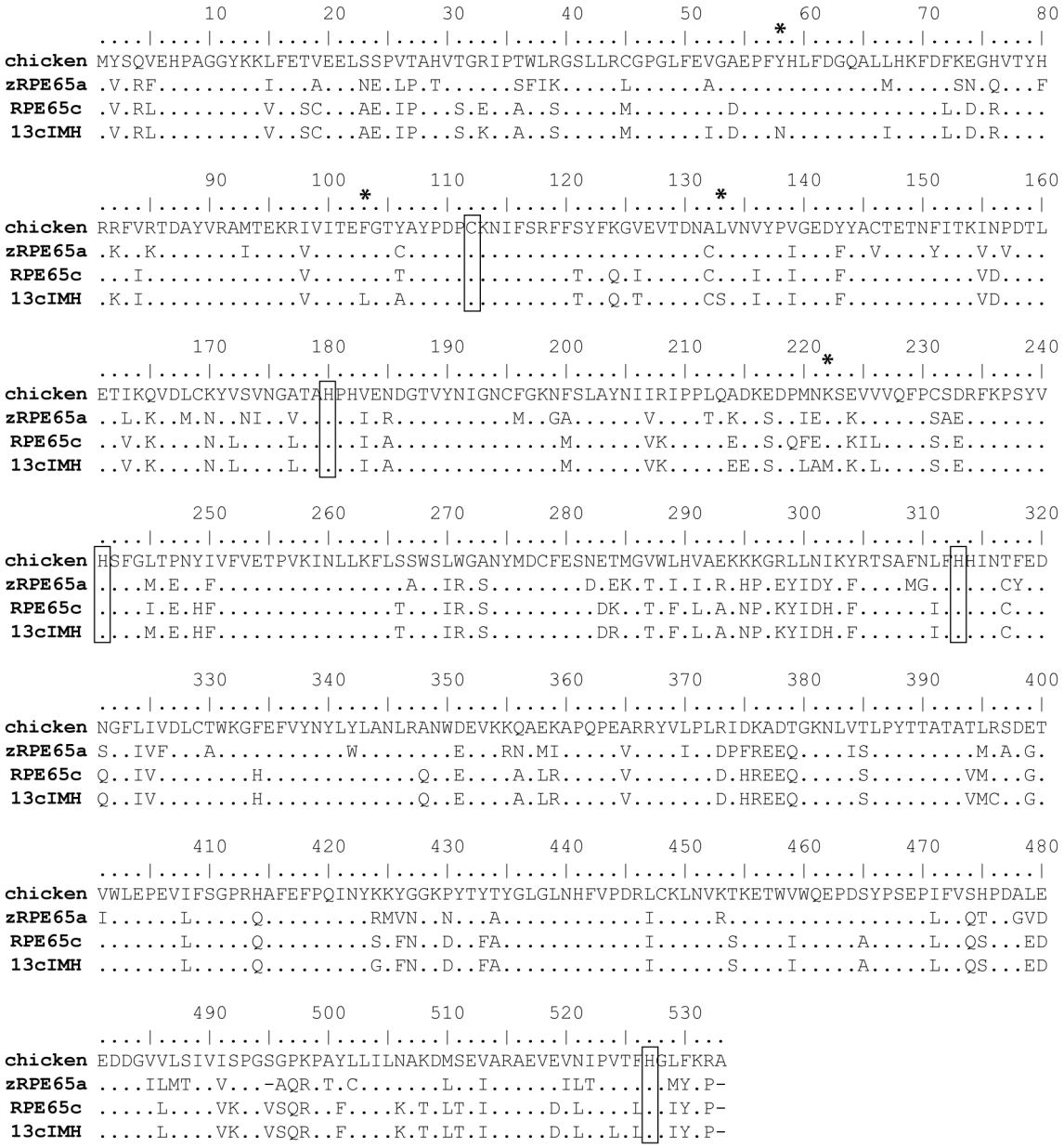



**Figure 1. Amino acid comparison of RPE65 and its homologs**

Amino acid sequences of chicken RPE65, zebrafish RPE65 (zRPE65a), RPE65c and 13cIMH were aligned. Amino acid residues identical to chicken RPE65 are indicated by dots ".", while only the different residues are shown. The known key residues (four His residues forming the iron binding site and a palmitylated Cys residue (7, 11, 27)) were boxed. The four amino acid residues in 13cIMH (at position 58, 103, 133 and 222) which may contribute to exclusive 13cROL production of 13cIMH were indicated by stars "*".

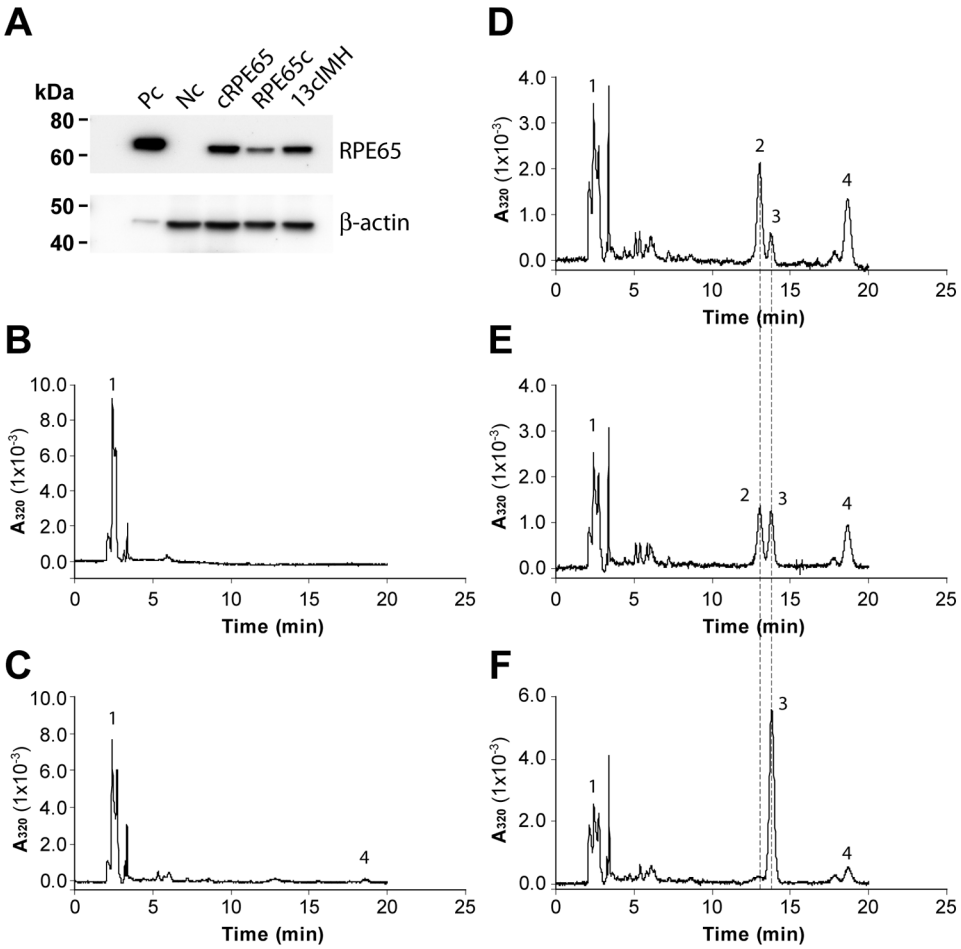
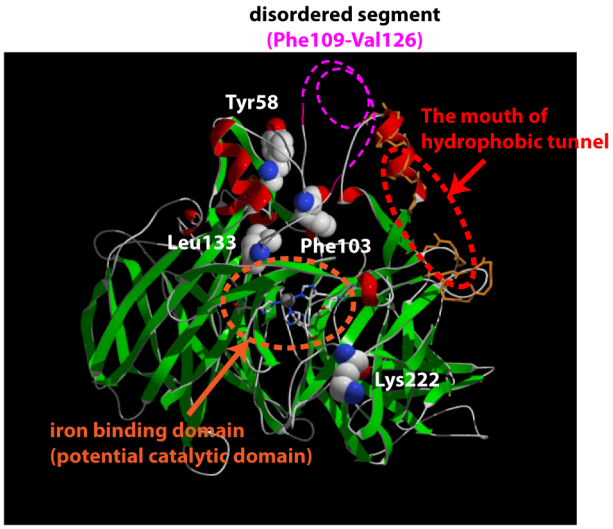



**Figure 2. HPLC profiles showing products from the isomerohydrolase assay of RPE65 and its homologs**

The 293A cells were separately transfected with the plasmids expressing wt chicken RPE65, zebrafish RPE65c and 13cIMH, and cultured for 48 hrs. The cells were harvested, and the cell lysate was incubated with liposomes containing atRE (250 μM lipids, 3.3 μM atRE) for 1 hr at 37°C, and the generated retinoids were analyzed by HPLC. Protein expression was confirmed by Western blot analysis **(A), Pc**, positive control (2.5 μg of bovine microsomal fraction); **Nc**, negative control (25 μg of total cellular protein of 293A cell expressing Rfp); **Chicken**, chicken RPE65. The HPLC profiles are presented as follows: **(B)** without cell lysate (only atRE incorporated in the liposome); **(C)** negative control cell lysate (atRE-liposome and cell lysate expressing Rfp); **(D)** wt chicken RPE65; **(E)** zebrafish RPE65c; **(F)** 13cIMH. The peaks were identified as follows: **1**, retinyl esters; **2**, 11cROL; **3**, 13cROL; **4**, atROL.




| Residue | Distance (Å) carbon-α to iron-II |
|---|---|
| Tyr58 | 19.15 |
| Phe103 | 14.56 |
| Leu133 | 10.79 |
| Lys222 | 25.91 |

**Figure 3. The locations of unique residues in the 3-D structure of RPE65**

The locations of the four residues are shown in the model of crystal structure of bovine RPE65 (PDB accession: 3FSN). The iron-binding site which may be a part of the catalytic domain is indicated by an orange dotted circle. Tyr58, Phe103 and Leu133 which exhibited impacts on the isomerization specificity are located in the random coils, which may be a part of “substrate cleft” or “hydrophobic tunnel” contributing to the isomerization specificity of RPE65 (15) are highlighted by a broad pink line. The disordered segment (Phe109-Val126), which contains a palmitylated Cys residue (Cys112) that may be responsible for its membrane association, is shown by a pink dotted-line (10, 27). The potential mouth of substrate entry/product exit of the hydrophobic tunnel (10) is indicated by a red dotted-line. The Lys222 residue which exerted minor effect on the isomerization specificity is located in α helix-4.

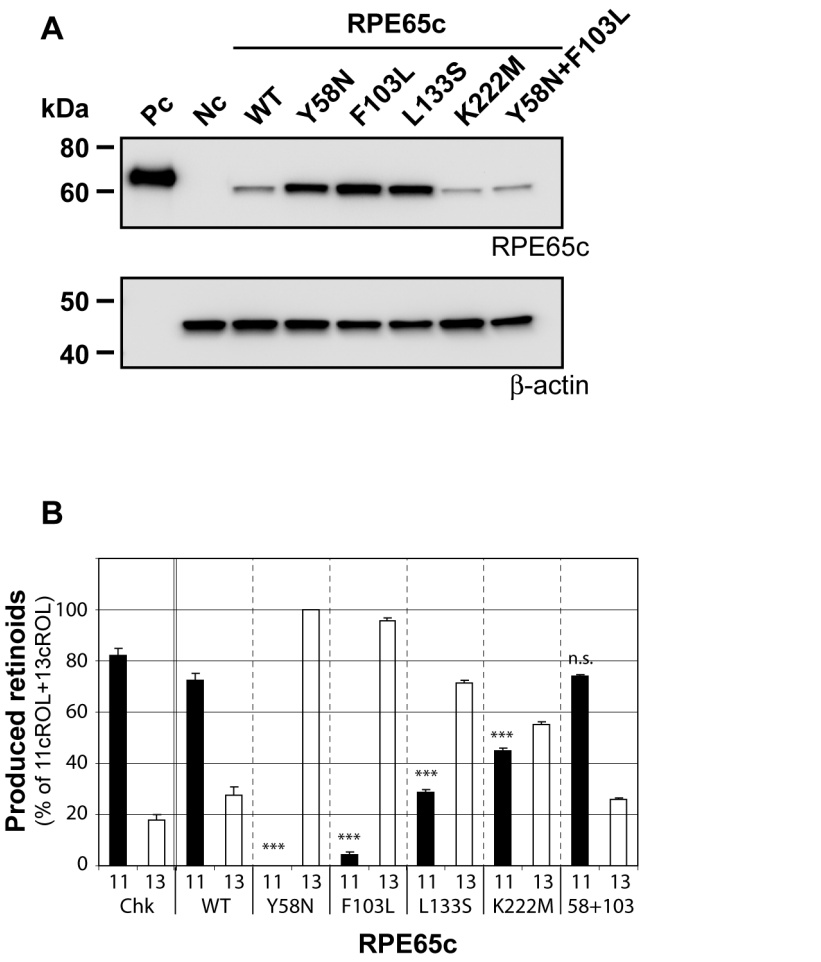



**Figure 4. Site-directed mutagenesis of zebrafish RPE65c and the impacts on its isomerization specificities**

Four candidate residues in RPE65c were replaced by their respective counter parts in 13cIMH. (**A**) The expression plasmids were separately transfected into 293A cells, and the protein expression was confirmed by Western blot analysis. **Pc**, positive control (2.5 μg of bovine microsomal fraction); **Nc**, negative control (25 μg of total cellular protein of 293A cell expressing Rfp); **WT**, wild-type RPE65c; **Y58N**, mutant Tyr58 of RPE65c replaced by Asn; **F103L**, **L133S**, **K222M** and **58+103** (the double mutant of RPE65c containing Y58N and F103L). Equal amounts of total cellular proteins (125 μg) from the cells expressing zebrafish RPE65c and its mutants were incubated with liposomes containing atRE (250 μM lipids, 3.3 μM atRE) for 1 hr at 37°C, and the generated retinoids were analyzed by HPLC. The produced 11cROL and 13cROL were separately quantified from the areas of the 11cROL and 13cROL peaks, respectively (mean ± SD, n = 3). (**B**) The produced retinoids by wt chicken RPE65 (**Chk**), RPE65c and its mutants were expressed as the percent of produced 11cROL and 13cROL (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s, $p > 0.05$).

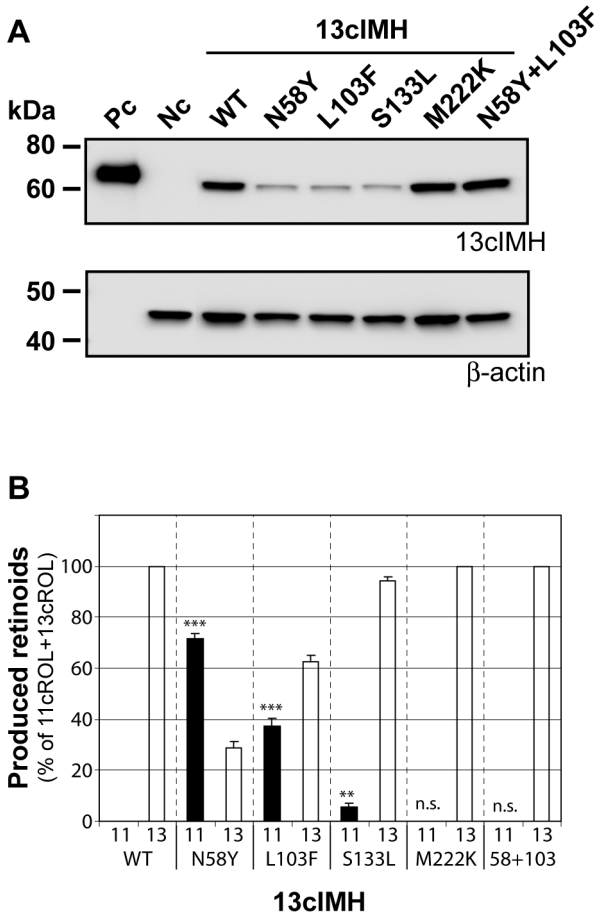



**Figure 5. The reciprocal mutants of zebrafish 13cIMH and the impacts on its isomerization specificities**

Similar to the RPE65c mutants, the reciprocal mutants of 13cIMH for the four candidate residues were generated and separately expressed in 293A cells. (**A**) The protein expression was confirmed by Western blot analysis. **Pc**, positive control (BMF); **Nc**, negative control (Rfp); **WT**, wild-type 13cIMH, and **58+103** (a double mutant of 13cIMH containing N58Y and L103F). Equal amounts of total cellular proteins (125 μg) from the cells expressing zebrafish 13cIMH and its mutants were incubated with liposomes containing atRE for 1 hr at 37ºC, and the generated retinoids were analyzed by HPLC. The produced 11cROL and 13cROL were separately quantified from the area of the 11cROL and 13cROL peaks, respectively (mean ± SD, n = 3). (**B**) The produced retinoids by 13cIMH and its mutants were expressed as the percent of the produced 11cROL and 13cROL (* $p < 0.05$; ** $p<0.01$; *** $p<0.001$; n.s, $p>0.05$).

**Table 1**

The primer sets for site-directed mutagenesis in this study.

| Primer names | Sequences |
|---|---|
| RPE65c Y58N Fwd | 5′-GAGATGAACCATTTAATCATCTTTTTGATGGCC-3′ |
| RPE65c Y58N Rev | 5′-GGCCATCAAAAAGATGATTAAATGGTTCATCTC-3′ |
| RPE65c F103L Fwd | 5′-GTTGTGATCACAGAGCTTGGCACCACTGCATATC-3′ |
| RPE65c F103L Rev | 5′-GATATGCAGTGGTGCCAAGCTCTGTGATCACAAC-3′ |
| RPE65c L133S Fwd | 5′-GTGACAGACAATTGCTCTGTAAACATTTACCC-3′ |
| RPE65c L133S Rev | 5′-GGGTAAATGTTTACAGAGCAATTGTCTGTCAC-3′ |
| RPE65c K222M Fwd | 5′-CCGATCAGTTTGAGATGTCAAAGATTTTGG-3′ |
| RPE65c K222M Rev | 5′-CCAAAATCTTTGACATCTCAAACTGATCGG-3′ |
| 13cIMH N58Y Fwd | 5′-GAGATGAACCATTTTACCATCTTTTTGATGGCC-3′ |
| 13cIMH N58Y Rev | 5′-GGCCATCAAAAAGATGGTAAAATGGTTCATCTC-3′ |
| 13cIMH L103F Fwd | 5′-GTTGTGATTACAGAATTCGGCACCGCTGCATATC-3′ |
| 13cIMH L103F Rev | 5′-GATATGCAGCGGTGCCGAATTCTGTAATCACAAC-3′ |
| 13cIMH S133L Fwd | 5′-GTTACAGACAACTGTCTTGTAAACATTTACCC-3′ |
| 13cIMH S133L Rev | 5′-GGGTAAATGTTTACAAGACAGTTGTCTGTAAC-3′ |
| 13cIMH M222K Fwd | 5′-CTGATCCACTTGCGAAGTCAAAGGTTTTGG-3′ |
| 13cIMH M222K Rev | 5′-CCAAAACCTTTGACTTCGCAAGTGGATCAG-3′ |

**Table 2**

Product isomerization specificities of RPE65 and tested mutants in this study.

| | 11cROL (%) | 13cROL (%) | 11cROL (pmol/hr) | 13cROL (pmol/hr) | % of substrate isomerization* |
|---|---|---|---|---|---|
| Wt chicken | 87.3 ± 3.9 | 12.7 ± 3.9 | 43.1 ± 13.2 | 6.1 ± 1.9 | 7.5 |
| Wt RPE65c | 72.2 ± 3.0 | 27.8 ± 3.0 | 24.8 ± 8.8 | 9.3 ± 2.6 | 5.2 |
| Y58N | 0 | 100 | n.d. | 51.6 ± 2.9 | 7.8 |
| F103L | 4.3 ± 1.1 | 95.7 ± 1.1 | 1.6 ± 0.3 | 35.1 ± 2.0 | 5.6 |
| L133S | 28.7 ± 1.1 | 71.3 ± 1.1 | 7.3 ± 0.5 | 18.2 ± 1.4 | 3.9 |
| K222M | 44.9 ± 1.2 | 55.1 ± 1.2 | 13.9 ± 1.0 | 17.1 ± 0.4 | 4.7 |
| Y58N+F103L | 74.1 ± 0.5 | 25.9 ± 0.5 | 30.2 ± 1.3 | 10.6 ± 0.3 | 6.2 |
| Wt 13cIMH | 0 | 100 | n.d. | 48.4 ± 7.1 | 7.3 |
| N58Y | 71.3 ± 2.4 | 28.7 ± 2.4 | 11.2 ± 3.2 | 4.4 ± 0.9 | 2.4 |
| L103F | 37.5 ± 2.6 | 62.5 ± 2.6 | 8.1 ± 1.3 | 13.5 ± 1.0 | 3.3 |
| S133L | 5.6 ± 1.4 | 94.4 ± 1.4 | 1.2 ± 0.3 | 20.4 ± 1.1 | 3.3 |
| M222K | 0 | 100 | n.d. | 45.9 ± 6.7 | 7.0 |
| N58Y+L103F | 0 | 100 | n.d. | 41.1 ± 0.7 | 6.2 |

*Each reaction (200 μL) contains 660 pmol of atRE (3.3 μM atRE) and “percent of substrate isomerization in 1 hr” was the sum of 11cROL and 13cROL production divided by amount of atRE substrate. “n.d.” indicates non-detectable.

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