Appendix 1. Supplemental methods for:

REGULATION OF THE HUMAN TYROSINASE GENE IN THE RPE CELLS: THE SIGNIFICANCE OF TRANSCRIPTION FACTOR OTX2 AND ITS POLYMORPHIC BINDING SITE

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METHODS

Cloning of the human tyrosinase promoter and construction of promoter deletions. To assess the role of known and putative DNA elements (deduced in studies with melanoma cells and transgenic mice) that are responsible for expression of tyrosinase mRNA in the RPE, we cloned various fragments of the human *tyrosinase* gene promoter (Genbank NT_008984.17) from genomic DNA. First, we cloned the fragment -1995/+74 which contains MITF binding sites *M* (-104 to -94) and *E boxes* (-12 to -7) in the proximal promoter and the distal element *TDE* (-1861 to -1842) [1]. We also cloned a DNA region which was highly homologous to the mouse *tyrosinase* gene (-2525 to -1995) and a region corresponding to a mouse distal *enhancer* [2] (-9860 to -8741; termed here Enh; Genbank AY367052) so as to cover as much as possible of the human *tyrosinase* gene promoter for our experiments. Human genomic DNA for cloning was isolated from whole blood (Qiagen DNA kit, Hilden, Germany). The fragment -1995/+74 (designated -1995) was amplified with sequence-specific primers (forward 5´-TTCGAACGCGTAGAAAAGAAT TATG-3´ and reverse 5´-GAGCATTAGATCTC TAGTCCTCACAAG-3´; *Mlu*I and *Bgl*II sites for cloning underlined), and Phusion DNA polymerase (Finnzymes). Amplified PCR products were digested with *Mlu*I and *Bgl*II and cloned into the *Mlu*I/*Bgl*II-digested pGL3-Basic luciferase reporter vector (Promega). Deletions -462/+74 (designated as -462) and -152/+74 (designated as -152) were digested from the -1995 construct with *EcoR*I/*Mlu*I or *Eco147*I/*Mlu*I, respectively, followed by blunting with Klenow fragment (MBI Fermentas) and self-ligation. A promoter fragment -2525/-1817 which was highly homologous to the mouse *tyrosinase* promoter was amplified with specific primers (forward 5´-CTCTACGCGT GTCTGCAAGTAAATAC-3´ containing a *Mlu*I site and reverse 5´- GCAGTGTGTGGGTG AAGAGG-3´). Amplified and digested PCR products were cloned into the -1995 vector that was partially digested with *Nco*I and *Mlu*I, providing the construct -2525/+74 (designated as -2525). The fragment -9860 to -8741 containing the corresponding human sequence to the mouse distal enhancer [2] was amplified with specific primers (forward 5´-TCTCTTGCGGTACCATC ATTTCCTACAC-3´ and reverse 5´-GTCA ACGCGTGTGCCTCCCTTAG-3´) containing *Kpn*I and *Mlu*I sites. The digested PCR product was ligated into *Kpn*I/*Mlu*I digested -2525 vector resulting in a construct designated Enh/-2525.

Preparation of nuclear extracts and EMSA reactions. Nuclear extracts were prepared from ARPE-19 cells cultured on 100 mm plates for 14 days because expression of tyrosinase, OTX2 and MITF mRNAs were elevated at this time (see Figure 1). After washing with ice-cold PBS, cells were scraped into 1-2 mL of ice-cold PBS and centrifuged for 5 min at 800 g at +4°C. The cell pellet was suspended into 200 µL of lysis buffer [20 mM Hepes, pH 8.0, 20 mM NaCl, 0.5% Nonidet P-40, 1.0 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2.0 µg/ml leupeptin], incubated on ice for 5 min and nuclei were spun down for 1 min at 15 100 g. The nuclear pellet was suspended into 40 µL of nuclear buffer [20 mM Hepes, pH 7.9, 25% v/v glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.5% Nonidet P-40, 1.0 mM DTT, 0.5 mM PMSF, 2.0 µg/ml leupeptin], incubated for 30 min on ice with repeated pipetting and

centrifuged for 1 min at 15 100 g. The supernatant was diluted with ice-cold 20 mM Hepes, pH 8.0 (2 volumes) and protein concentration of the nuclear extract was quantified by using Bio-Rad (Hercules, CA) protein assay.

The EMSA binding reactions (20 µl) were separately optimized for MITF and OTX2 probes and contained 5% glycerol, 100 mM KCl, 1.7 mM $MgCl_2$, 200 mM NaCl, 30 mM Hepes (pH 8.0), 0.8 mM DTT, ^{32}P-labeled probe (100,000 cpm) and poly(dI-dC) (Amersham Biosciences, Sunnyvale, CA) (0.5 µg for OTX2; 1.5 µg for MITF). Optimized nuclear extract concentrations were used for each experiment (5 µg for MITF; 10 µg for OTX2). For competition experiments, only 6-fold molar excess of unlabeled probes were added. Supershift assays were performed by using nuclear extracts from ARPE-19 cells (5 µg) cultured for 14 days or from D407 cells (0.75 µg) reverse-transfected with Flag-tagged OTX2 vector (Origene). For supershift analyses, 5 µg of specific antibodies against OTX2 [3], Flag-tag or control antibody (RET-P1; NeoMarkers, Fremont, CA) were pre-incubated with the nuclear extract for 2 hours on ice prior to initiation of the EMSA reaction by addition of labeled probe. After 20 minute incubation at room temperature, reactions were loaded on 8% non-denaturing gels and run in 0.5 X TBE buffer for 3 hours at 220-250 V. Emitted energy was collected by exposing gels to Phosphor Storage Screens (Amersham Biosciences) which were scanned with the Typhoon 9400 Scanner (Molecular Dynamics, Sunnyvale, CA).

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