Fluorescence in situ hybridization (FISH)

Chromosome slides for metaphase and interphase FISH analysis were obtained from short term murine fibroblast cultures established from ear explants as described in (Jefferson and Volpi, Methods Mol Biol. 2010; 659:409-26). Briefly, the cells were cultured in Dulbecco’s Modified Eagles Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 1% L-Glutamine at 37°C in a 5 % CO₂ incubator. Three hours before harvesting they were treated with Colcemid (Gibco BRL) at a final concentration of 0.2 μg/ml. They were then resuspended in hypotonic solution (0.034 M KCl/ 0.017 M TriSodium Citrate) at 37°C for 5 minutes and fixed in three changes of 3:1 methanol: acetic acid. For FibreFISH analysis the cells were grown until confluent, then centrifuged at 195g for 5 minutes and resuspended to a density of 2 x 10⁶ cells/ ml in 1 X PBS. 10µl of this solution was dried on a microscope slide. The slides were assembled in a cover-plate holder (Shandon), and the cells were lysed using a Sodium Hydroxide solution (5 volumes 0.07M NaOH with 2 volumes ethanol) and then fixed with methanol.

To identify the chromosome(s) holding the transgenic insertion, a ~39Kb clone (G248P88405H3) for the OPN1LW gene and its upstream region from a human fosmid library was labeled by nick-translation (Abbott Molecular) with Biotin-16-dUTP (Roche) and detected with Texas Red-conjugated Streptavidin (Molecular Probes), and initially co-hybridised with a panel of FITC-labelled mouse chromosome-specific probes (Cambio or Metasystems). Once the chromosome holding the transgenic insertion was identified, the OPN1LW probe was co-hybridised with a number of clones from mouse
fosmid and BAC libraries mapping on the region of interest. Clones were selected for their potential interest on the basis of their cytogenetic band location and gene content. They were labeled by nick-translation (Abbott Molecular) with Digoxigenin-11-dUTP (Roche) and detected using a Rhodamin anti-Digoxigenin antibody (Roche), while in this instance the biotin-labelled transgene was detected with FITC-conjugated Streptavidin (Molecular Probes).

The hybridization procedure followed a standard protocol. Briefly, the genomic probes were ethanol precipitated in a mix of Salmon testis DNA (Gibco BRL), Escherichia coli tRNA (Boehringer) and 3M sodium acetate. They were then dried on a heating block at 60°C with a 50X excess of either Human or Mouse Cot-1 DNA (Gibco BRL) and resuspended at 20 ng/µl in hybridisation solution (50% formamide, 10% dextran sulphate, 2x SSC). The probes were denatured at 72°C for 5 minutes and pre-annealed at 37°C for 30 minutes, before being applied to the denatured slides. The slides were denatured in 70% formamide at 70°C for 2 minutes, quenched in 2xSSC at 4°C and then dehydrated in an ethanol series. Following hybridisation, the slides were washed in 50% formamide at 42°C for 10 minutes and 2 X SSC at 42°C for 5 minutes. The FITC directly labelled mouse chromosome-specific probes (or “paints”) were obtained commercially and hybridized following the manufacturer’s instructions. The slides were mounted with Vectashield (Vector Laboratories) containing 4’, 6-diamidino-2-phenylindole (DAPI) for chromosome counterstaining.
Image capture and analysis were carried out on a CytoVysion system (Genetix) consisting of an Olympus BX-51 epifluorescence microscope coupled to a JAI CVM4+ CCD camera.

**Morphological**

In order to perform a morphological characterization of cone survival, eyes from transgenic and wild type animals were harvested at P80, P140 and P245 time points. Eyes were enucleated and fixed in 4% paraformaldehyde (PFA) for 30 minutes during which time the anterior segment and lens were removed. The posterior segment was cryoprotected overnight at 4°C in 30% sucrose and embedded in OCT. 16µm Cryosections were taken sagittally through the optic disc and immunostained for either SWS opsin (1:1500 dilution; Santa Cruz, SC-14363) or MWS opsin (1:1000 dilution; Millipore, AB5405). Antibody details can be found in table 1 below. Flat-mounted eyes were fixed as above and the retina detached; immunostaining was performed free floating and the primary antibody incubation period extended to 4 days. Cone number was quantified on flat-mounted retinas (n=3 mice per group) by analysis of multiple non-overlapping fields (x20 objective) covering ventral/dorsal and temporal/nasal opsin gradients. Automated quantification of cone number was carried out using ImageJ software. Automated counts were validated and found to be highly consistent with manual counts.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Host, Antibody, Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>ML opsin</td>
<td>Rabbit, primary anti-ML, 1:1000</td>
<td>Millipore (1mg/ml, AB5405)</td>
</tr>
<tr>
<td>Primary anti-ML</td>
<td>Donkey, secondary 555nm, 1:300</td>
<td>Invitrogen (2mg/ml, A-31572)</td>
</tr>
<tr>
<td>S opsin</td>
<td>Goat, primary anti-S opsin, 1:500</td>
<td>SantaCruz (200µg/ml, Sc-14363)</td>
</tr>
<tr>
<td>Primary anti-S</td>
<td>Donkey, secondary 350nm, 1:300</td>
<td>Invitrogen (2mg/ml, A-21081)</td>
</tr>
</tbody>
</table>
ERG

Prior to testing mice were dark adapted for 4 hours. All animal preparation and set-up were conducted under dim red illumination. Mice were anesthetized with a single intraperitoneal injection of Dormitor (medetomidine hydrochloride, 1mg/kg body weight) and Ketamine (60mg/kg body weight). Pupils were dilated with 1% tropicamide. Animals were placed on a heated platform, maintained at 38°C by a circulating pump-water bath. A custom-made silver-coated nylon contact lens active electrode was manually positioned on the study eye. Hypromellose eye drops (0.5% methylcellulose) were used to maintain electrical conductivity and prevent corneal desiccation. Subdermal platinum needle electrodes were placed in the scruff (reference) and at the base of the tail (ground). Prepared animals were positioned inside the Ganzfeld dome of the Espion E2 system (Diagnosys LLC, Cambridge, UK). Stimulus timing, duration and intensity were all controlled by the Espion software (except for the UV-LED intensity, which was controlled manually by a potentiometer). Stimuli were rendered full field through reflection in the Ganzfeld dome or, in the case of the UV-LED array, by proximity of an extended source close to the test eye. All recordings were made in a custom-made, light-tight Faraday cage. ERG signals were differentially amplified and digitized at a rate

<table>
<thead>
<tr>
<th>Primary anti-opsin</th>
<th>Terminal β-galactose (Cone photoreceptor lectin sheath)</th>
<th>Donkey, secondary 555nm, 1:300</th>
<th>Invitrogen (10µg/ml, A-21432)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Arachis hypogaea (peanut), 488nm, 1:100</td>
<td></td>
<td>Invitrogen (L21409)</td>
</tr>
</tbody>
</table>

Table 1. Antibody Details. The blocking agent for opsin staining was Normal Donkey Serum (10% NDS during blocking, 1% NDS antibody incubation). The blocking agent for PNA staining was Bovine Serum Albumin (0.5mg/ml)
of 5 KHz (bandpass filtered 0-100Hz) using the Espion E2 system. Recording sessions began with the dark-adapted flash ERG intensity series, progressed to dark-adapted flicker ERG recording, and concluded with light-adapted flash ERG testing (after at least 10 min exposure to the rod-saturating background).