Appendix

Retinal Ganglion Cell Maturation and Maintenance (RGCM) Media

Most iPSC-derived retinal organoid differentiation protocols, contain a specific window during which the retinoids begin a process of lamination into the prospective inner and outer retinal segments. RGC nerve fiber layers can be seen around day 40 (Figure A1A), and generally undergo degeneration beyond day 60 of differentiation under the most widely used protocols. The degeneration of the RGC layer beyond this time point is generally attributed to the lack of neurotrophic support that would normally arise from RGC innervation of visual centers of the brain (which is obviously lacking in-vitro). We have devised a modified protocol in which, through media supplementation of RGC-specific trophic factors, we can maintain viable RGC nerve fiber layers for much longer periods of time (Figure A1B), and can thus stabilize and mature this cell layer while other retinal layers develop. To initiate early stages of retinal differentiation, we employ the strategy described by Zhong et al (Zhong et al., 2014) up to week five of the protocol. Subsequently, to promote retinal ganglion cell maturation and maintenance, during the sixth week of the differentiation, suspension culture of retinal organoids are switched to an RGC maintenance medium containing DMEM/F12 (3:1), supplemented with Forskolin (10µM), ciliary neurotrophic factor (CNTF, 10ng/ml), brain derived neurotrophic factor (BDNF, 50ng/ml), Y-27632 (10µM) and 1X Glutamax-I. We have been able to maintain our retinal organoids in this RGCM protocol beyond 12 weeks of differentiation while still maintaining a viable nerve fiber layer and RGCs (Figure A1B).

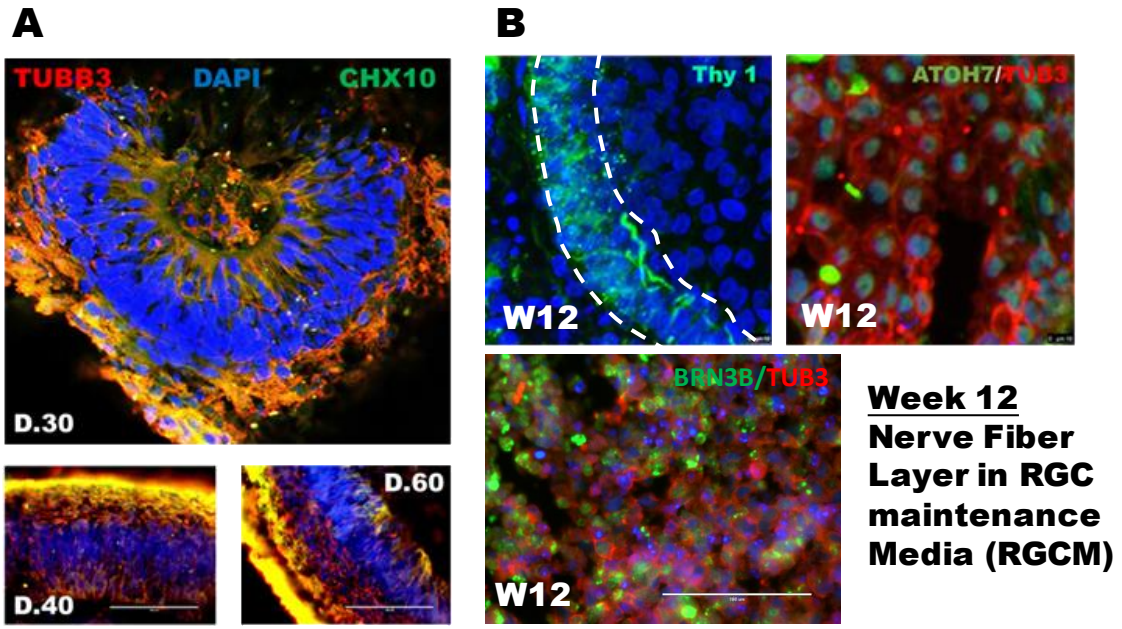

Figure A1: Immunohistochemical images of (A) Progressive lamination of retinal organoids at various developmental stages up to Day 6o of differentiation protocol described by Zhong et al. (B) Nerve fiber layer (outlined by Thy1-positive cells) and RGC-specific markers within this layer are still present at 12 weeks of differentiation when organoids are switched to, and maintained in, RGCM media at week 6 of differentiaiton.

Functional Response to Glutamate Stimulation in iPSC Retinal Organoid-Derived RGC

In order to assess the formation of a functional RGC layer in our organoid differentiation protocol, we isolated organoid-derived RGCs by magnetic bead soring of Thy1-positive cells and performed a standard glutamate stimulation assay on them. Thy1-isolated cells were incubated at room temperature with 20 µM Fluo-4 AM (Life Technologies, Grand Island, NY) in PBS containing 0.1% Pluronic F-127 (Sigma-Aldrich, St. Louis, MO) for 20 min. Following dye loading, the cells were washed with fresh Ca2+ free PBS and further incubated for 25 minutes at room temperature to allow for full acetoxymethyl (AM) ester cleavage.

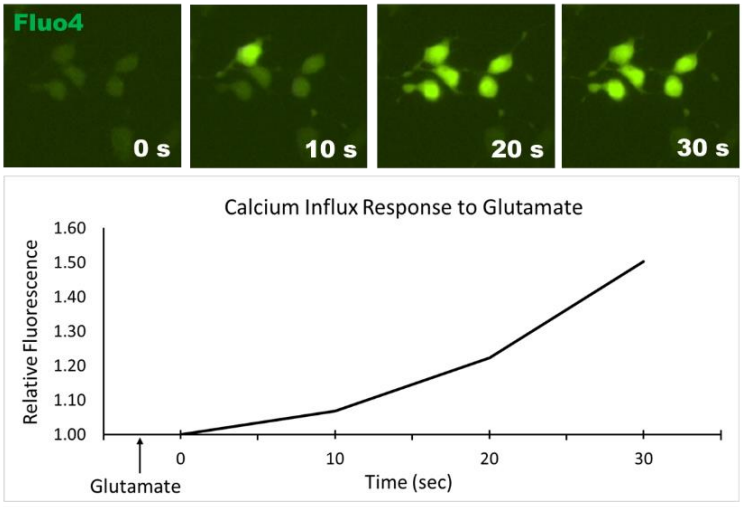

Figure A2: Fluorescent images and quantitation of intracellular calcium influx in response to glutamate stimulation of organoid-derived RGCs isolated by Thy1 immunoreactivity.

Subsequently, 10µM final concentration of glutamate was added to the cells. Cells were imaged using a Nikon Eclipse Ti inverted fluorescent microscopes equipped with a high-resolution monochrome CCD camera. Image sequences were processed using ImageJ. Average fluorescence values of cells in each frame were computed using region of interest (ROI) analysis for each frame. The fluorescent intensities were measured as the mean grey value for the regions and normalized with respect to the averaged fluorescence value of the first frame (ΔF/F0, where ΔF = F-F0). Retinal Organoid-derived RGCs present a functional response to glutamate as assessed by a positive influx of intracellular calcium into the RGCs (Figure A2). We are currently working on the electrophysiological Characterization of these cells at various time points. Using our novel solution, we will be able to establish developmental cues and timeframe for RGC functional maturation.

<u>Establishment of RB1 knockout iPSC cell lines (RB1KO)</u>

Induced pluripotent stem cells (iPSC) cultured in our lab were modified using a CRISPR/Cas9 guide-RNA (gRNA) knockout targeting the retinoblastoma tumor-suppressor gene (RB1). One group of iPS cells was transfected with RB1 gRNA vector 1 (gRNA1 sequence: GCCCAAAACCCCCCGAAAAA) in pCas-Guide vector and a GFP-Puro functional cassette; the other group of cells received the RB1 gRNA vector 2 (gRNA2 sequence: TCGCTCACCTGACGAGAGGC) in pCas-Guide vector and a GFP-Puro functional cassette (Figure A3A). The system is designed to create an excision removal of the first 2 exons of the RB1 gene, and insert a custom-designed cassette through recombination of the right and left homologous arms (LHA and RHA) of the insert (Origene cat # KN206933) (Figure A3A). The modified chromosome 13q14.2 will then contain a Puro selection cassette under constitutive phosphoglycerate kinase (PGK) promoter, and a GFP cassette driven by the native RB1 promoter region (Figure A3A). After 7 days of puromycin selection and cell expansion, western blot analysis was performed to validate the absence of RB1 encoded protein (Figure A3B).

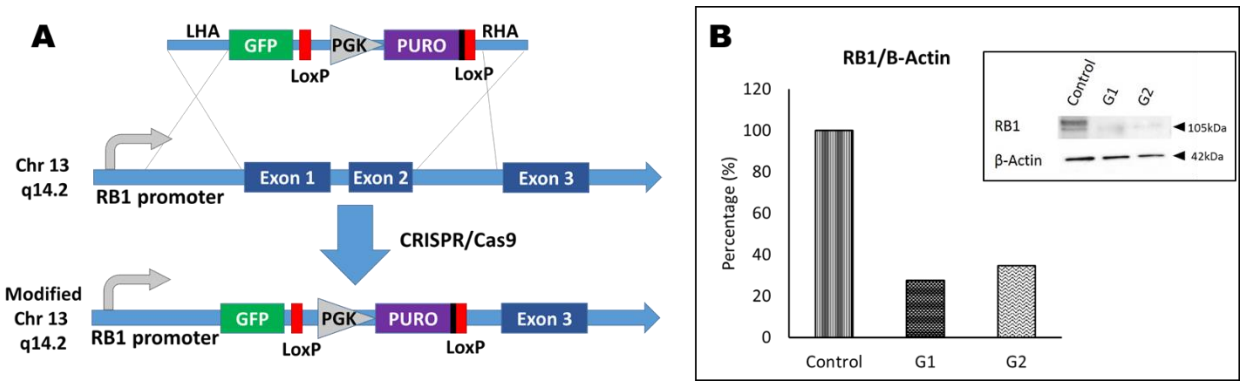

Figure A3: Establishment of RB1-knockout (RB1KO) iPSC cell lines. (A) Schematic of CRISPR/Cas9 system used to create RB1KO lines. (B) Western blot quantitation of RB1 protein in RB1KO cells generated using 2 guide RNA constructs (G1 and G2) showing strong knockout in protein translation.

Initiation of self-organizing retinal cup differentiation of RB1KO iPSCs, was performed following the protocol described in Zhong et al (Zhong et al., 2014) (Figure A4). In this platform, we are able to trace the activation of the RB1 locus at various developmental time point by live, or fixed-imaging of GFP fluorescent RB1KO cells (Figure A4).

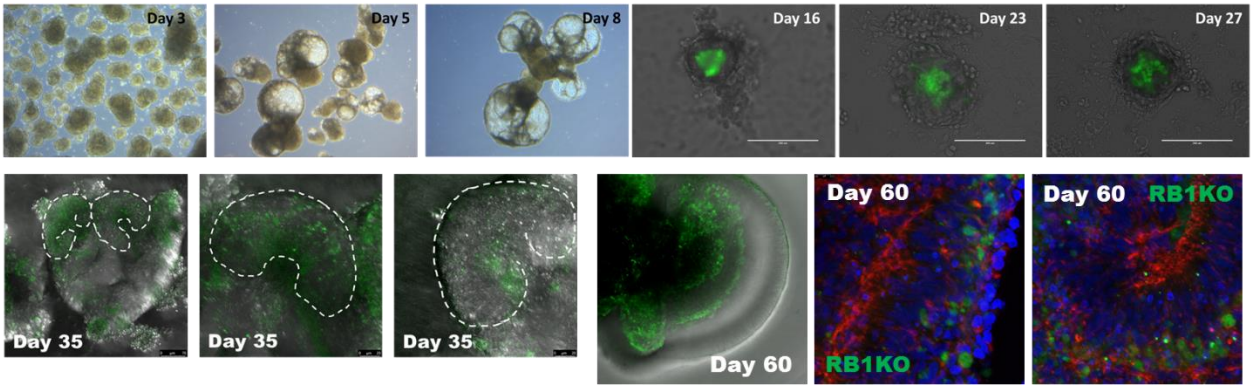

Figure A4: Live cell-fluorescent images of RB1KO cells undergoing retinal organoid differentiation showing developmental stages of RB1 locus activation (GFP) through 60 days of differentiation protocol.

Similarly, we are able to use DIC-Fluorescent imaging in our confocal system to track single cells in the RB1KO retinal organoids for our proposed studies into the cell of origin, and progression of retinoblastoma tumors in developing retinas (Figure A5, arrowheads).

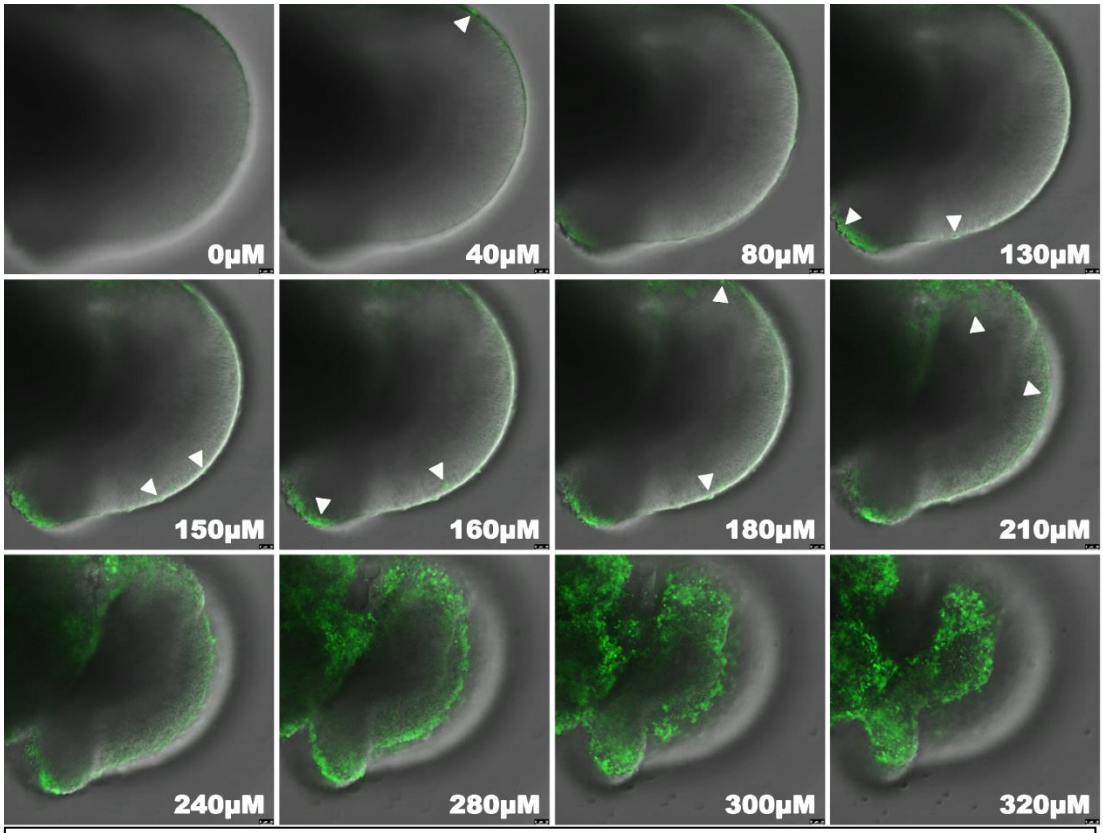

Figure A5:Cross-sectional planes (z-stack) of live-cell imaging of RB1KO retinal organoids at day 60 of differentiation. RB1KO GFP-positive cells can be seen integrated into the developing retinas and can be traced individually (arrowheads) over time in culture.