# AAV-based evaluation of novel *in silico*-based promoters to drive expression in rod photoreceptors

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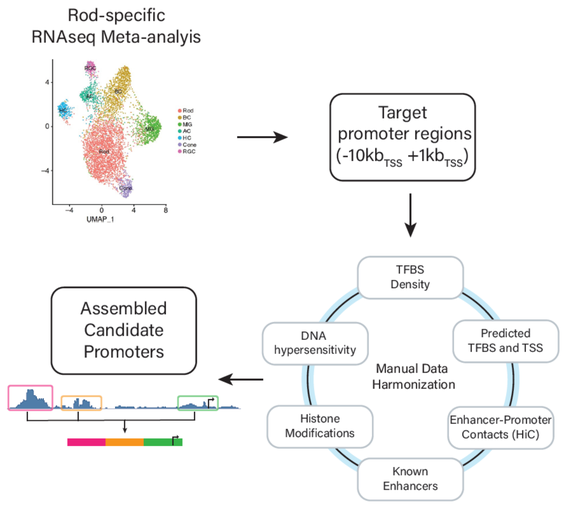
## Introduction:

Overall, rod photoreceptors outnumber cone photoreceptors by a ratio of 1:20 or greater in most mammalian retinas. Defects are common in rods and lead to various ocular diseases. To date, there's been little progress in designing a strong rod-specific promoter derived from human DNA sequences. Therefore, we sought to design and test novel promoters to drive expression specifically and at high expression levels in both mouse and human rods. These would be essential for the success of any future gene therapy strategies targeting rod photoreceptors.

## Methods:

Rod specific promoters were rationally designed by intersecting multiple genomic and epigenomic datasets. Several types of data were used, including DNA accessibility assays, transcription factor binding affinity, histone markers, validated transcription start sites and regions with cis-regulatory elements predicted to contact the promoter of interest. To pinpoint the genomic regions with active promoter activity in rod cells, the analysis was restricted to a 10kb upstream-1kb downstream region of the transcription start sites (TSS) of interest. The selection of TSSs was conducted by cross-referencing current literature and single cell sequencing data from the human retina. Regions harbouring an increased number of regulatory elements were isolated and combined with their target core promoter. Additionally, following an enrichment analysis for transcription factors regulating a set of rod-specific genes (TRANSFAC/MAST), we in-silico determined an optimal set of transcription factor binding sites (TFBS) predicted to have a significant impact in regulating a rod-specific promoter when placed withing the same genomic context.




Using the approach described above, nine novel promoter sequences were designed, cloned into an AAV backbone carrying eGFP and finally packaged into AAV5 or AAV7m8, alongside vectors carrying the commonly used rhodopsin kinase (RK or GRK1) promoter driving eGFP expression. To assess promoter activity in the murine retina, wild type mice received subretinal injections with the AAV5 vectors. Four weeks post vector administration, eyes were harvested for immunohistochemical analysis and qPCR expression analysis to determine specificity and expression levels, respectively. In parallel, AAV7m8 vectors were used to assess promoter activity in human induced pluripotent stem cell (hiPSC-) derived retinal organoids. Three weeks post transduction organoids were dissociated into single cells for flow cytometry analysis or for immunohistochemistry. For murine eyes, sections were stained with Peanut Agglutinin (PNA) which selectively labelled cone photoreceptor cells. Sections of hiPSC retinal organoids were stained with L/M opsin to selectively identify cone populations and assessed for eGFP expression.

## Results: Promoter activity in the murine retina

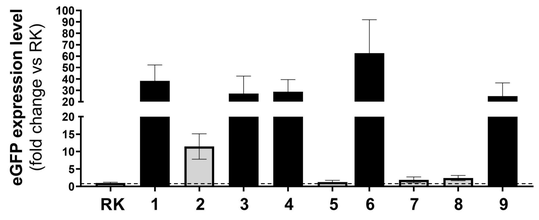



**Figure 1.** qPCR analysis of eGFP expression in mouse retina

Adult WT C57BL/6 mice were subretinally injected (4x10$^9$ VG/eye) with AAV5 vectors expressing eGFP under a range of rod specific promoters. Retinas were dissected 4 weeks post injection and analyzed using on-target qPCR. Data displayed are normalized to eGFP expression levels driven by AAV5 containing an RK promoter (dashed line). The top 5 promoters that exceeded RK expression levels are indicated by black columns, while those excluded from further consideration are represented in grey. Mean +/- SEM (n=4 per group).

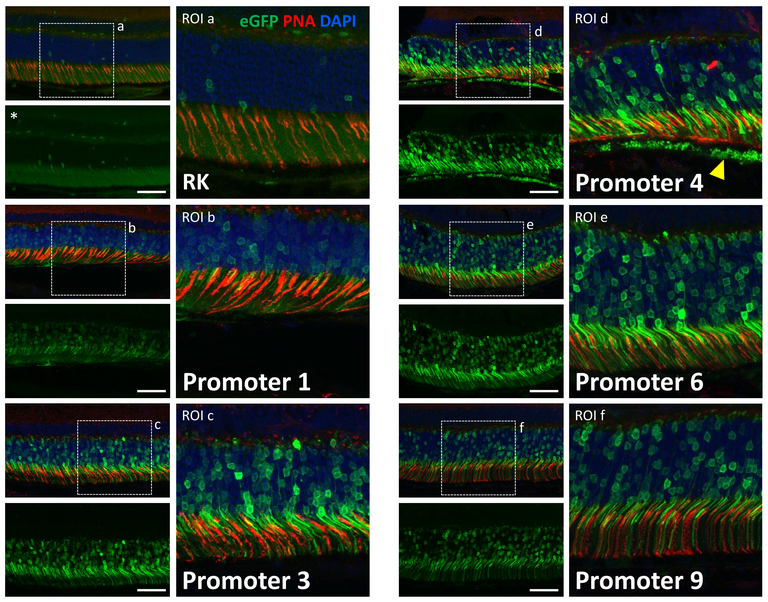



**Figure 2.** Immunohistochemical analysis of eGFP expression in mouse eyes

Mice were treated as described previously, whole eyes were enucleated, fixed in 4% PFA and embedded in OCT. 18µm sections were stained with PNA (cone photoreceptor marker) and DAPI. n=4 per group, representative images are shown. Promoter 4 showed some un-specific transduction in the RPE (yellow arrowhead). Promoters 1, 3, 6 and 9 showed highly specific rod transduction as indicated by the lack of colocalization with a cone-specific marker (PNA). *Note that image acquisition settings were set higher in the eGFP channel for RK group. Scale bar 50µm.

## Results: Promoter activity in hiPSC retinal organoids

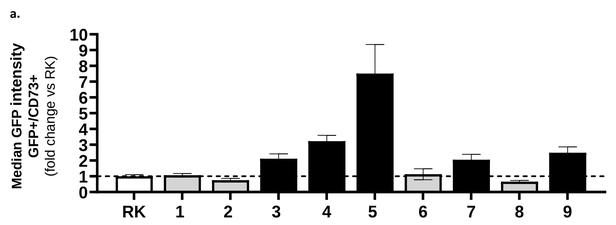



**Figure 3.** Flow cytometric analysis of eGFP intensity in retinal organoids

a. hiPSC-derived retinal organoids (week 21) were transduced with AAV7m8 vectors expressing eGFP under the same rod-specific promoters used *in vivo* at a dose of 1E11 VG per organoid. Three weeks post transduction, organoids were dissociated and stained with photoreceptor specific marker CD73. Median eGFP intensity in eGFP positive/CD73 positive photoreceptors (between 20-61% of CD73 positive cells) was normalized to expression in organoids transduced with RK-eGFP and expressed as a fold change relative to RK (dashed line). n=5 organoids from N=3 experiments. b. Example organoid 3 weeks post transduction with 7m8 promoter 5 eGFP, live imaging and post sectioning. Scale bar 100 µm and inset 10 µm.




**Figure 4.** Immunohistochemical analysis of eGFP expression in hiPSC retinal organoids

Retinal organoids were fixed in 4% PFA and cryosectioned at 7µm. Sections were stained to identify eGFP, L/M opsin and nuclei (DAPI, blue). n=2 per group, representative images are shown. Promoters 3, 4, 5, 7 and 9 showed highly specific rod transduction as indicated by the lack of colocalization with a cone-specific marker (L/M opsin). *Note that image acquisition settings were set higher in the eGFP channel for RK group. Scale bar 40µm.

**Conclusions:** Lead rod-specific promoter candidates were identified on the basis of their expression levels and how they compare to a photoreceptor-specific promoter with a known clinical track record (RK or GRK1 promoter) as well as their specificity for rod photoreceptors (lack of colocalization with a cone-specific marker), determined by immunohistochemistry. In mice, promoter strength was assessed by measuring expression levels using qPCR (Fig.1) and in human retinal organoids expression levels were assessed using flow cytometry (Fig.3).

In the murine retina, four promoters (1, 3, 6 and 9) showed a marked increase compared to the RK baseline in the expression levels and a strong rod-specific tropism (Fig.2) . In human retinal organoids, five promoters (3, 4, 5, 7 and 9) showed a marked increase compared to the RK baseline in the intensity of eGFP fluorescence and a strong rod-specific tropism (Fig.3, 4). Comparing data from both platforms indicates that promoters 3 and 9 provide high levels of specific expression in both mouse and human rod photoreceptors. The differential expression profile of certain candidates highlights the need to perform promoter screens in experimental platforms from multiple species. High performance in both, is critical for achieving translation of treatments from preclinical to clinical setting.

Access to a potent rod-specific gene therapy toolkit allows not only for treatment of defective rods but could also prolong survival of cones that otherwise degenerate due to a by-stander effect.