Parallel Assessment of RGC Regenerative & Neuroprotective Targets

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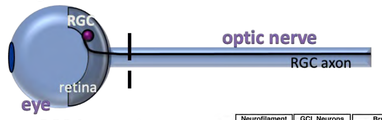
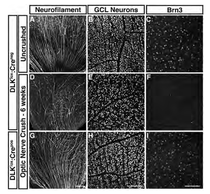
The Need for More Efficient *in vivo* Assessment of RGC Interventions

Insults to retinal ganglion cell (RGC) axons, as occurs in glaucoma, disrupt the connection between the eye and the brain. Restoration of vision will require preservation of these neurons and regeneration of their axons.



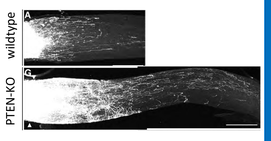
RGC Apoptosis:

Optic axon injury results in the deaths of the majority of RGCs. Though a variety of interventions, such as knockout of the Dual Leucine-zipper Kinase (DLK), can promote varying degrees of neuroprotection, **the *in vitro* discovery and individual *in vivo* validation of each is laborious and costly**, as is investigation of their potential additive effects or other targets in its pathway.



Optic axon regeneration:

Severed optic axons, like other CNS axons, fail to regenerate. Extensive efforts over many years have uncovered multiple interventions, such as PTEN knockout, that stimulate RGC axon regeneration, some of which are additive. **Assessment of optimal combinations and new candidates can require substantial time and resources.**



Images from Watkins *et al.*, *PNAS* 110(10):4039 (2013)

Strategy for Parallel Barcode Readout of Regenerative and Apoptotic Phenotypes

Figure 1. RNA barcode enrichment or depletion as a readout for interventions that promote regeneration or protection against Wallerian degeneration. 100% of wildtype RGC axons undergo Wallerian degeneration following optic nerve crush, depleting the large majority of axonally-targeted RNA barcodes. Barcodes associated with axon regeneration-enabling interventions will be most strongly replenished in the optic nerve just distal to the injury. Axonally-targeted barcodes that protect against axon degeneration will be preserved throughout the nerve and target tissue.

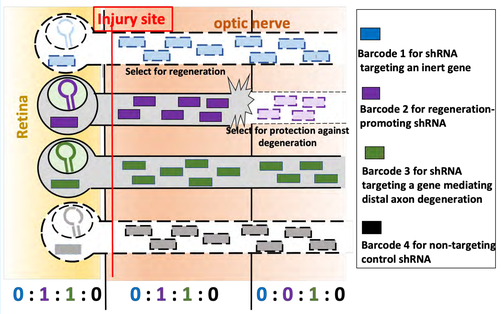
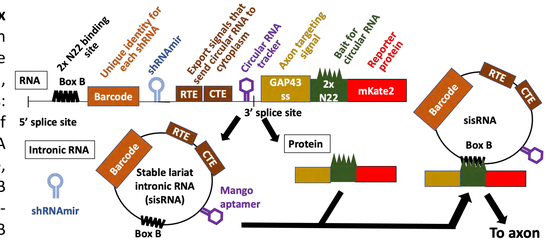


Figure 2. RNA barcode enrichment or depletion as a readout for interventions that promote regeneration or protection against Wallerian degeneration. 100% of wildtype RGC axons undergo Wallerian degeneration following optic nerve crush, depleting the large majority of axonally-targeted RNA barcodes. Barcodes associated with axon regeneration-enabling interventions will be most strongly replenished in the optic nerve just distal to the injury. Axonally-targeted barcodes that protect against axon degeneration will be preserved throughout the nerve and target tissue.

Design of a multi-functional construct for targeting stable RNA barcodes to axons

Figure 3. A protein-RNA complex targeted to axons. Our concise design for AAV packaging generates a single pre-mRNA that, when fully processed, generates three functional components: (1) an shRNAmir targeting a candidate of interest; (2) a stable intronic lariat RNA (sisRNA) harboring an RNA barcode, nuclear export sequences, and BoxB binding sites; and (3) an axonally-targeted protein that tightly binds BoxB to carry the sisRNA down the axon.



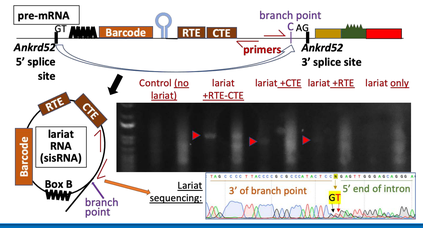
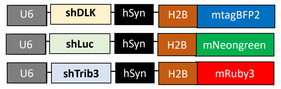


Figure 4. Generation of stable cytoplasmic lariat RNA barcodes using *Ankrd52* splice sites and viral nuclear export sequences. PCR of cytoplasmic and nuclear fractions confirms the presence of stable intronic sequence RNA (sisRNA), aka, lariat RNA, with optimal nuclear export using a combination of both viral sequences CTE and RTE.

Proof-of-principle tests using AAV pools expressing fluorescent proteins

To establish the conditions under which we can generate a mosaic of interventions within the retina and a benchmark by which to assess RNA barcode readouts, we began with proof-of-principle experiments using nuclear-targeted (H2B-fused) fluorescent proteins associated with control (shLuc) or experimental (shDlk and shTrib3) interventions.



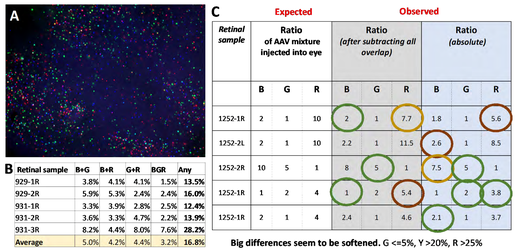
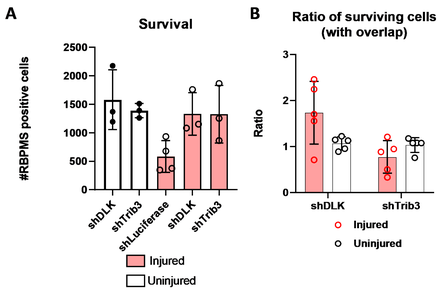


Figure 5. Transduction of RGCs with ratios of pooled AAVs. A first unexpected challenge was the tendency of AAVs within a pool to co-transduce the same few RGCs, even when injected at low titer. **(A-B)** Optimization of buffers and handling reduced aggregation such that less than 17% of RGCs exhibited co-transduction. **(C)** Varying initial transduction ratios among TagBFP (B), mNeonGreen (G), and mRuby3 (R) AAVs demonstrates that expected differences can be detected and quantified.

Figure 6. RGC neuroprotection by classic shRNAs, as assessed by standard and novel methods. (A) Individual AAV at high-titer for expression of shRNA targeting DLK or Trib3 improves RGC survival of RGCs immunostained for RBPMS 2 weeks after optic nerve crush. (B) Pooled AAV results in higher ratio of mTagBFP2, shDLK-expressing neurons to mNeonGreen, shLuciferase-expressing neurons, relative to uninjured control (1:1:1 initial AAV ratio). Imperfect RGC targeting with hSyn1 promoter and reduced shRNA effectiveness at low titer may account for discrepancies in shTrb3 results.



Multiplexed shRNAmir as a tool for combinatorial interventions

A potential use for this system is efficient comparison of various combinations of neuroprotective and axon regenerative interventions. We have therefore optimized the use of sisRNA-localized shRNAmirs for target knockdown to enable within the small AAV packaging size:
(1) RGC-specific knockdown of desired targets
(2) Knockdown of multiple targets at once
(3) Co-expression of "overexpression" interventions

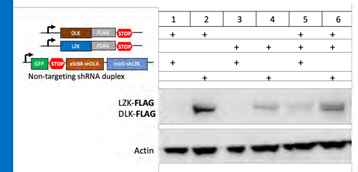


Figure 7. Individual and multiplex knockdown of the injury-responsive MAP kinases DLK and LZK. Expression of multiplexed eSIBR and mir-E-based shRNAmir reduces the expression of FLAG-tagged DLK (lane 1) and LZK (lane 3), or both together (lane 5) relative to non-targeting control (lanes 2, 4, and 6).

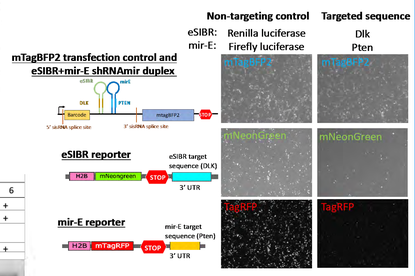


Figure 8. Optimized duplex shRNAmirs for knockdown of multiple targets in the context of sisRNA introns harboring RNA barcodes. mNeonGreen and TagRFP reporters harboring 3'UTR target sequences from genes of interest (Dlk, Pten) were expressing in 293T cells along with sisRNA intronic multiplexed eSIBR- and mir-E-based shRNAmirs.

CONCLUSIONS and NEXT STEPS

- **A strategy for assessing multiple potential RGC regenerative & neuroprotective interventions in parallel and in combination.** We have created a mosaic of RGCs expressing distinct interventions, each associated with a different marker that can be assessed in the neuronal cell body (retina) and in the axon (optic nerve).
- **Proof-of-principle experiments.** As a benchmark for the use of RNA barcodes, we have evaluated a mosaic of different fluorescent proteins to assess how RGC survival assessment compares to traditional approaches.
- **Critical technical improvements for the next generation of pooled AAVs include:**
  - Proper handling of AAV to reduce aggregation and co-transduction within pooled AAVs.
  - The use of FlpO-dependent expression vectors (fDIO) in vGlut2.IRES.FlpO knock-in mice to restrict expression to RGCs.
  - Optimized miRNA-based shRNA backbones (mir-E, eSIBR) for RGC-specific, multiplexed knockdown that can be combined with overexpression interventions for assessment of combinatorial strategies.

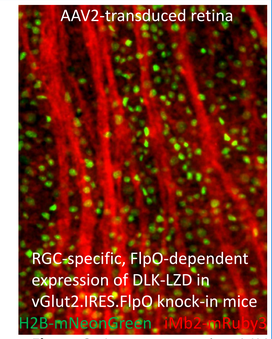


Figure 9. A next-generation AAV expresses dominant-negative DLK along with nuclear mNeonGreen and membrane-targeted mRuby3 to specifically label RGCs (green) and their axons (red) in retinas of vGlut2.IRES.FlpO knock-in mice.

ACKNOWLEDGEMENTS

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