Raver2: A Novel Regulator Of sFlt1 Production - 2390/A407

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Abstract

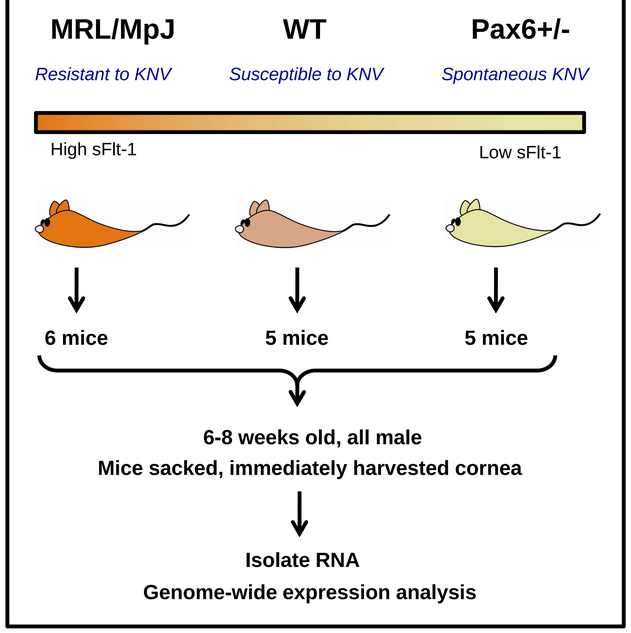
Purpose: Pathologic neovascularization (NV) underlies several sight-threatening ocular diseases. VEGF is a key regulator of angiogenesis and misregulation of VEGF signaling has been implicated in the pathophysiology of NV. Our previous work has shown that the soluble isoform of tyrosine kinase receptor Flt1 (sFlt1) plays a critical role in preserving avascularity in the cornea and outer retina, and that disruption of endogenous sFlt1 can recapitulate certain human NV disease states. sFlt1 production relies upon a specific form of alternative RNA processing known as intronic polyA activation (IPA). In this study, we investigate the molecular mechanisms underlying this key RNA processing event.

Methods: siRNA knock-down was done via corneal plasmid injection and transfection into HUVEC cultures. mRNA expression levels were assayed by RT-PCR, and protein levels determined by Western and ELISA. Molecular associations were investigated via standard immunoprecipitation (IP) methods, including co-IP and RNA IP (RIP).

Results: Prior microarray analyses identified Raver2 as a factor whose expression profile mirrored that of sFlt1, suggesting that it might promote sFlt1 production. Indeed, knock-down of Raver2 correlated with significantly decreased sFlt1 levels both *in vivo* (mouse cornea) and *in vitro* (HUVEC cells). We also observed marked corneal NV following Raver2 knock-down in WT mice. Given the functional importance of Raver2, we sought to determine if the factor acted at the level of Flt1 pre-mRNA. RIP experiments showed robust enrichment of Flt1 RNA, implicating a direct association of Raver2 with Flt1 pre-mRNA. To further characterize Raver2 function, IP of cellular extracts was performed, which showed specific enrichment for PTB, a well-studied factor involved in alternative splicing and polyadenylation. RIP for PTB also enriched Flt1 RNA, and RIP following Raver2 knock-down showed significantly reduced PTB occupancy.

Conclusions: We identify for the first time a specific endogenous factor, Raver2, which promotes sFlt1 production. Furthermore, we demonstrate that Raver2 and PTB localize to Flt1 pre-mRNA and provide evidence that Raver2 promotes PTB association with Flt1 pre-mRNA. Taken together, our data suggest a model in which Raver2-mediated recruitment of PTB promotes specific IPA resulting in expression of the sFlt1 isoform.

Experimental Design



Raver2 Promotes sFlt1 mRNA Expression

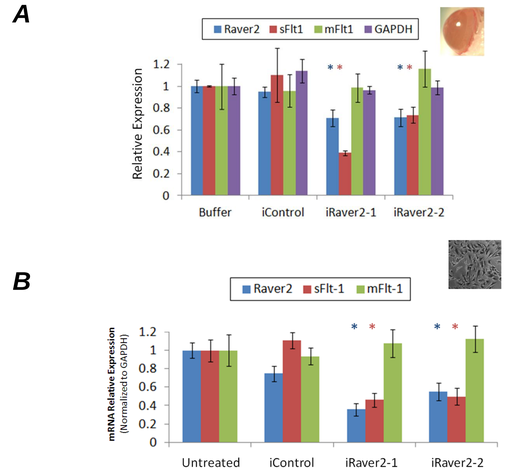


Figure 3. siRNA-mediated Raver2 Knock-Down Leads to Decreased sFlt1 at mRNA Level. Knock-down of Raver2 in (A) mouse cornea and (B) HUVEC resulted in decreased sFlt1 mRNA levels. * p<0.05

Raver2 and PTB Localize to Flt1 mRNA

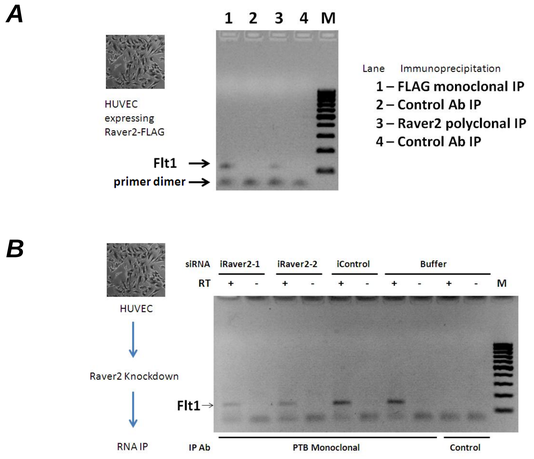
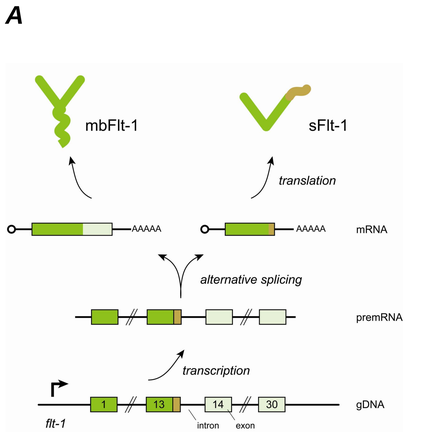


Figure 5. RNA Immunoprecipitation (RIP) Localizes Raver2 and PTB to Flt1 mRNA. (***A***) RIP shows association of Raver2 with Flt1 mRNA (***B***) RIP following Raver2 knock-down shows PTB associates with Flt1 mRNA in a Raver2-dependent fashion.

Background



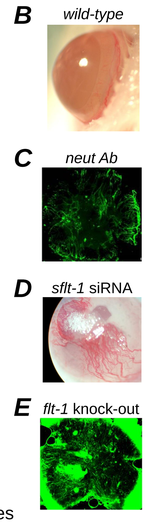


Figure 1. sFlt-1 is the key preserver of corneal avascularity. (***A***) The VEGF receptor Flt-1 undergoes alternative splicing to produce either membrane-bound (mbFlt-1) or soluble (sFlt-1) isoforms which augment or antagonize VEGF signaling, respectively. While normal corneas are avascular (***B***), disruption of sFlt-1 expression at the genetic (***C***), mRNA (***D***), or protein (***E***) level results in corneal neovascularization.

Genes Enriched in MRL/MpJ

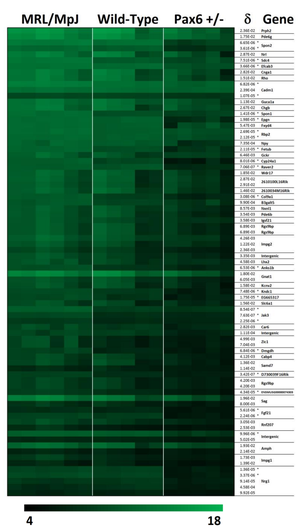


Figure 2. Genes Enriched in MRL/MpJ. Microarray expression data represented at heat map after log2 transformation. Columns represent biological replicates, rows represents microrarray probes. δ indicates Benjamini and Hochberg False Discover Rate.

Raver2 Promotes sFlt1 Protein Expression

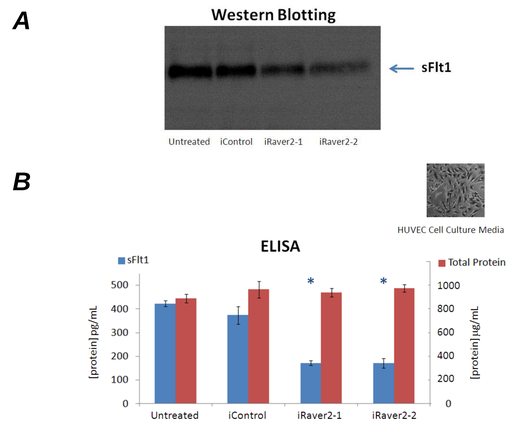


Figure 4. siRNA-mediated Raver2 Knock-Down Leads to Decreased sFlt1 at Protein Level. Knock-down of Raver2 in HUVEC leads to decreased secreted sFlt1 protein in culture media by (A) Western Blot and (B) ELISA, error bars represent standard error* p<0.05

Conclusions

- Genome-wide microarray analysis identifies Raver2 as candidate regulator of sFlt1 expression
- sFlt1 expression is Raver2-dependent in murine cornea and HUVEC cell culture models
- Raver2 and PTB localize to Flt1 mRNA

Future Directions

- Investigation of other candidate regulators identified in genome-wide screen
- *In vivo* studies of Raver2 and other candidate regulators in murine models of corneal neovascularization
- Studies into the molecular mechanisms by which Raver2 and other candidate genes regulate sFlt1 expression