stop-PRISM integration allele in the *rb1* tumor suppressor recapitulates developmental and cellular neural progenitor loss of function phenotypes

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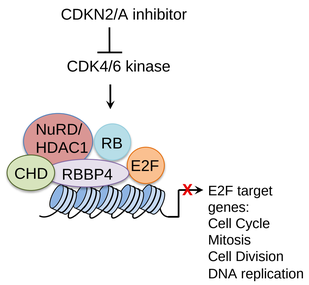
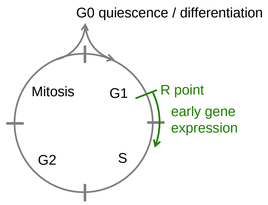
Abstract

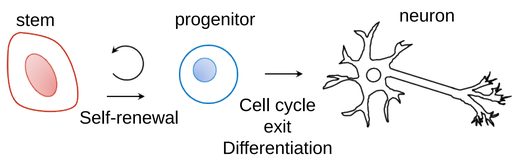
To simplify screening and genotyping of mutant embryos we generated a set of vectors for CRISPR/Cas9 targeted integration called pPRISM, which contain secondary markers that allow for simple visual genotyping (plasmids for Precise Integration with Secondary Markers). The plasmids are designed for integration using short homology arms as we reported previously (Wierson et al., 2019 bioRxiv). The stop-PRISM vectors have a cassette for transcriptional termination followed by a secondary marker for heart, lens or hatching gland-specific fluorescent reporter expression. Targeted integration into an early exon is predicted to cause early transcription termination and create a loss of function mutation. We isolated an *rb1-stop-PRISM-cmlc2:GFP* line and tested whether the integration allele behaved as a loss of function mutation in trans with our previously published 7 bp indel allele *rb1Δ7is54*. The gross morphology of *rb1-stop-PRISM-cmlc2:GFP/rb1Δ7is54* trans heterozygotes is relatively normal, however the larvae fail to develop a swim bladder and are lethal, similar to *rb1Δ7is54/Δ7is54* homozygotes. To examine if the *rb1-stop-PRISM-cmlc2:GFP* allele disrupts *rb1* function at the cellular level we used live embryo confocal imaging of H2A.F/Z:GFP chromatin in the developing optic tectum to follow neural progenitor cell cycle dynamics. At 5 dpf, *rb1-stop-PRISM-cmlc2:GFP/rb1Δ7is54* mutant neural progenitors re-enter the cell cycle and condense their chromatin. The cells then appear to arrest in prophase, and fail to align the chromosomes at the metaphase plate and progress through the cell cycle, as we previously found for *rb1Δ7is54/Δ7is54* homozygotes (Schultz et al., 2018). PCR genotyping of the imaged embryos revealed the expected genotypes matched the observed phenotypes. Quantification of the neural progenitor mutant phenotype is in progress. Future analysis will compare phenotype and quantification data between our *rb1-stop-PRISM-cmlc2:GFP/rb1Δ7is54* trans heterozygotes and homozygous *rb1-stop-PRISM-cmlc2:GFP/stop-PRISM-cmlc2:GFP* embryos. Our results confirm integration of the pPRISM cassette can create loss of function mutations that recapitulate the phenotype of standard indels in the tumor suppressor *rb1*, both at the morphological and cellular level. stop-PRISM loss of function alleles will be useful genetic tools for rapid genotyping in combination with more complex edited alleles to study tissue or cell type specific gene function.

1. RB tumor suppressor regulates cell cycle entry, progression, and cell cycle exit

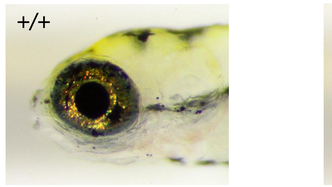


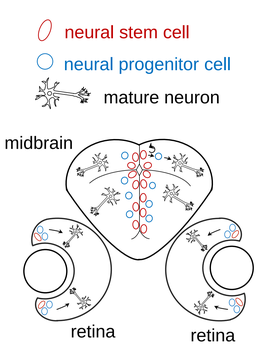


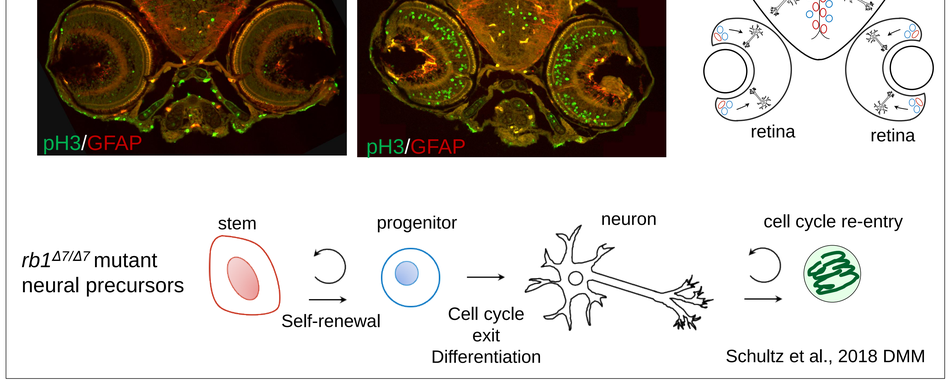
RB promotes neural stem cell proliferation, drives neural progenitor cell cycle progression, and maintains G0 arrest in differentiated neurons



2. RB is required for neural progenitor cell cycle exit and differentiation during zebrafish neural development

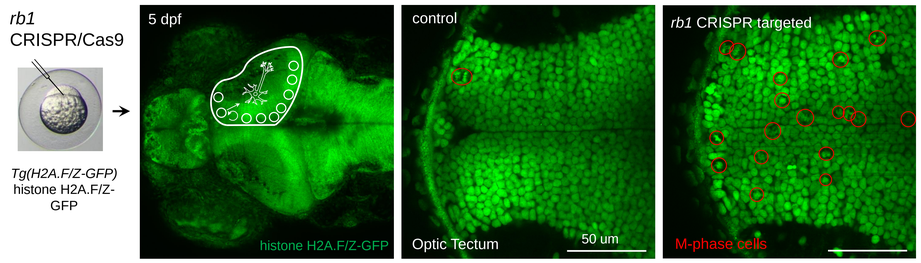


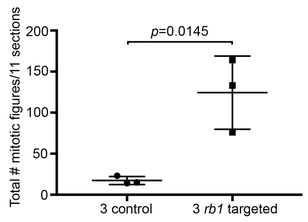


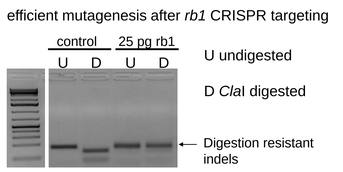


3. H2A.F/Z-GFP live imaging-somatic CRISPR assay reveals *rb1* mutant neural precursors re-enter the cell cycle, condense chromatin and arrest in M-phase

rb1 exon 2 CRISPR sgRNA target site 5′-GGAGAGGGAGATCAGATCGATGGAC-3′ (*ClaI*, PAM)



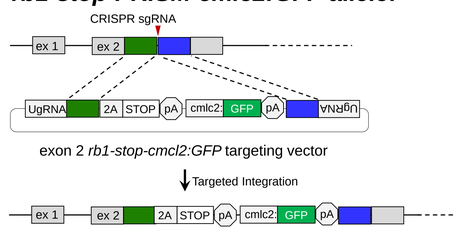


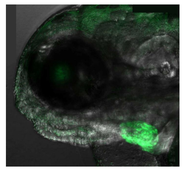


Schultz et al., 2018 DMM

4. *rb1* exon2 –*stop-PRISM-cmlc2:GFP* allele generated by CRISPR short homology directed targeted integration recapitulates *rb1* mutant neural progenitor cell cycle defects

***rb1-stop-PRISM-cmlc2:GFP* allele:**



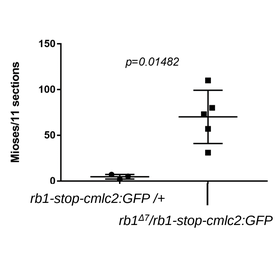


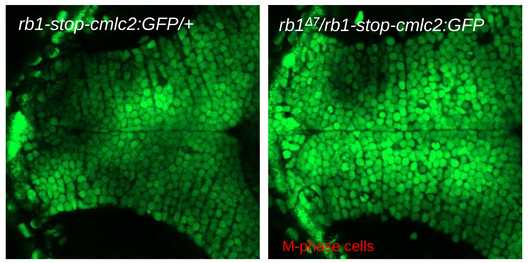
4 dpf Tg(*rb1-stop-cmcl2:GFP)* larva

Welker et al. *in prep*

***rb1*^{Δ7}*/rb1-stop-cmlc2:GFP* trans-heterozygotes display cell cycle re-entry phenotype:**

rb1-stop-cmlc2:GFP/+ X *rb1*^{Δ7}*/+ ; H2A-GFP*





PCR genotyping confirms:

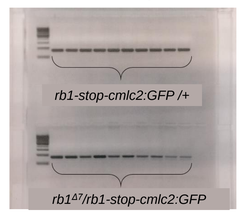
- larva with normal neurogenesis are heterozygous *rb1-stop-cmlc2:GFP/+*
- larva showing the neural cell cycle entry mutant phenotype are *rb1*^{Δ7}*/rb1-stop-cmlc2:GFP* trans-heterozygotes

BclI

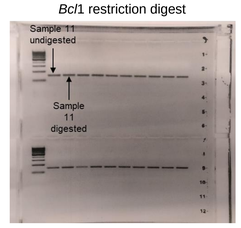
+/+ 5′-CCATCTGTGATCATGCATGGAGAA-3′

rb1^{Δ7} 5′-CCATCTGTGA-------TGGAGAA-3′

rb1-stop-cmlc2:GFP 5′ junction PCR



rb1 exon 2 PCR individuals 11-20 *Bcl*1 restriction digest



rb1 exon 2 PCR individuals 1-10 *Bcl*1 restriction digest

Summary

- Knock-in stop-PRISM *rb1-stop-cmlc2:GFP* allele generated by precision targeted integration 1. allows for simple visual genotyping and 2. produces loss of functions phenotypes consistent with *rb1* indel mutation.
- Visual genotyping plus live imaging allows phenotyping of rb1 mutant neural cellular phenotypes – two color alleles offer potential for genotyping without PCR.
- Work is ongoing to determine the interaction of *rb*1 and *tp53* in neural progenitor proliferation and differentiation, and the pathways blocking progression and differentiation of *rb1* mutant neural progenitors.

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