



# Ca<sup>2+</sup>/calmodulin-activated adenylyl cyclase 8 is required for SDF-1 signaling during zebrafish retinal axon guidance

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## Introduction

Axons are actively guided to their targets in the nervous system during development by chemoattractive and chemorepulsive guidance cues.

Slit2, signaling via the receptor Robo2, is a powerful chemorepulsive cue for retinal ganglion cell (RGC) axons (Nicolou et al. 2000, Fricke et al. 2001). The chemokine SDF-1, binding to its receptor CXCR4, activates an 'anti-repellent' signaling pathway that antagonizes the repellent activity of Slit2 (Chalasani et al. 2003). In a bioassay for repellent activity, when SDF-1 is present, Slit2 is eight times less effective as a repellent (Chalasani et al. 2003).

SDF-1 signaling has been shown to elevate cAMP levels; therefore, it is reasonable to hypothesize that cAMP synthetic enzymes, the adenylyl cyclases (ADCYs), are activated by SDF-1 (Chalasani et al. 2003). Furthermore, blocking calcium/calmodulin has been shown to interfere with SDF-1 signaling (Chalasani et al. 2003).

I hypothesized that the Ca<sup>2+</sup>/calmodulin-activated ADCY8 is a necessary component of the SDF-1 anti-repellent signaling pathway. I therefore predicted that knock down of ADCY8 would prevent SDF-1 antagonism of Slit2 repellent signaling.

## Methods

**Morpholinos:** Zebrafish embryos were collected and the blocking splicing was injected at the one cell stage with either a control morpholino (MO) or two MOs that target separate intron-exon borders in the ADCY8 pro-RNA. The ADCY8 MOs block splicing and induce premature stop codons in the mRNA.

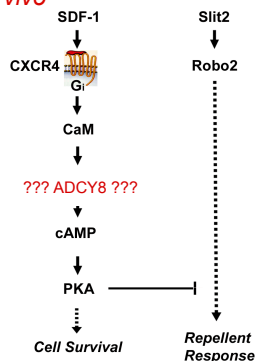
**Zebrafish retinal cultures:** Retinal explants were prepared from 2 dpf ADCY8 MO or Control MO injected zebrafish and cultured in supplemented L15 media on laminin (80 µg/ml) and poly-lysine (200 µg/ml) coated MatTek dishes. The explants were cultured for approximately 24 hours at 28.5 degrees Celsius.

**Protein preparation:** Human Slit2 and Zebrafish SDF-1a were made as secreted proteins by a stable 293T cell line or transfected 293T cells. Crude supernatants containing the proteins were collected and protein content was confirmed by Western blot analysis.

**Live imaging:** Control MO and ADCY8 MO containing axons were maintained on the heated stage of an inverted microscope. At the start of the experiment and after 75 minutes they were photographed using phase optics. Either 125 µl hSlit2, 250 µl zSDF-1a, or 125 µl hSlit2 plus 250 µl zSDF-1a were added to the media. The extension of individual axons during the initial control period and the treated period was compared between conditions.

## Results

1. Previous experiments show that SDF-1 signaling antagonizes axonal response to the repellent Slit2, *in vitro* and *in vivo*



2. Retinal axons containing Control MO or ADCY8 MO extend similar distances with or without SDF-1 treatment

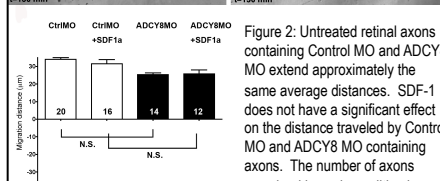
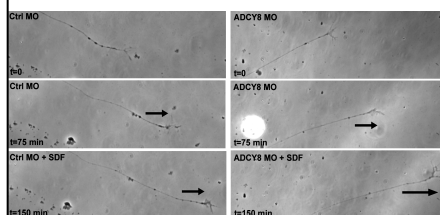


Figure 2: Untreated retinal axons containing Control MO and ADCY8 MO extend approximately the same average distances. SDF-1 does not have a significant effect on the distance traveled by Control MO and ADCY8 MO containing axons. The number of axons examined in each condition is shown on the graph. Error bars indicate the SEM. Levels of significance were estimated using a Mann-Whitney two-tailed test.

3. Knock down of ADCY8 does not interfere with axon responsiveness to the repellent Slit2

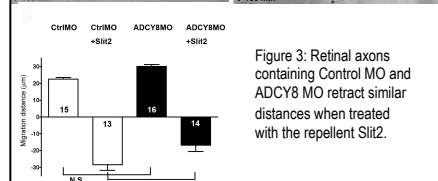
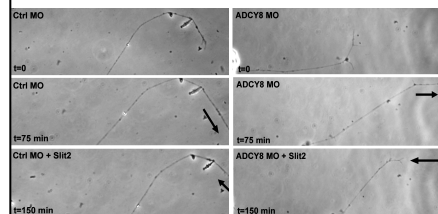


Figure 3: Retinal axons containing Control MO and ADCY8 MO retract similar distances when treated with the repellent Slit2.

4. Knock down of ADCY8 blocks SDF-1 antagonism of the Slit2 repellent pathway

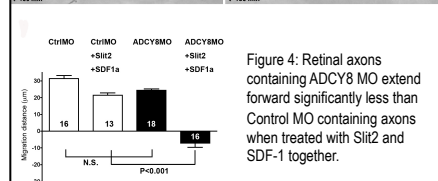
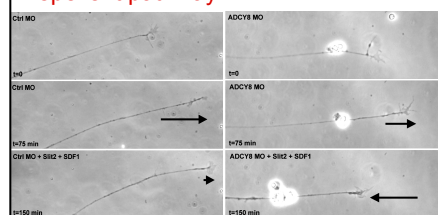


Figure 4: Retinal axons containing ADCY8 MO extend forward significantly less than Control MO containing axons when treated with Slit2 and SDF-1 together.

5. Knock down of ADCY8 perturbs retinal axon crossing of the midline

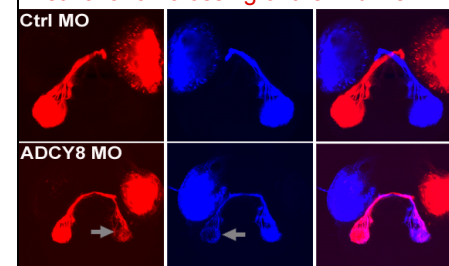


Figure 5: Dil and DiI were injected into the left and right eyes of 5 day post fertilization zebrafish to label retinal axons. Retinal axons treated with a Control MO correctly cross the midline and project to the contralateral tectum. Retinal axons treated with ADCY8 MO sometimes fail to cross the midline and mis-project ipsilaterally. The ipsilateral projections in the ADCY8 morphants still follow normal axon tracts and project to the tectum (Xu and Raper, unpublished).

## Conclusions

- These results are consistent with our prediction that ADCY8 is a necessary component of the SDF-1 pathway that antagonizes Slit2 repellent activity.
- ADCY8 MO containing retinal axons are able to extend normally compared to Control MO axons.
- Knocking down ADCY8 has no effect on axonal response to the repellent Slit2.
- Morpholinos against ADCY8 cause zebrafish to develop with several retinal axon guidance errors including intermittent failure to cross the midline and aberrant projection to the ipsilateral rather than contralateral tectum (Xu and Raper, unpublished).
- We hypothesize that midline guidance errors in the developing fish are caused by hypersensitivity of retinal axons to midline repellents.

## Literature Cited

1. Chalasani SH, Sabelko KA, Sunshine MJ, Littman DR, Raper JA (2003) A Chemokine, SDF-1, reduces the effectiveness of multiple axonal repellents and is required for normal axon pathfinding. J of Neuroscience 23(4): 1360-1371.
2. Fricke C, Lee J, Geiger-Rudolph S, Bonhoeffer F, Chien C (2001) Astray, a Zebrafish roundabout homolog required for retinal axon guidance. Science 292(5516): 507-515.
3. Nicolou SP, Jia L, Raper JA (2000) Slit2 is a repellent for retinal ganglion cell axons. J of Neurosci 20(13): 4962-4974.