Fin to Function: Using CRISPR/Cas9 genome editing to investigate the role of *Dlx* homeobox genes in zebrafish

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Background

Members of the *Distal-less homeobox (Dlx)* gene family play an important role in the differentiation and migration of GABAergic interneurons[1]. These inhibitory interneurons are crucial in neural circuitry and activity in the developing nervous system of many organisms. In vertebrates, *Dlx* genes are organized in convergently transcribed bigene clusters[1] (Fig 1). Each cluster includes a short intergenic region harbouring cis-regulatory elements (CREs) that influence *Dlx* expression and forebrain development[1]. Therefore, characterization of *Dlx* genes is important to understand their function and regulation during vertebrate neurogenesis.

Bigene Cluster

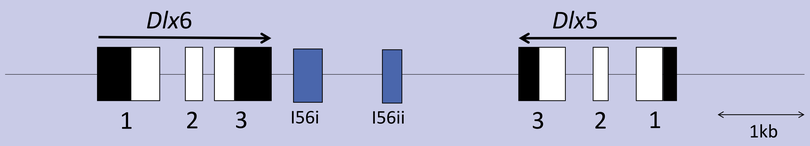

Figure 1: Genomic organization of the *Dlx*5/*Dlx*6 bigene cluster. Located on chromosome 19. White boxes represent exons, black boxes represent non-coding regions, and blue boxes represent CREs important for gene regulation.

Methodology

CRISPR-based applications using RNA-guided nucleases (e.g. Cas9) are promising genome editing and engineering tools with exciting potential in molecular biology and genetics. By designing specific guide RNA (gRNA) sequences, the CRISPR/Cas9 system can precisely target and remodel defined endogenous gene loci. In this project, the CRISPR/Cas9 system was used to delete the *I56i – I56ii* intergenic region of the *dlx5a/dlx6a* bigene cluster to create a mutant zebrafish line.

Target
- Retrieved the nucleotide sequence for the targeted deletion using Ensembl genome browser 91.

Guide RNA
- Designed two 20 nucleotide gRNA sequences and three primers using ZiFit, ChopChop, and Primer BLAST software.

Cleavage
- Microinjected a Cas9 protein and gRNA mix into ≈ 200 single-cell embryos for target specific cleavage.

Extraction
- Extracted DNA from fin clippings and amplified samples using PCR technology.

Electrophoresis
- Assessed the presence or absence of the target deletion using gel electrophoresis.

Sequencing
- Confirmed the presence or absence of the target deletion using sequencing technology.

Amplicons

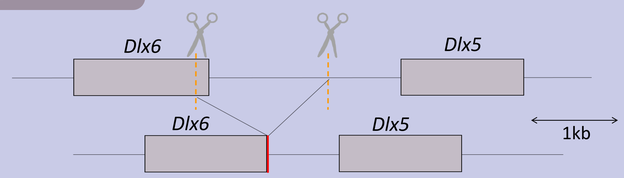

Figure 2: Principle of the *I56i – I56ii* deletion. Cas9 cuts the DNA at the location of the guide RNA in the 3' UTR of *Dlx6* and between *I56ii* and the 5' UTR of *Dlx5* (orange line). The break is repaired by non-homologous end-joining resulting in an insertion/deletion mutation (red line).

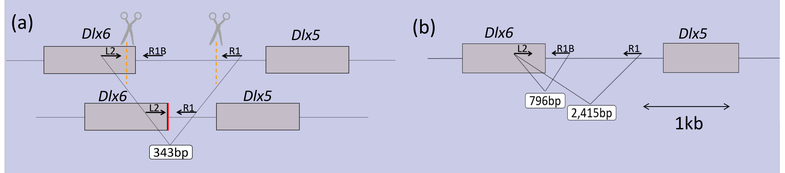

Figure 3: Possible PCR amplification products after CRISPR/Cas9 deletion mutagenesis using three primers. (a) Amplification from primers L2 + R1 yielding a 343bp product; successful deletion. (b) Amplification from primers L2 + R1B or L2 + R1 yielding a 706bp or 2,415bp product respectively; unsuccessful deletion.

Results

Of the 55 F0 adults screened, 5 transmitted the mutation to F1 progeny. Sibling-pairs of adult F1 fish were crossed to produce F2 progeny. The F2 generations of each line were screened to determine the proportion of heterozygous and homozygous adults (Fig 4). Table 1 shows the genotyping data from one of the five lines.

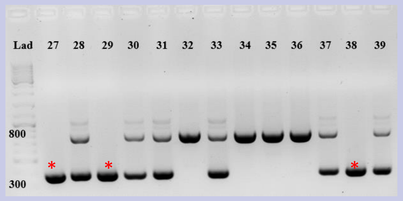

Figure 4: Gel electrophoresis results for deletion mutagenesis. Fin samples from F2 (#27 – 39) from Male #19 using L2+R1-B+R1; 95°C for 30s + 60.8°C for 30s + 72 °C for 55s and 3 min denaturation at 95°C for step 1 (X35). Red stars indicate fish homozygous for the mutation.

Table 1: Genotyping data of F1 and F2 progeny from F0 Male #19

	Homozygous Wild-Type	Heterozygous	Homozygous Mutant
F1	119	12	-
F2	11	28	12

CRISPR/Cas9

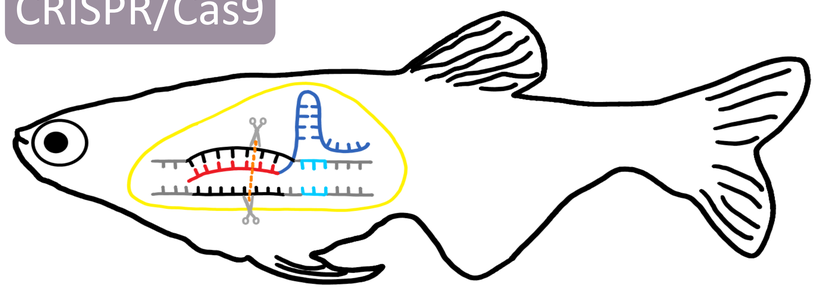

Figure 5: Schematic representation of the CRISPR/Cas9 system. A single gRNA strand (red) binds complimentary to the target sequence (black) located upstream of the PAM sequence (light blue). The Cas9 protein (yellow) with DNA endonuclease activity interacts with the hairpin structure of the gRNA (dark blue). The complex creates a double-strand break (orange) at the target site which will be repaired by non-homologous end joining (NHEJ), resulting in an insertion/deletion mutation.

Conclusion

The CRISPR/Cas9 system was used to successfully delete the intergenic region of the *dlx5/dlx6* bigene cluster in zebrafish. From the ≈ 200 embryos injected, 5 mutant lines were bred to the F2 generation. The transmission of the mutation approximately followed a Mendelian inheritance pattern leading to an approximate 1:2:1 ratio of homozygous wild-type, heterozygous, and homozygous mutants.

Figure 6: Mutant zebrafish homozygous for the *I56i-I56ii* deletion mutation. F2 #27 from Male #19.

Next Steps

The production of *I56i – I56ii* mutants yields exciting potential for the ultimate characterization of *Dlx* genes in neuronal development. Possible follow-up studies include:

- Breeding F2 sibling-pairs to produce an F3 homozygous generation for the *I56i – I56ii* deletion mutation
- Phenotypic studies to investigate the effect of the *I56i – I56ii* deletion on neural function
- Cross-breeding with other *Dlx* mutants to elucidate relationships between members of the *Dlx* gene family

References

[1]Zhao, Pengcheng. Phenotype characterization of mice with targeted deletions of *Dlx* enhancers. Faculty of Graduate and Postdoctoral studies. *University of Ottawa*. 2018.

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