Using Zebrafish as a Model System for Studying the Autism Risk Gene *adnp*

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Introduction

Autism Spectrum Disorders (ASD) are a classification of developmental disorders which are characterized by intellectual disability, difficulties with social interaction, impairments in verbal and nonverbal communication, and restricted repetitive behaviors. ASD affects as many as 1 in every 59 children worldwide and is an incredibly complex and genetically diverse group of disorders, with a single gene being mutated in less than 1% of the ASD population. Thus, there is an increasing demand in ASD research for genetic screenings as well as research on novel genetic mutations[1–3].

One of the more common forms of ASD is ADNP syndrome. This disorder is characterized by intellectual disability, facial dysmorphia, and multiple organ system deficits. It is caused by *de novo* mutations in the gene ADNP; and is believed to occur in at least 0.17% of ASD patients. A clinical study of patients with ADNP syndrome, found that frame shift mutations in ADNP lead to a loss of the C-terminus of the protein; a region responsible for recruiting components of the BAF chromatin remodeling complex[3–5].

An effective method for *in vivo* investigation of ADNP syndrome is the zebrafish model organism. Zebrafish embryos are transparent and have short generation times making them an excellent model for developmental and genetic analysis. Mutations identified in zebrafish mimic those in human disease; allowing for the identification of medically relevant phenotypes during embryogenesis[6].

Zebrafish have two paralogs of *adnp*; *adnpa* and *adnpb*. While knockdown of *adnp* in zebrafish has implicated these two proteins in erythropoiesis, additional research is needed to fully understand their functional roles in zebrafish as well as the effect of mutations *in vivo*[7]. We sought out to generate knockout and overexpression zebrafish models for *adnp* and to characterize the role of *adnp* during embryonic development. In addition to overexpressing full-length *adnpa* in zebrafish embryos; we injected a truncated *adnpa* mRNA; one that contains an early stop codon in a conserved region of the protein known to contain pathogenic frameshift mutations in patients with ADNP syndrome[3].

Materials and Methods

CRISPR Mediated Knock-Out and Generation of Stable Transgenic Lines

CRISPR/Cas9 was utilized to target and cut the promoter and fourth exon of *adnpa* and the fourth exon of *adnpb*. sgRNAs were designed using the CRISPRScan online program[8] . To ensure the efficiency of cutting in the promoter region of ADNPA, the promoter was amplified by PCR and sequenced. Considering the level of polymorphisms that occur outside of coding sequences, these sequences were a more reliable reference for designing guide RNAs. sgRNAs and cas9 mRNA were generated by *in vitro* transcription. mRNA was injected into zebrafish embryos at the one cell stage. Through the generation of simultaneous double stranded breaks; approx. 2.5kb was deleted from *adnpa* and 1.2kb in *adnpb*. F0s were genotyped by PCR to confirm cutting of the guide RNAs and deletion of the gene fragments. Mosaic F0s were raised and subsequently intercrossed to generate putative ADNP F1s.

***Adnpa* Full Length and Truncated mRNA Preparations and Injections**

RNA was extracted from zebrafish embryos at 6 hours post fertilization(hpf). cDNA was synthesized and used to amplify the *adnpa* full length and truncated coding sequences by PCR. PCR products were cloned into PCS2 and *in vitro* transcribed using an sp6 kit. 150 pg of *adnpa* mRNA was injected into zebrafish embryos at a one cell stage.

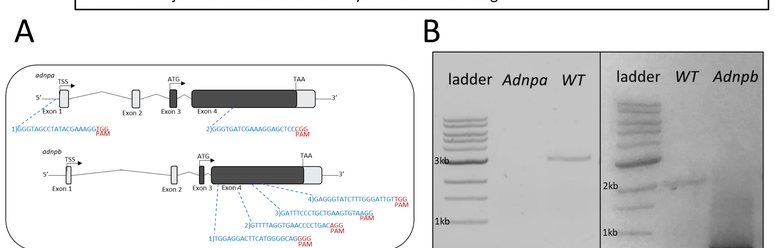


Figure 1: Strategy for Generating a Null Allele Using CRISPR/Cas9 for *adnpa* and *adnpb*. a) For *adnpa,* 2 sgRNAs were designed to target upstream from the TSS and in exon 4. Generating a large deletion would result in loss of the TSS, 5' UTR, ATG and a large fragment of the coding sequence; as well as result in a frameshift. This should result in complete loss of transcription and avoid any problems with genetic upregulation of paralogous genes[9]. For *adnpb* 4 sgRNAs were designed spanning the coding sequence; in order to generate a large deletion and frameshift resulting in a null allele. b) Results from PCR genotyping of *adnpa* and *adnpb* injected embryos. PCR products were sequenced to confirm the deletions.

Results

3 sets of *adnpa* mosaic F0s were intercrossed to generate putative *adnpa* F1s. Within single clutches of F1 offspring, 24% displayed cardiac edema and reduced head size compared to phenotypically wildtype embryos. Embryos exhibiting the cardiac edema phenotype also showed deficits in motor function and sensory responses. *Adnpb* F0 embryos also exhibited strikingly similar cardiac edema beginning at 24 hpf. These embryos did not have any noticeable differences in head size. Overexpression of *adnpa* mRNA in wildtype embryos caused forebrain expansion and mild cardiac edema; beginning at 22 hpf. Injection of truncated *adnpa* mRNA (*adnpa* p.(790*)) also caused forebrain expansion but additionally; injected embryos displayed cyclopia. Taken together these findings support an essential role of adnp in both cardiovascular and neurological development.

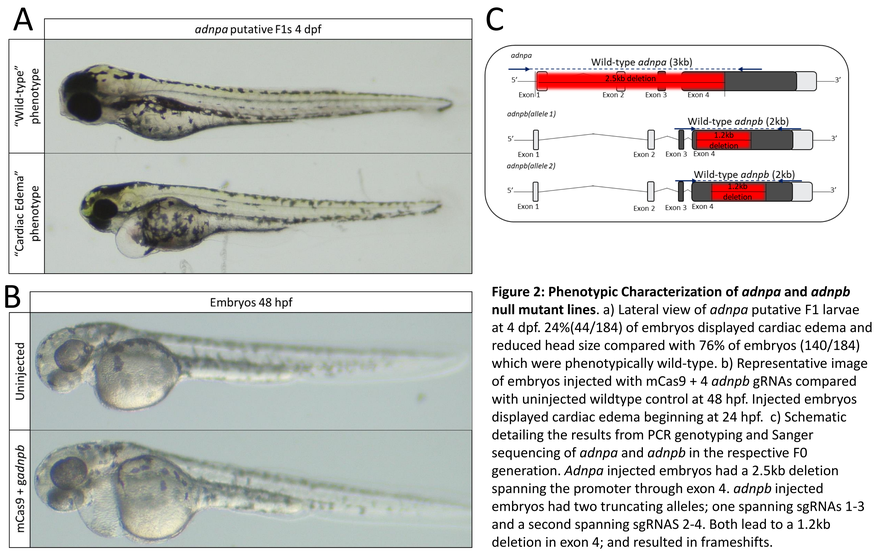
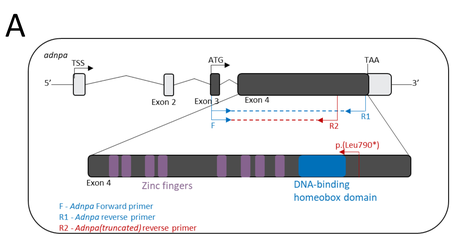
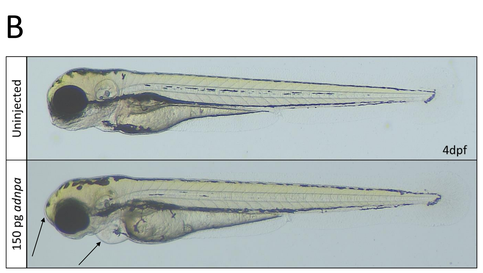


Figure 2: Phenotypic Characterization of *adnpa* and *adnpb* null mutant lines. a) Lateral view of *adnpa* putative F1 larvae at 4 dpf. 24%(44/184) of embryos displayed cardiac edema and reduced head size compared with 76% of embryos (140/184) which were phenotypically wild-type. b) Representative image of embryos injected with mCas9 + 4 *adnpb* gRNAs compared with uninjected wildtype control at 48 hpf. Injected embryos displayed cardiac edema beginning at 24 hpf. c) Schematic detailing the results from PCR genotyping and Sanger sequencing of *adnpa* and *adnpb* in the respective F0 generation. *Adnpa* injected embryos had a 2.5kb deletion spanning the promoter through exon 4. *adnpb* injected embryos had two truncating alleles; one spanning sgRNAs 1-3 and a second spanning sgRNAS 2-4. Both lead to a 1.2kb deletion in exon 4; and resulted in frameshifts.





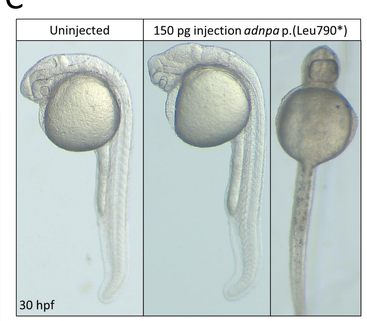


Figure 3: Overexpression of *adnpa* in wildtype embryos. a) Schematic of *adnpa* and the encoded protein domains within exon 4. Truncated *adnpa* mRNA was generated by using a reverse primer containing a stop codon. A conserved sequence known to harbor pathogenic frameshift mutations in patients with ADNP Syndrome was chosen as the site for truncation. b) Comparison of embryos injected with 150 pg *adnpa* mRNA with uninjected controls. Representative images were taken at 4 dpf. Arrows are pointing to the expanded forebrain and cardiac edema. c) Lateral and anterior ventral view of embryos injected with 150 pg *adnpa* p.(Leu790*) compared with uninjected control at 30 hpf. Injected embryos developed forebrain expansion, cyclopia and cardiac edema beginning at 22 hpf.

Conclusions

Our work on ADNP in Zebrafish thus far, has suggested that ADNPA/B may play a role in angiogenesis as well as brain development. Preliminary data shows that CRISPR/Cas9 knockout of *adnpa* leads to reduced blood flow and the development of cardiac edema in F1 embryos at 48 hours-post fertilization(hpf). 24% of embryos within a single clutch of ADNPA F1s exhibited this phenotype in addition to showing a reduction in head size and severe deficiencies in motor function and sensory responses. ADNPB F0s show a similar phenotype beginning at 24 hpf. At 6 days post-fertilization all embryos with this phenotype develop fatal hemorrhages around the heart. Adult ADNP +/- fish have been identified by genotyping and show no changes in development or behavior. These findings support the idea that ADNP loss of function leads to the development of cardiac and neurological deficits in patients with ADNP syndrome. ADNP overexpression recapitulates the role in both cardiogenesis and neurogenesis. Embryos injected with 150 pg of *adnpa* mRNA develop cardiac edema and an expanded forebrain; beginning at 22 hpf.

ADNP syndrome results from frameshift mutations in and around the DNA binding domain of ADNP; leading to a loss of the C-terminus of the protein. Studies have shown that the C-terminus is required for recruitment of components of the BAF chromatin remodeler[5]. However; more research is needed to fully understand the consequence of *adnp* truncation in early development. Here we have shown that overexpression of *adnp* p.(790*); leads to cyclopia in developing embryos. Bifurcation deficits are often the consequence of dysregulation of critical pathways like *shh, NODAL, and wnt*[10]. In future studies; we aim to investigate *adnp's* role in regulation of gene regulatory networks during early embryonic development.

References

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