Mutational Screening of a Known Atoh1 Enhancer

Tuba Ege [1], Celia Bloom [1], and Litao Tao [1]

Biomedical Sciences Department, School of Medicine, Creighton University, Omaha, NE 68178, USA

Introduction

Atoh1 is a crucial regulator in sensory hair cell development and, therefore, critical for auditory function. Despite its importance, the upstream transcriptional regulators of Atoh1 remain largely unknown. Understanding the regulation of Atoh1 is vital for unraveling the complex network governing hair cell development. To address this, we focus on a previously characterized Atoh1 3' enhancer, in which we have identified numerous binding motifs and putative transcription factor regulatory sites. We propose an innovative approach that combines saturation mutagenesis and STARR-seq to conduct large-scale mutagenesis screening of the Atoh1 3' enhancer. By identifying the mutations that impact Atoh1 expression most strongly, we will be able to identify the most critical transcriptional regulators of Atoh1. This technique aims to identify key regulators by assessing the functional impact of enhancer mutations on Atoh1 expression levels **(Figure 1)**. This comprehensive analysis seeks to elucidate Atoh1's transcriptional control and explore its potential for regenerative therapies in hearing loss.

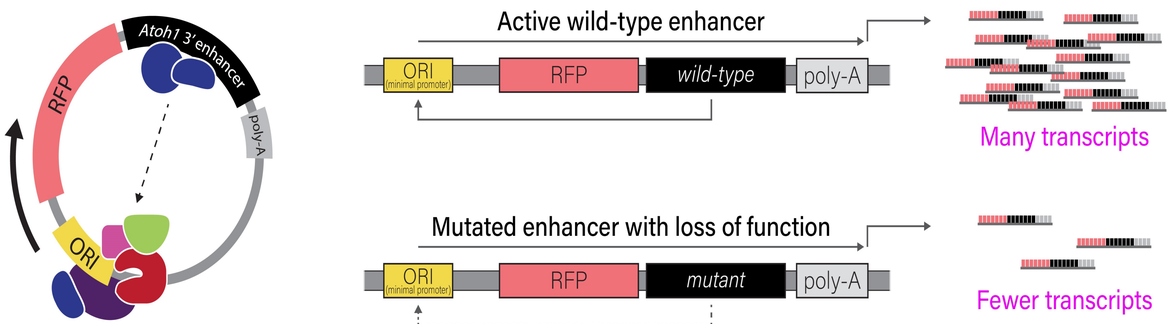

Figure 1: The principle of STARR-seq is to measure enhancer activity. An active wild-type enhancer will upregulate its mRNA expression by stimulating a minimal promoter, generating many transcripts (top). Specific mutations that disrupt enhancer functionality will result in fewer transcripts (bottom).

Method

Our study utilizes a combination of saturation mutagenesis and STARR-seq to investigate enhancer functionality and mutational impact of the Atoh1 3' enhancer. Starting with error-prone PCR, we amplify the enhancer, introducing diverse mutations to create a comprehensive library **(Figure 2)**. Each mutant enhancer is cloned into a modified STARR-seq vector, replacing the original luciferase reporter with RFP for high-throughput functional screening.

The backbone for our screening vector is derived from Addgene plasmid #99297, which we have customized to suit our specific experimental requirements. Through meticulous PCR amplification and subsequent In-Fusion cloning, we've exchanged the luciferase reporter with an RFP coding sequence. This allows for a more direct and observable readout of enhancer activity. Our saturation mutagenesis approach, facilitated by a Qiagen kit and strategically designed primers, ensures a broad spectrum of mutations. Importantly, the Atoh1 3' enhancer is flanked by sequence adapters, optimizing it for efficient downstream sequencing and analysis.

Postnatal mouse cerebellar granule progenitor (CGP) cells containing the active Atoh1 3' enhancer and GFP-tagged Atoh1 will serve as our model system. These cells require no external induction for Atoh1 expression and are suitable for studying cellular and developmental processes. We will harvest cerebella from P0 C576/J mice, dissociate them, and sort them by GFP expression. The mutant library will be delivered to the CGP cell by electroporation. Cells will be harvested and purified for expression of both GFP and RFP, after which RNA extraction and sequencing will be performed **(Figure 2)**. Bioinformatic tools will be used to process our data and quantify our transcripts.

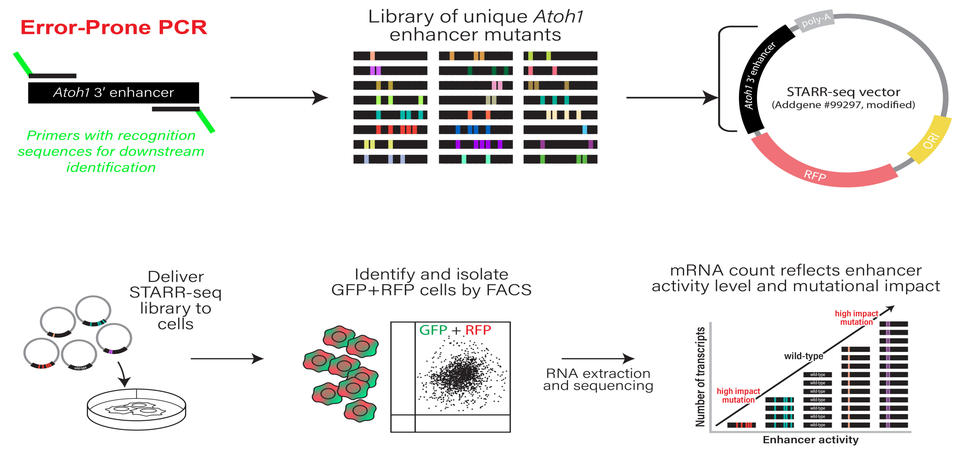

Figure 2:Modified STARR-seq plasmid to clone mutant enhancer library and simplified experimental workflow and analysis.

Results

Our comprehensive mutagenesis experiment aimed to map the mutational effects on the functionality of the 3' Atoh1 enhancer, a pivotal regulatory element in auditory hair cell development. Subsequent analysis was designed to elucidate the relationship between enhancer mutations and their influence on gene expression.

Figure 3 represents the mutational landscape of the Atoh1 3' enhancer region following saturation mutagenesis. Each row corresponds to an individual DNA sequence from our library, and the x-axis represents the nucleotide positions within the Atoh1 enhancer. Red marks indicate the presence of mutations compared to the reference sequence. We display the mutation distribution from the heatmap, showing a high density of mutations covering the entirety of the enhancer region, which suggests a successful introduction of diversity in our mutagenesis process. We have also validated GFP and RFP expressions by our CGP cells **(Figure 4)** and affirmed an optimal culturing protocol.

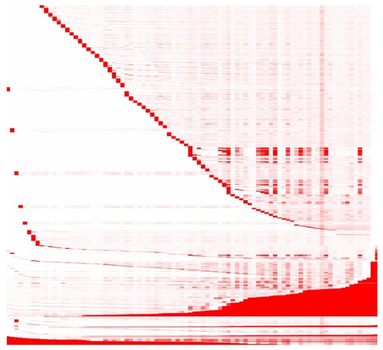

Figure 3:Mutation heatmaps of Atoh1 3' enhancer sequences.

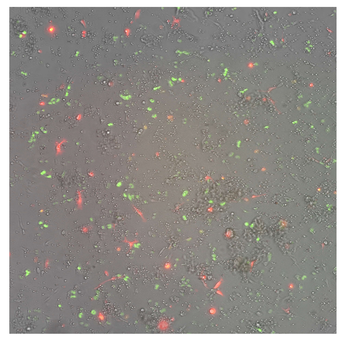

Figure 4: Validation of GFP-expressing mouse cerebellar granule progenitor cells and successful electroporation.

Future Directions

Our future research will prioritize the experimental validation of the predicted transcription factor binding sites within the Atoh1 3' enhancer, involving ATOH1, SOX2, SIX1, and EYA1. We aim to identify and characterize these key sites comprehensively, enhancing our understanding of Atoh1-mediated hair cell programming. This focused exploration will confirm the role of these transcription factors in regulating Atoh1 and help determine the complex network of genetic interactions essential for auditory function.

Acknowledgment

This research was supported by the Postdoctoral Research Award of Dr. Richard J. Bellucci.