Distribution Agreement

In presenting this thesis as a partial fulfillment of the requirements for a degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter now, including display on the World Wide Web. I understand that I may select some access restrictions as part of the online submission of this thesis. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

Jianing Wang April 8th, 2025

ncBAF Function in iPSCs: Investigating a Feedback Loop Between BICRA and BICRAL

by

Jianing Wang

Dr. David U. Gorkin

Adviser

Biology Department

Dr. David U. Gorkin

Adviser

Dr. Leila Rieder

Committee Member

Dr. Arri Eisen

Committee Member

2025

ncBAF function in iPSCs: Investigating a feedback loop between BICRA and BICRAL

By

Jianing Wang

Dr. David U. Gorkin

Adviser

An abstract of

a thesis submitted to the Faculty of Emory College of Arts and Sciences

of Emory University in partial fulfillment

of the requirements of the degree of

Bachelor of Science with Honors

Biology

2025

Abstract

ncBAF function in iPSCs: Investigating a feedback loop between BICRA and BICRAL

By Jianing Wang

Chromatin accessibility is regulated by a group of proteins called chromatin remodelers. The BRG1/BRM-Associated Factor (BAF) family of chromatin remodeling complexes play pivotal roles in regulating gene expression by repositioning nucleosomes at non-coding cis-regulatory elements. Recently, a new subtype of BAF complexes was discovered and are referred as non-canonical BAF (ncBAF) complexes. These ncBAF complexes contain some subunits not found in other BAF subtypes, including the proteins BICRA and BICRAL. Importantly, loss-of-function mutations in *BICRA* cause a rare syndromic form of intellectual disability, which suggests that BICRA and the ncBAF complexes that contain it play an important role in brain development. To further investigate the role of BICRA in brain development, I helped a graduate student in the lab, Ziben Zhou, engineer human induced pluripotent stem cells (hiPSC) with loss-of-function mutations in BICRA, which could then be differentiated to neurons *in vitro*. In the process of characterizing these cells, I discovered that BICRA null hiPSC show increased protein levels of BICRA's paralog, called BICRAL. In my thesis research, I found that BICRAL protein *and* mRNA levels are increased in BICRA null hiPSCs, suggesting a possible transcriptional feedback loop. I further discovered that this feedback loop is not reciprocal. I found that BICRA protein and mRNA levels are unchanged in hiPSCs with BICRAL mutations. Together, the data suggest the existence of a previously-unknown feedback loop in which BICRAL mRNA expression level is responsive to BICRA abundance. In the future, I plan to investigate the mechanism of BICRAL mRNA upregulation and whether it is conserved in different cell types.

ncBAF function in iPSCs: Investigating a feedback loop between BICRA and BICRAL

By

Jianing Wang

Dr. David U. Gorkin
Adviser

A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Science with Honors

Biology Department

2025

Acknowledgements

I would like to thank my amazing thesis committee. Dr. Gorkin, my PI, has always been an incredible mentor throughout my undergraduate research career, including guiding me to pick a research topic, interpreting research data, and writing the discussion section. Dr. Rieder, my genetics and science communication class instructor, has provided me a lot of writing support. Dr. Eisen, my epigenetics class instructor, guided me to learn critical thinking and teaching skills. Thank you for always believing in me and encouraging me throughout this process.

Thank you to all the members in the Gorkin lab. Ziben has always been supportive in teaching me lab techniques and data analysis. Susan, our lab technician, helped me to learn wet lab. Alex, helped me a lot with writing. Alan, supported me with grad school application and proofreading. Also the former students, Devin, Sona, and Sara, thank you all for giving me helpful guidance on this thesis. Thank you to my family, my mom, dad, and grandparents, for supporting me financially and emotionally. I truly won't have accomplished this without everyone's support.

Table of Contents

List of Figures and Tables

Chapter 1: Background and Introduction

1. 1 Eukaryotic chromosome organization

DNA and histones are carefully organized in every single mammalian cell nucleus as chromatin, and its basic unit is a nucleosome (Uhler & Shivashankar, 2017). A nucleosome consists of 8 histone monomers with DNA wrapped around them. (Cutter & Hayes, 2015) each nucleosome contains two copies each of the histone monomers H2A, H2B, H3, and H4.. About 150bp of DNA revolves around each histone, and some linker DNA with lengths between 20 to 90 bp connect different nucleosomes on the same chromosome. (Venkatesh & Workman, 2015) Histone tails can undergo various chemical modifications that influence gene expression. For example, inhibitory marks such as H3K9Me3 and activating marks like H3K4Ac are recognized by specific reader proteins, which in turn recruit other factors to activate or repress transcription.. Occasionally, histone variants such as H2A.Z and H3.3 replace usual histones and bring in different histone marks that could affect the tightness of DNA around histones. (Szerlong & Hansen, 2011)

While chromatin is organized in nucleosomes, and they exhibit continuous accessibility states.(Klemm et al., 2019) Accessible chromatin are the naked DNA strand in between nucleosomes, while inaccessible chromatin are those DNA wrapped around condensed histones. (Mansisidor & Risca, 2022) Chromatin accessibility correlates with nucleosome spacing and epigenetic factors such as DNA methylation and histone modifications. Gene regions with inhibitory histone marks such as H3K27Me3 are 30% less accessible, while those with activating histone marks are 30% more accessible (Chereji et al., 2019). DNA methylation gathers together to form CpG islands, which silencing gene expression and condense heterochromatin. CpGs correlate with condensed chromatin, especially in the centromeres and telomeres. (Han et al., 2008) Nucleosome spacing interplays with epigenetic regulations, for example, the HP1 protein recognizes H3K9Me3, compacts chromatin, creating new areas of closed chromatin. (Verschure et al., 2005) Overall, these factors makes DNA differentially accessible to diverse nuclear proteins, including transcription factors (TFs), RNA polymerases, and chromatin remodeling enzymes.

1.2 Chromatin accessibility at cREs

Chromatin is a dynamic structure with variable accessibility across the genome, encompassing both genes and cis-regulatory elements (cREs). The accessibility of cREs varies across cell types and developmental stages, and can change rapidly in response to external stimuli. These changes are often initiated by pioneer transcription factors, which can bind to nucleosomal DNA and facilitate chromatin opening, enabling the recruitment of additional regulatory proteins. (Shlyueva et al., 2014) Recently, ATAC-sequencing (ATAC-seq) has been used to cleave open chromatin regions and identify active cRE sites, contributing to our understanding for enhancer activity and chromatin accessibility changes over time. (Smith et al., 2022) (figure 1)

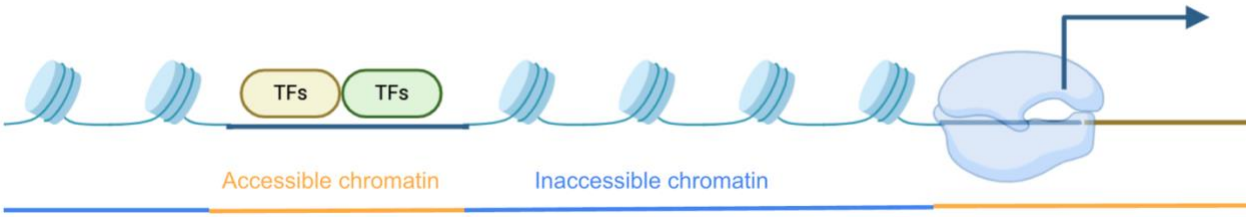


Figure 1: Model of accessible and inaccessible chromatin.

cRE accessibility accounts for differential gene expression in different cell types and developmental stages. Human pluripotent stem cells (ESCs) direct expression of pluripotency genes through maintaining highly active enhancers that tend to interact with pioneer TFs such as OCT4, SOX2, and NANOG. (Barakat et al., 2018) When ESCs become early blastocysts, they use SOX2 to occupy preaccessible enhancers and make them accessible to TFs such as TFAP2C and NR5A2. This pioneering binding is stable over time and carried on to later epiblasts. (Li et al., 2023) For hematopoietic stem and progenitor cells (HSPCs), the enhancer at the beta-globin locus control region (LCR) can contrast either the fetal-type (HBG) or adult-type beta-globin genes (HBB), depending on the type of TFs it interacts with. LCR turns on HBG with fetal erythroid-specific TFs like GATA1, but in adults, it turns on HBB because

GATA1 are less expressed. (Peslak et al., 2023) Depending on the embryonic coordination of developmental gene expressions and cell fate determination, these bivalent promoters become either activating or inhibitory, and chromatin accessibility changes are kept accordingly. (Macrae, 2023)

1.3 BAF family of chromatin remodelers

Chromatin remodelers reposition, slide, eject, or edit nucleosomes in an ATP-dependent manner, and their ATP dependency is highly conserved in eukaryotes, including yeast, mice, fruit flies, and humans. (Mashtalir et al., 2018) Human chromatin remodelers include four main subtypes: imitation switch (ISWI), chromodomain helicase DNA-binding (CHD), switch/sucrose non-fermentable (SWI/SNF), and INO80. They are different structurally and genomically, in which the (Clapier et al., 2017)

Among these chromatin remodelers, we are particularly interested in SWI/SNF, also known as BAF complexes, because they are a heterogenous group and have important implications in cancer and neurodevelopmental diseases (NDDs). Mechanistically, SWI/SNF promotes the formation of euchromatin, and are considered as part of the trithorax group (TrxG) which competes with the repressive effect of polycomb repressive complex (PRC) to maintain the balance between euchromatin and heterochromatin. (Bögershausen & Wollnik, 2018) BAF complexes first hydrolyze ATP and use its energy to interact with histones, then make new open chromatin areas by sliding histones around, ejecting subunits or the whole histone. (Mashtalir et al., 2018) SWI/SNF chromatin remodelers share functionally conserved domains, including the SMARCA2/4 ATPase domains, PBM1, BRD7, BRD9 bromodomain-containing proteins, and DPF1/2/3, PHF10 PHD-finger containing proteins. (Centore et al., 2020) Some of these conserved domains have mutually exclusive paralogs, which is the same gene duplicated during evolutionary history and kept in this generation. (Deem & Brisson, 2024)

Maintaining proper chromatin accessibility is crucial for embryonic development, nervous system maturation, and cell or tissue growth. Chromatin remodeler gene mutations dysregulate open and closed chromatin areas, causing a wide range of diseases, such as cancers and neurodevelopmental diseases (NDDs). (Kadoch et al., 2015) About 5-7% of human cancers are caused by SMARCA4 loss-of-function

(LoF) mutations. SMARCA4 is the ATPase subunit of BAF complex, it powers the complex and interacts with genes like BRG1 and BRCA1. If it is unfunctional, BAF complexes cannot open chromatin at tumor suppressor gene sites, leading to lower expression of tumor suppressor genes. (Mardinian et al., 2022) Meanwhile, the most frequent mutation in human NDDs are BAF complex subunit nonsense, missense, and predominantly heterozygous mutations. These NDDs are also called SWI/SNF related intellectual disability disorders (SSRIDDs), a group of NDDs characterized by syndromic autism, intellectual disability, and developmental delay. (Valencia et al., 2023)They are are caused by mutations in either of the genes *ARID1B, ARID1A, ARID2, SMARCA4, SMARCB1, SMARCE1, SOX11*, or *DPF2*, and ARID1A/B is the most common one. (Bögershausen & Wollnik, 2018)

1.4 The non-canonical BAF contains BICRA and BICRAL

Around 2018, multiple independent studies identified a distinct SWI/SNF (BAF) complex variant, the non-canoncial BAF (ncBAF) complex. (figure 2) The ncBAF is compositionally and functionally distinct from the cBAF and pBAF complexes. The first study to identify ncBAF was a sucrose gradient analysis on HEK239T cells. They discovered ncBAF's unique subunits: BRD9, GLTSCR1 (glioma tumor suppressor candidate region gene 1, also known as BICRA, BRD4 Interacting Chromatin-Remodeling Complex Associated Protein), and its paralog GLTSCR1L (or BICRAL).

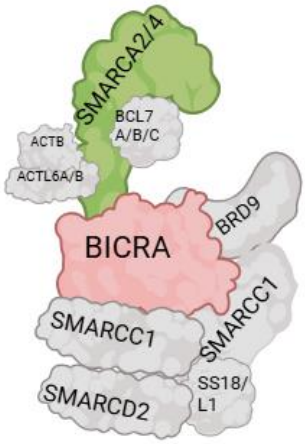


Figure 2: Structure of the ncBAF complex. Created with Biorender.

(Alpsoy & Dykhuizen, 2018) Following that, more research has been done to characterize ncBAF as a distinct complex. Mass spectrometry and Co-IP on BRD9-containing ncBAF complexes showed that BRD9 and GLTSCR1 co-occur in ncBAF complexes in several other types of human cells. (Wang et al., 2019) (Michel et al., 2018) In mouse embryonic stem

cells (ESCs), their ncBAF uses its BRD9 subunit to interact with BRD4 to open chromatin sites at pluripotency genes. (Gatchalian et al., 2018)

Previous studies have shown that the BICRA/L paralogs are thought to be mutually exclusive in ncBAF, but little is known about the functional relationship between BICRA and BICRAL. The study by Alpsoy and Dykhuizen used a doxycycline-inducible FLAG-tagged system to overexpress BICRAL protein in wild type HEK239K cells. They observed decreased BICRA protein expression from the Co-IP on ncBAF, and insignificant change in BICRA mRNA level from RT-qPCR. Vice versa, BICRA overexpression led to reduced BICRAL protein levels. This suggests a feedback loop relationship between BICRA and BICRAL proteins, in which the overexpression of one protein leads to decreased stability of its paralog.

Besides biochemical characterization of ncBAF, researchers also looked at *BICRA* mutations and diseases. Before knowing BICRA/L are mutually exclusive obligate subunits of ncBAF, it was mostly seen to be enriched in diffuse gliomas, giving it the other name *GLTSCR1*. (Tu et al., 2023) Then, several clinical reports have shown a causal relationship between BICRA mutation and Coffin-Siris syndrome (CSS), a type of SSRIDDs characterized by NDD symptoms and dysmorphic hand and facial features. In 2020, Barish et al. first identified nine Coffin-Siris syndrome patients with de novo missense *BICRA* mutation. Following that, two clinical case reports revealed novel heterozygous variant in *BICRA* exon 6 that causes CSS (Asadauskaite et al., 2022) (Tu et al., 2023).

These clinical research inspired people to study the mechanism of BICRA mutation leading to NDDs. Han et al. created a BICRA knockout mice to discover the molecular mechanisms of BICRA leading to neurodevelopmental defects. Instead of finding out that BICRA knockout mice we exhibit mid and late gestation death due to cardiac defects like ventricular septal defect and double outlet right ventricle. However, these defects are caused by genetic dysregulation of cardiac progenitor cells, rather than cell death. Normally, BICRA blocks the promoter and enhancer and prevents natriuretic peptide type A (NPPA) expression, so the loss of BICRA promotes NPPA overexpression. Mutation of NPPA usually leads to heart mophological defects, and NPPA is an diagnostic and prognostic biomarker for

cardiovascular diseases. (Han et al., 2022) Consistent with Alpsoy's research on BICRA knockout cell survival, mouse BICRA knockout did not affect myocardial cell proliferation and apoptosis.

1.5 Research is needed for understanding the functional relatedness of BICRA and BICRAL

While previous research has shown a reciprocal feedback loop between BICRA and BICRAL at the protein level, the regulatory logic behind this mechanism remains unknown. If overexpressing one paralog lowers the expression level of the other, then what happens when one is completely lost? Can the remaining paralog compensate, and if so, how? This question has significant clinical relevance. Since BICRA loss-of-function (LoF) mutations lead to SSRIDDs in children, understanding how BICRA/L compensate for one other, especially in human induced pluripotent stem cells (iPSCs), could help elucidate SSRIDDs mechanism or therapeutic strategies.

Chapter 2: Evidence of a feedback loop between BICRA and BICRAL in iPSCs

2.1 Overview

Induced pluripotent stem cells (iPSCs) are commonly used as models for disease modeling because they can unlimitely expand and differentiate to diverse cell types or organoids. iPSCs share similar self regeneration and differentiation capacity with ESCs, so they are important model for understanding the effects of genetic mutations on developing embryos or organs. (Cerneckis et al., 2024) The traditional genetic method to study gene function is to knock out the gene and observe phenotypes, so my lab used CRISPR-Cas9 to create BICRA and BICRAL knockout iPSCs, and genotyped them with Sanger sequencing and Western blot. We want to know whether knocking out BICRA or BICRAL will change the expression of its paralog in iPSCs.

2.2 Generation of BICRA and BICRAL knockout iPSCs

CRISPR-Cas9, developed in 2013, is a commonly used technique to edit mammalian cell genome. Compared to other gene editing methods, it is cheaper and easier. It works by delivering a guide RNA

(gRNA) and Cas9 protein through electroporation to iPSCs, and the gRNA will bind to the target gene editing site, while the Cas9 creates a double stranded break. (Sanjurjo-Soriano et al., 2022) The two gRNA sites for BICRA knockouts (KO) are around BICRA exon 9. (Figure 2A) The three gRNA sites for BICRAL KO flanked exon 6. (Figure 2B) The gene editing then creates two double stranded DNA breaks that cuts off a whole exon. This creates an early stop codon in the target gene, and its mRNA transcripts will undergo nonsense-mediate decay (NMD), a mechanism that rapidly degrades mRNA with premature termination codon. (Lejeune, 2022) After NMD, the mRNA cannot make the protein product. Therefore, the CRISPR-generated loss-of-function mutation leads to a complete knockout of the gene. The CRISPR-Cas9 edited iPSCs clones are then cultured to 70% confluency, and then used for future experiments.

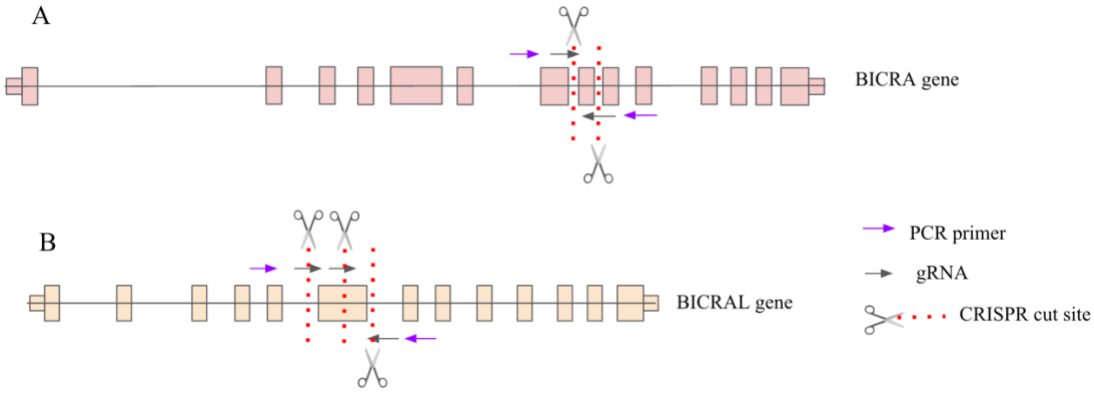


Figure 3: CRISPR editing schematic for *BICRA* and *BICRAL* gene. A. *BICRA* exon 9 was knocked out by two sets of gRNAs. B. *BICRAL* exon 6 were knocked out by three sets of gRNAs.

2.3 BICRA and BICRAL KO genotyping

iPSCs DNA are isolated with the Qiagen DNA extraction toolkit. To select desired clones, the cells were genotyped with PCR followed by Sanger sequencing. The PCR primers are designed to amplify a 800 bp region that flanks the edited exons in wildtypes (figure 4), show light signals in the heterozygous KO, and no signal in the homozygous KO. The PCR products are verified by gel electrophoresis, amplified with TOPO cloning, and the clones are sent to sequencing company, where the bacterial

plasmids were isolated and sequenced with Sanger sequencing technique. Sanger sequencing involves incorporating labeled dNTPs lacking a 3′-OH (ddNTP) into the ~1500 bp TOPO cloning plasmids. (Rodriguez & Krishnan, 2023) Although it is more costly than next generation sequencing, it is more accurate, has higher read length, and commonly used for resequencing small genome regions or genotyping PCR products. (Verma et al., 2017)

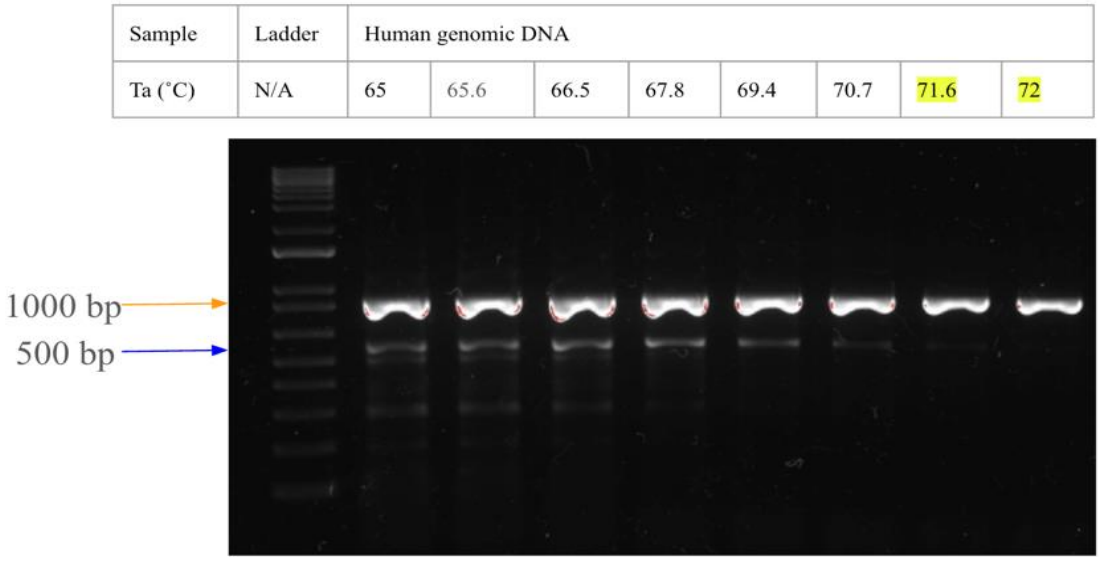


Figure 4: BICRA exon 9 genotyping primer efficiency confirmed. The PCR products were run on a 1.5% agarose gel, and target product size was 891 bp.

We screened 47 BICRA knockout clones (figure 5) and 48 BICRAL knockout clones (figure 6) with PCR and gel electrophoresis. From that, we picked 2 homozygous KOs, 2 heterozygous KOs, and 2 wildtypes for both BICRA and BICRAL, which enhances data validity and accounts for biological variation. (figure 7)

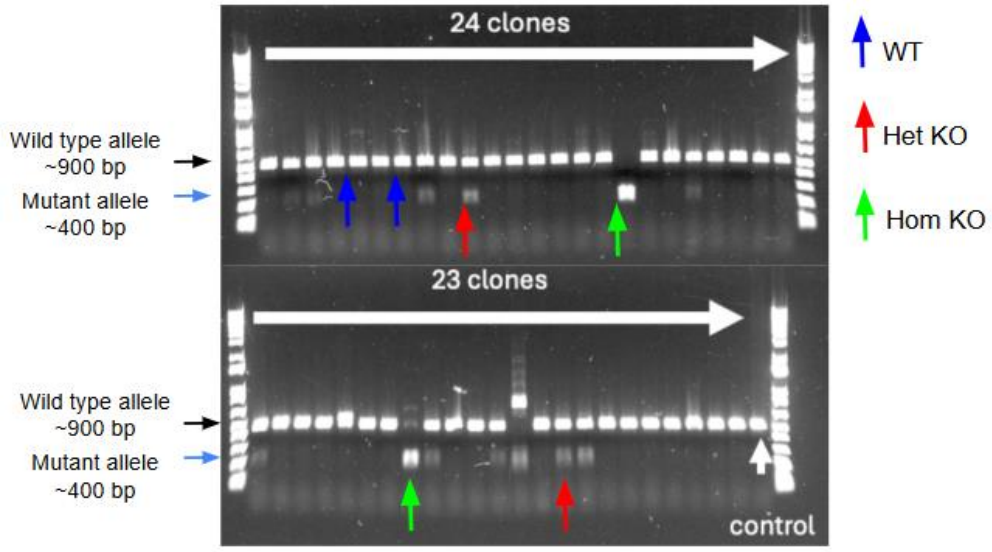


Figure 5: BICRA knockout clone selection and identification. The wildtype allele product size is around 900 bp, but the mutant allele is around 400 bp. Two homozygous KOs, two heterozygous KOs, and the wildtypes were used to generate them were selected for further experiments. (Figure from Ziben Zhou)

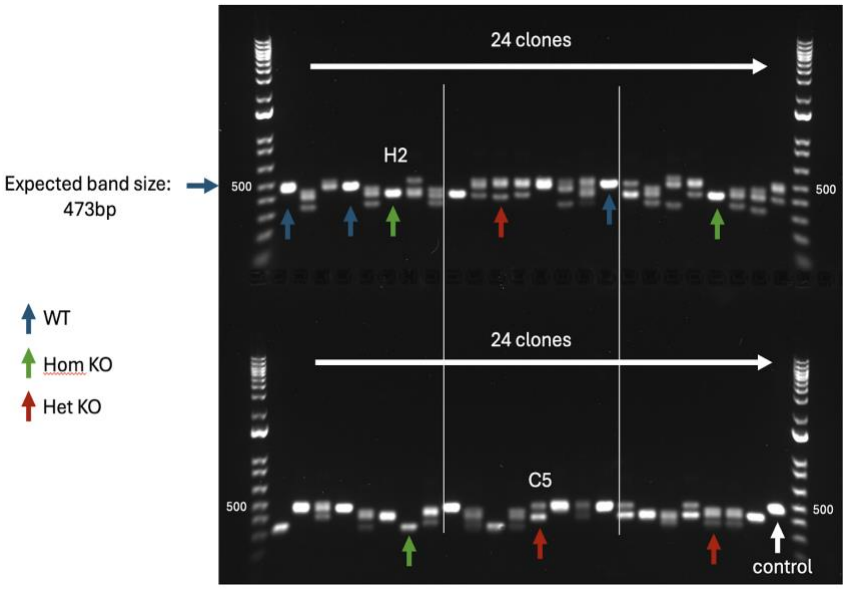


Figure 6: BICRAL knockout clone screening and selection. the DNA of BICRAL clones were isolated, amplified with PCR primer set 2, then ran on 1.5% agarose gel. The expected band size for wildtype allele was 473 bp, and that for mutant was about 200 bp. (Figure from Ziben Zhou)

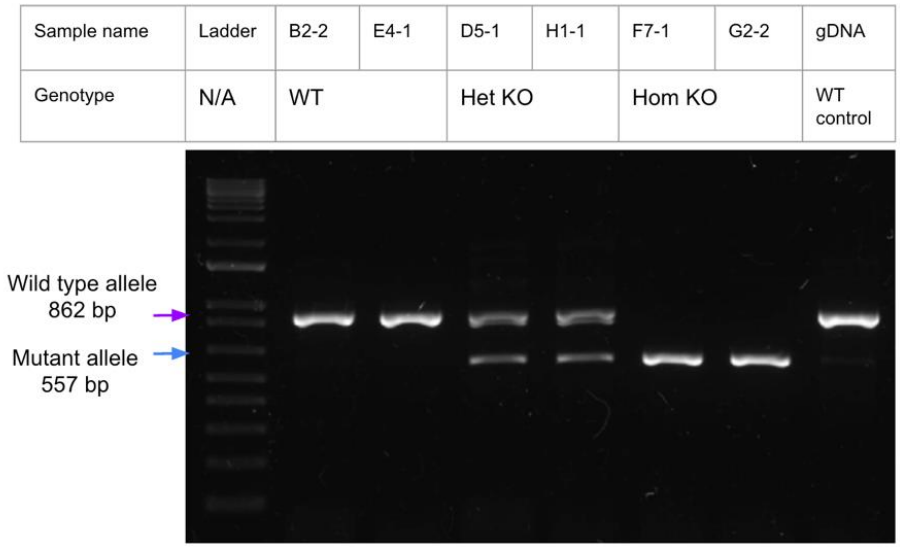


Figure 7: Confirmed BICRA wildtype and heterozygous clones. The cells' gDNA was amplified with PCR using primers in figure 4, and products were run on 1.5% agarose gel.

To confirm whether the heterozygous knockouts had one truncated BICRA allele and one normal one, instead of a mixture of homozygous knockouts and wildtypes, we used SNP phasing to test whether the gene editing site is on the same alleles for all clones. We picked a SNP in BICRA intron 9, amplified a 1000 bp region including the SNP with PCR, and sequenced the PCR product. The result shows that all the truncated BICRA allele has a G for the SNP, and the normal ones had A, and this SNP is heterozygous in the wildtype.

2.4 BICRA and BICRAL KO Western Blot

Based on the genotyping results, two homozygous KO, two heterozygous KO, and two wild-type of both BICRA KO and BICRAL KO iPSCs were selected (figure 7). Then, their phenotypes were verified by quantitative Western blot (WB). Since the target proteins BICRA and BICRAL were mostly located in the nucleus, the WB was done on the nuclear extracts. WB involves a protein SDS-PAGE followed by protein wet transfer, antibody incubation, imaging, and ImageJ based densitometry measurements. The blot quantifies protein level by using a primary antibody (Ab) to bind with the target protein and its

housekeeping protein control, a secondary Ab and imaging reagent to amplify the primary Ab signals, and calculating he blot size and intensity of the target protein relative to the housekeeping protein. (Taylor & Posch, 2014)

The WBs confirmed that BICRA and BICRAL knockout were successful, and BICRAL protein is upregulated in BICRAL homozygous knockouts. (Figure 8A and 9A) Homozygous knockouts make no target, and heterozygous knockout makes less target protein than the wild type. (Figure 8B and 9B) Surprisingly, BICRA homozygous knockouts express significantly more BICRAL protein (figure 8B), but BICRAL knockouts express the same level of BICRA protein as the wildtypes. (figure 9B)

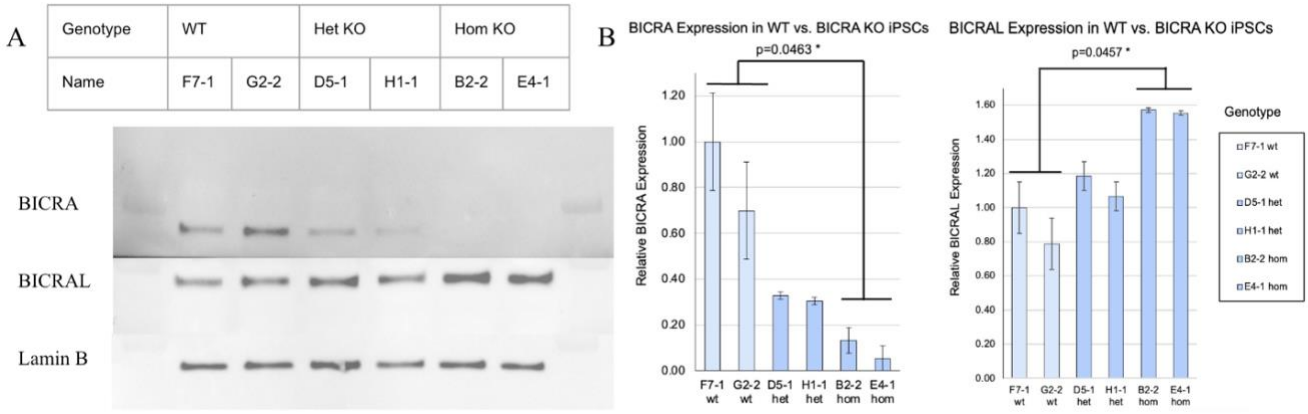


Figure 7: Western blot on BICRA knockout iPSCs. A. Whole Western blot membrane B. Quantification of BICRA and BICRAL protein expression level, protein signal was normalized to lamin B using ImageJ.

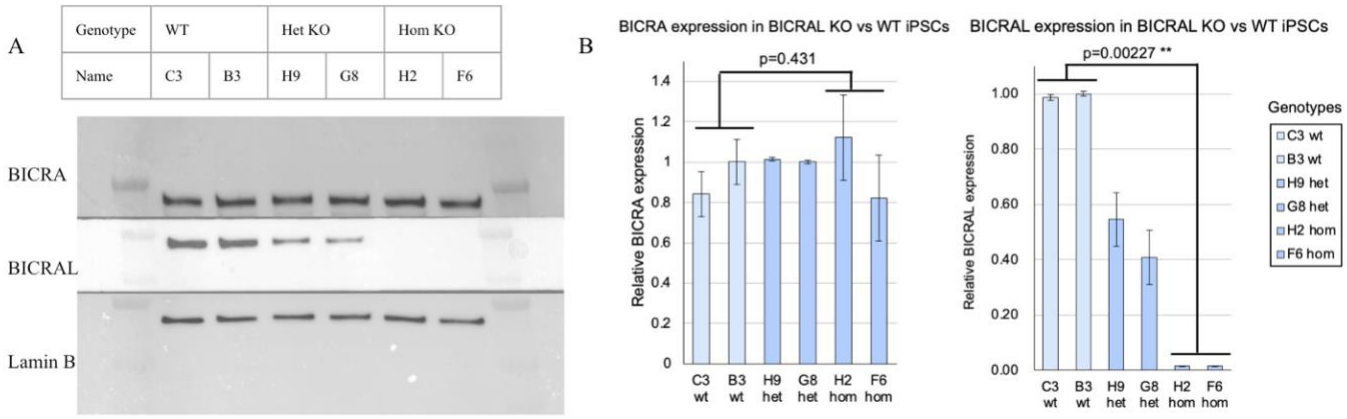


Figure 8: Western blot on BICRAL knockout iPSCs. A. Whole Western blot membrane B. Quantification of BICRA and BICRAL protein expression level, protein signal was normalized to lamin B using ImageJ.

2.5 Conclusion: BICRAL protein level is increased in BICRA KO iPSCs

Data has shown that BICRAL protein is upregulated in the absence of BICRA especially in the homozygous knockouts, but BICRA expression is not changed in BICRAL knockouts. This surprising finding made us ask the question on whether BICRAL is transcriptionally upregulated in BICRA KOs.

Chapter 3: BICRAL is transcriptionally upregulated in BICRA knockouts

3.1 Overview

Reverse transcription-quantitative PCR (RT-qPCR) is a commonly used method to quantify the abundance of a specific mRNA transcript. (Bong et al., 2024) iPSCs were harvested at 70% confluency, snap frozen, and total RNA was isolated. RNA quality was measured by Agilent 2100 tape station, which uses a 1-10 value system to assess RNA integrity, and a larger number indicated more intact RNA. (Walker et al., 2024) All six RNA samples had high integrity score, and are thus proceeded to reverse transcription. (Figure 10)

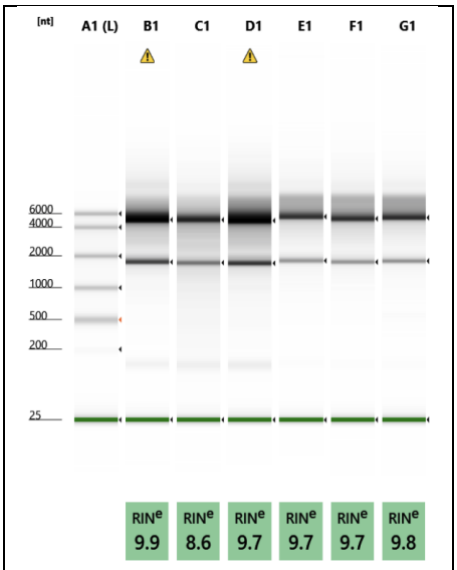


Figure 10: RNA quality check in BICRA KO clones. RNA integrity scores were measured with RNA tape station.

Since the cell's total RNA is unstable, it is first treated with DNase to eliminate DNA, then treated with Superscript IV reverse transcriptase to synthesize complementary DNA (cDNA). The Superscript IV enzyme can hydrolyze the DNA:RNA hybrid during RT, thus having high sensitivity, yield, and precision. (Verwilt et al., 2023) cDNA from mature mRNA only has exons because the introns are spliced during maturation. The RT-qPCR probes flank two exons of a single gene, ensuring that it only binds to cDNA synthesized from mRNA.

3.2 Testing BICRAL probe specificity

The BICRAL probe specificity was measured by running the qPCR reaction with the wild-type iPSC cDNA, BICRAL qPCR probe, and NebNext DNA polymerase Master Mix. The products' specificity were confirmed with gel electrophoresis, and were TOPO cloned and sequenced. The sequencing result showed that the probe amplified the target amplicon and had no off-target effects.

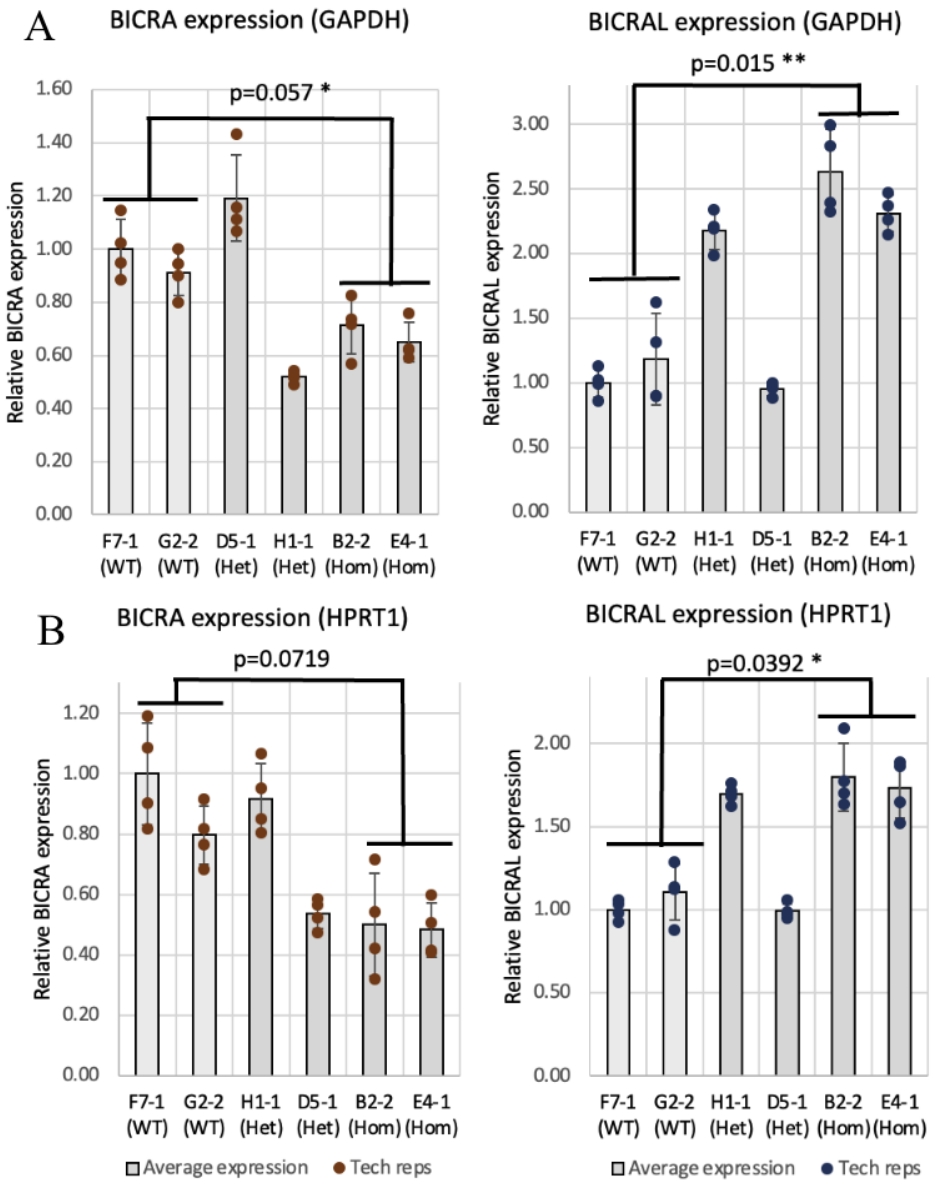
3.3 BICRAL is transcriptionally upregulated in BICRA KO iPSCs

To test the mRNA transcript abundance of BICRA and BICRAL in the iPSCs, I used reverse transcription quantitative PCR (RT-qPCR). I extracted the total mRNA from BICRA KO iPSCs, reverse transcribed to cDNA, and quantified BICRA, BICRAL, GAPDH, and HPRT1 transcript level. The RT-qPCR on BICRA KO vs WT iPSCs were done twice with either GAPDH or HPRT1 as control to ensure data reproducibility.

In these experiments, BICRA homozygous knockouts had a significant increase in BICRAL expression compared to the wild-type. Although less significant, the BICRA knockouts have less BICRA mRNA. Interestingly. there is a high degree of variation between the two heterozygous knockouts. (Figure 10) H1-1 is more similar to the homozygous knockout as it has higher BICRAL and lower BICRA, but D5-1 is more similar to the wild type in terms of mRNA expression. (Figure 10)

3.4 Summary

The RT-qPCR confirmed that the feedback loop happens one way round: BICRAL is transcriptionally increased in BICRA knockouts. The CRISPR knockout was successful, as it lowered BICRA mRNA to about 50% of that in the wild-type and eliminated BICRA protein. One possibility is that not all BICRA mRNA in the homozygous knockouts have undergone NMD, making them detectable by RT-qPCR. The variation at BICRA mRNA level of the heterozygous knockouts might be accounted by intracellular pathways that result in differences mRNA stability.



=

Figure 11: RT-qPCR on BICRA and BICRAL mRNA expression in BICRA knockouts versus wild type iPSCs. A. BICRA and BICRAL expression levels in iPSCs using GAPDH as control. B. BICRA and BICRAL expression levels using HPRT1 as control. A t-test was done on the average expression levels of the wildtypes and homozygous knockouts to compare the significance of expression differences.

Chapter 4: Materials and Methods

4.1 Cell Culture

Two biological replicates of the BICRA homozygous knockout, BICRA heterozygous knockout, and wild type iPSC were cultured according to protocol from the stem cell core. As they reach ~50% confluency, the iPSCs were harvested and snap frozen.

4.2 CRISPR-Cas9 and genotyping

The knockout was verified by PCR, TOPO cloning, and Sanger sequencing. The PCR primers sequences were designed with SnapGene. (table 1) They were around the knocked-out exon of BICRA/L, and the product was verified by gel electrophoresis (1.5% agarose). Verified product was cloned in E. Coli following the manufacturer's protocol for Zero Blunt TOPO PCR cloning kit (invitrogen, 450031). After overnight competent E. coli culture, ten single clones containing the same transgene were picked for Sanger sequencing, and data was visualized with SnapGene.

Primer	Sequence
BICRA genotyping forward primer	GACTTCCAGCTCCAGTTCCC
BICRA genotyping reverse primer	GGCAAAAGCCTTGTTCTCGG
BICRA phasing forward primer	AATCATCCTCCAGAACAAGGCTGGG
BICRA phasing reverse primer	CACCGTCTCCGTGTTTCTCCCTCAT
BICRAL genotyping forward primer	AATGCTGATCCTAAGTCATCCC
BICRAL genotyping reverse primer	CCAGTGATGGACTGCCACTT

Table 1: Genotyping primer sequences

4.3 RNA extraction, quality validation, and reverse transcription

Total mRNA was isolated with the RNeasy MinElute Kits (Qiagen, 74204). RNA quality was assessed using RNA HS ScreenTapes (Agilent, 5067-5579) and stored at -80ºC. Reverse transcription was done following the manufacturer's protocol of SuperScript IV VILO Master Mix with ezDNAse Enzyme (ThermoFisher Scientific, 11766050), and cDNA samples were stored at -20 ºC. RNA quality validation was done with the Agilent technologies 4200 tapestation machine with the Agilent high sensitivity RNA screen tape assay protocol.

4.4 RT-qPCR

cDNA sample concentration was measured by the Qubit flex fluorometer from Thermo Fisher using the dsDNA HS assay kit. cDNA was diluted with molecular biology grade water to ensure even concentration across samples. The BICRA, BICRAL, GAPDH, and HPRT1 probes are all ordered from thermofisher and were tested to be effective.There were three technical replicate for each sample and genotype, and each replicate is 20 uL, including 1 uL cDNA (~5 ng), 10 uL TaqMan™ Fast Advanced Master Mix (ThermoFisher Scientific, 4444556) from appliedbiosystems, 1 uL TaqMan probe, and 8 uL molecular biology grade water. The qPCR was run on the biorad 1000 series thermal cycling platform with the following protocol. *GAPDH* and *HPRT1* Ct values were used as the reference to measure *BICRA/L* gene expression levels. The average ΔCt value of BICRA/L for every sample was the average of three technical replicates' target gene Ct minus reference gene Ct. The ΔΔCt value for each sample was calculated by subtracting the average reference gene Ct values of the wildtype from the average ΔCt of each sample's target gene. Relative target gene expression level in each sample was calculated with $2^{-\Delta\Delta Ct}$ divided by the average of the target gene expression of the two wild type samples. The average expression levels of wildtype and knockouts was calculated and plotted, and the error bars are the target gene expression of two biological replicates.

4.5 Western blot

The protein concentration of iPSC nuclear extracts were evaluated with the BCA assay. Then, their concentration was normalized to 20 ng/uL.10 uL samples plus 10 uL 2*Laemmli sample buffer from biorad were loaded to each well of the mini-PROTEAN TGX stain-free precast gels from biorad. The gel was run at 200 V with the 1x running buffer with SDS, and proteins were transferred to the PVDF membrane in the transfer buffer at 100 V in 4 degrees for one hour. The membrane was equilibrated with the transfer buffer, then blocked with 1x TBST with 5% milk for an hour, cut according to protein sizes, then incubated with primary antibodies at 4 degrees overnight. The primary rabbit antibodies for

BICRA/L and lamin B are from cell signaling technology, and the concentrations were: GLTSCR1 1:1000, GLTSCR1L 1:500, and lamin B 1:2000. Lamin B was used as the internal control to check for equal protein sample loading. After that, the membrane was incubated with the secondary anti-rabbit antibody at concentration of 1:2000 for 1 hour at room temperature. The membrane was washed with 1x TBST buffer, incubated in 1:1 mixed ECL prime Western blotting detection reagents from cytiva for 2 minutes, and imaged with colormetric and chemiluminescent imaging to take picture. ImageJ was used to quantify the sizes of protein band.

Chapter 5: Discussion and conclusions

5.1 Summary and limitations

I found that BICRAL is transcriptionally upregulated in BICRA knockout iPSCs, as shown by a ~2 fold increase in the mRNA, and ~1.5 fold increase in the protein level. The feedback loop does not happen on the other way round: in the absence of BICRAL, BICRA protein do not upregulate. Given prior evidence that BICRAL protein stability is decreased in BICRA overexpression cells, and vice versa, I think that the upregulation of BICRAL in BICRA knockouts is likely due to two reasons: BICRAL transcription is increased, and BICRAL protein is stabilized.

However, my experiments have technical limitations. In the RT-qPCR experiments, I tried both GAPDH and HPRT1 for housekeeping reference gene, but I ended up only using data from HPRT1. The GAPDH Ct value varied more than the HPRT1 control, indicating that the GAPDH expression level was probably affected by BICRA gene knockout, making it less desirable as a control gene. Confirming the transcriptional changes of BICRA in BICRAL absent cells will rule out the possibility that BICRA is transcriptionally upregulated but its protein degraded fast.

In the Western blot experiments, the Santa Cruz BICRA antibody signal was relatively lower, because it is not optimized for human BICRA protein. It would be better to have stronger primary antibodies, like

the BICRA antibody from cell signaling technology. While having two clones for each genotype eliminates concerns for biological variability in between two clones of the same genotype, we could have more clones to get more reproducible data. Biologically, measuring protein expression level in the iPSC nuclear extract through Western blot only tells information about total protein level at a given time, rather than how long it takes for iPSCs to make more BICRAL protein in response to the loss of BICRA.

5. 2 Potential mechanism for BICRAL upregulation in BICRA knockouts and future experiments

Following up on the consistent observation of increased BICRAL mRNA expression in BICRA KO iPSCs, I think it is important to investigate how this happens and whether this is specific to iPSCs. The two steps of making mature mRNA that is detectable with RT-qPCR include: first, transcribing BICRAL DNA into pre-RNA, and second, producing mature mRNA through alternative splicing. BICRAL transcription level is related to its cRE activities, and I hypothesize that BICRA KO changes the accessibility of its cREs, so the gene produces more BICRAL pre-RNA. It is also possible that BICRAL mRNA is more stable in BICRA KO iPSCs compared to the wildtype, and I speculate that BICRA KOs have less BICRAL-silencing siRNAs.

The future directions involve elucidating how BICRAL mRNA is upregulated in iPSCs and their derivatives. This can be done by comparing the ratio of BICRAL pre-RNA and functional mRNA in a single cell, and the relative amount of BICRAL pre-RNA in BICRA KO and WT. All require designing a qPCR probe that flanks a consecutive intron and exon in BICRAL pre-RNA. If I observe increased BICRAL pre-RNA, then I will investigate whether BICRA KO changes the chromatin accessibility landscape of BICRAL cREs by comparing their ATAC-seq data. If I only observe increased BICRAL mRNA, then I will look for potential mechanisms that increase the half-life or decrease degradation. I will look for changes in inhibitory RNAs from the RNA-seq data of the BICRA KO and WT, and I expect to see a decrease in RNAs that inhibit BICRAL mRNA.

5. 3 Future directions and broader impacts

Building on the transcriptional and translational upregulation of BICRAL in the absence of BICRA, a possible explanation is that: in the absence of BICRA, the demand for ncBAF structural integrity drives a compensatory increase in BICRAL expression. Alternatively, BICRA-containing ncBAF may play regulatory roles in limiting BICRAL transcription under normal conditions, and their absence could result in de-repression. I speculate that BICRAL upregulation happens naturally in BICRA knockout cells through transcriptional upregulation.

However, iPSCs have unique transcription features and nuclear organizations that make them different from neurons and glial cells. SSRIDDs, caused by the disruption of chromatin remodeler genes and with neurodevelopmental symptoms, are likely impairing brain cells. Therefore, it is worth investigating whether the BICRAL transcriptional upregulation trend is persistent in iPSC-derived neurons and glia. From that, we can gain deeper insights into how ncBAF structural shifts influence transcriptional regulation during neurodevelopment—and whether these shifts might serve as points of future therapeutic intervention. Overexpressing BICRAL may partly rescue NDD symptoms caused by defective BICRA-containing ncBAF.

Works Cited

1. Alpsoy A, Dykhuizen EC. Glioma tumor suppressor candidate region gene 1 (GLTSCR1) and its paralog GLTSCR1-like form SWI/SNF chromatin remodeling subcomplexes. J Biol Chem. 2018 Mar 16;293(11):3892–903.
2. Asadauskaitė G, Morkūnienė A, Utkus A, Burnytė B. Identification of a novel BICRA variant leading to the newly described Coffin-Siris syndrome 12. *Brain Dev*. 2023;45(3):185-190. doi:10.1016/j.braindev.2022.11.003
3. Barakat TS, Halbritter F, Zhang M, et al. Functional Dissection of the Enhancer Repertoire in Human Embryonic Stem Cells. *Cell Stem Cell*. 2018;23(2):276-288.e8. doi:10.1016/j.stem.2018.06.014
4. Barish S, Barakat TS, Michel BC, et al. BICRA, a SWI/SNF Complex Member, Is Associated with BAF-Disorder Related Phenotypes in Humans and Model Organisms. The American Journal of Human Genetics. 2020 Dec;107(6):1096–112.
5. Bögershausen N, Wollnik B. Mutational Landscapes and Phenotypic Spectrum of SWI/SNF-Related Intellectual Disability Disorders. Front Mol Neurosci. 2018 Aug 3;11:252.
6. Bong D, Sohn J, Lee SV. Brief guide to RT-qPCR. *Mol Cells*. 2024;47(12):100141. doi:10.1016/j.mocell.2024.100141
7. Cerneckis, J., Cai, H. & Shi, Y. Induced pluripotent stem cells (iPSCs): molecular mechanisms of induction and applications. *Sig Transduct Target Ther* 9, 112 (2024). https://doi.org/10.1038/s41392-024-01809-0
8. Centore RC, Sandoval GJ, Soares LMM, Kadoch C, Chan HM. Mammalian SWI/SNF Chromatin Remodeling Complexes: Emerging Mechanisms and Therapeutic Strategies. *Trends Genet*. 2020;36(12):936-950. doi:10.1016/j.tig.2020.07.011
9. Chereji RV, Eriksson PR, Ocampo J, Prajapati HK, Clark DJ. Accessibility of promoter DNA is not the primary determinant of chromatin-mediated gene regulation. *Genome Res*. 2019;29(12):1985-1995. doi:10.1101/gr.249326.119

10. Clapier CR, Iwasa J, Cairns BR, Peterson CL. Mechanisms of action and regulation of ATP-dependent chromatin-remodelling complexes. Nat Rev Mol Cell Biol. 2017 Jul;18(7):407–22.
11. Cutter AR, Hayes JJ. A brief review of nucleosome structure. *FEBS Lett*. 2015;589(20 Pt A):2914-2922. doi:10.1016/j.febslet.2015.05.016
12. Deem KD, Brisson JA. Problems with Paralogs: The Promise and Challenges of Gene Duplicates in Evo-Devo Research. *Integr Comp Biol*. 2024;64(2):556-564. doi:10.1093/icb/icae009
13. Gatchalian, J., Malik, S., Ho, J., Lee, D. S., Kelso, T. W. R., Shokhirev, M. N., Dixon, J. R., & Hargreaves, D. C. (2018). A non-canonical BRD9-containing BAF chromatin remodeling complex regulates naive pluripotency in mouse embryonic stem cells. Nature communications, 9(1), 5139. https://doi.org/10.1038/s41467-018-07528-9
14. Grandi FC, Modi H, Kampman L, Corces MR. Chromatin accessibility profiling by ATAC-seq. *Nat Protoc*. 2022;17(6):1518-1552. doi:10.1038/s41596-022-00692-9
15. Gratacós Arenas MA, Soler Portilla C, Carlo S, Arciniegas NJ. Neurodevelopmental Disorders and the Mystery of the Genes Involved: A Case Report of a BICRA Heterozygous Mutation Identified in Autism Spectrum Disorder. *Cureus*. 2024;16(8):e66442. Published 2024 Aug 8. doi:10.7759/cureus.66442
16. Han F, Yang B, Chen Y, Liu L, Cheng X, Huang J, et al. Loss of GLTSCR1 causes congenital heart defects by regulating NPPA transcription. Angiogenesis. 2023;26(2):217–32.
17. Han, Leng et al. “CpG island density and its correlations with genomic features in mammalian genomes.” *Genome biology* vol. 9,5 (2008): R79. doi:10.1186/gb-2008-9-5-r79
18. Kadoch C, Crabtree GR. Mammalian SWI/SNF chromatin remodeling complexes and cancer: Mechanistic insights gained from human genomics. *Sci Adv*. 2015;1(5):e1500447. Published 2015 Jun 12. doi:10.1126/sciadv.1500447
19. Klemm, S.L., Shipony, Z. & Greenleaf, W.J. Chromatin accessibility and the regulatory epigenome. *Nat Rev Genet* **20**, 207–220 (2019). https://doi.org/10.1038/s41576-018-0089-8

20. Lejeune F. Nonsense-Mediated mRNA Decay, a Finely Regulated Mechanism. *Biomedicines*. 2022;10(1):141. Published 2022 Jan 10. doi:10.3390/biomedicines10010141
21. Li, Lijia et al. “Multifaceted SOX2-chromatin interaction underpins pluripotency progression in early embryos.” *Science (New York, N.Y.)* vol. 382,6676 (2023): eadi5516. doi:10.1126/science.adi5516
22. Louise Smith, Emily et al. “Investigating chromatin accessibility during development and differentiation by ATAC-sequencing to guide the identification of cis-regulatory elements.” *Biochemical Society transactions* vol. 50,3 (2022): 1167-1177. doi:10.1042/BST20210834
23. Macrae TA, Fothergill-Robinson J, Ramalho-Santos M. Regulation, functions and transmission of bivalent chromatin during mammalian development. *Nat Rev Mol Cell Biol*. 2023;24(1):6-26. doi:10.1038/s41580-022-00518-2
24. Mardinian K, Adashek JJ, Botta GP, Kato S, Kurzrock R. SMARCA4: Implications of an altered chromatin-remodeling gene for cancer development and therapy. Mol Cancer Ther. 2021 Oct 12;10.1158/1535-7163.MCT-21–0433.
25. Mansisidor AR, Risca VI. Chromatin accessibility: methods, mechanisms, and biological insights. *Nucleus*. 2022;13(1):236-276. doi:10.1080/19491034.2022.2143106
26. Mashtalir N, D’Avino AR, Michel BC, Luo J, Pan J, Otto JE, et al. Organization and Assembly of SWI/SNF Family Chromatin Remodeling Complexes. Cell. 2018 Nov 15;175(5):1272-1288.e20.
27. Michel, B. C., D'Avino, A. R., Cassel, S. H., Mashtalir, N., McKenzie, Z. M., McBride, M. J., Valencia, A. M., Zhou, Q., Bocker, M., Soares, L. M. M., Pan, J., Remillard, D. I., Lareau, C. A., Zullow, H. J., Fortoul, N., Gray, N. S., Bradner, J. E., Chan, H. M., & Kadoch, C. (2018). A non-canonical SWI/SNF complex is a synthetic lethal target in cancers driven by BAF complex perturbation. Nature cell biology, 20(12), 1410–1420. https://doi.org/10.1038/s41556-018-0221-1
28. Peslak SA, Demirci S, Chandra V, et al. Forced enhancer-promoter rewiring to alter gene expression in animal models. *Mol Ther Nucleic Acids*. 2023;31:452-465. Published 2023 Jan 31. doi:10.1016/j.omtn.2023.01.016

29. Rodriguez R, Krishnan Y. The chemistry of next-generation sequencing. *Nat Biotechnol*. 2023;41(12):1709-1715. doi:10.1038/s41587-023-01986-3
30. Sanjurjo-Soriano C, Erkilic N, Mamaeva D, Kalatzis V. CRISPR/Cas9-Mediated Genome Editing to Generate Clonal iPSC Lines. *Methods Mol Biol*. 2022;2454:589-606. doi:10.1007/7651_2021_362
31. Schmetz A, Lüdecke HJ, Surowy H, et al. Delineation of the adult phenotype of Coffin-Siris syndrome in 35 individuals. *Hum Genet*. 2024;143(1):71-84. doi:10.1007/s00439-023-02622-5
32. Shlyueva, D., Stampfel, G. & Stark, A. Transcriptional enhancers: from properties to genome-wide predictions. Nat Rev Genet 15, 272–286 (2014). https://doi.org/10.1038/nrg3682
33. Szerlong HJ, Hansen JC. Nucleosome distribution and linker DNA: connecting nuclear function to dynamic chromatin structure. *Biochem Cell Biol*. 2011;89(1):24-34. doi:10.1139/O10-139
34. Taylor SC, Posch A. The design of a quantitative western blot experiment. *Biomed Res Int*. 2014;2014:361590. doi:10.1155/2014/361590
35. Tu Y, Fang C, Xu J, Zhou Y, Liang M, Yang Z. A de novo variant of BICRA results in Coffin-Siris syndrome 12. *Mol Genet Genomic Med*. 2023;11(11):e2250. doi:10.1002/mgg3.2250
36. Usluer S, Hallast P, Crepaldi L, et al. Optimized whole-genome CRISPR interference screens identify ARID1A-dependent growth regulators in human induced pluripotent stem cells. *Stem Cell Reports*. 2023;18(5):1061-1074. doi:10.1016/j.stemcr.2023.03.008
37. Uhler, C., Shivashankar, G. Regulation of genome organization and gene expression by nuclear mechanotransduction. *Nat Rev Mol Cell Biol* 18, 717–727 (2017). https://doi.org/10.1038/nrm.2017.101
38. Valencia AM, Sankar A, van der Sluijs PJ, et al. Landscape of mSWI/SNF chromatin remodeling complex perturbations in neurodevelopmental disorders. *Nat Genet*. 2023;55(8):1400-1412. doi:10.1038/s41588-023-01451-6

39. Venkatesh, S., Workman, J. Histone exchange, chromatin structure and the regulation of transcription. *Nat Rev Mol Cell Biol* 16, 178–189 (2015). https://doi.org/10.1038/nrm3941

40. Verma M, Kulshrestha S, Puri A. Genome Sequencing. *Methods Mol Biol*. 2017;1525:3-33. doi:10.1007/978-1-4939-6622-6_1

41. Verwilt J, Mestdagh P, Vandesompele J. Artifacts and biases of the reverse transcription reaction in RNA sequencing. *RNA*. 2023;29(7):889-897. doi:10.1261/rna.079623.123

42. Walker JE, Oliver JC, Stewart AM, et al. Measuring Up: A Comparison of TapeStation 4200 and Bioanalyzer 2100 as Measurement Tools for RNA Quality in Postmortem Human Brain Samples. *Int J Mol Sci*. 2023;24(18):13795. Published 2023 Sep 7. doi:10.3390/ijms241813795

43. Wang, X., Wang, S., Troisi, E.C. et al. BRD9 defines a SWI/SNF sub-complex and constitutes a specific vulnerability in malignant rhabdoid tumors. Nat Commun 10, 1881 (2019). https://doi.org/10.1038/s41467-019-09891-7