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SANTA CRUZ

DISCOVERY OF A NOVEL ROLE FOR TRNA GENES IN THE REGULATION OF OVERLAPPING PROTEIN-CODING GENE TRANSCRIPTION

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in

BIOMOLECULAR ENGINEERING AND BIOINFORMATICS

by

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Abstract

DISCOVERY OF A NOVEL ROLE FOR TRNA GENES IN THE REGULATION OF OVERLAPPING PROTEIN-CODING GENE TRANSCRIPTION

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This thesis explores the intricate regulatory mechanisms involving embedded tRNA genes within the introns and 3' untranslated regions (UTRs) of protein-coding genes during neural development. Transfer RNAs (tRNAs), essential non-coding RNAs integral to mRNA translation, have recently been found to significantly regulate neighboring protein-coding genes. This study focuses on four $tRNA^{Gly(GCC)}$ genes embedded within the introns of VAC14 and the $tRNA^{Arg(TCT)}$ gene within the 3'UTR of HES7, elucidating their regulatory functions.

The first chapter employs computational and evolutionary methods to investigate the regulatory role of $tRNA^{Gly(GCC)}$ genes within VAC14 introns. Using published DNase-seq, RNA-seq, and ChIP-seq data, I mapped chromatin accessibility changes and transcription factor binding during mouse neuronal differentiation. Functional and comparative genomic analyses revealed that the tRNA genes in the first intron appear to function as enhancer elements, and Zic transcription factors play a previously unrecognized suppressive regulatory role in VAC14 gene transcription during neuronal maturation.

The second chapter provides an experimental analysis of the transcriptional regulation of VAC14 by its intronic $tRNA^{Gly(GCC)}$ genes. Utilizing CRISPR/Cas9-mediated homology-directed recombination (HDR) in HEK293T cells, I deleted and mutated the tRNA genes to assess their influence on VAC14 expression. ChIP-seq analysis and western blotting demonstrated that these tRNA genes impact VAC14 expression through transcriptional interference, revealing an interaction between Pol II and Pol III. These

findings offer new insights into the transcriptional control mechanisms involved in regulating VAC14.

The third chapter examines the regulatory potential of the $tRNA^{Arg(TCT)}$ gene within the HES7 3'UTR, hypothesizing its influence on HES7 expression through chromatin architecture modulation and transcriptional interference. Luciferase reporter assays, along with ChIP-seq and RNA-seq data, demonstrated that the UTR-overlapping $tRNA^{Arg(TCT)}$ gene significantly modulates HES7 expression, shedding light on broader regulatory networks essential for neural development and potential impacts on developmental disorders.

This thesis provides novel insights into the complex regulatory networks involving tRNA genes and their impact on host protein-coding genes. Understanding these regulatory mechanisms sheds light on pol3-driven RNA gene regulation of key protein-coding genes during neural development, offering potential therapeutic targets for neurodevelopmental disorders.

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Part I

Chapters

Chapter 1

Introduction

1.1 Overlapping genes and their regulatory roles

In eukaryotic genomes, overlapping genes are common despite the small protein-coding gene-to-genome size ratio (~25,000 genes/3.2 billion nucleotides = 1 gene every 128,000 nucleotides). Approximately a quarter of all human protein-coding genes overlap at least one other gene (C.-H. Chen, Pan, and W.-c. Lin 2019). Two genes occurring either partially or entirely in the same sequence space define these. Overlaps located within the intron of another gene are relatively common in the human genome but studied most commonly are cis-natural antisense transcripts (cis-NATs), which are complementary mRNAs transcribed from opposite strands of DNA at the same genomic locus (Huber et al. 2016; Osato et al. 2007). The different arrangements of overlapping genes can contribute to the regulation of gene expression by several mechanisms, involving insulators of enhancer elements, regulators of promoter-associated RNA polymerase II (Pol II), the antisense transcripts, and the process of overlapping transcription (Pelechano and Steinmetz 2013). Examples in mammals include lncRNA Wrap53, which can regulate human p53 gene expression by direct interaction with p53 mRNA (Saldaña-Meyer et al. 2014); also, the lncRNA antisense of NOS1 is a negative regulator of NOS2 and NOS3 protein expression but not the mRNA (Korneev et al. 2015), indicating post-transcriptional interference. Some small non-coding RNAs (ncRNAs) such as miRNAs, snoRNAs, and tRNAs are found overlapping with protein coding genes (Dill et al. 2012; Smith and Steitz 1998; Yeganeh et al. 2017), while studies specifically examining the complex gene regulatory networks mediated by small ncRNAs and their implications in various biological processes and diseases are limited.

1.2 Known regulatory roles of tRNAs, tDRs, and tRNA genes

Transfer RNAs (tRNAs), best known for their function in protein translation, are encoded by over 500 human genes. However, tRNAs also have essential extra-translational functions, such as reducing the translation of viral proteins in a codon usage-dependent manner, interacting with RNA Pol II genes and enhancers by DNA loops, and antagonizing the stability of oncogenic transcripts in breast cancer

cells (Goodarzi, X. Liu, et al. 2015; Goodarzi, Nguyen, et al. 2016; Van Bortle, Phanstiel, and Snyder 2017). tRNA-derived small RNAs (tDRs) or tRNA fragments (tRFs) also regulate genes at both the chromatin level (P. Kumar, Kuscu, and Dutta 2016) and mRNA levels (Goodarzi, X. Liu, et al. 2015; H. K. Kim et al. 2017). For example, 5' tRF-Gly-GCC controls noncoding RNA biogenesis and global chromatin organization by modulating heterochromatin-mediated transcription repression (Boskovic et al. 2020), while 3' tRF-Leu-CAG binds directly to ribosomal protein mRNAs, and inhibition of this tDR suppresses tumor growth (H. K. Kim et al. 2017). tDRs from $tRNA^{Glu}$, $tRNA^{Asp}$, $tRNA^{Gly}$, and $tRNA^{Tyr}$ suppress the stability of oncogenic transcripts in breast cancer cells by displacing their 3' UTRs from the RNA-binding protein YBX1 (Goodarzi, X. Liu, et al. 2015).

Many human genes are clustered genomically (Chan and Lowe 2016), with some overlapping enhancer or promoter region of other genes, contributing to nearby gene expression regulation. Experiments have shown that actively transcribed tRNA genes can be targeted by Argonaute 2 (AGO2) to repress mRNA expression levels among adjacent genes (Jl, Bl, and Ke 2015). Some tRNA genes also function as barrier insulators, blocking enhancers from activating Pol II transcribed promoters (Raab et al. 2012). Notably, tRNA genes ($tRNA^{Tyr}$ and $tRNA^{Pro}$) located upstream of Pol II promoters can influence the Pol II gene transcription in a position- and orientation-dependent manner (Sheng et al. 2014). However, the collaborative regulatory mechanisms of these factors remain to be fully understood.

1.3 Pol II and Pol III interference

The interplay between Pol II and Pol III is a significant area of study in gene expression regulation. These polymerases are responsible for transcribing different sets of genes, and their interactions can lead to transcriptional interference, affecting gene expression levels and cellular functions. Pol III elements within their promoter regions influence ANG and RNASE4 transcription (Sheng et al. 2014). An antisense Pol III transcribed gene can affect Polr3e gene expression at the transcription elongation level (Yeganeh et al. 2017). The enhancer Pol III element controls Fos transcription through its transcription interacting

with the distal Poll gene promoter region, which is necessary for brain development (Policarpi et al. 2017).

The interaction between Pol II and Pol III involves complex mechanisms of transcriptional interference, competition for promoter usage, and gene-specific regulation (Barski, Chepelev, et al. 2010; Gao, Herrera-Carrillo, and Berkhout 2018; Gerber et al. 2020; Lukoszek, Mueller-Roeber, and Ignatova 2013; Oler et al. 2010). These interactions are influenced by chromatin structure and can be harnessed for precise gene expression control in biotechnological applications. Understanding these dynamics is crucial for optimizing gene expression systems and developing targeted gene therapies.

1.4 tRNA genes nested within protein-coding genes

There are 53 instances of tRNA genes, which are transcribed by RNA polymerase III (Pol III), located within annotated human gene introns and UTR regions, of which 24 tRNA genes occur inside 14 experimentally confirmed protein-coding genes in humans (Akkuratov et al. 2014). In mice, only two experimentally confirmed annotated protein-coding genes have overlapping tRNA genes. The tRNA-Gly-GCC genes overlap with the VAC14 gene intron, and the tRNA-Arg-TCT overlaps with the HES7 gene 3'UTR region, both be found in human and mouse, are highly occupied by Pol III.

HES7 and VAC14 gene expression is essential for brain development (Bessho, Sakata, et al. 2001; Stutterd et al. 2017; Y. Zhang, Zolov, et al. 2007). ENCODE ChIP-seq data shows Pol II accumulation preceding Pol III at the HES7^{tRNA} and VAC14^{tRNA} loci in multiple human cell lines (Yeganeh et al. 2017). However, the impact of this Pol II and Pol III accumulation on the tRNA genes affecting the related protein-coding gene expression remains unknown.

While the general regulatory effects of tRNAs and tRNA genes are varied, the tRNA gene's role in the overlapping protein-coding gene expression has yet to be well understood. Here, I examined the potential roles of these nested tRNA genes in VAC14 and HES7 gene expression regulation.

Chapter 2

The Role of VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ Genes in Regulating VAC14 Expression

2.1 Abstract

Transfer RNAs (tRNAs) are essential for mRNA translation across all life forms and have been implicated in regulating neighboring protein-coding genes. Four $tRNA^{Gly(GCC)}$ genes overlap with VAC14 introns, a conserved feature among mammals, and exhibit high occupancy by both Pol II and Pol III. Given the association between VAC14 loss and neurodegeneration, this study investigates the transcriptional regulatory role of $VAC14^{tRNA\text{-}Gly\text{-}GCC}$ genes on the VAC14 gene during neuronal development. This chapter employs computational and evolutionary approaches to explore chromatin accessibility at $tRNA^{Gly(GCC)}$ gene loci and VAC14 gene expression in developing mouse neurons. Published DNase-seq data were used to map the accessibility of $VAC14^{tRNA\text{-}Gly\text{-}GCC}$ genes, while RNA-seq assessed VAC14 transcript abundance during mouse neuronal differentiation. Chromatin at $VAC14^{tRNA\text{-}Gly\text{-}GCC}$ genes loci was observed to open during the development of mouse neurons, as verified by H3K27ac ChIP-seq data. Functional and comparative genomic analyses reveal that the tRNA genes in the first intron appear to function as enhancer elements. Additionally, Motif discovery within the upstream of $VAC14^{tRNA\text{-}Gly\text{-}GCC}$ genes, combined with Zic 1/2 ChIP-seq and knockdown data, demonstrated the suppressive regulatory role of the Zic family transcription factors on VAC14 gene transcription during neuronal maturation. Together, these data reveal that VAC14 transcription is regulated by the accessibility of $VAC14^{tRNA\text{-}Gly\text{-}GCC}$ upstream Zic 1/2 during mouse neuronal development.

2.2 Background

2.2.1 Four $tRNA^{Gly(GCC)}$ genes overlapped with the introns of the VAC14 gene

tRNAs are essential for mRNA translation across all life forms and have been implicated in regulating neighboring protein-coding genes. Among the protein-coding genes that overlap with Pol III-transcribed tRNA genes, VAC14 is notable. Four copies of the $tRNA^{Gly(GCC)}$ genes are nested inside two introns of VAC14, and two of them are conserved in mammals and highly occupied by Pol III. Pol III

transcription can interfere with Pol II gene expression through transcriptional interference, particularly when Pol III genes are embedded within Pol II genes (Lukoszek, Mueller-Roeber, and Ignatova 2013; Yeganeh et al. 2017). The chromatin landscape and epigenetic marks associated with Pol III-transcribed genes resemble Pol II templates, suggesting that active chromatin can gate Pol III accessibility to the genome (Barski, Chepelev, et al. 2010; Oler et al. 2010). So, this study aims to explore the precise mechanisms by which these tRNA genes regulate VAC14 gene expression by examining the chromatin landscape and epigenetic marks.

2.2.2 VAC14 gene and its function

VAC14 is a widely expressed scaffold protein critical for membrane trafficking of endosomal vesicles and implicated in neurodegenerative disorders. It plays a crucial role in regulating phosphoinositide signaling pathways, particularly those involving phosphatidylinositol 3,5-bisphosphate (PI(3,5)P2) (Jin et al. 2008; Sbrissa et al. 2008). Loss of VAC14 leads to vacuolation of neuropilin in the brain, particularly the basal ganglia, in mice and humans (Stutterd et al. 2017; Y. Zhang, Zolov, et al. 2007). Mutations or dysregulation of VAC14, or its associated pathways, have been linked to neurodegenerative disorders, including dystonia, neurodegeneration, and neuropathy (Baumann et al. 2020; Hertz et al. 2016; Karaoğlu and Köse 2021; Kaur et al. 2019; Lenk et al. 2016; Y. Zhang, McCartney, et al. 2012). Additionally, VAC14 is crucial for regulating the endolysosomal pathway and brain iron (Baumann et al. 2020; Gusmao et al. 2019; Lyon et al. 2019). These findings suggest that VAC14 contributes to brain development by regulating signaling pathways involved in neuronal development, function, and survival. Furthermore, an antisense transcript within VAC14 (VAC14-AS1) is only expressed in the brain (ENSG00000214353.3 Gene Expression from GTEx). From ENCODE ChIP-seq data, a transcriptional insulator element, CTCF (CCCTC-binding Factor / 11-zinc finger binding factor), has been found to bind in the 10th intron of VAC14. CTCF sites are crucial for genome regulation, acting as insulators, organizing chromatin, maintaining stability, and modulating epigenetics to ensure accurate gene expression and prevent diseases (S. Kim, Yu, and Kaang 2015; B.-K. Lee and Iyer 2012; F. Liu, Wu, and X. Wang 2019; Yao et al. 2010). Understanding how VAC14

transcription is regulated during neural development could provide insights into potential therapeutic targets for these debilitating diseases.

2.2.3 Zic motif and function

The Zic family of zinc-finger proteins is crucial for neural development, playing critical roles in neurogenesis, neurulation, and CNS morphogenesis (Aruga 2004), with mutations linked to cerebellar disorders and neural tube defects (Grinberg et al. 2004). The Zic family of transcription factors plays a crucial role in cerebellar granule neuron maturation by binding to cis-regulatory elements, as confirmed by ChIP-seq, and coordinating mature neuronal gene expression patterns, as demonstrated by knockdown experiments (Frank et al. 2015). These findings highlight the importance of Zic 1/2 in regulating chromatin accessibility and gene expression necessary for neuronal differentiation and cerebellar development (Aruga and Millen 2018).

2.2.4 Investigating the regulation role of VAC14$^{\text{tRNA-Gly-GCC}}$ genes for VAC14 gene

Further research into how VAC14$^{\text{tRNA-Gly-GCC}}$ genes regulate VAC14 expression during brain development will provide valuable insights into normal neurodevelopment and the pathogenesis of neurodevelopmental disorders. To fill this knowledge gap, I examined the interaction between VAC14$^{\text{tRNA-Gly-GCC}}$ genes and VAC14 expression during mouse brain development. Our objective was to understand how these tRNA genes, embedded within the introns of VAC14, influence the gene's transcriptional regulation through chromatin accessibility and transcription factor binding.

In this study, I used published DNase-seq data to map chromatin accessibility at the VAC14$^{\text{tRNA-Gly-GCC}}$ gene loci and RNA-seq to measure VAC14 transcript abundance during mouse neuronal differentiation. I also utilized Frank's published ChIP-seq dataset to analyze histone modifications and transcription factor binding. Specifically, I investigated the binding of Zic transcription factors to the VAC14$^{\text{tRNA-Gly-GCC}}$ region using Frank's Zic 1/2 ChIP-seq and assessed the effects of Zic knockdown on VAC14 expression. These

methods allowed me to comprehensively explore the regulatory role of VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ genes in VAC14 transcription during neuronal development.

2.3 Results

2.3.1 tRNA genes nested within protein-coding genes

Among the annotated human genes, 53 tRNAs are located within introns and untranslated regions (UTRs). Of these, 24 tRNAs are found within 14 experimentally confirmed protein-coding genes (Table 1.1). Notably, when comparing this distribution to the mouse genome, overlaps between tRNAs and protein-coding genes are observed only in the VAC14 and HES7 genes (Table 1.2). These overlaps in VAC14 and HES7 genes suggest possible evolutionary conservation of these genomics arrangements and raise questions about their functional significance in gene regulation and expression.

2.3.2 Four tRNA$^{Gly(GCC)}$ genes overlapping with the introns of the VAC14 gene are conserved in mammals and highly occupied by Pol II and Pol III.

In the genomes of most vertebrate species, four copies of tRNA$^{Gly(GCC)}$ genes are nested within two introns of the VAC14 gene. Specifically, two of these tRNA genes are oriented in an antisense direction within the first intron of VAC14, while the other two are oriented in a sense direction within its ninth intron (Figure 2.1 A). The first two tRNA genes also overlap with the mouse VAC14 gene (Figure 2.1A). To investigate the conservation of tRNA$^{Gly(GCC)}$ sequences within the VAC14 gene across different species, I analyzed the mature tRNA$^{Gly(GCC)}$ sequences from the VAC14 gene in 100 species, including mammals, birds, reptiles, amphibians, and fish. Sequence alignment results indicate that the tRNA-Gly-GCC-2 sequence within the VAC14 gene is highly conserved across all studied vertebrates (Figure 2.1B).

Most tRNA$^{Gly(GCC)}$ gene sequences within the introns of the VAC14 gene exhibit a remarkable level of conservation across all mammalian species. This conservation is evident in the sequence similarity

Gene	# of tRNA/s	tRNA	location	Pol II and Pol III overlap
VAC14	4	Gly-GCC	intron, sense and antisense	Yes
FNBP1L	1	Cys-GCA	intron, antisense	No
ULK2	1	Gly-CCC	intron, antisense	No
HES7	1	Arg-TCT	UTR, antisense	Yes
PARVB	1	Sec-TCA	intron, sense	No
CTC1	3	Ile-AAT, Ser-AGA, Thr-AGT	UTR, sense	Yes
FBXO31	1	Met-CAT	intron, sense	No
ARPC2	1	Tyr-ATA	intron, sense	No
PPFIBP1	1	Lys-TTT	intron, sense	No
CEP95	1	Thr-AGT	intron, antisense	No
ATP5A1	1	Lys-CTT	intron, sense	No
SHF	3	His-GTG	UTR and intron, antisense and sense	Yes
MTRNR2L10	1	nmt-Leu-TAA	sense	No
BCAS3	1	Sup-TTA	intron, sense	No

Table 2.1: tRNA genes nested within protein-coding genes in the human genome.

Gene	# of tRNA/s	tRNA	location	Pol II and Pol III overlap
VAC14	2	Gly-GCC-2-4, Gly-GCC-2-5	intron, antisense	Yes
HES7	1	Arg-TCT-2-1	UTR, antisense	Yes

Table 2.2: tRNA genes nested within protein-coding genes in the mouse genome.

tree, where the mature $tRNA^{Gly(GCC)}$ genes display strikingly similar sequences (Figure 2.1 B). To assess the extent of evolutionary conservation, I analyzed PhyloP (Phylogenetic analysis, compute p-values for conservation or acceleration, either lineage-specific or across all branches) scores, which measure the conservation level at each human genomic position (Pollard et al. 2010). Positive PhyloP scores signify significant conservation, whereas negative scores indicate accelerated evolution (Thornlow, Hough, et al. 2018). Notably, the tRNA sequences within the VAC14 loci exhibit a notably high conservation, as reflected by the average PhyloP score, contrasting with the downstream intron region (Figure 2.1C).

ENCODE ChIP-seq signal for TATA-binding protein (TBP), obtained from HeLa-S3 (a cervical carcinoma cell line) (ENCODE Project Consortium 2012), reveals high occupancy of Pol III on both the tRNA-Gly-GCC-2-4 and tRNA-Gly-GCC-2-5 genes. Pol II, known to bind at protein-coding gene promoter regions (Scheidegger, Dunn, et al. 2019; G. Wang et al. 2010) was enriched in the VAC14 transcription start site (TSS) from ENCODE ChIP-seq analysis. Surprisingly, Pol II accumulations overlapped with Pol III peaks at $tRNA^{Gly(GCC)}$ loci in HeLa-S3 cells (Figure 2.1 D). To assess the generality of this phenomenon, I analyzed Pol III ChIP-seq peaks and signal data for $tRNA^{Gly(GCC)}$ loci across five human cell lines (Figure 2.1 E) and Pol II ChIP-seq peaks and signal data across 21 human cell lines (Figure 2.1 F) from the ENCODE Project. Not all tRNA genes are equally active; some exhibit higher Pol III signals than others. Specifically, tRNA in VAC14 is active in certain cell types. Pol II signals do not directly correlate with Pol III peaks. Only the tRNA gene overlapping with a Pol II gene (marked in red) shows a positive correlation with Pol III peaks. Notably, nine out of 21 human cell lines display prominent Pol II peaks within the tRNA-Gly-GCC-2-4 gene, making it the most enriched among all $tRNA^{Gly(GCC)}$ genes. Pol II and Pol III overlap were detected at VAC14 tRNA-Gly-GCC-2 loci in A549, ECC-1, H1-hESC, HCT-116, and MCF-7 cell lines.

Human tRNA genes can function as chromatin insulators, acting as genetic roadblocks between active and silenced chromatin domains (Raab et al. 2012). A strong peak of Pol II pauses just before the Pol III accumulation at tRNA-Gly-GCC-2-4 and tRNA-Gly-GCC-2-5 loci. These loci exhibit high conservation across vertebrates, implying that these tRNA genes may play a regulatory role in VAC14 transcription.

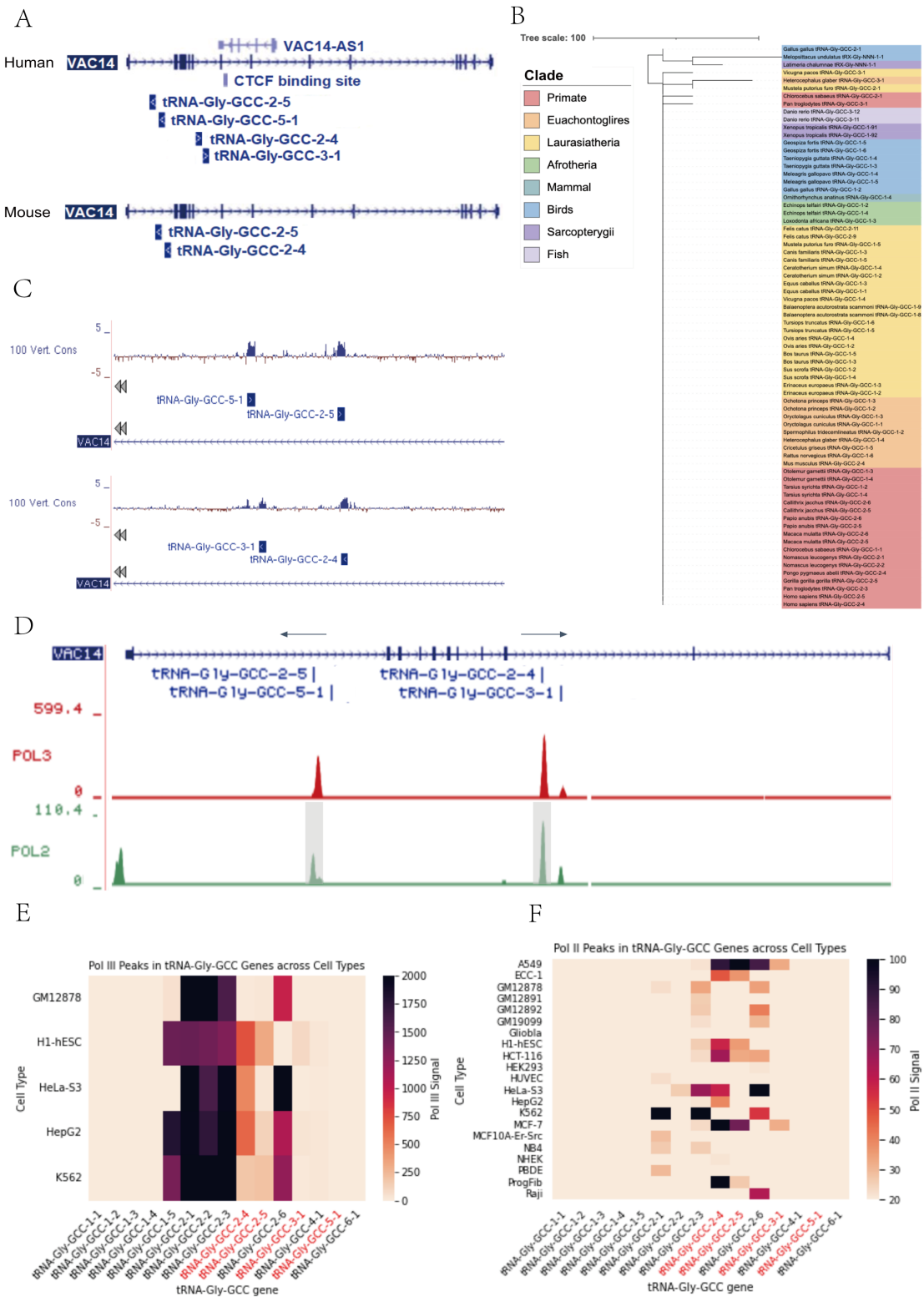

A
Human
VAC14
VAC14-AS1
CTCF binding site
tRNA-Gly-GCC-2-5
tRNA-Gly-GCC-5-1
tRNA-Gly-GCC-2-4
tRNA-Gly-GCC-3-1
Mouse
VAC14
tRNA-Gly-GCC-2-5
tRNA-Gly-GCC-2-4
B
Tree scale: 100
Clade
Primate
Euachontoglires
Laurasiatheria
Afrotheria
Mammal
Birds
Sarcopterygii
Fish
C
100 Vert. Cons
tRNA-Gly-GCC-5-1
tRNA-Gly-GCC-2-5
VAC14
100 Vert. Cons
tRNA-Gly-GCC-3-1
tRNA-Gly-GCC-2-4
VAC14
D
VAC14
tRNA-Gly-GCC-2-5
tRNA-Gly-GCC-5-1
tRNA-Gly-GCC-2-4
tRNA-Gly-GCC-3-1
599.4
POL3
0
110.4
POL2
0
E
Pol III Peaks in tRNA-Gly-GCC Genes across Cell Types
GM12878
H1-hESC
HeLa-S3
HepG2
K562
Cell Type
Pol III Signal
2000
1750
1500
1250
1000
750
500
250
0
tRNA-Gly-GCC gene
F
Pol II Peaks in tRNA-Gly-GCC Genes across Cell Types
A549
ECC-1
GM12878
GM12891
GM12892
GM19099
Gliobla
H1-hESC
HCT-116
HEK293
HUVEC
HeLa-S3
HepG2
K562
MCF-7
MCF10A-Er-Src
NB4
NHEK
PBDE
ProgFib
Raji
Cell Type
Pol II Signal
100
90
80
70
60
50
40
30
20
tRNA-Gly-GCC gene

Figure 2.1: Four tRNA$^{Gly(GCC)}$ genes overlapping with the introns of the VAC14 gene are conserved in mammals and highly occupied by Pol II and Pol III.

(A) *The genomic arrangement of the human and mouse VAC14 gene and the overlapping tRNA$^{Gly(GCC)}$ genes. Four copies of the tRNA Gly-GCC genes nested inside the first and ninth introns of VAC14 in humans. Two tRNA genes are antisense to VAC14, and two are sense. The first two tRNAs still overlap with mouse VAC14.* ***(B)*** *Phylogenetic tree based on tRNA$^{Gly(GCC)}$ sequence alignments in VAC14 intron across 100 vertebrates.* ***(C)*** *The average PhyloP score (comparing humans to 100 vertebrate species) is plotted for each position within the tRNA$^{Gly(GCC)}$ and flanking region across the tRNAs in VAC14 introns.* ***(D)*** *ENCODE ChIP-seq data for Pol III and Pol II distribution are shown in the human VAC14 gene region. The genomic arrangement of the beginning of the human VAC14 gene is shown at the top. tRNA-Gly-GCC-2-5 and tRNA-Gly-GCC-5-1 genes are oriented in an antisense direction within the first intron of VAC14. tRNA-Gly-GCC-2-4 and tRNA-Gly-GCC-3-1 genes are oriented in a sense direction within the ninth intron of VAC14. The bottom part shows a University of California at Santa Cruz (UCSC) genome browser view of Pol III and Pol II ChIP-seq profiles in a human cervical carcinoma cell line. Pol II accumulations overlap with Pol III peaks at tRNA$^{Gly(GCC)}$ loci. Tracks are from ENCODE (RPC1) and Liu et al.* ***(E)*** *Heatmap illustrating the ENCODE ChIP-seq Pol III signal distribution at all human tRNA$^{Gly(GCC)}$ loci across five human cell types.* ***(F)*** *Heatmap illustrating the ENCODE ChIP-seq Pol II signal distribution at all human tRNA$^{Gly(GCC)}$ loci across 21 human cell types.*

2.3.3 VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ region is opening and bears the epigenetic hallmarks of enhancers

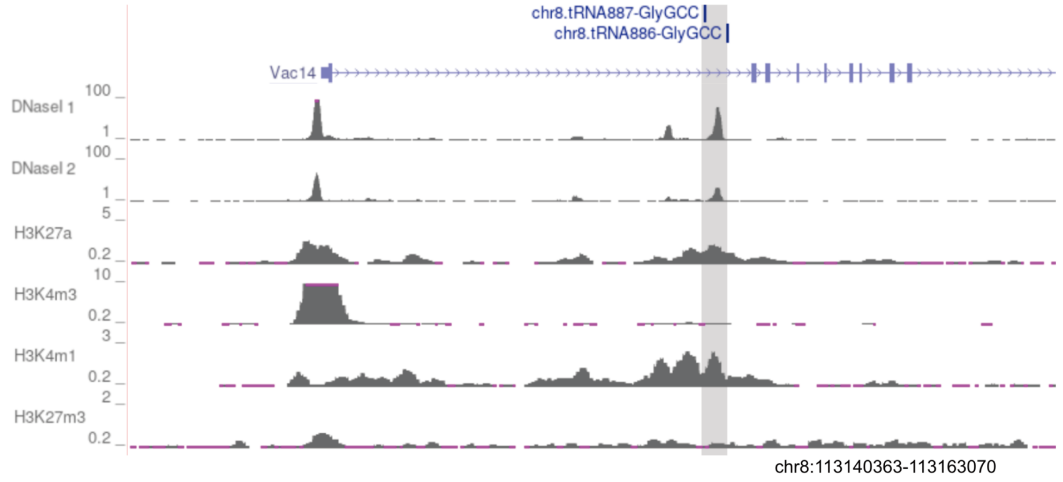
DNase I hypersensitive (DHS) sites indicate nucleosome-depleted regions that are universal markers of gene regulatory elements, including promoters, enhancers, insulators, and most transcription factor binding sites (Yamakawa and Itakura 2019). To determine if the VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ genes can be DHS sites (e.g., enhancers, promoters, and silencers), specific histone modifications ChIP-seq datasets in the Mouse ENCODE release 3 were mapped to the VAC14 locus. DNase I, hypersensitive to the VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ locus (Figure 2.2A), indicates that this locus is open in the adult cerebellum. Histone modifications reflect the activation state of cis-regulatory elements, with active enhancer/promoter marker Histone H3 lysine 27 acetylation (H3K27ac), promoter marker Histone H3 at Lysine 4 Trimethylation (H3K4me3), enhancer marker Histone H3 lysine 4 mono-methylation (H3K4me1) and repressive marker

Histone H3 lysine 27 Trimethylation (H3K27me3) (Barski, Cuddapah, et al. 2007; Bernstein et al. 2006; Creyghton et al. 2010; Hawkins et al. 2011; Heintzman et al. 2009; T. H. Kim, Barrera, et al. 2005; Rada-Iglesias et al. 2011)used to identify the potential function of this locus. The VAC14 TSS was verified as a promoter region by combining the enrichment of H3K27ac and H3K4me3 histone marks. In contrast, the absence of H3K4me3 and H3K27me3 signals, combined with the colocalization of H3K27ac and H3K4me1 marks, particularly enriched at the VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ site, indicates that this site functions as an active enhancer in adult cerebellum (Figure 2.2A).

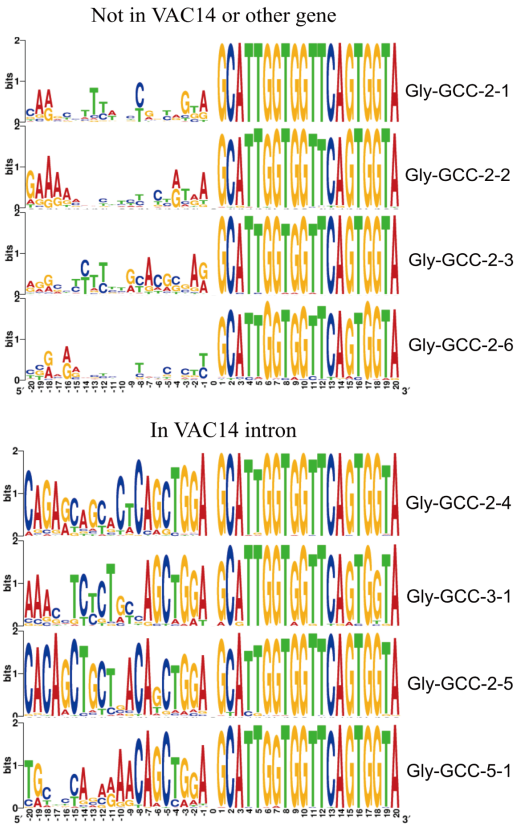
2.3.4 Zic motifs were found upstream of VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ genes

The regulatory chromatin landscape is believed to be determined by the accessibility of gene regulatory elements to transcription factor binding (Sheffield et al. 2013; Song et al. 2011; Stergachis et al. 2013). Therefore, I set out to identify transcription factors by searching for the enrichment of transcription factor motifs near the VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ site. Compared to other tRNA$^{Gly(GCC)}$ genes, the tRNA$^{Gly(GCC)}$ gene in VAC14 exhibits higher conservation in the upstream region from the sequence logo for 100 vertebrates (Figure 2.2B). Active tRNA genes typically show high upstream sequence variation (Thornlow, Hough, et al. 2018; Thornlow, Armstrong, et al. 2020), consistent with the results in the tRNA-Gly-GCC-2 genes that do not overlap with protein-coding genes. Surprisingly, the upstream sequences of the VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ gene show a highly conserved motif across 100 vertebrate species. A motif was found at -10 from the start of the tRNA genes, with a consensus pattern (5' - ACAGCTGGA - 3'). Tomtom motif comparison results indicate similarity to known zinc-finger in the cerebellum (Zic) transcription factor family motifs (Figure 2.2C).

A



B



C

motif matches	P-value	TF name
	3.11e-05	Zic1_secondary
	1.39e-04	Zic2_secondary
	1.62e-04	TWIST1
	1.83e-04	ETV2::FIGLA
	3.44e-04	TFAP4::FLI1
	4.36e-04	Zic3
	4.68e-04	Zic2
	8.22e-04	MSC
	9.69e-04	ZBTB18
	1.13e-03	FIGLA
	1.94e-03	Zic1::Zic2
	4.32e-03	Zic3_secondary

Zic family motifs

Basic helix-loop-helix factors (bHLH)

Figure 2.2: VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ region bears the epigenetic hallmarks of enhancers and Zic motif

(A) *UCSC genome browser images for VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ and overlapping VAC14 gene region of mouse MGSCv37/mm9 genome, highlighting that VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ locus opens during development by DNase-seq and overlaps with H3K27ac and H3K4me1 enrichment (gray bar) in adult (P56) cerebellum.* ***(B)*** *Sequence Logos for tRNA$^{Gly(GCC)}$ in Vertebrates. Top: Sequence logos for tRNA$^{Gly(GCC)}$ within the VAC14 gene (20 bp upstream and followed by 20 bp of the tRNA gene sequence for 100 vertebrates). Bottom: Control sequence logos for tRNA$^{Gly(GCC)}$ outside the VAC14 gene.* ***(C)*** *Top 12 MEME Similar Motifs in Published Libraries (Tomtom) results for VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ upstream consensus sequence. Transcription factor (TF) names for identified motifs are listed with E-values < 10, as per the default settings for the Tomtom motif comparison tool.*

To verify the presence of Zic motifs upstream of VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ genes in the human genome, a position-specific scoring matrix (PSSM) (J. G. Henikoff and S. Henikoff 1996) for Zic motifs was acquired from JASPAR (Castro-Mondragon et al. 2022) in MEME format. These PSSMs were then used to scan the VAC14 gene region using FIMO (Grant et al., 2011). The FIMO output, displayed as the UCSC genome browser track, confirmed Zic motifs upstream of VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ genes (Figure 2.3A).

2.3.5 Zic 1/2 proteins bind to VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ gene locus and control the VAC14 transcription during the development of mouse cerebellum

Zic transcription factors play a pivotal role in neural development by regulating chromatin accessibility at neuronal enhancers and coordinating gene expression patterns crucial for cerebellar granule neuron maturation (Aruga and Millen 2018; Frank et al. 2015). VAC14 is vital for neural development, as its deficiency causes severe neurodegeneration, and mutations are linked to childhood-onset striatonigral degeneration (Stutterd et al. 2017; Y. Zhang, Zolov, et al. 2007; Y. Zhang, McCartney, et al. 2012). To investigate how chromatin accessibility changes in the VAC14 locus during cerebellar differentiation and whether Zic transcription factors bind to this region, Zic 1/2 ChIP-seq and DNase seq datasets from P7 to P60 cerebellum were analyzed.

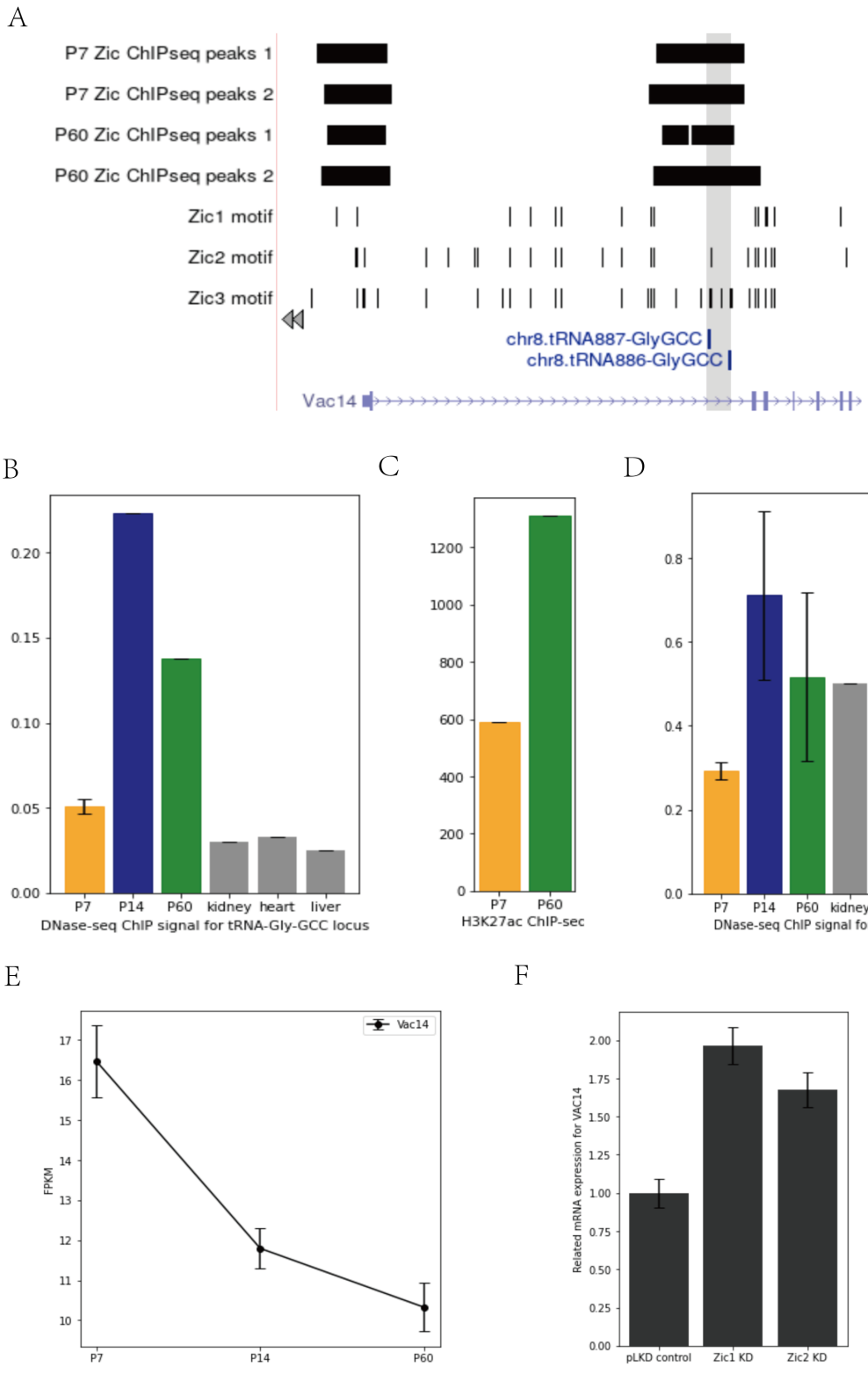

A
P7 Zic ChIPseq peaks 1
P7 Zic ChIPseq peaks 2
P60 Zic ChIPseq peaks 1
P60 Zic ChIPseq peaks 2
Zic1 motif
Zic2 motif
Zic3 motif
chr8.tRNA887-GlyGCC
chr8.tRNA886-GlyGCC
Vac14
B
P7 P14 P60 kidney heart liver
DNase-seq ChIP signal for tRNA-Gly-GCC locus
C
P7 P60
H3K27ac ChIP-sec
D
P7 P14 P60 kidney heart liver
DNase-seq ChIP signal for VAC14 TSS
E
Vac14
FPKM
P7 P14 P60
F
Related mRNA expression for VAC14
pLKD control
Zic1 KD
Zic2 KD

Figure 2.3: VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ region is opening, and Zic 1/2 protein binds to VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ gene loci during the development of mouse cerebellum

(A) *Zic ChIP-seq signal peak and Zic motifs at VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ locus.* ***(B)*** *DNase-seq signal in VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ locus that opens from P7 to P60 and closes for normal tissues.* ***(C)*** *H3K27ac ChIP-seq signal in VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ locus in P7 or P60 cerebellum* ***(D)*** *DNase-seq signal in VAC14 TSS across cerebellum development and normal tissues.* ***(E)*** *RNA-seq analysis of VAC14 mRNA expression levels across developmental time.* ***(F)*** *RNA-seq analysis of VAC14 mRNA expression levels in P7 mice following Zic1 and Zic2 knockdown by shRNA, compared to control mice transfected with an empty vector (pLKO). Error bars represent the standard error of the mean based on three cultures.*

During mouse cerebellum development, a 4-fold increase in DNase signal indicates the opening of the tRNA$^{Gly(GCC)}$ gene locus after day 14 postnatal (Figure 2.3B), consistent with the increase in H3K27ac signal (Figure 2.3C). However, RNA-seq data revealed a decrease in Vac14 mRNA expression, similar to observations in human brain organoids (Figure 2.3E). The VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ gene locus appears to close after mouse cerebellum development, as evidenced by a decline in ChIP signals in the kidney, heart, and liver compared to brain development (Figure 2.3B). Meanwhile, the VAC14 transcription start site remained open (Figure 2.3D).

A 50% increase in the Zic signal at the VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ locus, accompanied by a two-fold increase in the signal for vac14 TSS, was observed (Figure 2.3A). To elucidate the impact of Zic transcription factor binding on VAC14 expression during cerebellar development, I examined RNA-seq data of the VAC14 gene and Zic 1/2 knockdown neurons during neural development. Following Zic 1/2 protein knockdown, VAC14 mRNA exhibited a two-fold increase compared to the control (Figure 2.3F), suggesting a regulatory role for Zic transcription factors in VAC14 expression during cerebellar development.

2.4 Discussion

Four glycine tRNAs are embedded within the intron of the Vac14 gene and are highly conserved across vertebrate genomes. Previous studies have highlighted the intricate relationship between Pol III

and Pol II transcription, where Pol III genes can function as enhancers or insulators, impacting nearby Pol II gene transcription through mechanisms such as transcriptional interference and chromatin architecture modulation (Policarpi et al. 2017; Raab et al. 2012; Raha et al. 2010; Yeganeh et al. 2017). This study focused on the VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ genes to map chromatin accessibility changes occurring during development. ChIP-seq data reveal significant changes in chromatin accessibility of VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ during neuronal maturation. The enrichment of H3K4me1 and H3K27ac signals indicates VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ genes function as enhancer elements. Genehancer analysis has also predicted this locus as an enhancer, suggesting the functional significance of the VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ genes in gene regulation (Fishilevich et al. 2017). Additionally, Zic transcription factors were found to bind to the VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ genes region, regulating VAC14 transcription during neural development. This identification of the VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ genes' function is novel and raises the possibility that these genes play a critical role in neural development.

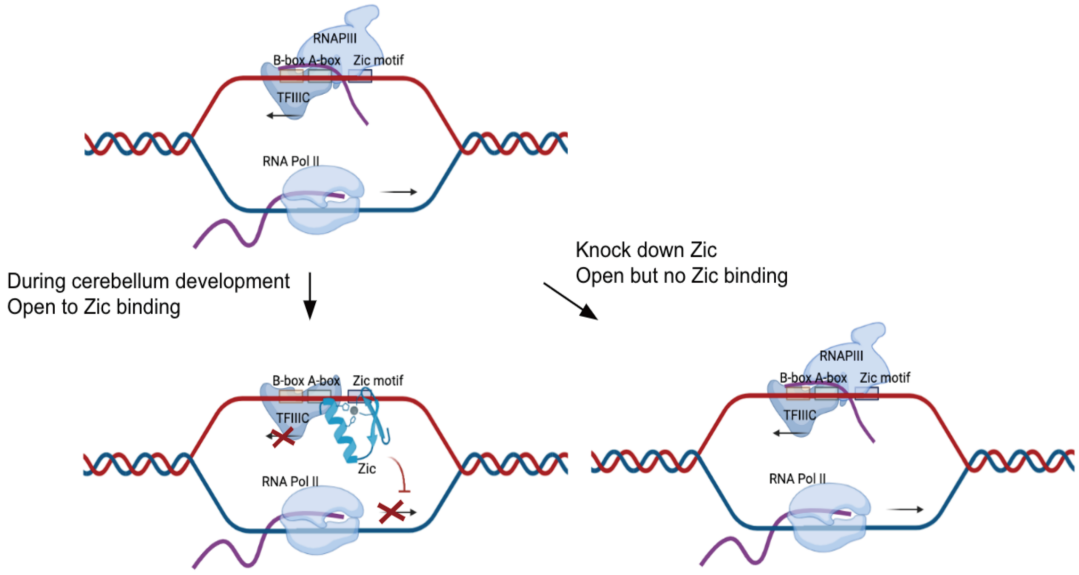


Figure 2.4: Schematic of chromatin states of VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ locus during cerebellar development illustrating a simple model of Zic binding protein-mediated VAC14 gene expression regulation.

The PhyloP scores across 100 vertebrates indicate that this genomic arrangement has been maintained and retained throughout evolution in various vertebrate species. This conservation suggests

that this regulatory mechanism might serve essential functions in gene regulation and is likely biologically significant across diverse vertebrate organisms. Half of the 21 tested human cell lines exhibit prominent Pol II peaks within the tRNA-Gly-GCC-2-4 gene. This particular $tRNA^{Gly(GCC)}$ gene shows the highest level of Pol II occupancy compared to all other $tRNA^{Gly(GCC)}$ genes in these cell lines. Notably, all the $tRNA^{Gly(GCC)}$ genes with Pol II peaks are around or overlap with the protein-coding gene (Figure 2.1E labeled in red). The tissue-specific expression of the tRNA gene and VAC14 raise the possibility that the regulation and impact of Pol II transcription within the tRNA-Gly-GCC-2-4 gene may be particularly significant and could be cell-type specific, further emphasizing the complexity of the interplay between Pol III and Pol II in different cellular contexts.

The differential chromatin architecture of the VAC14 gene enabled us to anticipate and validate the binding of the Zic transcription factor to this locus and its regulatory role in neuronal development. During neuronal maturation, the open chromatin state of $VAC14^{tRNA\text{-}Gly\text{-}GCC}$ gene locus facilitates the binding of Zic proteins. Deleting Zic1 and Zic2 in mice results in increased VAC14 transcription, underscoring the necessity of Zic proteins in VAC14 regulation during cerebellar development (Figure 2.4). This research sheds light on how the $VAC14^{tRNA\text{-}Gly\text{-}GCC}$ gene modulates VAC14 gene expression.

In summary, the presence of four glycine tRNAs within the intron of the Vac14 gene, highly conserved across vertebrate genomes, underscores a potentially significant regulatory mechanism in VAC14 expression. This study has illuminated the intricate relationship between Pol III and Pol II transcription, revealing how Pol III genes, such as the $VAC14^{tRNA\text{-}Gly\text{-}GCC}$ genes, can function as enhancers impacting nearby Pol II gene transcription during neuronal maturation. Moreover, identifying Zic transcription factors binding to the $VAC14^{tRNA\text{-}Gly\text{-}GCC}$ genes region and their role in regulating VAC14 transcription adds a new layer of complexity to our understanding of neural development. The conservation of this genomic arrangement across vertebrate species and the tissue-specific Pol II signal patterns observed underscore the importance of further investigating the functional implications of these findings.

Future research should focus on the precise manipulation of the $VAC14^{tRNA\text{-}Gly\text{-}GCC}$ gene locus

or Zic binding sites in this locus using advanced technologies like CRISPR-Cas9, which holds promise for unraveling the detailed mechanisms underlying tRNA-mediated regulation of VAC14 expression and its significance for neuronal development. Additionally, investigating whether substituting glycine tRNAs with other tRNAs or upstream motifs can impact gene regulation and expressing zinc finger proteins in normal cells could provide deeper insights into the regulatory networks governing gene expression and development.

2.5 Methods

2.5.1 Data Sets and Alignment Analyses

The $tRNA^{Gly(GCC)}$ gene sequences in VAC14 intron from 100 vertebrates were downloaded from GtRNAdb (October 2015 release) (Chan and Lowe 2009). Complete genomic $tRNA^{Gly(GCC)}$ gene upstream sequences for all the vertebrates were acquired from NCBI RefSeq and UCSC Genome Browser Vertebrate Multiz Alignment track (Blanchette et al. 2004; Pruitt, Tatusova, and Maglott 2007). Multiple sequence alignments to generate consensus sequences were performed using Jalview v.2.11 (Waterhouse et al. 2009). The phylogenetic trees were constructed based on the alignment data and were generated using Jalview, employing the neighbor-joining method based on percent identity; the file was processed at the Interactive Tree of Life (iTOL) Web site (http://itol.embl.de) for graphic representation.

2.5.2 Transcriptional factor binding sites analysis

Alignments of the 20-nt upstream region of tRNAs gave us an initial approximation of the consensus motif pattern. The upstream generic consensus motifs were searched using MEME with a 3rd-order Markov model background (T. L. Bailey and Elkan 1994) within the 100-nt upstream region of VAC14 tRNA genes in humans and mice.

The top 1 DNA motif discovery hits, ranked by e-value, was selected and aligned to known

motifs in the JASPAR core vertebrates and UniPROBE mouse databases using Find Similar Motifs in Published Libraries (Tomtom) (Gupta et al. 2007). Additionally, a Position-specific scoring matrix (PSSM) (J. G. Henikoff and S. Henikoff 1996) for Zic motifs was acquired from JASPAR (Castro-Mondragon et al. 2022) in MEME format. These PSSMs were then utilized to scan the 25-nt upstream region of all tRNA genes to verify the presence of the tRNA upstream Zic motif using Find Individual Motif Occurrences (FIMO) (Grant, Timothy L. Bailey, and Noble 2011). Sequence logos of consensus Zic motifs were then generated using WebLogo (Crooks et al. 2004).

2.5.3 Pol II and Pol III signal and peaks

The ENCODE Project Consortium employed ChIP-seq data (Kharchenko, Tolstorukov, and Park 2008; Q. Li et al. 2011; ENCODE Project Consortium 2012) extracted from the UCSC Genome Browser (Kent et al. 2002) to identify specific binding regions for regulatory factors such as TBP, Pol 3, and Pol 2 transcription factors within the human genome. Regions displaying higher signal intensities indicate more frequent binding of the respective transcription factors (ENCODE Project Consortium 2011). The peak calls provided by Peakseq indicate significant enrichment of transcription factors in the target regions (Rozowsky et al. 2009). We identified the strongest Pol2 signal for each $tRNA^{Gly(GCC)}$ gene and assessed the presence of a significant peak in the tRNA region across 21 human cell lines. Additionally, I calculated the average PhyloP score for all the $tRNA^{Gly(GCC)}$ genes in the VAC14 intron and the intronic control region, utilizing the PhyloP conservation data (Pollard et al. 2010) available in the UCSC Genome Browser.

2.5.4 Histone Makers and Zic ChIP-seq Datasets

DNase, H3K4me1, H3K4me3, and H3K27ac ChIP-seq data for P56 (postnatal day 56) cerebellum were retrieved from the Mouse ENCODE project release 3 (August 2012) created by Bing Ren's lab at the Ludwig Institute for Cancer Research (LICR) (**mouse_encode_consortium_encyclopedia_2012**). Available peak files were used in this study to determine chromatin open status. ChIP-seq datasets for DNase, H3K27ac, and Zic 1/2, RNA-seq data at P7, P14, and P60 mouse cerebellum, and Zic1 and Zic2 knockdown

data were retrieved from a previously published dataset, and the publicly available data can be found at GEO: GSE60731 (Frank et al. 2015).

Chapter 3

Altering the intronic tRNA$^{Gly(GCC)}$ genes can affect VAC14 expression

3.1 Abstract

To further elucidate the transcriptional regulation role of VAC14$^{tRNA\text{-}Gly\text{-}GCC}$ genes in the VAC14 gene, I employed CRISPR/Cas9-mediated homology-directed recombination (HDR) to delete and mutate the tRNA genes within HEK293T cells. Through ChIP-seq analysis and western blotting, I demonstrated that these tRNA genes influence VAC14 expression through transcriptional interference. This study provides the first experimental validation of a previously proposed mechanism, revealing the interaction between Pol II and Pol III, where the transcription of embedded sense or antisense Pol III genes can regulate a Pol II gene at the level of transcription elongation. These findings underscore the intricate relationship between Pol III and Pol II transcription in regulating VAC14, offering new insights into the transcriptional control mechanisms involved.

3.2 Background

3.2.1 Pol III Genes regulate overlapping Pol II gene transcription

Pol III genes have been shown to function as enhancers or insulators, impacting Pol II gene transcription. For instance, a mammalian interspersed repeat (MIR) within the Polr3e gene can influence its expression through transcriptional interference, disrupting Pol II transcription elongation (Yeganeh et al. 2017). Similarly, enhancer SINEs interact with Pol II at gene promoters, linking Pol III and Pol II transcription (Policarpi et al. 2017). Additionally, tDNA elements transcribed by Pol III can act as insulators, blocking enhancers from activating Pol II promoters and maintaining distinct chromatin domains (Raab et al. 2012). These examples highlight the complex interplay between Pol III and Pol II transcriptional machinery.

3.2.2 Pol II peak overlap with Pol III tRNA$^{Gly(GCC)}$ genes nested within VAC14 gene

Four copies of the tRNA$^{Gly(GCC)}$ genes, nested inside two introns of VAC14, are conserved in mammals and highly occupied by Pol III. Two of these tRNA genes are oriented in the sense direction within the VAC14 intron, while the other two are antisense. ENCODE ChIP-seq data ((ENCODE Project Consortium 2012)) visualized within the UCSC genome browser show accumulations of Pol II before the Pol III signal at both tRNA-Gly-GCC-2-4 and tRNA-Gly-GCC-2-5 loci across multiple cell lines. However, the consequences of this accumulation of Pol II protein and its relation Pol III protein at these tRNA genes and their effect on VAC14 expression remain unknown.

3.2.3 Investigating the Role of tRNA$^{Gly(GCC)}$ Genes in VAC14 Expression

To investigate the potential roles of the four nested intronic tRNA$^{Gly(GCC)}$ genes in regulating VAC14 gene expression, I employed CRISPR/Cas9-mediated homology-directed recombination (HDR) to delete and mutate these tRNA genes within HEK293T cells. By using methods such as ChIP-seq analysis and western blotting, I aimed to explore how these tRNA genes might influence VAC14 transcription through transcriptional interference. This approach is designed to address the gap in understanding regarding the regulatory impact of these tRNA genes on VAC14 expression, building on previous research that suggests Pol III genes can regulate Pol II genes through various mechanisms.

3.3 Results

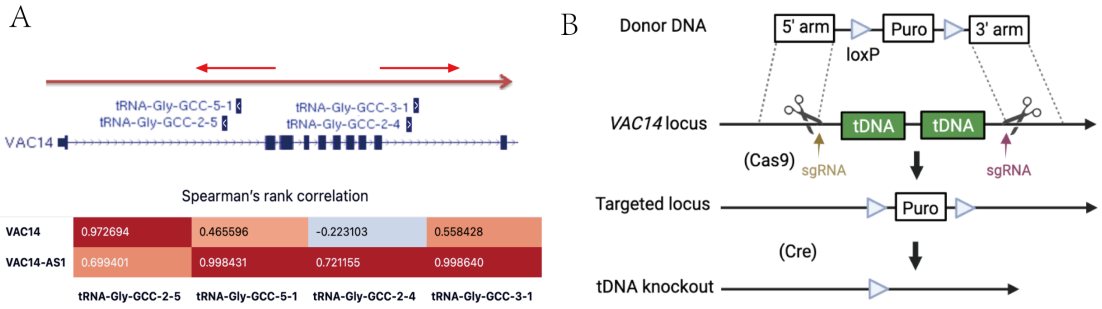
3.3.1 Correlation Analysis of tRNA Genes and VAC14 Gene Expression

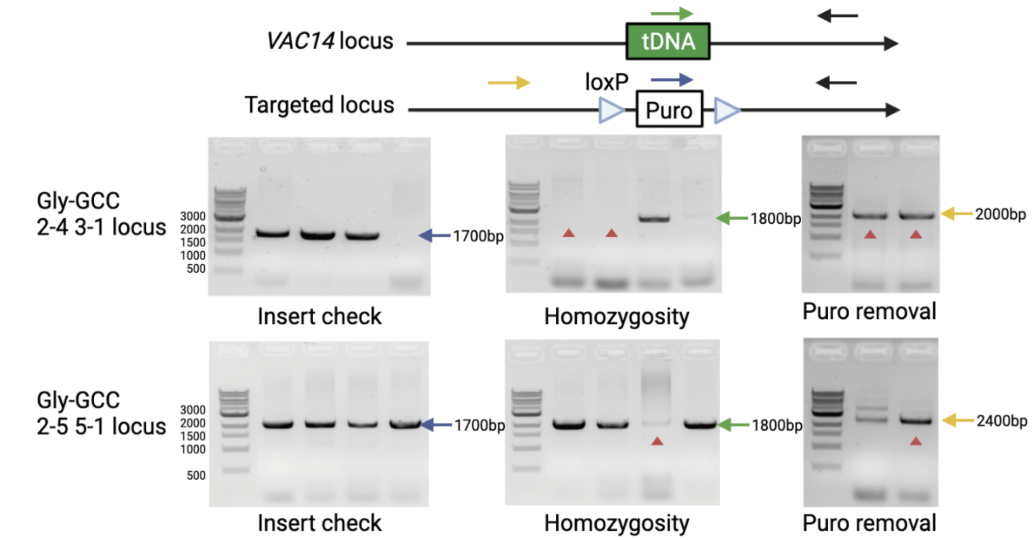
The mature tRNA-Gly-GCC-2-5 and tRNA-Gly-GCC-2-4 have the same sequence, but their pre-tRNA sequences differ. To determine whether one or more of the tRNA genes in the intron of VAC14 correlate with the expression of VAC14, pre-tRNA and mRNA expression data during human neuronal development were used to calculate the correlation coefficients between the tRNA genes and VAC14.

Spearman's rank correlation coefficient of 0.97 indicates a strong positive association between tRNA-Gly-GCC-2-5 and VAC14 expression. In contrast, tRNA-Gly-GCC-2-4 exhibits a negative correlation with VAC14 expression, with a correlation coefficient of -0.22 (Figure 3.1A). These findings suggest differential regulatory roles for these tRNA genes in VAC14 transcription during neuronal development.

3.3.2 CRISPR/Cas9-mediated deletion of antisense tRNA genes leads to decreased expression of VAC14, while deletion of sense tRNA genes leads to increased expression of VAC14

To determine the effect of the intronic tRNA genes on the expression of VAC14, I used a genome editing approach to knock out the tRNA genes. By combining CRISPR/Cas9-mediated genome editing with the Cre/LoxP system, I designed a two-step strategy to make the tRNA gene knock out cell lines (Figure 3.1B). I generated two homozygous double $tRNA^{Gly(GCC)}$ gene deletion knockout HEK293T cell lines: tRNA-Gly-GCC-2-4 + 3-1 KO and tRNA-Gly-GCC-2-5 + 5-1 KO. PCR genotyping (Figure 3.1C) and DNA sequencing (Figure 3.1D) were used to validate their specific deletion. I investigated how the double tRNA gene deletions affected VAC14 gene transcription by RT-qPCR. Loss of tRNA-Gly-GCC-2-5 + 5-1 locus caused a pronounced reduction in VAC14 mRNA expression levels compared to control HEK293T cell (Figure 3.2A), while the specific deletion of tRNA-Gly-GCC-2-4 + 3-1 locus resulted in a significant increase of VAC14 mRNA content compared to wild-type cells. Next, western blot analysis was used to investigate the change of VAC14 protein expression level for these tRNA gene knock-out cells. The VAC14 protein expression was decreased in the tRNA-Gly-GCC-2-5 + 5-1 knockout cell lines, whereas the VAC14 protein expression was increased in the tRNA-Gly-GCC-2-4 + 3-1 knockout cell lines, consistent with the mRNA change pattern (Figure 3.2B, C). Thus, cutting out the antisense intronic $tRNA^{Gly(GCC)}$ genes chunk can effectively suppress VAC14 expression, whereas cutting out the sense intronic $tRNA^{Gly(GCC)}$ genes chunk can enhance VAC14 expression.





D

tRNA-Gly-GCC-2-4 and 3-1 locus Sequencing

untargeted 5' GAGGTGTGCCATGAGGAACGTGG------TGGCTGCTTCGGCAAAGGAGGACA

sgRNA targeted 5' GAGGTGTGCCATGA---------- CAAAGGAGGACA

tRNA-Gly-GCC-2-5 and 5-1 locus Sequencing

untargeted 5' CACTGGTCATCGCACAGGAGGAGT------CACCAGCCCAGAGCGAGACCTATT

sgRNA targeted 5' CACTGGTCATCG---------- GCGAGACCTATT

tRNA-Gly-GCC-2-4 and 3-1 locus 5' LoxP site Sequencing

untargeted 5' GTGCCATGAGGA----------ACGTGGTGCTGT

sgRNA targeted 5' GTGCCATGAGGAggatcctagtgaacctcttcgagggacctaataacttcgtatagcatacattatacgaagttat

The PAM sequences are red. Homolougous arms are green. tRNA genes are yellow and LoxP site are blue.

Figure 3.1: CRISPR/Cas9-mediated deletion of the tRNA genes

(A) *Correlation for VAC14 mRNA and tRNA$^{Gly(GCC)}$ pre-tRNA expression during the development of Human Cortical Organoid.* ***(B)*** *Schematic depiction of the two-step strategy for generating tRNA-Gly-GCC-2-4 + 3-1 or tRNA-Gly-GCC-2-5 + 5-1 gene knockout cell line. The first step is to replace the tRNA genes with a drug-resistance gene (PGK-Puromycin) cassette via CRISPR/Cas9-catalyzed high-fidelity homology-directed repair (HDR) (Chen, Y, 2015). CRISPR/Cas9 and dual-sgRNA cut the tRNA gene at both sites. HDR repaired these targeted double-strand breaks in co-transfected donor plasmid to knock in PGK-Puromycin genes. Including a removable drug-resistance gene (PGK-Puromycin) cassette facilitates the rapid generation of tRNA gene knock-out cell lines. The second step is to remove the drug-resistance cassette. Upon treatment with Cre, the drug-resistance gene recombines, thus resulting in a frame-shift of the targeted sequence and creating an empty locus at the site. tRNA gene (tDNA) is shown as green boxes. Vertical arrows indicate sgRNA targeting sites. Donor DNA: Puro, a puromycin-resistance gene with phosphoglycerate kinase promoter; Blue triangles represent LoxP sites.* ***(C)*** *Validation of tRNA knock-out cell lines. PCR genotyping of knockout tRNA-Gly-GCC-2-4 + 3-1 locus (top panels) and tRNA-Gly-GCC-2-5 + 5-1 locus (bottom panels) clones in HEK293T cell is displayed. The expected PCR products for correctly targeting and insertion are 1700 bp (left panels, blue arrows). Those clones with PCR products of about 1800 bp are heterozygous (middle panels, blue arrow), and those clones without these PCR products are homozygous (middle panels, red triangle). The expected PCR products for Puro removal are around 2000 bp (in tRNA-Gly-GCC-2-4 + 3-1 locus, right panel, yellow arrows) or 2,400 bp (in tRNA-Gly-GCC-2-5 + 5-1 locus, right panel, yellow arrows), respectively.* ***(D)*** *Representative sequencing of targeted homozygous clones in the VAC14 locus using HDR. The PAM sequences are labeled in red. The homologous arms are labeled in green. tRNA genes are yellow, and the LoxP site is blue.*

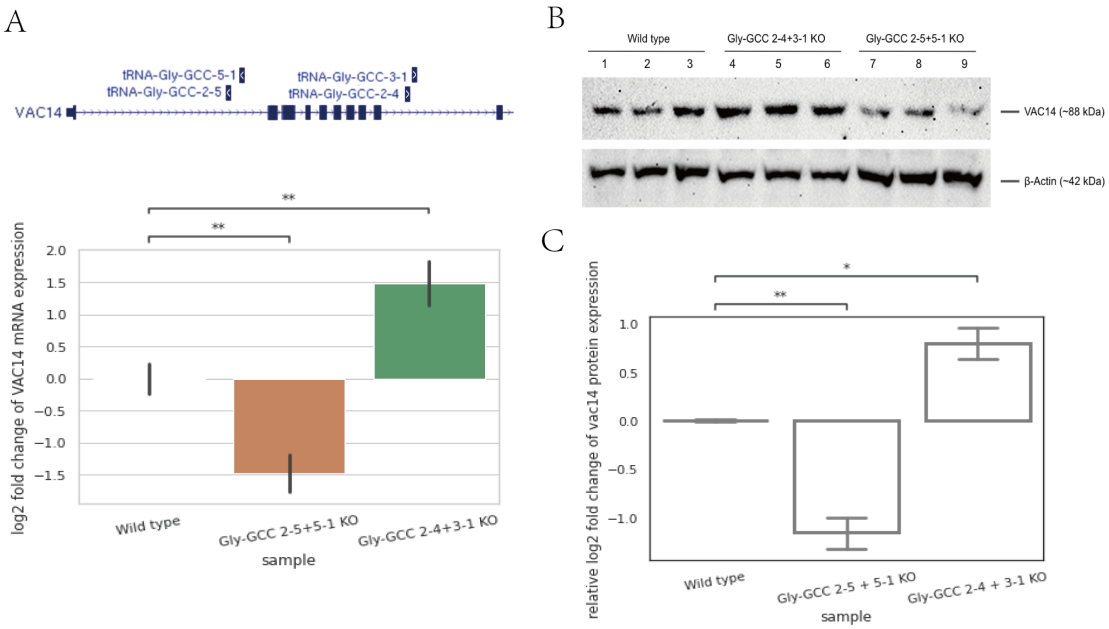


Figure 3.2: Deletion of antisense tRNA genes leads to decreased expression of VAC14, while deletion of sense tRNA genes leads to increased expression of VAC14

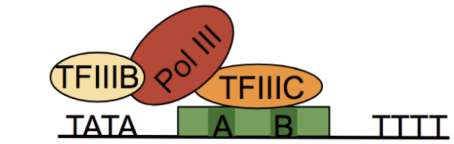
(A) *RT-qPCR shows VAC14 mRNA expression after knockout of the tRNA genes. The qPCR results were normalized to β-Actin mRNA, and error bars indicate ± SEM. The sample size was n = 3, and P-values were calculated using Student's t-test relative to the wild type.* ***(B)*** *Western blotting was performed with anti-VAC14 and anti-β-Actin antibodies with protein extracts from tRNA genes knockout and wild-type HEK293T cell lines.* ***(C)*** *The VAC14 band intensities were quantified and normalized to their corresponding β-Actin band intensities. Error bars are as in A.*

3.3.3 Disrupting the promoter of antisense tRNA genes results in decreased expression of VAC14, while disrupting the promoter of sense tRNA genes leads to increased expression of VAC14

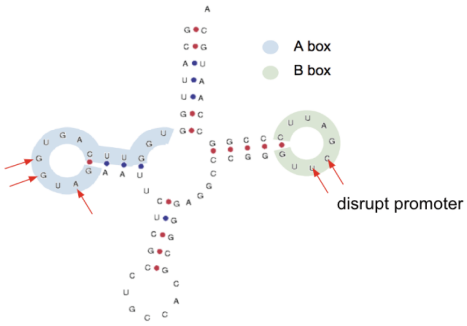
I employed CRISPR/Cas9 and HDR technology in the tRNA gene knockout cells to excise the tRNA gene pairs and the intron between them. To specifically investigate the impact of the tRNA gene itself, I utilized CRISPR/Cas9 and HDR to introduce targeted mutations on the A and B boxes of the tRNA gene genomic sequence. Eukaryotic tRNA gene transcription relies on the internal promoter, comprising the A and B boxes, which are bound by RNA Pol III transcription factors TFIIIC and TFIIIB, along with

the upstream transcription modulatory region (Figure 3.3A top) (Baker and Hall 1984; Geiduschek and Tocchini-Valentini 1988). By introducing 5-base mutations in these regions, I aimed to maximize the reduction in promoter efficiency while minimizing the impact on tRNA processing (Figure 3.3A right). This approach is designed to elucidate the influence of Pol III binding on these tRNA genes. The selection of specific mutations in the tRNA gene was informed by prior studies (Gaëta, S. J. Sharp, and Stewart 1990; Knapp et al. 2019; S. Sharp et al. 1981) that systematically evaluated the effects of mutations in the promoter regions, including the A and B box tRNA sequences and the semi-invariant G-T-psi-C sequence, on both promoter activity and tRNA processing capabilities. A schematic diagram depicting the strategy for the tRNA gene mutation in the VAC14 locus is presented in Figure 3.3B.

A

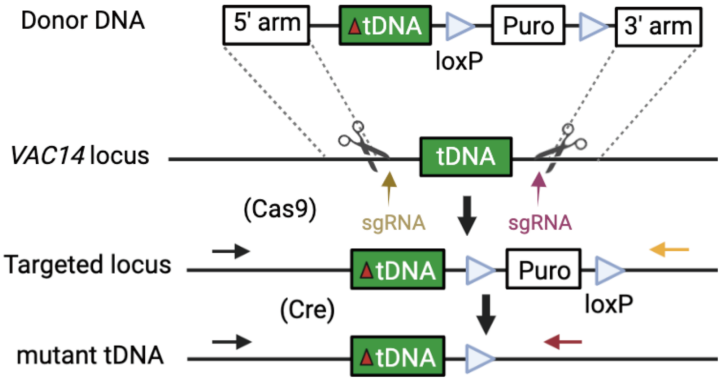


tRNA Promoter

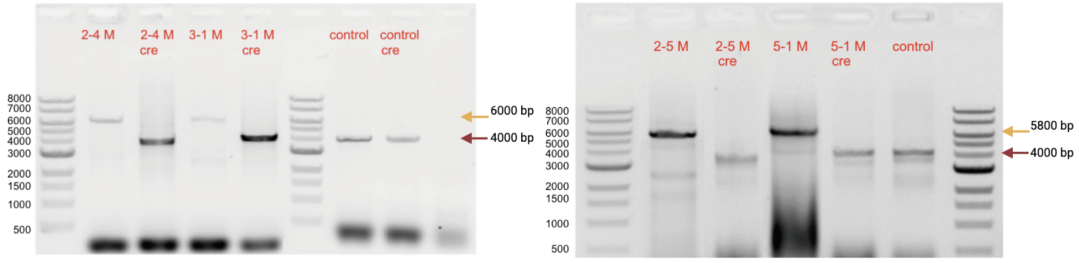


A box		B box	
15 16 17 18 19 20 21		54 55 56 57 58 59 60	
GTTC**AGTGGTA**GAAT		CCGGG**TTCGATT**CCCGG	
GTTC**AGT**T TTGGAAT		CCGGGT**CA**G**ATT**CCCGG	

B



C



D

tRNA-Gly-GCC-2-4 locus Sequencing

untargeted 5' GCATTGGTGGTTCAGTGGTAGAATTCTCGCCTGCCACGCGGGAGGCCCGGGTTCGATTCCCGGCCAATGCA

sgRNA targeted 5' GCATTGGTGGTTCAGTTTTGGAATTCTCGCCTGCCACGCGGGAGGCCCGGGTCAGATTCCCGGCCAATGCA

tRNA-Gly-GCC-2-5 locus Sequencing

untargeted 5' GCATTGGTGGTTCAGTGGTAGAATTCTCGCCTGCCACGCGGGAGGCCCGGGTTCGATTCCCGGCCAATGCA

sgRNA targeted 5' GCATTGGTGGTTCAGTTTTGGAATTCTCGCCTGCCACGCGGGAGGCCCGGGTCAGATTCCCGGCCAATGCA

tRNA sequences are green. Mutations are red.

Figure 3.3: CRISPR/Cas9-mediated mutation of the tRNA genes

(A) *Strategy to make mutations on A, B box of tRNA gene to disrupt the promoter region and make mutant cell lines. (A top left) The internal promoter, which consists of the A and the B box in the tRNA gene, is bound by the RNA Pol III transcription factors TFIIIC and TFIIIB. Mutations are introduced in the A and B boxes of the tRNA gene. Changes are indicated in red.* ***(B)*** *Schematic diagram depicting the strategy for the tRNA gene mutation in the VAC14 locus. Donor DNA: Puro, a puromycin-resistance gene with phosphoglycerate kinase promoter; blue triangles represent LoxP sites; tDNA with A B box mutations are shown as green box; red triangle indicates mutations* ***(C)*** *Validation of tRNA mutant cell lines. PCR genotyping of tRNA mutant HEK293T clones is displayed. Those clones with PCR products of around 6000 bp are homozygous (orange arrows). The expected PCR products for Puro removal are around 4000 bp (red arrows).* ***(D)*** *Representative sequencing of targeted homozygous clones in the VAC14 locus using HDR. The PAM sequences are labeled in red. The homologous arms are labeled in green. tRNA genes are yellow, and the LoxP site is blue.*

I generated $tRNA^{Gly(GCC)}$ gene mutant HEK293T cell lines: Gly-GCC 2-4 M, Gly-GCC 3-1 M, Gly-GCC 2-5 M, and Gly-GCC 5-1M and validated their specific mutations by PCR genotyping (Figure 3.3C) and DNA sequencing (Figure 3.3D). Subsequently, I assessed VAC14 mRNA expression levels in the different mutant cell lines and wild-type cells by performing RT-qPCR with qPCR primers in the first and second exons of the VAC14 transcription unit. Notably, Gly-GCC 2-4 M and Gly-GCC 3-1 M cell lines exhibited elevated VAC14 mRNA levels compared to wild-type cells, while Gly-GCC 2-5 M and Gly-GCC 5-1 M cell lines resulted in a decrease in VAC14 expression (Figure 3.4A). I observe related changes in the level of total VAC14 protein by Western blot (Figure 3.4B, C). When the promoter of antisense tRNA genes is disrupted, it results in decreased transcription of VAC14, implying that these antisense tRNA genes may have a regulatory role in promoting VAC14 transcription. Conversely, when the promoter of sense tRNA genes is disrupted, it leads to increased transcription of VAC14, suggesting that these sense tRNA genes might play a role in repressing VAC14 transcription or acting as negative regulators of VAC14 transcription. These findings highlight tRNA genes' potential bidirectional regulatory impact on VAC14 expression, depending on their orientation relative to the target gene's promoter.

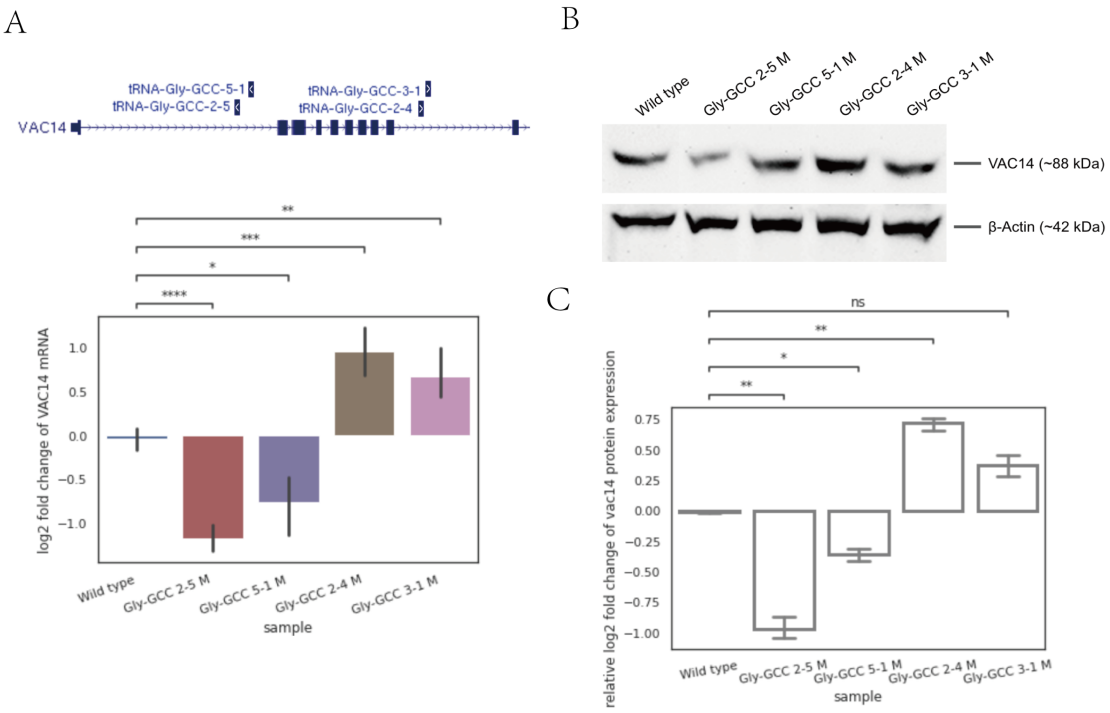


Figure 3.4: Inactive transcription of tRNA-Gly-GCC 2-4 and tRNA-Gly-GCC 3-1 leads to an increase of VAC14 expression, while Inactive transcription of tRNA-Gly-GCC 2-5 and tRNA-Gly-GCC 5-1 leads to decrease of VAC14 expression.

(A) *RT-qPCR shows VAC14 mRNA expression after mutant the tRNA genes. The qPCR results were normalized to β-Actin mRNA, and error bars indicate ± SEM. The sample size was n = 3, and P-values were calculated using Student's t-test relative to the wild type.* ***(B)*** *Western blotting was performed with anti-VAC14 and anti-β-Actin antibodies with protein extracts from mutant tRNA genes and wild-type HEK293T cell lines.* ***(C)*** *The VAC14 band intensities were quantified and normalized to their corresponding β-Actin band intensities. Error bars are as in E.*

3.3.4 The tRNA gene effect on VAC14 is mediated by transcriptional interference

The results in Figure 2.5 A demonstrated significant Pol II accumulation at tRNA Gly-GCC-2-4 and tRNA Gly-GCC-2-5 loci, indicating the presence of another layer of gene expression regulation by the interplay between RNA polymerases. To investigate this further, ChIP-qPCR assays were performed in wild-type and mutant cell lines using antibodies against the RPC32 (POLR3D) subunit of Pol III and Pol II. Three pairs of qPCR primers were designed to cover different regions of each tRNA gene. Among these

primers, I selected the pair that produced the highest peak in the wild-type cell during qPCR analysis. This primer pair was used to measure VAC14 mRNA expression levels in the various mutant cell lines and wild-type cells. As expected, robust Pol III signals were detected at the tRNA gene regions in the wild-type cells, demonstrating active transcription. In contrast, in each mutant cell line, the Pol III signal notably decreased at the respective tRNA gene regions, indicating a disruption in Pol III binding (Figure 2.5D). With the anti-Pol II antibody, I observed similar Pol II enrichment on the tRNA gene region in the wild-type cell. However, in the tRNA gene mutant cell lines, I observed a reduction of Pol II binding after decreasing Pol III binding, suggesting that suppressing the Pol III binding relieves Pol II pausing.

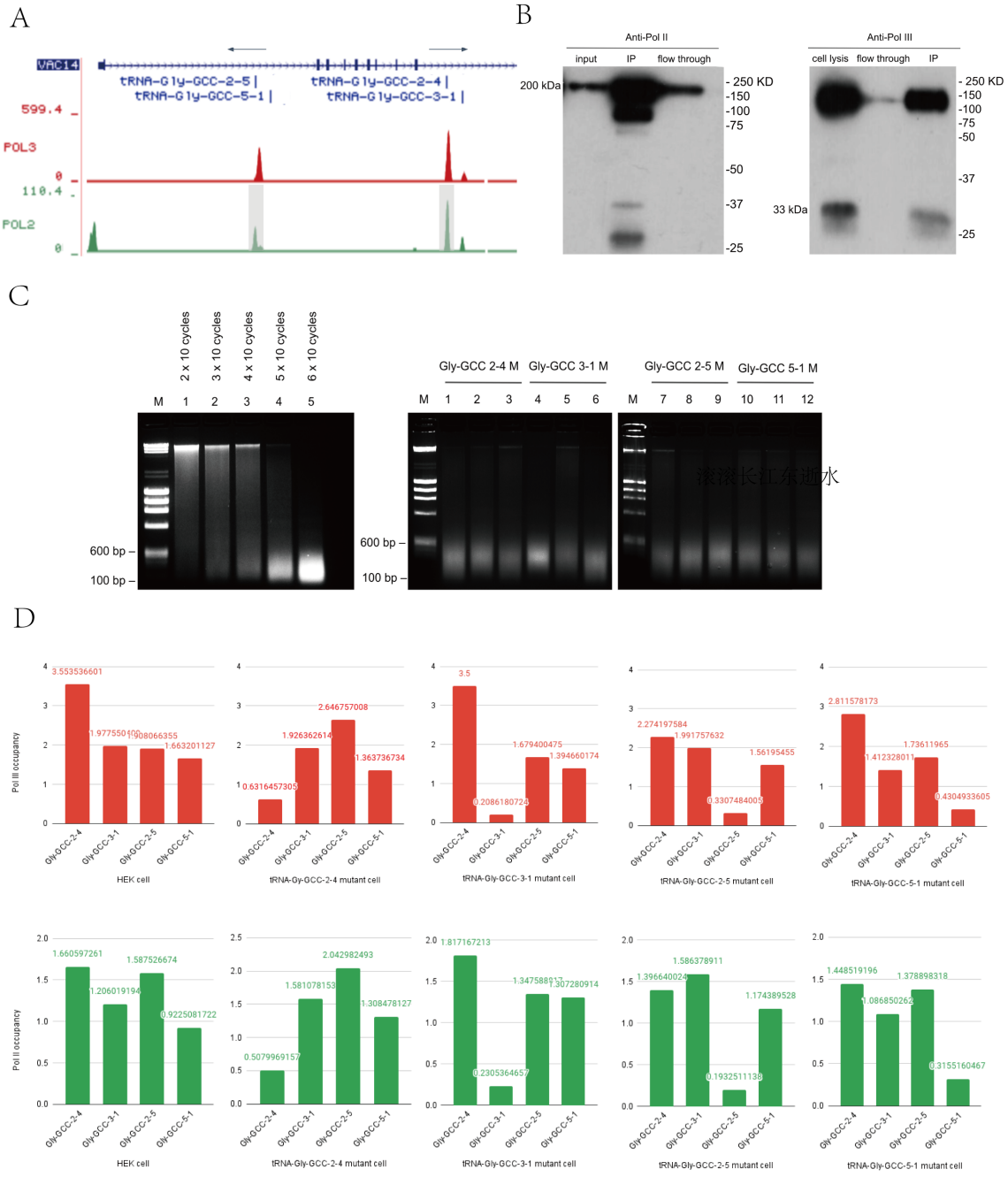

A
VAC14
tRNA-Gly-GCC-2-5
tRNA-Gly-GCC-5-1
tRNA-Gly-GCC-2-4
tRNA-Gly-GCC-3-1
599.4
POL3
0
110.4
POL2
0
B
Anti-Pol II
input
IP
flow through
200 kDa
250 KD
150
100
75
50
37
25
Anti-Pol III
cell lysis
flow through
IP
33 kDa
250 KD
150
100
75
50
37
25
C
2 x 10 cycles
3 x 10 cycles
4 x 10 cycles
5 x 10 cycles
6 x 10 cycles
M 1 2 3 4 5
600 bp
100 bp
Gly-GCC 2-4 M
Gly-GCC 3-1 M
Gly-GCC 2-5 M
Gly-GCC 5-1 M
M 1 2 3 4 5 6
M 7 8 9 10 11 12
600 bp
100 bp
滚滚长江东逝水
D
Pol III occupancy
3.553536601
1.977550109
1.908066355
1.663201127
HEK cell
0.6316457305
1.926362614
2.646757008
1.363736734
tRNA-Gly-GCC-2-4 mutant cell
3.5
0.2086180724
1.679400475
1.394660174
tRNA-Gly-GCC-3-1 mutant cell
2.274197584
0.3307484009
1.991757632
1.56195455
tRNA-Gly-GCC-2-5 mutant cell
2.811578173
1.412328011
1.73611965
0.4304933605
tRNA-Gly-GCC-5-1 mutant cell
Pol II occupancy
1.660597261
1.206019194
1.587526674
0.9225081722
HEK cell
0.5079969157
1.581078153
2.042982493
1.308478127
tRNA-Gly-GCC-2-4 mutant cell
1.817167213
0.2305364657
1.347588817
1.307280914
tRNA-Gly-GCC-3-1 mutant cell
1.396640024
0.1932511138
1.586378911
1.174389528
tRNA-Gly-GCC-2-5 mutant cell
1.448519196
1.086850262
1.378898318
0.3155160467
tRNA-Gly-GCC-5-1 mutant cell
Gly-GCC-2-4
Gly-GCC-3-1
Gly-GCC-2-5
Gly-GCC-5-1

Figure 3.5: ChIP qPCR for Pol II and Pol III near tRNA genes.

(A) *ENCODE ChIP-seq data for Pol III and Pol II distribution are shown in the human VAC14 gene region. The genomic arrangement of the beginning of the human VAC14 gene is shown at the top. tRNA-Gly-GCC-2-5 and tRNA-Gly-GCC-5-1 genes are oriented in an antisense direction within the first intron of VAC14. tRNA-Gly-GCC-2-4 and tRNA-Gly-GCC-3-1 genes are oriented in a sense direction within the ninth intron of VAC14. The bottom part shows a University of California at Santa Cruz (UCSC) genome browser view of Pol III and Pol II ChIP-seq profiles in a human cervical carcinoma cell line. Pol II accumulations overlap with Pol III peaks at* $tRNA^{Gly(GCC)}$ *loci. Tracks are from ENCODE (RPC1) and Liu et al.* ***(B)*** *Validation of Pol II and Pol III antibodies by IP/Western blot. The expected protein size for RNA Pol II is around 200 kDa. The predicted protein size for RNA Pol III is around 33 kDa* ***(C)*** *the chopped pattern of chromatin from* $tRNA^{Gly(GCC)}$ *mutant cell lines* ***(D)*** *ChIP-qPCR performed with anti-Pol II and anti-Pol III antibodies to show active Pol II and Pol III occupancy in the tRNAs region as fold enrichment.*

3.4 Discussion

This study provides compelling evidence that the $tRNA^{Gly(GCC)}$ genes within the introns of VAC14 play a significant regulatory role in the expression of VAC14 during neuronal development. By employing CRISPR/Cas9-mediated deletion and targeted mutagenesis, I demonstrated that these tRNA genes influence VAC14 transcription through transcriptional interference, highlighting the complex interplay between Pol III and Pol II transcriptional machinery.

The correlation analysis revealed that tRNA-Gly-GCC-2-5 positively correlates with VAC14 expression, whereas tRNA-Gly-GCC-2-4 negatively correlates with VAC14 expression. This suggests that different tRNA genes within the same intronic region can have opposing regulatory effects on the host gene. Our results indicate that the presence of these tRNA genes is crucial for the precise modulation of VAC14 expression.

The CRISPR/Cas9-mediated knockout experiments provided further insights into the functional roles of these tRNA genes. Deleting the antisense $tRNA^{Gly(GCC)}$ genes resulted in decreased VAC14 expression while deleting the sense $tRNA^{Gly(GCC)}$ genes led to increased VAC14 expression. These findings

suggest that the antisense tRNA genes may act as enhancers, promoting VAC14 transcription, whereas the sense tRNA genes may function as repressors, inhibiting VAC14 transcription. The effect on protein expression was significant (2-fold), in line with the result of recent systematic studies in humans, where sense tDNA-containing fragments in chromosome 19 acted as enhancer-blockers, leading to reduced downstream reporter protein expression (Raab et al. 2012) and where Pol III elements located upstream of Pol II promoters in the forward orientation can decrease Pol II transcription (Sheng et al. 2014). However, the result contrasts with the regulation effect of antisense MIR Pol III transcription in humans, which appears to suppress the overlapping Pol II gene expression (Yeganeh et al. 2017). Instead, a genome-wide study revealed that antisense ncRNA transcription could switch off corresponding sense genes under low, but not high, expression (Xu et al. 2011).

Furthermore, disrupting the internal promoter regions of these tRNA genes resulted in similar patterns of VAC14 expression changes, reinforcing the idea that the transcriptional activity of these tRNA genes directly impacts VAC14 expression. The reduction of Pol II binding in the tRNA gene mutant cell lines suggests that Pol III binding at these loci may interfere with Pol II transcriptional elongation, thereby modulating VAC14 transcription. A previous study has also highlighted the significance of the tRNA promoter region, where the tRNA gene functions as an enhancer blocker only in the presence of a functional B-box promoter (Raab et al. 2012). These findings emphasize the critical role of the tRNA gene and its promoter elements in mediating the transcription interference phenomenon, showcasing the intricate regulatory interplay between various components in gene expression.

Our findings underscore the importance of tRNA genes as regulatory elements within protein-coding genes, contributing to the intricate regulatory networks that govern gene expression during development. The bidirectional regulatory roles of the $tRNA^{Gly(GCC)}$ genes within the introns of VAC14 reveal a novel layer of gene expression control that could be pivotal for understanding the transcriptional regulation of other genes with similar genomic arrangements.

Future research should focus on further elucidating the mechanisms underlying this transcrip-

tional interference, including the potential involvement of chromatin remodeling and other transcription factors across different cell types or tissues. Additionally, exploring the broader implications of these findings in other biological contexts and developmental processes could provide valuable insights into the general principles of gene regulation by intronic tRNA genes.

In conclusion, this study demonstrates that the $tRNA^{Gly(GCC)}$ genes within the introns of VAC14 have distinct and significant regulatory effects on VAC14 expression. These results provide direct experimental evidence that Pol III-occupied genes can suppress or enhance Pol II gene expression, offering valuable insights into the intricate interactions between these two RNA polymerases and their impact on gene regulation in the cellular context. These findings contribute to our understanding of the complex interplay between Pol III and Pol II transcription and highlight the potential of tRNA genes as crucial regulatory elements in gene expression.

3.5 Methods

3.5.1 Construction of donor plasmids, sgRNAs, and Cas9 plasmids

pCas9-GFP and pCAG-Cre were obtained from Addgene (plasmid #44719, plasmid #13775). Oligos, including targeting sequences, were cloned into BbsI restriction sites of the Cas9 sgRNA vector obtained from Addgene (plasmid #68463) (Table S2.1). To generate the donor plasmid, DNA fragments of about 5 kb in length were amplified from the genomic DNA of the targeted tRNA genes and the upstream sequences. The DNA fragments of about 1 kb in length from downstream of tRNA genomic DNA were also amplified. These two fragments were then cloned into the multiple cloning sites of plasmid PL552 (Addgene plasmid #68407), upstream and downstream of the Puro gene, respectively. Site-directed mutagenesis was then used to make tRNA mutation and deletion donor plasmid. Primers used for cloning can be found in Table S2.2.

3.5.2 CRISPR/Cas9-mediated HDR genome editing

Pairs of sgRNA plasmids, Cas9 plasmids, and donor plasmids were co-transfected into HEK293T cells using calcium phosphate transfection. 72 hr later, puromycin was added to the cell. Seven days later, puromycin-resistant cells were diluted and plated at low density to allow for individual cell growth. Genomic DNA was extracted from 1 x 10$\hat{6}$ cells of single-cell colonies. Genomic PCR was performed using primer pairs that flanked the edited gene to detect the success of genome deletion and mutation.

3.5.3 RNA extraction, RT-qPCR, and Western blot

Total RNA was extracted using TRIzol reagents (Ambion) according to the manufacturer's protocol. One microgram of RNA was reverse transcribed with reverse transcriptase (Invitrogen) with random hexamer primer. iTaq SYBR Supermix protocol (Bio-Rad) was used for qPCR. The sequences of the qPCR primers are listed in Supplemental Table S2.3. The primary antibodies for Western blots (anti-VAC14, C-10 lot #A2114) and anti-β Actin ACTBD11B7 LOT #J1116 [Santa Cruz Biotechnology]) were used at 1:1000 dilutions.

3.5.4 Antibody Validation

RNA Pol II (Santa Cruz Biotechnology, D0521 8WG16) and Pol III RPC32 (Santa Cruz Biotechnology, L0419 C32-1) antibodies were validated by both immunoprecipitation followed by Western blot (IP/Western blot).

3.5.5 ChIP-qPCR

ChIP experiments were performed following the methodology outlined in (Yamakawa and Itakura 2019). For each ChIP experiment, ChIP DNA was prepared from 5 million formaldehyde cross-linked cells. Chromatin was sheared into 100 - 500 bp fragments using a Bioruptor sonicator (Diagenode). Immunoprecipitation was conducted with antibodies against Pol II and Pol III. The ChIP DNA was subse-

quently purified through reverse crosslinking at 65 °C overnight, followed by RNase, proteinase K, and phenol/chloroform extraction treatments. The purified DNA fragments were then subjected to qPCR analysis. qPCR was performed using the iTaq SYBR Supermix protocol (Bio-Rad). The primers used are listed in Table S2.4.

3.5.6 RNA-seq and tRNA Expression Datasets

VAC14 mRNA expression data were retrieved from the study by Fiddes (Fiddes et al. 2018). The pre-tRNA expression data during human neuron development were obtained from Alex Bagi's ARM-seq data generated in our lab.

Chapter 4

$tRNA^{Arg(TCT)}$ gene within HES7 3'UTR can suppress upstream gene expression

4.1 Abstract

HES7, a crucial member of the Hes family of basic helix-loop-helix (bHLH) transcriptional repressors, is well-known for its involvement in somitogenesis and the segmentation clock. Recent studies, however, highlight its significant role in neural development. The 3'UTR of HES7 is vital for regulating its expression by influencing mRNA stability, localization, and translation efficiency. This region contains regulatory elements that bind to RNA-binding proteins and microRNAs, modulating mRNA degradation and translational repression. Our study investigates the potential regulatory roles of one particular $tRNA^{Arg(TCT)}$ gene within the 3'UTR of HES7, hypothesizing that it modulates HES7 expression through chromatin architecture modulation and transcriptional interference. Using published DNase-seq, RNA-seq, and ChIP-seq data, I mapped chromatin accessibility changes and transcription factor binding during mouse cerebellar development. Luciferase reporter assays demonstrated that the UTR-overlapping $tRNA^{Arg(TCT)}$ gene significantly modulates upstream gene expression. Our results demonstrate that the $tRNA^{Arg(TCT)}$ gene within the HES7 3'UTR plays a critical role in regulating HES7 expression, with implications for understanding the broader regulatory networks essential for neural development and their potential impacts on developmental disorders.

4.2 Background

4.2.1 $tRNA^{Arg(TCT)}$ gene overlapped with the 3'UTR region of the HES7 gene

Besides VAC14, the Pol II and Pol III binding-signal overlap can also be observed in many other tRNAs, which are partially or entirely nested in protein-coding gene introns or UTRs, such as HES7 (Yeganeh et al. 2017). One $tRNA^{Arg(TCT)}$ gene is nested inside the 3'UTR region and is antisense to the HES7 gene.

4.2.2 HES7 gene and function

HES7 is a crucial gene within the HES family of basic helix-loop-helix (bHLH) transcriptional repressors. HES7 is well-known for its involvement in somitogenesis and the segmentation clock (Bessho, Miyoshi, et al. 2001; Hirata et al. 2004). Beyond this, Hes7's importance extends to neural development (Kageyama, Ohtsuka, and Kobayashi 2008; Kobayashi and Kageyama 2014; Ochi et al. 2020).

4.2.3 HES7 and 3'UTR regulation

The 3' untranslated region (3'UTR) of Hes7 plays a crucial regulatory role in gene expression by influencing mRNA stability, localization, and translation efficiency (Mayr 2017; Nitanda et al. 2014). Regulatory elements within the 3' UTR can bind to RNA-binding proteins and microRNAs, which modulate mRNA degradation and translational repression (Marc Robert Fabian, Sonenberg, and Filipowicz 2010; Marc R. Fabian and Sonenberg 2012). Additionally, studies have shown that axial-skeleton defects in Hes7 knockout and 3'UTR mutant mice further underscore the critical role of Hes7 in developmental processes (Fujimuro et al. 2014; Harima et al. 2013; Hubaud and Pourquié 2014). In the context of Hes7, these regulatory mechanisms are essential for fine-tuning its expression during development, ensuring proper somitogenesis and neural differentiation processes are tightly controlled (Gong and Maquat 2011; Kageyama, Shimojo, and Isomura 2018; Nitanda et al. 2014). The research on how the overlapping $HES7^{tRNA\text{-}Arg\text{-}TCT}$ gene in the 3'UTR region affects HES7 transcription and influences its role in development will further illuminate the complex regulatory networks essential for precise gene expression control and developmental outcomes.

4.3 Results

4.3.1 A tRNA$^{Arg(TCT)}$ gene overlapped with the 3'UTR region of the HES7 gene is conserved in mammals and highly occupied by Pol II and Pol III

In the genomes of most vertebrate species, a tRNA$^{Arg(TCT)}$ gene is nested within the 3'UTR region at the end of the longest isoform of the HES7 gene on the antisense strand (Figure 4.1A). To investigate the evolutionary conservation of the tRNA$^{Arg(TCT)}$ sequence within the HES7 gene across different species, the PhyloP score for this region was analyzed. Notably, the tRNA sequence within the 3'UTR region exhibits a high degree of conservation in contrast to the 3'UTR region and the last exon of HES7 (Figure 4.1A).

I examined the Pol III and Pol II signals to determine whether the tRNA$^{Arg(TCT)}$ gene in the UTR region plays a regulatory role in upstream gene transcription. ENCODE ChIP-seq data for HeLa-S3, H1-hESC and K562 cells (ENCODE Project Consortium 2012) reveal high Pol III occupancy on the tRNA-Arg-TCT-2-1 gene and Pol II accumulation stopping just before the Pol III peak (Figure 4.1B). This pattern suggests the tRNA gene's potential transcriptional regulatory role on HES7 expression.

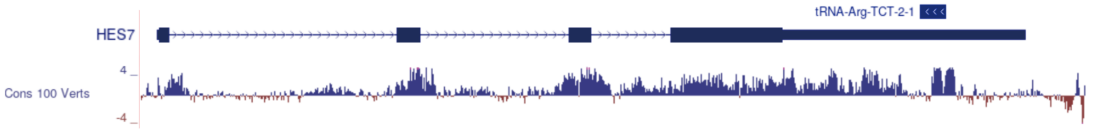
4.3.2 HES7$^{tRNA\text{-}Arg\text{-}TCT}$ region is opening and bears the epigenetic hallmarks of promoter

DNase I hypersensitive (DHS) sites indicate nucleosome-depleted regions that are universal markers of gene regulatory elements, including promoters, enhancers, insulators, and most transcription factor binding sites (Yamakawa and Itakura 2019). To determine if the HES7$^{tRNA\text{-}Arg\text{-}TCT}$ gene can be DHS sites (e.g., enhancers, promoters, and silencers), I mapped specific histone modifications ChIP-seq datasets in the Mouse ENCODE release 3 to the HES7 locus. DNase I is hypersensitive to the HES7$^{tRNA\text{-}Arg\text{-}TCT}$ locus (Figure 4.1C), which means this locus is open in the adult cerebellum. Histone modifications reflect the activation state of cis-regulatory elements, so the active enhancer/promoter marker Histone H3 lysine

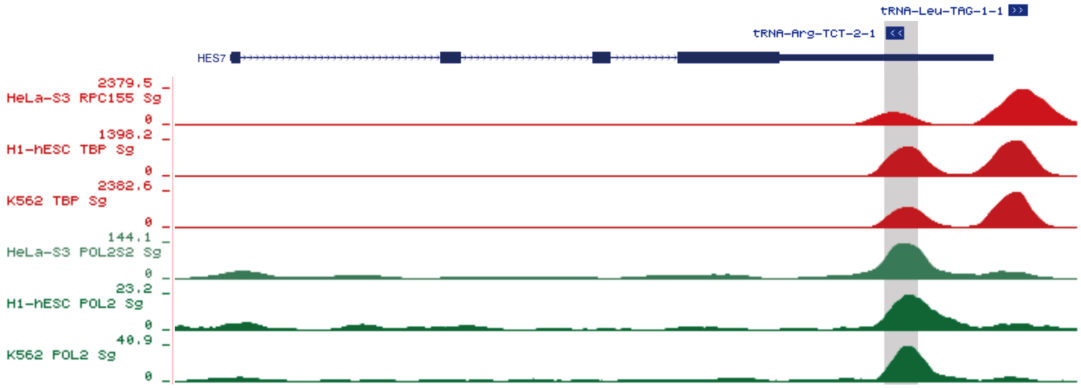
27 acetylation (H3K27ac), the promoter marker Histone H3 at Lysine 4 Trimethylation (H3K4me3), the enhancer marker Histone H3 lysine 4 mono-methylation (H3K4me1) and the repressive marker Histone H3 lysine 27 Trimethylation (H3K27me3) (Barski, Cuddapah, et al. 2007; Bernstein et al. 2006; Creyghton et al. 2010; Hawkins et al. 2011; Heintzman et al. 2009; T. H. Kim, Barrera, et al. 2005; Rada-Iglesias et al. 2011) were used to identify the potential function of this locus.

The enrichment of H3K27ac and H3K4me3 histone marks, combined with the absence of the absence of H3K4me1 and H3K27me3 signals, indicates that the HES7$^{tRNA\text{-}Arg\text{-}TCT}$ site functions as a promoter region in adult cerebellum, heart and liver (Figure 4.1C).

A


tRNA-Arg-TCT-2-1
HES7
Cons 100 Verts
4
-4

B


tRNA-Leu-TAG-1-1
tRNA-Arg-TCT-2-1
HES7
2379.5
HeLa-S3 RPC155 Sg
0
1398.2
H1-hESC TBP Sg
0
2382.6
K562 TBP Sg
0
144.1
HeLa-S3 POL2S2 Sg
0
23.2
H1-hESC POL2 Sg
0
40.9
K562 POL2 Sg
0

C

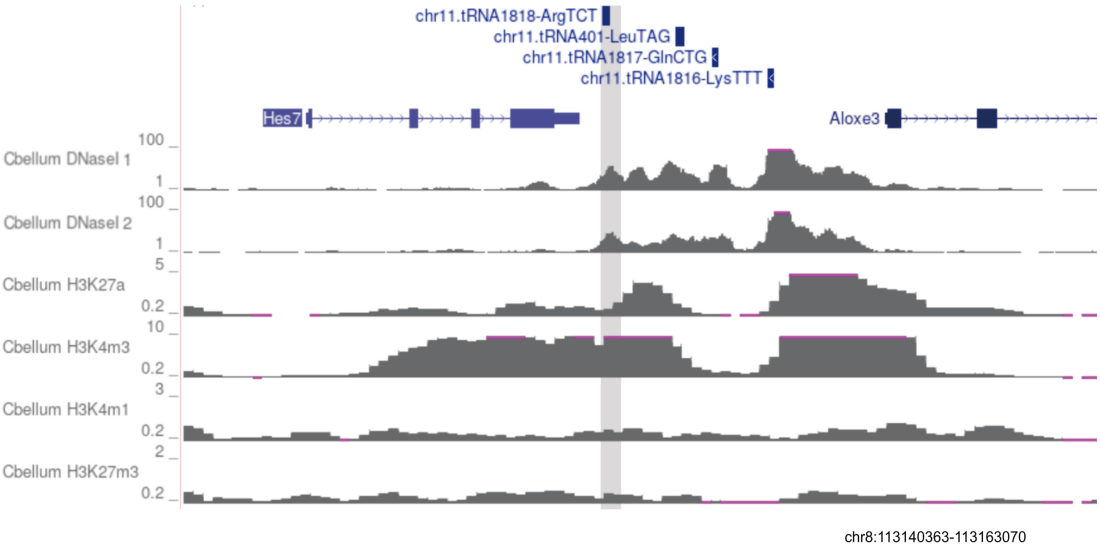

chr11.tRNA1818-ArgTCT
chr11.tRNA401-LeuTAG
chr11.tRNA1817-GlnCTG
chr11.tRNA1816-LysTTT
Hes7
Aloxe3
Cbellum DNaseI 1
100
1
Cbellum DNaseI 2
100
1
Cbellum H3K27a
5
0.2
Cbellum H3K4m3
10
0.2
Cbellum H3K4m1
3
0.2
Cbellum H3K27m3
2
0.2
chr8:113140363-113163070

Figure 4.1: Conservation and regulatory potential of a tRNA$^{Arg(TCT)}$ gene overlapping the 3'UTR of HES7 gene in vertebrates

(A) *The average PhyloP score (comparing humans to 100 vertebrate species) is plotted for each position within the tRNA$^{Arg(TCT)}$ and 3'UTR region and the last intron sequence in the HES7 gene.* ***(B)*** *ENCODE ChIP-seq data for Pol III and Pol II distribution are shown in the human HES7 gene region. The genomic arrangement of the human HES7 gene is shown at the top. tRNA-Arg-TCT-2-1 gene is oriented in an antisense direction within the 3'UTR region of the HES7 gene. tRNA-Leu-TAG-1-1 gene is oriented in a sense direction after the 3'UTR region of the HES7 gene. The bottom part shows a University of California at Santa Cruz (UCSC) genome browser view of Pol III and Pol II ChIP-seq profiles in human HeLa-S3, H1-hESC and K562 cell lines. Pol II accumulations overlap with Pol III peaks at the tRNA$^{Arg(TCT)}$ locus. Tracks are from ENCODE (RPC1) and Liu et al.* ***(C)*** *UCSC genome browser images for tRNA-Arg-TCT-2-1 gene and HES7 gene region of mm9 genome highlighting that tRNA-Arg-TCT-2-1 locus opens during development by DNase-seq and overlaps with H3K27ac and H3K4me3 enrichment (gray bar) in adult (P56) cerebellum.*

4.3.3 Zic 1/2 Protein Binding to HES7 Gene Region and Regulation of HES7 Transcription During Mouse Cerebellum Development

Zic transcription factors play a pivotal role in neural development by regulating chromatin accessibility at neuronal enhancers and coordinating gene expression patterns crucial for cerebellar granule neuron maturation (Aruga and Millen 2018; Frank et al. 2015). Hes7 is essential for neural development, influencing mRNA stability and translation through its 3'UTR and playing a significant role in processes such as somitogenesis and neural differentiation (Bessho, Miyoshi, et al. 2001; Hirata et al. 2004; Kageyama, Ohtsuka, and Kobayashi 2008; Kobayashi and Kageyama 2014; Ochi et al. 2020). To investigate how chromatin accessibility changes in the HES7 locus during cerebellar differentiation and whether Zic transcription factors bind to this region, I explored Zic 1/2 and DNase ChIP-Seq dataset from P7 to P60 cerebellum. I aimed to describe the DNA landscape encompassing the HES7$^{tRNA\text{-}Arg\text{-}TCT}$ locus and HES7 TSS.

During mouse cerebellum development, I observed a 5-fold increase in DNase signal, indicating the opening of the tRNA Arg-TCT gene locus after day 14 postnatal (Figure 4.2A gray bars), consistent with

the increase in H3K27ac signal (Figure 4.2B). However, RNA-seq data revealed decreased HES7 mRNA expression (Figure 4.2D). Notably, the HES7 TSS appeared to entirely shut down after mouse cerebellum development, as evidenced by ChIP signals dropping to zero in the kidney, heart, and liver compared to brain development (Figure 4.2A). Meanwhile, the HES7$^{tRNA\text{-}Arg\text{-}TCT}$ gene locus partially closed (Figure 4.2A).

Concurrently, Zic ChIP-seq data revealed that Zic 1/2 protein binds to the HES7 3'UTR region during the development of the human cerebellum (Figure 4.2C). To elucidate the impact of Zic transcription factor binding on HES7 expression, I examined RNA-seq data of the HES7 gene and Zic 1/2 knockdown neurons during neural development. Knockdown Zic 1/2 protein completely shut down the HES7 mRNA expression (Figure 4.2E), suggesting a regulatory role for Zic transcription factors in development expression during cerebellar development.

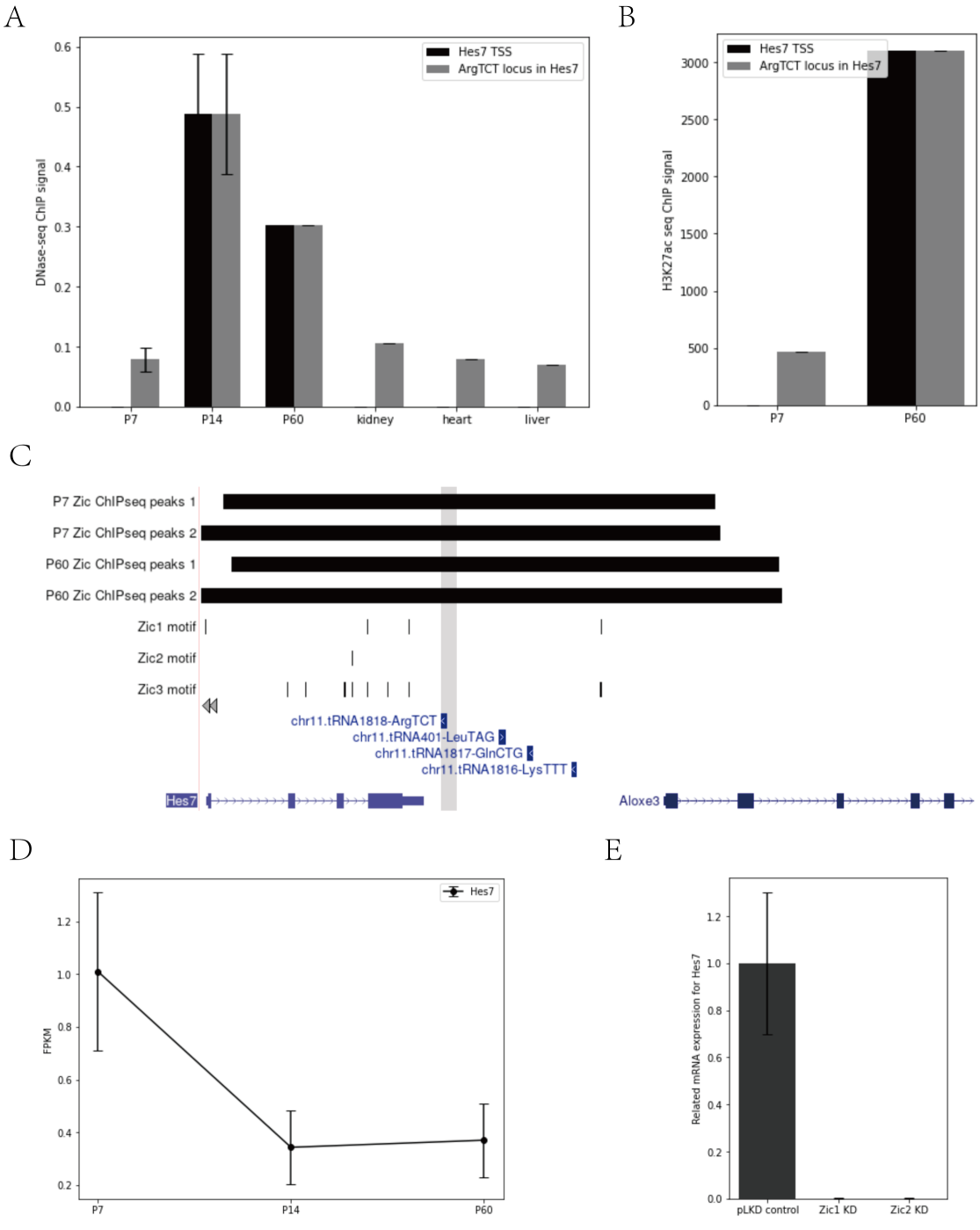

A
Hes7 TSS
ArgTCT locus in Hes7
DNase-seq ChIP signal
0.0
0.1
0.2
0.3
0.4
0.5
0.6
P7
P14
P60
kidney
heart
liver
B
Hes7 TSS
ArgTCT locus in Hes7
H3K27ac seq ChIP signal
0
500
1000
1500
2000
2500
3000
P7
P60
C
P7 Zic ChIPseq peaks 1
P7 Zic ChIPseq peaks 2
P60 Zic ChIPseq peaks 1
P60 Zic ChIPseq peaks 2
Zic1 motif
Zic2 motif
Zic3 motif
chr11.tRNA1818-ArgTCT
chr11.tRNA401-LeuTAG
chr11.tRNA1817-GlnCTG
chr11.tRNA1816-LysTTT
Hes7
Aloxe3
D
Hes7
FPKM
0.2
0.4
0.6
0.8
1.0
1.2
P7
P14
P60
E
Related mRNA expression for Hes7
0.0
0.2
0.4
0.6
0.8
1.0
1.2
pLKD control
Zic1 KD
Zic2 KD

Figure 4.2: Chromatin accessibility and Zic transcription factor binding at the HES7 locus during cerebellar development

(A) *DNase-seq signal in HES7$^{tRNA\text{-}Arg\text{-}TCT}$ locus and HES7 TSS. HES7$^{tRNA\text{-}Arg\text{-}TCT}$ locus opens from P7 to P60 and closes for normal tissues. HES7 TSS is open during neuronal differentiation, whereas it's silent in normal tissue.* ***(B)*** *H3K27ac ChIP-seq signal in HES7$^{tRNA\text{-}Arg\text{-}TCT}$ locus and HES7 TSS in P7 or P60 cerebellum.* ***(C)*** *Zic ChIP-seq signal peak and Zic motifs at HES7$^{tRNA\text{-}Arg\text{-}TCT}$ locus.* ***(D)*** *RNA-seq analysis of HES7 mRNA expression levels across developmental time.* ***(E)*** *RNA-seq analysis of HES7 expression levels in P7 mice following Zic1 and Zic2 knockdown by shRNA, compared to control mice transfected with an empty vector (pLKO). Error bars represent the standard error of the mean based on three cultures.*

4.3.4 tRNA$^{Arg(TCT)}$ gene and the proximal 3'UTR can suppress upstream gene expression

3'UTR region of the HES7 gene plays a critical role in regulating its expression by influencing mRNA stability, localization, and translation efficiency (Mayr 2017; Marc R. Fabian and Sonenberg 2012). I aim to explore the potential role of the tRNA$^{Arg(TCT)}$ gene within this 3'UTR region in the expression of its host protein-coding gene. This UTR region is only expressed during development, making direct testing in typical cell types unfeasible. Based on my studies of tRNA gene modulation of VAC14, I designed a reporter to test the effects of the tRNA$^{Arg(TCT)}$ gene embedded in the HES7 3'UTR on a reporter gene. I created a panel of Renilla luciferase reporters with no 3'UTR as a negative control, the wild-type HES7 3'UTR, and various 3'UTR mutants. These mutants included removing the overlapping tRNA$^{Arg(TCT)}$ gene and truncating the UTR to identify regions important for translational regulation.

The luciferase expression data, displayed in the bar plot Figure 3.3, indicate the regulatory role of the internal tRNA gene within the HES7 3'UTR. When there is no insertion in the Renilla luciferase plasmid, Renilla expression is high. Inserting the UTR region decreases luciferase expression to 10% of the control. Removing the tRNA gene increases expression to 25% or even 80% of the control.

To further investigate the significance of the tRNA gene, I reinserted it into left and right

UTR truncations. Removing the tRNA gene alone resulted in a 6-fold increase in luciferase expression. Reintroducing the tRNA gene into the left truncation decreased luciferase expression back to low levels, while reintroducing it into the right truncation also resulted in a 10-fold decrease in luciferase expression. These results suggest that both the tRNA gene and the left region of the UTR are essential for regulating HES7 expression.

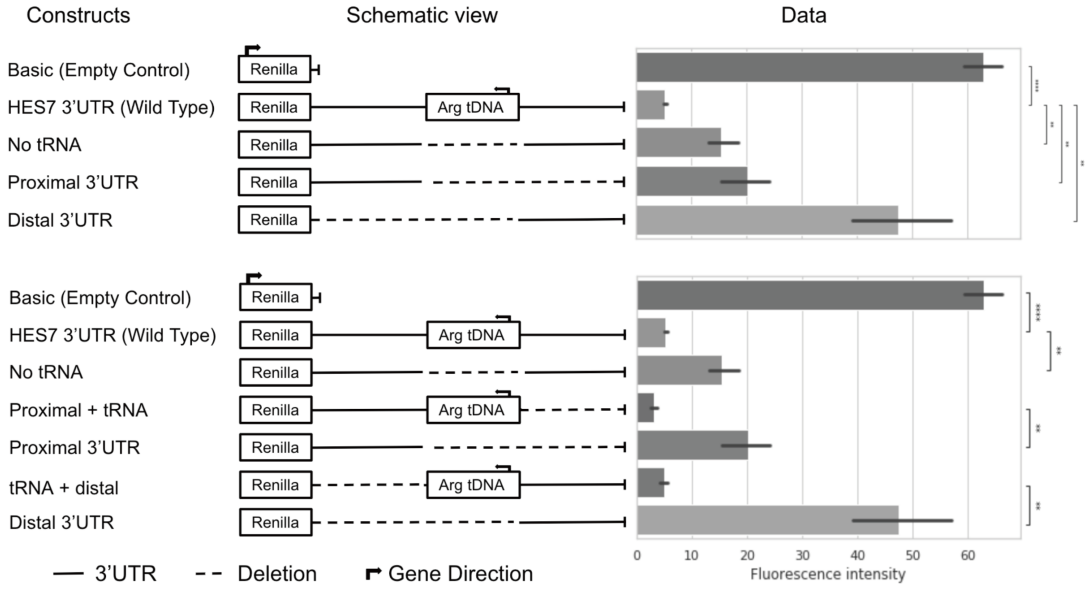


Figure 4.3: Luciferase reporter activity of serial deletion of human HES7 3'UTR. The left panel is the schematic view of the deletion constructs.

These constructs were co-transfected into HEK293 cells with a Firefly luciferase plasmid as a transfection control to test the influence of the tRNA-Arg(TCT) gene on HES7 gene expression. Luciferase expression was measured using a plate reader. Levels of Renilla luminescence were normalized to Firefly luminescence and compared to determine the effects of these truncations on HES7 3'UTR regulation of Renilla expression. The bar graphs at the right are luciferase activities of these constructs normalized to firefly control plasmid. The data shown are the means ± S.D. of three independent experiments. (bottom) Effect of $tRNA^{Arg(TCT)}$ on reporter activities. $tRNA^{Arg(TCT)}$ was combined with different parts of human HES7 3'UTR truncations, and the luciferase reporter system examined the luciferase reporter activity of each construct. The data shown are the means ± S.D. of three independent experiments.

4.4 Discussion

This study reveals the regulatory potential of the $tRNA^{Arg(TCT)}$ gene within the 3'UTR of the HES7 gene, highlighting a novel mechanism by which tRNA genes can influence protein-coding gene expression. Our results suggest that the $tRNA^{Arg(TCT)}$ gene plays a significant role in modulating HES7 expression through chromatin architecture and transcriptional interference.

Luciferase reporter assays provided additional evidence for the regulatory function of the HES7 3'UTR. Constructs containing the full-length 3'UTR significantly reduced luciferase expression, whereas truncations lacking the tRNA gene or specific regions restored higher expression levels. This indicates that both the tRNA gene and particular sequences within the 3'UTR are essential for regulating HES7 expression. The ChIP-seq and RNA-seq data further support this hypothesis, showing that the $tRNA^{Arg(TCT)}$ gene influences HES7 expression by altering transcription factor binding and chromatin accessibility at the HES7 promoter region.

Using ChIP-seq data, I observed that the $HES7^{tRNA\text{-}Arg\text{-}TCT}$ locus opens during mouse cerebellum development, with a notable increase in DNase signal and H3K27ac enrichment. This chromatin accessibility is crucial for the binding of transcription factors such as Zic1/2, which were shown to bind to this region and regulate HES7 expression. Knockdown experiments of Zic1/2 further confirmed their regulatory role, as the absence of Zic1/2 proteins led to a significant decrease in HES7 mRNA expression. These findings align with previous research suggesting that tRNA genes can act as regulatory elements, influencing the expression of neighboring genes through various mechanisms, including enhancer activity and transcriptional interference.

Overall, our findings highlight the complex regulatory mechanisms involving the HES7 3'UTR and its embedded $tRNA^{Arg(TCT)}$ gene. These elements are crucial for fine-tuning HES7 expression during development, particularly in the neural context. Understanding these regulatory interactions provides valuable insights into the broader mechanisms of gene expression control and their implications for

developmental processes and diseases.

4.5 Methods

4.5.1 Data Sets and Alignment Analyses

The $tRNA^{Arg(TCT)}$ gene sequences in HES7 3'UTR from 100 vertebrates were downloadable from GtRNAdb (October 2015 release) (Chan and Lowe 2016). Multiple sequence alignments to generate consensus sequences were performed using Jalview v.2.11 (Waterhouse et al. 2009).

4.5.2 Pol II and Pol III signal and PhyloP score calculation

The ENCODE Project Consortium employed ChIP-seq data (Kharchenko, Tolstorukov, and Park 2008; Q. Li et al. 2011; ENCODE Project Consortium 2012) extracted from the UCSC Genome Browser (Kent et al. 2002) to identify specific binding regions for regulatory factors such as TBP, Pol 3, and Pol 2 transcription factors within the human genome. Regions displaying higher signal intensities indicate more frequent binding of the respective transcription factors (ENCODE Project Consortium 2011). Additionally, I calculated the average PhyloP score for all the $tRNA^{Arg(TCT)}$ genes in the HES7 3'UTR region and the whole 3'UTR region and the last exon region as control, utilizing the PhyloP conservation data (Pollard et al. 2010) available in UCSC Genome Browser.

4.5.3 Histone Makers and Zic ChIP-seq Datasets

DNase, H3K4me1, H3K4me3, and H3K27ac ChIP-seq data for P56 (postnatal day 56) cerebellum were retrieved from the Mouse ENCODE project release 3 (August 2012) created by Bing Ren's lab at the Ludwig Institute for Cancer Research (LICR) (ENCODE Project Consortium 2012). Available peak files were used in this study to determine chromatin open status. ChIP-seq datasets for DNase, H3K27ac, and Zic 1/2, RNA-seq data at P7, P14, and P60 mouse cerebellum, and Zic1 and Zic2 knockdown data were retrieved from a previously published dataset, and the publicly available data can be found at GEO: GSE60731

(Frank et al. 2015).

4.5.4 Luciferase Reporter Construct

Firefly luciferase control plasmid (pls0) and Renila luciferase plasmid (pls2) were obtained from Addgene (Addgene plasmid #12178, Addgene plasmid #12177). PCR amplified a 3746 bp vector fragment of pls2 from the pls2 plasmid. The primer sequences were: forward, 5' - gcggccgcttcgagcagacatgataag - 3', and reverse, 5' - tctagaacaactagtagtgatatcgagaccggt - 3'. A 981 bp of human HES7 3'UTR was amplified by PCR from HEK293 genomic DNA and cloned into the XbaI and NotI site of pls2 by Gibson Assembly. The primer sequences were: forward, 5' - tgtctgctcgaagcggccgcAATTTACAACAAAACTAGTTT - 3', and reverse, 5' - atatcactactagttgttctagaGCCTTGGGGGGTGGTGGGGGC - 3'. Serial deletion constructs were prepared by Gibson Assembly using the full-length construct as the template. The primer sequences are shown in Table 3.1. All constructs were sequence confirmed.

4.5.5 Cell Culture, Transfection, and Reporter Assays

Human embryonic kidney 293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Transfections were performed using Lipofectamine 2000 when the cells reached 70% confluency. For transfection, 0.016 g of pls0 plasmid expressing firefly luciferase was co-transfected alongside 0.8 g of various target constructs expressing Renilla luciferase to serve as an internal control for transfection efficiency. Twenty-four hours post-transfection, luciferase activities were assessed using the Dual-Luciferase Reporter Assay. The Renilla luciferase activity was normalized to the firefly luciferase activity in each sample to account for variations in transfection efficiency.

Part II

Supplemental Data

Chapter 2: Supplemental Tables

Name	Target Gene	Sequence
5’ sgRNA primer1(2-4)	GlyGCC-2-4 3-1 downstream	GGACGAAACACCGAGGTGTG CCAT-GAGGAACGGTTTTAGAGCTA
3’ sgRNA primer1(2-4)	GlyGCC-2-4 3-1 downstream	TTTCTAGCTCTAAAACCGTTCCTC ATG-GCACACCTCGGTGTTTC
5’ sgRNA primer3(2-4)	GlyGCC-2-4 3-1 upstream	GGACGAAACACCGTACTCTGGCT GCTTCGGCAAGTTTTAGAGCTA
3’ sgRNA primer3(2-4)	GlyGCC-2-4 3-1 upstream	TTTCTAGCTCTAAAACTTGCCGA AGCAGCCAGAGTACGGTGTTTC
5’ sgRNA primer1(2-5)	GlyGCC 2-5 5-1 downstream	GGACGAAACACCGTCATCCACTGGT CATCGCACGTTTTAGAGCTA
3’ sgRNA primer1(2-5)	GlyGCC 2-5 5-1 downstream	TTTCTAGCTCTAAAACGTGCGAT GACCAGTGGATGACGGTGTTTC
5’ sgRNA primer3(2-5)	GlyGCC 2-5 5-1 upstream	GGACGAAACACCGACAAAATAG GTCTCGCTCTGTTTTAGAGCTA
3’ sgRNA primer3(2-5)	GlyGCC 2-5 5-1 upstream	TTTCTAGCTCTAAAACAGAGCGA GACCTATTTTGTCGGTGTTTC

Supplemental Table S2.1: sgRNA for gene target

Gene Amplified	Forward Primer	Reverse Primer
2-4 Mutation	gcgggaggcccgggtCA gattcccggccaatgcagcagctgaaagctttt	gtggcaggcgagaattcCa AAactgaaccaccaatgctccagctgagtgctgtt
3-1 Mutation	tggcaggcgagaattcC aAAactgaaccaccaatgctccagctggtaggga	cgcgggaggcccgggtCA gattcccggccagtgcagcctatctacctttta
2-5 Mutation	cgggaggcccgggtCA gattcccggccaatgcacccagatgtgagca	cgtggcaggcgagaattcCa AAactgaaccaccaatgcgcagcagaccttta
5-1 Mutation	gggcggccgggctCAg attcctggccaatgcatccagctgtttggt	gcatggcaggcgagaattcCa AAactgaaccaccaatgcatgaagtgttgctttt

Supplemental Table S2.2: The sequences of tRNA-Gly-GCC mutant primers

Gene Amplified	Forward Primer	Reverse Primer
VAC14 exon 1-exon 2	GCTGTACGAAAAGCGGAAGG	CAAACTCCTGGGACAGGGTC
VAC14 exon 2-exon 3	GACCCTGTCCCAGGAGTTTG	TCAGGTAGAGCCCTGAGTCC
ACTG	CCGCATCCTCCTCTTCTCTG	GAAGGTGGTCTCGTGGATGC

Supplemental Table S2.3: The sequences of VAC14 RT-qPCR primers

Gene Amplified	Forward Primer	Reverse Primer
Gly-GCC-2-4 1	CCGCACTAGCTTATCCCTCC	GCTGAAAGCTTTTTGGCAGC
Gly-GCC-2-4 2	AAAGCTTTCAGCTGCTGCAT	ACAGCACTCAGCTGGAGCAT
Gly-GCC-2-4 3	AGCTGAGTGCTGTTCTGCC	AGGTTACAGCTGTGCCATCA
Gly-GCC-2-4 4	GATTCACAGTGGTGATGGCAC	TCCCATAGGTTTGGCTGGTA
Gly-GCC-3-1 1	TGGTTAGGGTAATACTCTCCTGG	ACCTTTTTAGATGGTTTTCCTAAGC
Gly-GCC-3-1 2	TCCAGCTGGTAGGGATGTTT	AAGCCCGCTAGAAAGGAACC
Gly-GCC-3-1 3	TTTAGTTGAGGCCACGACCC	GCAAGAAGTCAAGCCCGCTA
Gly-GCC-2-5 1	CAGCATCAGAGACTGGCTTCA	GCACCTCCCTTTAACCCCATT
Gly-GCC-2-5 2	GTTCCATTCTTGCAACTGTGCT	TGGGCCTCCTTGCATATAAGTT
Gly-GCC-5-1 1	TGCCTTTTGCTGGCCAGTTT	TGGTGTAGTTGGAGGGACTGA
Gly-GCC-5-1 2	CCTGCAGATGCTCTACCAAACA	GGAAAACTGGCCAGCAAAAGG

Supplemental Table S2.4: The sequences of ChIP-qPCR primers

Appendix A

Glossary

AGO2: Argonaute 2

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

CTCF: CCCTC-binding Factor / 11-zinc finger binding factor

DHS: DNase I hypersensitive

FIMO: Find Individual Motif Occurrences

HDR: CRISPR/Cas9-mediated homology-directed recombination

H3K4me1: Histone H3 lysine 4 mono-methylation

H3K4me3: Histone H3 at Lysine 4 Trimethylation

H3K27ac: Histone H3 lysine 27 acetylation

H3K27me3: Histone H3 lysine 27 Trimethylation

PI(3,5)P2: phosphatidylinositol 3,5-bisphosphate

Pol II: RNA polymerase II

Pol III: RNA polymerase III

tDRs: The tRNA-derived small RNAs

TF: Transcription Factor

tRNAs: Transfer RNAs

TSS: transcription start site

UTR: untranslated region

Zic: zinc-finger in the cerebellum

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