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Investigation of histone modification and alternative splicing association in neural and non-neural tissues

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Abstract

Pre-mRNA splicing is a complex and vital process utilized in eukaryotes. Histone modifications are known to affect splicing through recruiting factors to the nascent pre-mRNA. They also influence splicing outcome through modulating the elongation rate of RNAPII by altering the degree of chromatin compaction. We hypothesized that the H3K36me3 histone modification in genes known to be neurally regulated would be enriched compared to non-neural tissues, as a potential contributor to tissue-specific splicing. Using publicly available ChIP-Seq and RNA-Seq data from embryonic and postnatal mouse tissues, we analyzed the differences in H3K36me3 signal between brain and non-neural tissues for select genes known to play an important role in brain function. We found that exon inclusion was associated with H3K36me3 across tissues in the Bin1 gene, although the correlation was less pronounced in other genes. Furthermore, we observed that changes in H3K36me3 during brain development were associated with changes in alternative splicing patterns at the same developmental time point. By identifying these associations, we postulate that H3K36me3 could serve as a key determinant of alternative splicing outcome.

Keywords: Chromatin, Epigenetics, RNA Splicing, H3K36me3

1. Introduction

DNA is the genetic material which universally codes for the proteins of almost all organisms. A singular diploid cell in the human body contains 6 billion base pairs, spanning 2 meters wide end-to-end. To compact it for packaging within the 10 micrometre nucleus, the DNA is wrapped around an octamer of positively charged proteins known as histones, which, when combined with associated RNAs, form chromatin. Chromatin can be modified in various ways, such as DNA methylation and histone modifications. Histone modifications are known to affect key properties of the chromatin, such as DNA accessibility, altering the extent to which proteins and transcription factors must compete with histones for DNA binding and utilisation. For example, accessibility is decreased by H3K27 methylation, and increased by acetylation or H3K36 methylation (Bannister & Kouzarides, 2011; Greer & Shi, 2012).

Alternative splicing is the process of producing multiple distinct mRNAs (isoforms) from the same pre-mRNA via joining exons (coding regions) in different combinations, used by higher eukaryotes for increased transcriptomic and proteomic diversity. It is mediated by splicing factors that guide the spliceosome, a complex assembled along the introns of small ribonuclear proteins, to the splice sites to cut out introns (non-coding regions). Alternative splicing preferentially occurs at suboptimal splice sites, giving rise to varying proportions of the variants, but splicing factors can bind to certain sequences to influence which splice site is used. This process is vital to eukaryotes, and its misregulation is implicated in cancers and neurological disorders (Marasco & Kornblihtt, 2023).

Recent studies have illuminated strong connections between histone modifications and alternative splicing (Yustis et al., 2024). The research in post-translational modifications, chromatin state, and elongation speed's ability to directly influence splicing has arisen, due to evidence that splicing occurs simultaneously to transcription, in other words, co-transcriptionally (Figure 1A)

Two models are prominent as the current consensus of how the chromatin landscape shapes splicing outcome: the recruitment and the kinetic model. The recruitment model describes chromatin and its modifications' interactions with splicing factors during cotranscriptional splicing (Yustis et al., 2024). The kinetic model proposes that the elongation rate of RNAP II (RNA Polymerase II) alters splice site selection and spliceosome assembly by modulating the availability of splice sites (Yustis et al., 2024). Histone modifications that induce chromatin compaction can also affect the elongation rate, leading to changes in splicing outcomes. Mechanistic models for particular modifications, such as H3K36me3, have been found, where they can regulate splicing through a chromatin reader protein, such as MRG15, which can then recruit splicing factors, such as polypyrimidine tract-binding protein (Figure 1B) (Luco et al., 2010). While the associations between histone modifications and alternative splicing in the brain are well studied, as the organ with a high frequency of alternative splicing in higher eukaryotes, the connections and comparisons of the same relationships in non-neural organs are not explored well.

In this study, we aimed to investigate the extent to which the histone modification H3K36me3 acted as a tissue-specific splicing regulator, examining its level of presence in exons that are distinctly spliced across tissues. An additional objective was to determine if H3K36me3 could be significant for developmental differences in exon inclusion percentages to assess its potential impacts on aberrant embryonic growth. These relationships were investigated in 4 select genes, Bin1, Mef2a, Cltb, and Pkm (elaborated in the next section). As alternative splicing is linked to cancer, cardiomyopathy, and autoimmune and neurological diseases, understanding of its regulators could provide valuable insights (Montes et al., 2019).

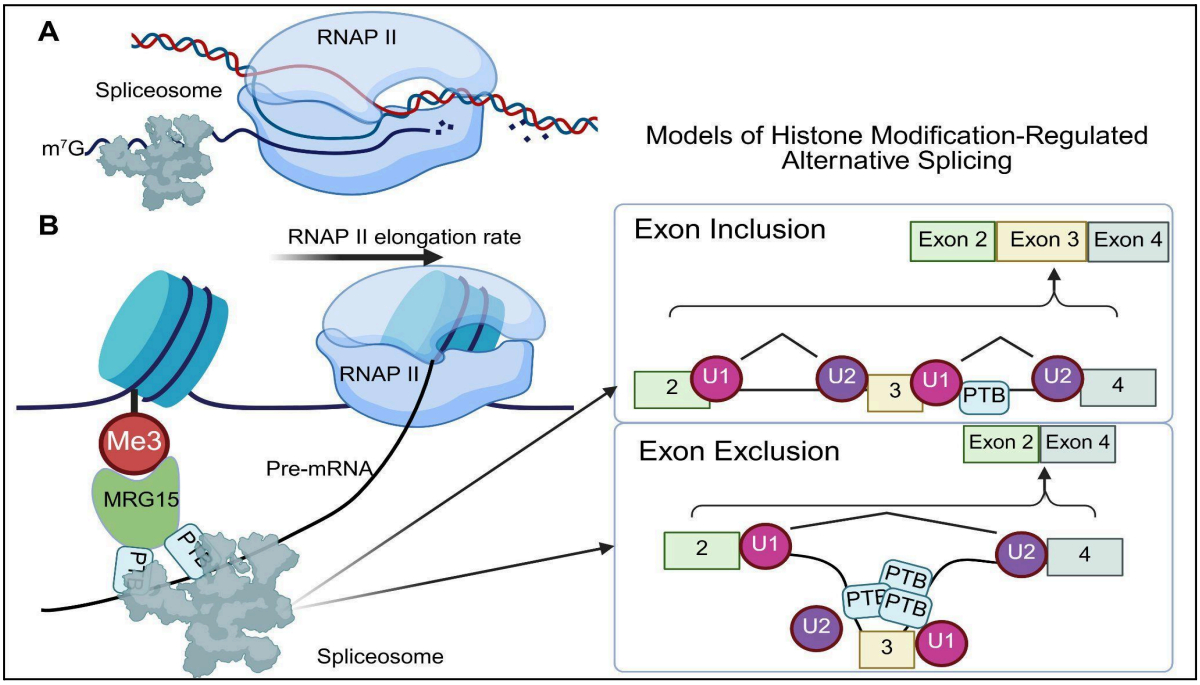

Figure 1: Cotranscriptional splicing and models for histone modification-regulated alternative splicing. (A) A schematic figure illustrating how the RNA Polymerase II (RNAP II) undergoes elongation simultaneously to splicing by the spliceosome, after the m^7G cap has been bound. **(B)** A schematic figure depicting the effects of chromatin states on differential splicing outcome through affecting RNAP II elongation speed and chromatin adaptors. H3K36me3 is shown here to be interacting with the chromatin adaptor protein MORF-related gene 15 (MRG15), which then recruits the polypyrimidine tract binding protein (PTB), adapted from (Luco et al., 2010). Subsequently, its location-dependent splicing outcomes are illustrated on the right, with the potential mechanisms. Adapted from (Chou et al., 2000). Figure generated using BioRender.

Methodology

Selection of H3K36me3 from other histone modifications

H3K36me3 was chosen as the only histone modification in this study due to its known role in influencing RNA splicing (Luco et al., 2010).

Selection of genes that contain differentially spliced exons

The genes analyzed in this study were manually selected, such that all 4 genes chosen were known to contain neurally regulated splicing events. This was verified by referring to the list provided in previous literature (Irimia et al., 2014).

Moreover, the genes were selected so that they can be compared against their histone modifications and their influence on alternative splicing in the brain. Specifically, Bridging Integrator 1 (Bin1, NM_001083334) is a neurally regulated gene with functions including regulating endocytosis, which has isoforms relevant to Alzheimer's disease, as well as being affected by DNA methylation (De Jager et al., 2014). Bin1 is also a tumour suppressor that is inactivated through attenuation or mis-splicing in human cancers. Its inactivation can particularly increase the likelihood of lung cancer (Chang et al., 2007). Myocyte enhancer factor 2A (Mef2a, NM_001291192) is a transcription factor that is typically expressed in the heart and neural cells, which can be affected by certain histone modifications, such as acetylation (Sharma et al., 2014). Clathrin light chain B (Cltb, NM_028870) is predicted to be involved in clathrin-dependent endocytosis and can similarly be affected by histone modification profiles (Forn et al., 2013). Pyruvate kinase, muscle (Pkm, NM_011099), is involved in canonical glycolysis and has been shown to be influenced by H3K36me3 (Luco et al., 2010). Splicing preference for the downstream mutually exclusive exon Pkm2 can promote tumour growth (Lin et al., 2021). The selection criteria were designed to ensure that the genes are alternatively spliced before comparing them across tissues. Furthermore, it could increase the probability that H3K36me3 can directly or indirectly regulate gene splicing, rather than merely correlating with it, although it will still naturally require wet lab experimentation to test any associations.

Epigenome browser visualisation of Tabula Muris and ENCODE data

UCSC Genome Browser was used to visualise the publicly available Tabula Muris single-cell (Fluorescence-Activated Cell Sorting (FACS) performed cells sequenced with SmartSeq2 protocol) RNA-Seq data and ENCODE 3 project's Histone Modification ChIP-Seq data in Mus musculus genome (December 2011 (GRCm38/mm10), GCA_000001635.2) (ENCODE Project Consortium, 2012; Perez et al., 2025; Schaum et al., 2018). Although many other modifications, such as H3K9ac and H3K27me3, have demonstrated effects on alternative splicing, they were not focused on here due to the scope of this study. (Agirre et al., 2021).

The H3K36me3 data from mice of age postnatal 0 (P0), the oldest available (out of E10.5, E11.5, E12.,5 E13.5, E14.5, E15.5,

E16.5, and P0), were used to understand the relationship between histone modifications and alternative splicing during development. Additionally, the signal from embryonic 14 (E14) mice was also quantified to complement the Cortexa data discussed later. We analysed the H3K36me3 signal ±200 bp from the intron-exon boundaries, which we decided upon from past literature (Agirre et al., 2021; Shindo et al., 2013). The tissues that the neuronal cell population is compared against were manually selected by observing if their expression levels were comparable to those of the neuron. Specifically, tissues were used if their flanking exons had an expression signature value of at least 10 % of the neuron's expression, to ensure that any differences in the splicing couldn't simply be attributed to the difference in expression. However, some tissues with RNA-Seq data could not be utilised for analysis due to the ENCODE dataset employed only contains 12 tissues.

Measuring developmental exon inclusion percentages

Cortexa database was used to analyse the changes in exon inclusion percentage in the cerebral cortex in mice with age (Weißbach et al., 2024). We searched the gene names "Bin1", "Pkm", "Cltb", and "Mef2a", which had the splicing events recorded. Although the coordinates of the splicing events in Cortexa didn't exactly match those in the GENCODE VM23 Transcript set used in the UCSC Browser, the corresponding events were found as those with equivalent length of the relevantly spliced exons.

Statistical analysis

To quantify the RNA-Seq and ChIP-Seq data, the exact coordinates of the desired region were found using UCSC's Table Browser, with the following configurations: Clade: Mammal, Genome: Mouse, Assembly: Dec. 2011 (GRCm38/mm10), Group: Expression and Regulation, Track: Histone Modifications, Table: H3K36me3 [Selected Organ] [Selected Age]. From the Table Browser, we obtained the summary statistics that included the mean and the standard deviation. Mean and standard deviation values were used to generate BioRender graphs and performed unpaired two-tailed t-tests with alpha< 0.05, variances of the samples being homogenous, and normal distribution.

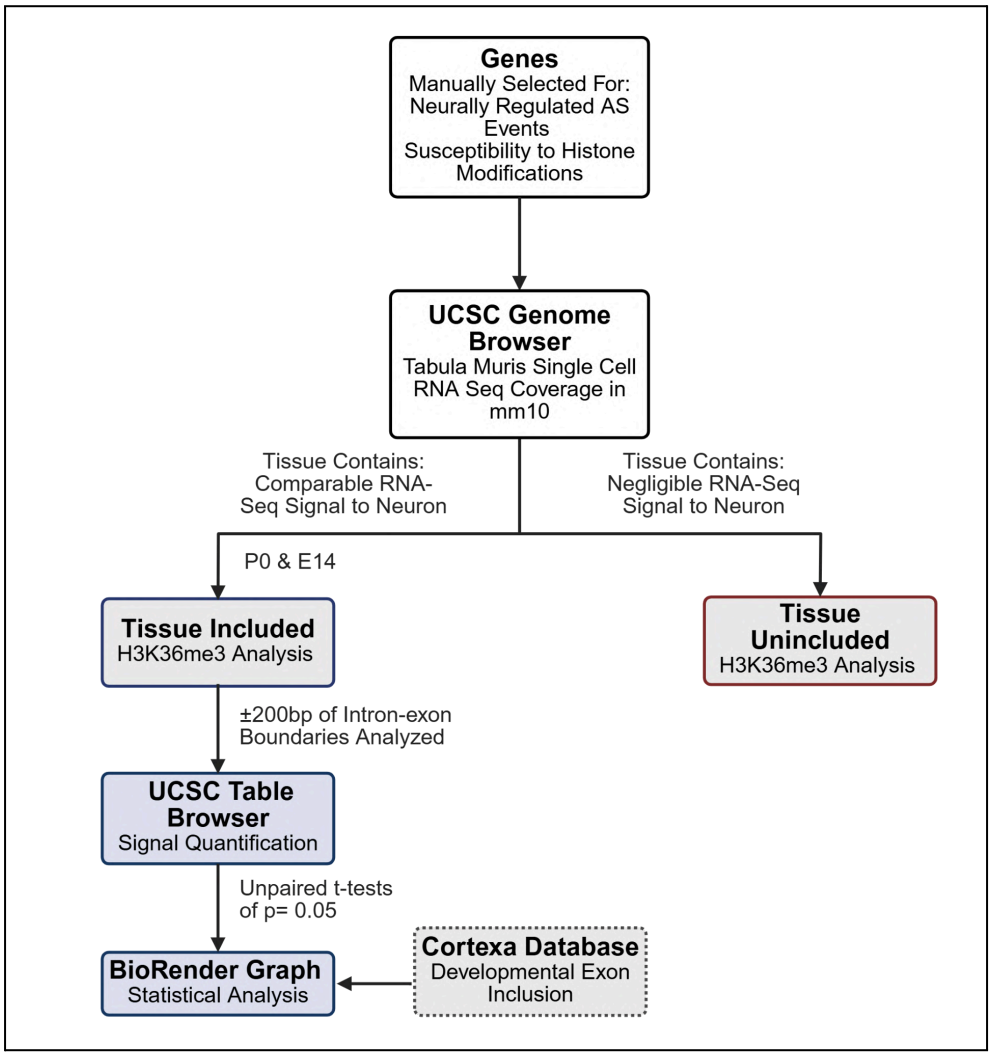

Figure 2: Methodological workflow of this study. Flowchart depicting the gene selection process, data visualisation and analysis methods. The genes were selected based on their susceptibility to alternative splicing in neurons. The tissues were chosen if they had sufficient flanking exon expression signal relative to neurons. The UCSC Table Browser was used to obtain summary statistics, which were analysed and plotted using the BioRender Graph tool. The Cortexa database was used to analyse the changes in developmental exon inclusion percentages with changes in H3K36me3.

2. Results

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Exon 7 of Bin1 is differentially spliced in neurons

Analysis of Exon 7 of Bin1 revealed differential splicing between neurons and lung endothelia. Both flanking exons are expressed to comparable levels in both neurons and the lung endothelia, while neurons exclusively have exon 7 included. Analysing the corresponding methylation pattern has shown H3K36me3 signatures in the lung near the exon, which are outside of the exon region (quantified in the red box, Fig. 3A), while the midbrain does have peaks both near and inside the region. Further quantification of H3K36me3 signal in the region displayed significant differences in H3K36me3 signal between the lung and all 3 areas of the fore, mid and hindbrain for Bin1, where the signal appears greater in all areas of the brain. However, among the genes analysed in this study, Bin1 is the only gene to display significant organ-specific H3K36me3 signals (Fig. 3B). We next asked if Exon 7 is developmentally regulated in the mouse brain. The inclusion level of the exon in the mouse brain increases significantly from E14 to E16 (78.6% to 93.8% respectively) (Fig. 3C); however, no significant differences were found between the H3K36me3 signals of the brain samples aged E14.5 to P0 for all 3 brain regions (Fig. 3D).

There is a limitation to this data, which is that the H3K36me3 data were derived from a bulk assay of many cell types within the tissue, while the RNA-Seq data are cell-specific. Thus, we have included the RNA-Seq data of all the types of brain cells for which the data are accessible in the Tabula Muris tracklist in part E, including the neuron, astrocyte, OPC, microglia, and oligodendrocytes. The splicing patterns are distinct, with neurons having a high degree of inclusion of exon 7, while OPC, astrocytes, and oligodendrocytes have much lower inclusion, and microglia have no observable inclusion. The expression of the gene itself varies widely, with the microglia and oligodendrocytes having much greater RNA-Seq signal than the rest. (Fig. 3E) This is in concordance with past literature on Bin1 (Dourlen et al., 2025).

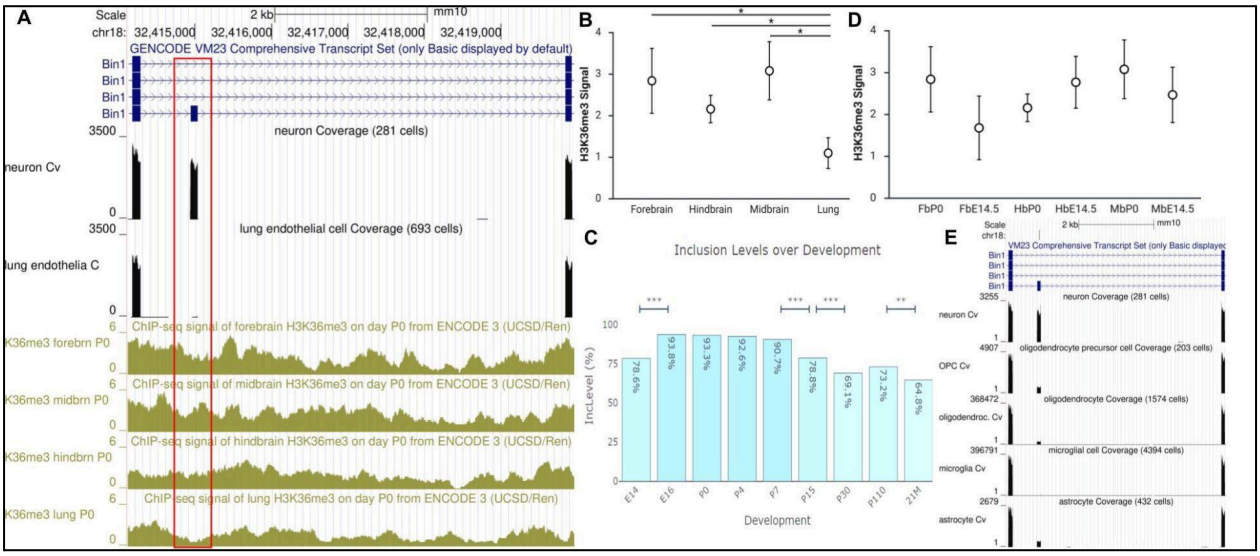

Figure 3: mRNA and H3K36me3 and alternative splicing analyses of Bin1 Exon 7. (A) The RNA-Seq data of neurons and lung endothelia visualised along with the associated tissues' H3K36me3 signals. **(B)** A graph where the circle represents the mean and the tails/error bars represent the standard deviation (SD) of the H3K36me3 signal of the 4 different tissues. P values: 0.0251 (Forebrain - Lung), 0.0123 (Midbrain - Lung), 0.0208 (Hindbrain - Lung). **(C)** The changes in inclusion levels over development, adapted from Cortexa(Weißbach et al., 2024). **(D)** Graph of H3K36me3 signal of the 3 brain regions (Forebrain - Fb, Midbrain - Mb, Hindbrain - Hb) from Age E14.5 to P0, where the circle represents the mean, and the error bars represent the SD (B and D, quantified region: chr18:32,414,746-32,415,240 | NM_001083334). **(E)** RNA-Seq data of the neuron, OPC, oligodendrocyte, microglia, and astrocyte. Their y-axes are not normalised at the microglia despite its 100-fold greater expression to emphasise the neuron-specific splicing pattern. P-values: * < 0.05, ** < 0.01, *** < 0.001. Note: Graphs without indicated statistical significance indicate a lack of significant differences.

Skipping of exon 9 of Mef2a is differentially regulated in neurons

Similar to Bin1, Exon 9 of Mef2a also displays differential splicing between the neuron and the lung endothelia, with the lung exclusively excluding it, while the cardiac muscle is spliced similarly to the neuron. The cardiac muscle is shown for comparison with the neuron. The flanking exons of all the neuron, heart, and lung are comparably expressed. All quantified regions have small peaks within them, with generally similar H3K36me3 signature patterns across the tissues in the visible areas. (Fig. 4A) Quantification of the regions indicated no significant differences between the H3K36me3 signals of any tissues, with all 5 tissues having very similar mean signatures. (Fig. 4B) We then asked whether Exon 9 was developmentally regulated in the mouse brain. Although there appears to be a general trend of increasing exon inclusion,

there were no significant differences between E14 and P0, the ages studied. (Fig. 4C) Similarly, we found no significant differences between the H3K36me3 signatures in all three brain regions. (Fig. 4D)

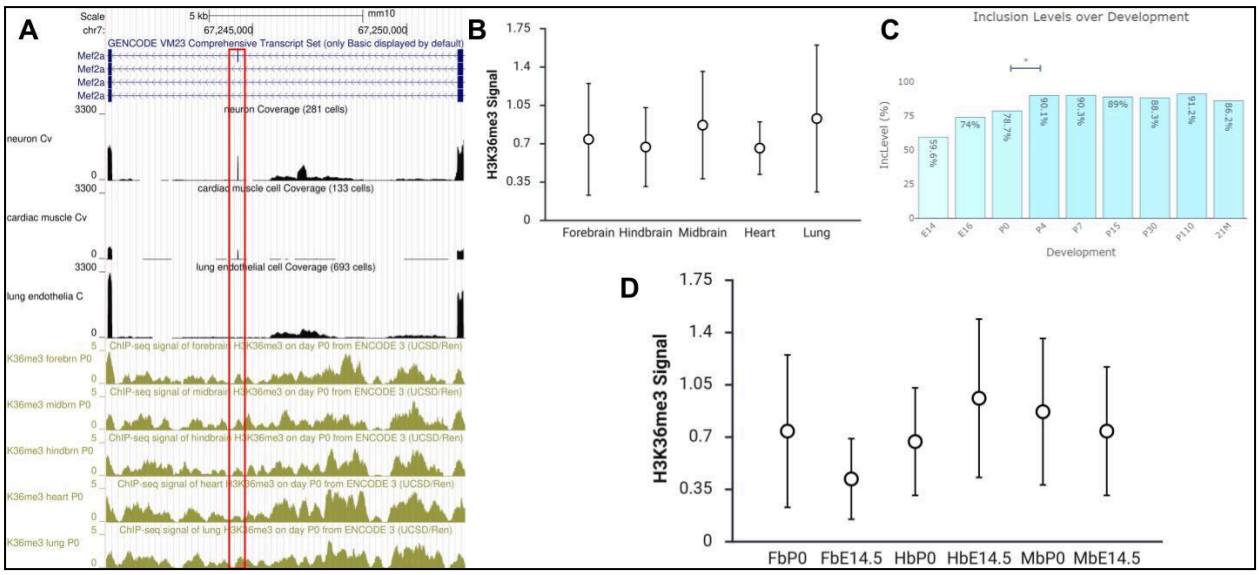

Figure 4: mRNA and H3K36me3 and alternative splicing analyses in Mef2a Exon 9. (A) The RNA-Seq data of neuron, cardiac muscle, and lung endothelia visualised along with the associated tissues' H3K36me3 signals. **(B)** A graph where the circle represents the mean and the tails/error bars represent the standard deviation (SD) of the H3K36me3 signal of the 5 different tissues. **(C)** The changes in inclusion levels over development, adapted from Cortexa(Weißbach et al., 2024). **(D)** Graph of H3K36me3 signal of the 3 brain regions from Age E14.5 to P0, where the circle represents the mean, and the error bars represent the SD (B and D, quantified region: chr7:67,244,319-67,244,743 | NM_001291192). P-values: * < 0.05, ** < 0.01, *** < 0.001. Note: Graphs without indicated significance indicate a lack of significant differences.

Exon 5 of Cltb is differentially spliced in neurons and developmentally regulated

Next, Exon 5 of Cltb also exhibited differential splicing, where neurons exclusively include it. Both flanking exons are comparably expressed in all tissues. Peaks are noticeable in the quantified regions of the hindbrain, liver, and lung. (Fig. 5A) Further quantification evinces the lack of significant differences between the tissues' trimethylation signatures. Forebrain and midbrain have markedly smaller standard deviations, suggesting that they don't contain peaks in the region. (Fig. 5B) When we next asked if Exon 5 was developmentally regulated, we found significant differences in inclusion in the analysed timeframe of E14 to P0, from 17.5% to 38.5% respectively. (Fig. 5C) Subsequent quantification demonstrated a significant difference between the H3K36me3 signature of E14.5 and P0 Forebrain, an increase with age, while the hind

and midbrain showed no significance. (Fig. 5D)

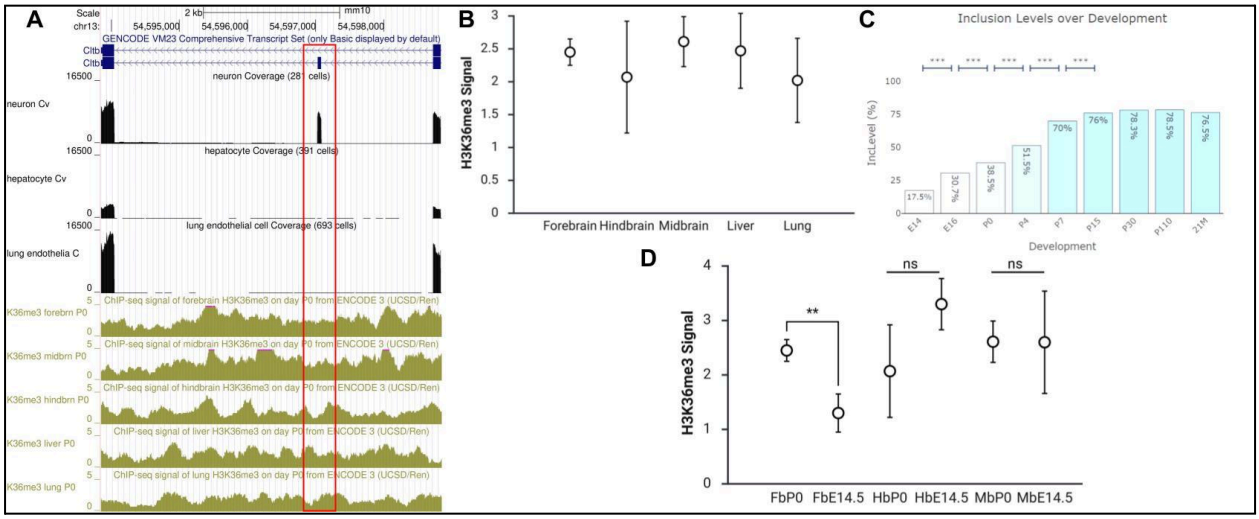

Figure 5: mRNA and H3K36me3 and alternative splicing analyses in Cltb Exon 5. (A) The RNA-Seq data of neuron, hepatocyte, and lung endothelia visualised along with the associated tissues' H3K36me3 signals. **(B)** A graph where the circle represents the mean and the tails/error bars represent the standard deviation (SD) of the H3K36me3 signal of the 5 different tissues. **(C)** The changes in inclusion levels over development, adapted from Cortexa(Weißbach et al., 2024). **(D)** Graph of H3K36me3 signal of the 3 brain regions from Age E14.5 to P0, where the circle represents the mean, and the error bars represent the SD (B and D, quantified region: chr13:54,596,823-54,597,278 | NM_028870). P value: 0.00781 (FbP0 - FbE14.5). P-values: * < 0.05, ** < 0.01, *** < 0.001. Note: Graphs without indicated significance indicate a lack of significant differences.

Mutually exclusive exons 9 and 10 of Pkm are differentially spliced in neurons

Exons 9 and 10 of Pkm (Pkm1 and 2, respectively) are mutually exclusive and exhibit differential splicing, with the neuron and cardiac muscle tending to include Exon 9 preferentially, while the lung endothelia appear to prefer inclusion of Exon 10. The trimethylation tracks display larger peaks in the quantified region of Exon 9 than in Exon 10. (Fig. 6A) In analysing both exons, we found no significant differences in either, although it may appear that lung may have a smaller signal in both than the neuron or cardiac muscle. (Fig. 6B & 6C) Following this, we examined whether these exons were developmentally regulated. The event did see significant changes in preference for inclusion with age from E14 to P0. Specifically, Exon 9 inclusion became more preferred with age (34.5% to 81.6% respectively), while Exon 10 inclusion preference decreased by equal proportions. (Fig. 6D) Additional quantification of the trimethylation signature indicated the absence of significant differences in the region of Exon 9, but also a significant difference and decrease in H3K36me3

from E14 to P0 Midbrain. The other regions did not signify any significant changes in Exon 10, however. (Fig. 6E & 6F)

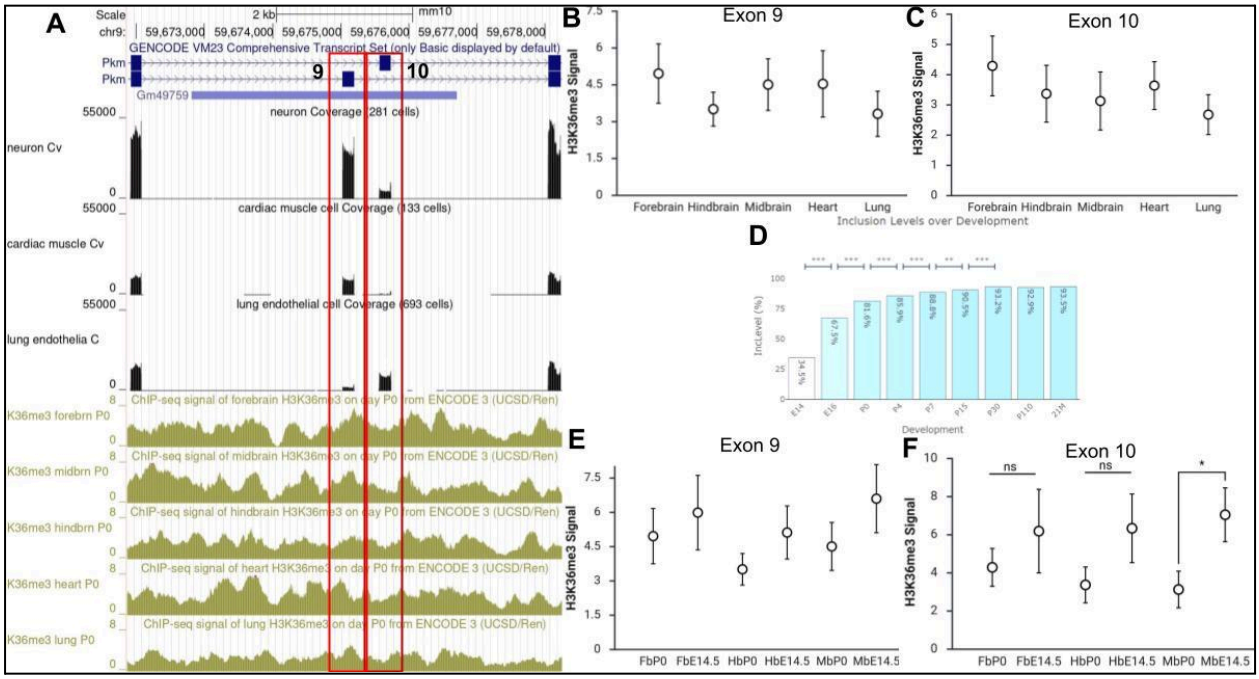

Figure 6: mRNA and H3K36me3 and alternative splicing analyses in Pkm Exons 9 and 10. (A) The RNA-Seq data of neuron, cardiac muscle, and lung endothelia visualised along with the associated tissues' H3K36me3 signals. **(B and C)** Graphs where the circle represents the mean and the tails/error bars represent the standard deviation (SD) of the H3K36me3 signal of the 5 different tissues. **(D)** The changes in inclusion levels over development, adapted from Cortexa (Weißbach et al., 2024). In this instance, the percentages are calculated such that the percentage inclusion of exon 9 (shown above) and the percentage inclusion of exon 10 add up to 100%. **(E and F)** Graph of H3K36me3 signal of the 3 brain regions from Age E14.5 to P0, where the circle represents the mean, and the error bars represent the SD (B, C, E and F, Quantified regions: Exon 9: chr9:59,674,826-59,675,395, Exon 10: chr9:59,675,364-59,675,930 | NM_011099). P value: 0.0164 (MbP0 - MbE14.5). P-values: * < 0.05, ** < 0.01, *** < 0.001. Note: Graphs without indicated significance indicate a lack of significant differences.

3. Discussion

To understand the co-regulation of H3K36me3 and alternative splicing, we investigated four genes. Our findings suggest that differences in splicing across tissues for neurally regulated alternative splicing events may be and in part, regulated by H3K36me3 in Bin1, but less so in other genes. Bin1 being correlated to H3K36me3 and Mef2a, and not Cltb, is consistent with the literature (Forn et al., 2013; Sharma et al., 2014; Shindo et al., 2013). Pkm2 was known to be affected by H3K36me3 in PNT2 cells, yet it does not appear to be a major factor for tissue-specific splicing (Luco et al., 2010). Other

factors, such as nucleosome occupancy, histone variants, and chromatin remodelling complexes, could be more significant factors that affect splicing outcome across organs. However, this study can only indicate correlation; thus, perturbation experiments are recommended to further understand H3K36me3's role in alternative splicing in interesting cases such as Bin1, as well as its potential functions in inter-tissue communication in regards to splicing.

There was no evidence of H3K36me3 being correlated with changes in developmental splicing outcomes in Bin1 and Mef2a, but it did so in Cltb and Pkm. The observation in Cltb is supported, as Cltb is known to be regulated by alternative splicing in a developmental stage-specific manner (Blue et al., 2018). The correlation is also corroborated by previous literature in mouse tissue (Hu et al., 2020). However, the study was based on predictions and data analysis, as was this study; thus, we once again recommend experiments be carried out to examine this relationship. Furthermore, as the Cortexa database used only covers neural alternative splicing events, similar research in other tissues and organs may also be fruitful. However, splicing, being known to affect the chromatin landscape in bilateral communications, could complicate the directionality of the relationship.

A limitation of this research is that, although H3K36me3 is typically known to enhance exon inclusion, there are modifications that are known to silence exons (Shindo et al., 2013; Zhou et al., 2014). These weren't able to be accounted for due to time and technological constraints; however, this could have also been affecting the splicing outcome by being increased or removed between tissues instead of H3K36me3. Moreover, no other modifications were studied in depth, even though others and various combinations of them are known to affect differential splicing (Chen et al., 2019). Additionally, the transcriptional elongation speed of RNAP II could have affected the splicing patterns, but they couldn't be accounted for in this study (Zhou et al., 2014). Lastly, the methylation data are not cell-specific, so perturbation experiments could be difficult to accurately perform.

The research on two cases of correlation between developmental alternative splicing and H3K36me3 may hopefully drive experimental studies. This could then illuminate H3K36me3 as a potential target of aberrant alternative splicing during development. Specifically, it could potentially address errors in embryo growth, as it is known that embryo growth retardation is likely caused by Pkm1 increase and Pkm2 decrease, as Pkm2 is highly expressed in embryos and essential for embryo development (Lin et al., 2021).

4. Conclusions

This study adds evidence for H3K36me3's potential for altering differential splicing across tissues in Bin1, while suggesting that other factors, in part, act as key contributors for future investigations, and uncovered two cases where differences in H3K36me3 were associated with differences in developmental splicing in this investigation of histone modifications' effects on differential splicing. Although there are certain constraints, such as counter-balancing modifications and elongation speed of RNAP II, perturbation experiments could fully uncover a connection between H3K36me3 and development, which could drive new perspectives to future studies.

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Data & Code Availability Statement

[If you used any scripts or datasets in your study, please add a link to a publicly available repository that contains them. For example, this can be a link to a GitHub repository, Google Drive folder, or Zenodo repository. This will help with the transparency and reproducibility of the results in your study, in case somebody wanted to use similar datasets or build upon your techniques. If your study contains no such scripts or datasets, please delete this section entirely.]

Tabula Muris Single Cell Data

https://genome.ucsc.edu/cgi-bin/hgTrackUi?hgsid=2303618686_hFf8vDaGo1RWvlEehhPpGvHhSniv&db=mm10&c=chr2&g=tabulaMuris

ChIP-Seq:

https://www.encodeproject.org/chip-seq/histone-encode3/

Cortexa:
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Mentor Contribution Statement

[You must attach a detailed mentor contribution statement of at most 300 words describing your mentor's explicit role(s) and contribution(s) in the writing of your manuscript. There should be one statement per mentor, and each statement should be written in third-person.]

[redacted] supervised my project. Specifically, he suggested suitable databases, reviewed my data analysis, and proofread my manuscript and figures.

Trust as the Foundation of Democracy:

Political Trust, Voter Turnout, and Democratic Legitimacy in Comparative Analysis

ABSTRACT

Voter turnout is a critical and unstable part of democracy. It reflects the trust and relationship that citizens have with their government. Many factors can influence citizens' desire to vote. Overall, there has been a decline in political participation. This research looks into the reasons behind this, suggesting that the weakening of political and social trust drives this trend. While traditional models often focus on socio-economic resources or institutional rules, this paper argues that trust is the main factor that determines whether citizens feel their vote truly matters. The research fills a gap in existing literature, which generally examines these factors separately instead of comparatively. It also responds to the current global decline in trust and its effects on democratic stability. The essay uses case studies from various countries to illustrate the trend of declining voter turnout worldwide and analyzes the differences in each country's institutions and their effects on turnout. The investigation also considers modern structural challenges, such as globalization, corporate influence, and the breakdown of national identity. Instead of providing alternative reasons for low turnout, the paper finds that these forces make national governments seem outdated or unresponsive, further damaging public trust in institutions. The study concludes that the current turnout crisis is essentially a crisis of faith. It argues that trying to increase participation through mechanical means will not work. Instead, democratic health relies on restoring the belief that political institutions are both legitimate and capable of serving the public good.

1. INTRODUCTION

In recent decades, a dramatic paradox has emerged within the world's democracies: despite the fact that the tools for political participation have proliferated and access to information has become universal, a fundamental act of citizenship, voting, has become less active. From the United States to European states, the trend of voter disengagement is a global phenomenon. For instance, in the 2022 French legislative elections, turnout fell to a record low (France: Voter Turnout in the Legislative Elections, 2024). There are also recent democracies facing similar issues: the 11.2% turnout in Tunisia's 2022 election demonstrated a deep-seated political disengagement (Tunisia Assembly of People's Representatives December 2022 Election, 2022). Rather than representing a short-lived issue, the decline that signifies a creeping crisis of democratic legitimacy is part of a growing crisis of democracy worldwide.

In this paper, I will argue that although factors such as civil society, economic conditions, and globalisation are substantial, the primary overarching phenomenon that determines voter turnouts in democracies–and, accordingly, contributes to the recent downturns–is political trust. This research is important, given the current global crisis of trust and its potential consequences for democratic stability; it fills a void in the current research that examines these factors in isolation rather than comparatively,

under a broader category such as political trust. To ground this research, the paper will focus on a broad comparative analysis of cases from Europe, Oceania, and Asia. Austria serves as an example of declining trust and turnout to demonstrate a great trend in Europe. Australia offers a contrasting model of stable engagement through an institutional framework that promotes voter participation. In comparison, Asian democracies, including Japan, South Korea, India, and Singapore, will show the varying relationships between trust and voting across different political cultures. Ultimately, the conclusion posits that trust is an essential precondition, as without it, other factors fail to sustain consistent electoral participation. Therefore, restoring trust is important to the vitality of democratic societies.

The erosion of voter turnout poses a significant statistical issue and a serious threat to the democratic health and equity of our society. Low and declining voter turnout leads toward a concentration of political power in mobilised and usually more affluent sections of the populace, which skews the outcomes of policy decisions and, therefore, weakens the principle of representation. This loss of legitimacy for governments elected through political processes also increases the likelihood of creating instability in the political system by widening the divide between a government and its electorate. Therefore, understanding the root causes of the erosion of voter turnout is critical to identifying the crisis currently faced by democratic systems.

This investigation centres on political and social trust as a multidimensional concept. Political trust refers to citizens' confidence in the impartiality, efficiency, and integrity of core institutions such as the government, parliament, the electoral commission, and the legal system (Vitale, 2018). Social trust is characterised as interpersonal trust that includes perceptions of honesty, the capacity to work together, and the existence of common social norms among other citizens (Kirby, 2025). The critical hypothesis is that these forms of trust will reduce perceived 'costs' of voting. If you believe your vote will be counted fairly and that you are part of a collective citizenry, you will be more likely to vote. Without this underlying basis, people will view their votes as irrelevant regardless of the particular circumstances.

The case studies selected for this paper are appropriate cases as they span a range of institutional settings and political cultures, which makes them well-suited for isolating the effect of trust from other potential confounding variables. Austria and Australia are both developed, stable democracies with established civil societies, yet they diverge sharply in turnout stability, which is due to differences in institutional design and the nature of the trust created through these institutions. The Asian cases add to the argument beyond the Western liberal democratic tradition, testing the universality of the trust-turnout hypothesis in contexts where participatory norms and state-society relationships are different. Crucially, while the analysis is focused on these specific cases, the evidence from the example cases refers to a global trend: when people lose faith in the institutions that govern them, the will to participate in the democratic process diminishes.

The essay is structured in four sections. First, it reviews the established literature on voter turnout and develops a theoretical rationale for political trust as the primary mediating variable influencing turnout rates. Second, it uses comparative empirical analysis through evidence from Austria, Australia, and Asia to test the relative explanatory strength of trust. Third, it examines additional structural forces, globalisation, corporate power, and the erosion of national boundaries and shows that they contribute to the decline of trust in politics. Finally, the conclusion synthesises the findings, arguing that while economic deprivation or weak civic networks can depress turnout, their impact is conditional on a bedrock of trust, the erosion of which presents the most fundamental challenge to electoral democracy globally.

2. LITERATURE REVIEW AND THEORETICAL FRAMEWORK

This literature review and theoretical framework will involve two related areas of scholarship: the extensive literature on the determinants of voter turnout and the evolving scholarship on political trust and social capital. The prior research has traditionally prioritised socio-economic models and institutional models of voting, while the latter treats trust as a background variable. This paper presents an argument for the importance of elector-focused political trust as the most significant proximate factor mediating the effects of more general, structural variables—civil society, economic conditions, and globalisation—on turnout in contemporary democracies. The existing studies on voter participation have generally followed two paths. The first path follows the rational choice and socioeconomic models, which views voting as a cost-benefit calculation that is influenced by the availability of resources (Blais et al., 2019). Sidney Verba, Kay Lehman Schlozman, and Henry Brady's 'Civic Voluntarism Model' remains foundational, arguing that turnout is a function of resources, psychological engagement, and recruitment networks (Verba et al., 1995). This explains correlations between higher turnout and factors like education and income. The second path, which is represented by the work of scholars including Arend Lijphart and Mark Franklin, offers an institutionalist perspective of the voter turnout with specific reference to the 'rules of the game', identifying how facilitative laws (Franklin, 2004), including proportional representation, automatic voter registration, and weekend voting, result in lower costs of voting and greater participation in elections (Lijphart, 1997). However, a growing body of work identifies a puzzle: even in democracies with facilitative institutions and rising education levels, turnout is often stagnant or declining. This leads to the conclusion that an independent factor is contributing to low turnout rates, which cannot be accounted for by resources or through the application of regulations. Scholars like Pippa Norris point to a shift from 'mobilising' citizens to 'critical' citizens, who have become distrustful of the system, resulting in their energy being diverted towards alternative forms of civic engagement (Noris, 2011).

This paper contends that while socio-economic models explain capacity and institutional models explain opportunity, they do not adequately explain an individual's will to participate in the electoral process. That will is fundamentally shaped by political

trust. It serves as the vehicle through which broader structural conditions impact an individual's decision to vote.

Political trust is a complex, multi-dimensional concept. Different scholars use different approaches to create a generalised understanding of this phenomenon. David Easton's systems theory creates a distinction between diffuse support, a generalised connection to the democratic regime as a whole, and specific support for leaders or policies (Easton, 2010). Marc Hetherington makes an important distinction between confidence in institutions and confidence in current officeholders, claiming that a lack of relative confidence in institutions is a more corrosive factor to civic engagement than a lack of confidence in politicians (Hetherington, 1998). Robert Putnam's work on social capital links interpersonal trust to political trust, but the causal relationship is still a point of contention regarding whether civic engagement helps to develop trust, or if pre-existing trust is necessary for civic engagement (Boix & Posner, 1996). Furthermore, Ronald Inglehart provides a cultural perspective that associates high levels of trust with post-materialist and safe societies (Inglehart, 1988). Yet critical theorists such as Claus Offe question whether trust can be extended beyond the relationship between individuals to abstract forms of political authority where citizens have limited means to monitor the actions of those authorities (Offe, 1999). These nuances reveal that 'low political trust' can mean many things: tension with a ruling party, cynicism about any politicians, or a widespread view that the political system has been rigged or is not responsive. The most damaging variation to turnout is mistrust of the system itself. Therefore this paper focuses on developing a focused conceptualization of political trust as it relates specifically to the electoral process and its expected fairness. The concept builds upon the perspective articulated by Adrian Leftwich — that politics is the main mechanism by which society arrives at collective decisions regarding the distribution of resources and values (Leftwich, 1993). When citizens lose confidence in this primary function, the incentive to participate in elections diminishes.

Here, political trust is defined as the citizen's eroding or sustained belief in the fairness, integrity, and functional efficacy of the electoral process and the key institutions that animate it. It encompasses two critical dimensions. First, belief in procedural fairness: the conviction that elections are free from manipulation and that all major interests have a roughly equal opportunity to compete for power. Second, belief in output responsiveness: the expectation that the government emerging from this process will function effectively and be responsive to public will. Erosion of this trust breeds a specific sense of electoral hopelessness; this hopelessness is the direct antagonist of voter turnout.

Electoral hopelessness does not automatically mean political apathy in all domains. Rather, Norris' 'critical citizens' thesis states that residents may withdraw entirely from participation as a classic abstainer or they will divert their energy and resources into alternative venues such as social movements or protests which they perceive as more genuine than the compromised electoral venue (Norris, 2011). This

represents a flight from participation rather than a flight from politics in general. Using this framework to understand the factors associated with diminished voter turnout could be useful in determining whether or not weak civil societies, economic distress, or globalization directly impact voter turnout, because the relationships among these variables do not have a direct influence on voter turnout. However, in using political trust as a filter, the economy has a detrimental impact on trust through "output" responsiveness, while the perception of elite capture has a corrosive effect on trust with regard to "procedural" fairness. Therefore, the state of electoral hopelessness is the most deterrent to voting. Consequently, in order to understand the crisis in voter participation, it is necessary to identify the crisis in trust in the electoral system itself.

3. ON WANING TRUST AND ERODING VOTES

Trust in political institutions is the foundational public belief in the integrity, competence, and legitimacy of democratic institutions. Once that trust declines, citizens will disengage because they consider democracy to be unresponsive to their needs and too far removed from them. The clearest way to measure this disengagement from the democratic system is through declining electoral participation. The instability in turnout is an indication of an individual's belief that their vote does not matter and is a reflection of electoral hopelessness. This section explores the link between political trust and voter turnout through a comparative lens. This will involve examining Austria as a European case study of unstable levels of political trust and electoral turnout, and then contrasting it with Australia's model of stability under compulsory voting, then finally analysing the different political trust and voter turnout trends across Asia, namely Singapore, India, Japan and South Korea. Ultimately, this section will conclude that effective and stronger institutional design can help sustain political trust in an age of democratic uncertainty.

3.1 Austria

Austria presents an interesting European model because it is not an extreme example of democratic failure, but rather a bellwether of broader continental trends. It is a stable, prosperous, democratic country; however, constant changes in the country give insight into some of the vulnerabilities within a stable democracy. Located in Central Europe, Austria faces both the integrationist pull of the EU and the disintegrationist impulses related to populism, immigration discussions, and complex coalition politics (Sus, 2016).

Voter turnout in Austrian national elections is declining and is very volatile. Austrian national election turnouts have been on a volatile, declining trajectory. Turnout was approximately 90% for much of the second half of the 20th century, but for the first time ever, has dropped below 80% at 77.5% in the 2008 elections, then rebounded to 84.3% in the 2017 elections likely due to an extremely polarising campaign, but fell again at the snap elections to only 75.6% in 2019 (Gaebler et al., 2020). The pattern of spikes in

turnout seems to occur when there is an apparent major crisis or when there is high-stakes polarisation. But it is difficult for spikes to sustain with an instrumentally motivated electorate who only votes when the stakes are sufficiently acute so as to break through their general disillusionment, but is unlikely due to deep-seated habitual voting or because of deep trust in their institutions.

The Austrian government's 2007 decision to lower the voting age to 16 has been referred to as an ambitious effort at advancing civic participation among young residents. The idea behind this was to create a stronger habit of voting and encourage civic engagement by allowing young people to vote as they develop their civic identities (Bronner & Ifkovits, 2019). However, the results have been mixed. While young people have been highly engaged in specific issues like climate, their overall turnout rates remain the lowest of any age group, for example in the United States, only 40% of 18 year olds registered as voters, compared to 80% of those aged 45 and above. This suggests that providing access does not automatically build confidence in participating in elections and that the underlying factors behind low turnout must be addressed, which in turn can be a symptom of low turnout. Thus, this intervention is addressing a symptom without addressing some of the larger issues underlying the cause

Austria's story is not unique. Rather, it plays out against the backdrop of a wider European tale of decline. Surveys, including the Eurobarometer and the European Social Survey, consistently show falling confidence in parliaments, political parties, and governments across the continent (European Commission, 2026). The EU technocracy's growing distance from citizens, the 2008 financial crisis and austerity measures, the 2015 migration crisis, and longstanding fears of inequality and globalisation have caused this decline of support (Scicluna, 2019). The turnout crisis is directly caused by the erosion of trust. When citizens see institutions as unresponsive or beholden to elites, the cost of voting, in time, effort and emotional burden, begins to exceed the benefits. In this way, abstention becomes a rational choice, a semantic protest, or apathy. The European pattern communicates clearly that an emptying trust reservoir is connected with the electoral participation rate. This creates a vicious cycle where turnout further delegitimises governing mandates and undermines public trust in the system's legitimacy and effectiveness.

3.2 Australia

Australia offers a clear and insightful contrast. Voter turnout in federal elections is consistently high, remaining around 90 to 95% for decades (*Participation in the 2025 Federal Election*, 2025). This impressive turnout reflects a strong trust in the democratic system. Australians generally believe in the system's fairness and legitimacy, which is known as procedural trust.

The foundation of this stability is compulsory voting, introduced in 1924. Its success however is not due to a modest fine (approximately $20 AUD). For many

citizens, the fine is financially insignificant. Instead, the law has a sociological and normative effect (Fowler, 2013). First, it encourages a lifelong habit of voting, making it a regular civic duty like paying taxes. Second, over generations, the norm of participating has become deeply embedded, so not voting is seen as unusual and neglectful of civic duty. Third, and most importantly, it changes political incentives. With the entire electorate voting, political parties must develop broad, centrist policies that appeal to all groups, not just their base. This strengthens the public's view of a responsive and inclusive system, which is the second pillar of political trust.

Consider a thought experiment: if Austria adopted a similar system with a meaningful fine, the result would likely be an immediate, mechanical increase in turnout. However, whether it would restore trust is a more complex question. It would force Austrian parties to appeal to a broader electorate, potentially mitigating polarisation. Yet, if citizens perceived compulsory voting as an authoritarian imposition on a system they already distrusted, it could breed resentment rather than renewed civic commitment. Australia's lesson is thus nuanced: compulsory voting appears most effective as a prophylactic measure. It sustains civic habit and social norms, preventing the trust-turnout spiral from starting in the first place, rather than serving as a simple cure for an already advanced condition of disengagement. The intervention works with trust, not despite its absence.

Australia's experience shows that compulsory voting works best as a preventive measure. It helps maintain civic habits and social norms, stopping the decline of trust and turnout before it starts rather than acting as a simple remedy for a serious problem of disengagement. This approach interacts with trust rather than ignoring its absence. Over generations, the norm of 'showing up' has become deeply rooted; not voting feels unusual and irresponsible. Most importantly, it changes political incentives. Knowing that everyone will vote, political parties must create broad, centrist policies and engage all groups, not just their core supporters. This enhances the public's view of a responsive and inclusive system, which is crucial for building political trust.

3.3 Asia

In Asia, the situation is more complex, with the diverse cultures and political structures across countries, this complicates the link between trust and turnout. It still emphasizes the importance of trust as a key factor, even when it looks different from the Western democratic framework.

In developed democracies like Japan and South Korea, there is a pattern of decreasing trust and lower voter turnout, especially among young people. This is driven by economic stagnation, political scandals, and a pervasive sense of unresponsive establishment politics. In Japan, turnout for lower house elections has fallen from over 70% in the 1990s to below 55% recently (Japan: Voting Rate at the Lower House Elections 1946-2024, 2024). South Korea shows similar trends. The mechanism

resembles the Austrian case: as trust in fairness and responsiveness declines, voting starts to feel pointless.

India presents an intriguing paradox. Voter turnout is usually high and often rising, fueled by strong partisan mobilization, identity politics, and a lively democratic culture. Yet trust in specific institutions like parliament, the civil service, and the judiciary can fluctuate and is often low (Ajay Gudavarthy, 2024). Here, high turnout reflects political engagement driven by social divisions and polarization, rather than broad support for the system. It's crucial to note that turnout can be high while democratic quality declines if participation is based on fear, grievance, or group identity instead of confidence in fair systems. India shows the limitations of using turnout numbers as a clear indicator of democratic health, as although there could be participation in the democratic system, it could have no relationship to political trust.

On the other hand, Singapore stands apart. It features consistently high voter turnout, with an average of 83.10% voter turnout and 92.47% turnout in the 2025 election, being within a system characterised by dominant-party rule and restricted civil liberties (IFES Election Guide Singapore, 2022). Trust here is based not on liberal democratic institutions but on an agreement about performance. Citizens have high confidence in the government's ability to deliver economic growth and social order. Turnout is high due to social pressure, the symbolic value of voting, and genuine support for governance outcomes. This model shows that high turnout can occur alongside a type of political trust focused on performance rather than on participation. Even in this unique case, trust is still the key factor; its nature simply differs from the procedural trust seen in liberal democracies.

3.4 Summary

Examining Austria's changing turnout, Australia's steady results, and Asia's diverse trends, demonstrates that political trust and voter turnout have a mutually reinforcing relationship. However, this relationship is shaped by political culture and institutional design. Austria illustrates that when trust diminishes, turnout becomes unpredictable which is a reflection of temporary crises rather than a solid foundation of democracy. Europe's broader challenges serve as a warning about this gradual decline. In contrast, Australia provides a powerful, practical lesson; simply the requirement to vote can cultivate a strong civic habit that protects the political system from the serious erosion of trust, making participation the norm rather than a calculated choice. Finally, the Asian experience reminds us that there is no universal equation: high turnout can indicate active democratic engagement, strong partisan mobilization, or a public agreement for competent governance. In all instances, some level of trust is essential. Democracies worried about declining trust should not just try to boost turnout with penalties; the deeper goal should be to create a responsive, legitimate, and effective political environment that encourages citizens to participate.

4. BEYOND POLITICAL TRUST

The previous section showed how political trust connects citizens to the electoral process. When trust in government declines, the motivation to vote often drops as well. This is evident from the evidence in Austria, Europe, and beyond. However, looking solely at political trust does not tell the whole story. A direct loss of faith in political institutions is clearly significant, but broader structural changes in society might also play a role. These changes can lead citizens to feel less dependent on their national government, making them less willing to participate in national elections, even if they do not have overly negative views of politicians or institutions. The question is whether these structural forces provide separate reasons for declining voter turnout or if they function through the same trust mechanism already identified.

Three interconnected phenomena are especially important in this context: globalization, the weakening of national boundaries, and the increasing reliance on markets and corporations to provide goods and services that were once the responsibility of the state. These forces do not replace political trust as the main explanatory factor. Instead, they work alongside it as additional contributors that guide citizens toward the same outcome, lowering turnout through the feelings of detachment and disincentive discussed earlier. This section will explore how these additional phenomena collectively reduce political trust and lessen the perceived importance of national elections, which contributes to lower electoral participation.

4.1 Globalisation and the Fragmentation of National Identity

The first factor is globalization, which has changed how people view their identity and their connection to their nation. In the past, the average person in a democratic country was more likely to identify with one national identity. They were born, educated, employed, and expected to live within the borders of a single country. This sense of belonging to a national community created a duty; voting was not just a right but an obligation to fellow citizens and the nation's future. Globalization has made this picture much more complex (Mahoney, 2022).

Increased mobility means that more people now hold dual or multiple citizenships. They may be born in one country, educated in another, and work in a third, with family members spread across various continents. For these individuals, figuring out where they truly belong can be complicated. This mix of identities can weaken the sense of responsibility toward any single national political system. If one's future is not tied exclusively to a specific country, the outcome of that country's election may seem less personally important. Besides the issue of multiple citizenships, globalization has also encouraged the growth of transnational identities. (Marko Mrakovčić et al., 2020) An environmental activist may feel a stronger connection with activists in other countries than with neighbors who do not share their concerns. A tech professional might feel more aligned with a global industry network than with political discussions in

their home country. These transnational identities are real and significant, but they do not easily lead to electoral participation.

Immigration and demographic change have also played a role. In many developed democracies, the population is now more diverse than ever before. This diversity enriches society in many ways, but it can complicate the relationship between citizenship and political participation. First-generation immigrants may face barriers to voting, even after gaining citizenship, such as not being familiar with the issues, candidates, or voting process, or coming from countries where elections were not meaningful or where political participation posed real dangers (Pratsinakis, 2017). More fundamentally, they may not yet feel a full sense of belonging to their new country. The feeling of being an outsider, even a permanent one, does not easily translate into the sense of civic duty that drives people to vote. The processes of globalization have created a world where a singular, unquestioned national identity is no longer the common experience it once was, weakening the sense of obligation to participate in the political life of any one nation.

4.2 Corporate Power and the Capture of Democratic Institutions

The second factor is the growing concentration of economic power among large corporations and the influence these companies have on politics. Increased market concentration accounts for about one-quarter of the recent decline in democracy worldwide (OECD, 2024). Large, profitable firms can turn their economic strength into political sway through campaign contributions, lobbying, and other forms of political involvement that ordinary citizens cannot access.

This situation creates a widespread belief that the political system favors wealthy interests, undermining the fairness of political trust identified in the theoretical framework. In the United States, for instance, a small group of wealthy donors makes up a significant portion of total election spending (Albert & Raja, 2020). Polls consistently show that many Americans feel donors have too much sway over Congress, while regular citizens have too little (Beshay, 2023). This view is not limited to the United States; citizens in many developed democracies are increasingly skeptical of the relationship between business and government. When people believe that the economy's rules benefit corporate interests over the public, faith in the political system's fairness naturally declines. Citizens who think that elections are mostly decided by the highest bidder have little motivation to take part. Their distrust often targets the system itself rather than individual politicians, as they see it as fundamentally compromised.

Besides directly influencing policy, corporations are also stepping into roles typically held by the government. They are taking positions on social issues, enhancing healthcare benefits, and providing social support to employees. While these actions may be well-intentioned, they can lower expectations for government responsibilities. This

distances citizens from political life and strengthens the belief that voting is not the best way to create meaningful change. This represents a decline in output responsiveness: if the private sector provides social benefits instead of elected officials, people's motivation to vote to influence public services goes down.

4.3 The Erosion of National Boundaries

The third factor involves the decreasing importance of national borders as globalization deepens and corporate power rises (Towfigh & Weyl, 2024). For much of the twentieth century, the nation-state was the main unit of political, economic, and cultural life (Held, 1989). Elections mattered because governments controlled power within clearly defined areas. Today, the sense of a bounded community has weakened. Capital flows freely across borders. Multinational media companies increasingly shape culture instead of national institutions. Even the experience of place has changed, as cities and regions connect more to global networks than to their own surrounding areas.

When national identity weakens, and people no longer see the nation-state as the natural framework for economic and social life, participating in a national election can seem less meaningful (Towfigh & Weyl, 2024). Citizens may feel a stronger connection to their region, profession, or transnational networks than to their fellow citizens. They might view global corporations as the main agents of change, not governments. In this situation, electoral politics can seem outdated, a remnant from a time when the nation-state truly mattered.

4.4 Summary: Ancillary Factors and the Trust Mechanism

In summary, the decline in voter turnout is not just about a loss of faith in government. It also stems from structural changes that have made the nation-state seem less relevant. Globalization has limited the policy choices available to national governments, forcing them to respond to external market pressures rather than the will of the people. The concentration of corporate power has created a perception that the political system serves wealthy interests. Corporations have taken on roles traditionally held by the state. Combined with the erosion of national boundaries, these factors have made national elections feel less significant to many citizens.

These elements do not function independently of political trust. Instead, they alter the environment in which political trust develops. Citizens do not simply think the government is performing poorly; they increasingly see it as unable to succeed because of global capital constraints, corporate agendas, or the redundancy of its traditional roles. This form of distrust comes from a belief that the government is ineffective and outdated—a collapse in output responsiveness shaped by structural conditions. In this way, globalization and corporate power do not compete with political trust; they are key

drivers of its decline. Their overall impact on voter turnout goes through the same trust mechanism that this paper emphasizes.

5. CONCLUSION

This paper aims to address a seemingly simple question: among the numerous factors that scholars propose to explain declining voter turnout in modern democracies, which is the most significant? A comparative analysis of Austria, Australia, and various Asian democracies, along with a theoretical exploration of globalization, corporate power, and the decline of national identity, points to a clear answer: political trust. Trust in institutions is not just one factor among many. It is the foundational element through which all other factors work—the lens that shapes whether economic conditions, civic infrastructure, or structural changes result in participation or abstention.

The findings can be summarized as follows. The literature review and theoretical framework show that existing voter turnout models—socio-economic and institutional—explain capacity and opportunity but fail to capture the will to participate. This willingness, the paper argues, is mainly influenced by political trust, defined as citizens' belief in the fairness of the electoral process and the responsiveness of the resulting governments. The comparative case studies then tested this framework empirically. Austria showed that a loss of institutional trust leads to erratic turnout instead of stable civic participation. It also demonstrated that measures like lowering the voting age fail if the underlying trust issues remain unaddressed. Australia showed the opposite: that institutional design can create conditions for trust—by normalizing participation across the electorate and encouraging parties to be responsive—thus preventing the trust-turnout spiral from starting. The Asian cases provided further insights, indicating that trust manifests differently across political cultures, from Singapore's focus on performance to India's turnout driven by mobilization. Still, every case highlighted trust as a crucial variable. Finally, Section 4 illustrates that the structural forces of globalization, corporate power, and diminishing national boundaries are not competing explanations for the turnout crisis. Instead, they are significant contributors to the erosion of institutional trust, confirming that trust is the central factor through which structural conditions influence individual political behavior.

This paper has two notable limitations, which deserve acknowledgement, along with reasons why its main findings remain compelling. The first limitation is the lack of detailed longitudinal quantitative data. A more thorough study would systematically use panel datasets like the World Values Survey, Comparative Study of Electoral Systems, and European Social Survey to connect trust scores with turnout rates across a broad, representative sample of democracies over time. This paper mainly relies on qualitative and structural comparisons. However, this method suits an exploratory, theory-building endeavor rather than a hypothesis-testing approach. The consistency of the trust variable in diverse contexts like Austria, Australia, Singapore, and India strongly

indicates it captures a real causal mechanism rather than just a contextual artifact—robustness that purely quantitative studies, which may struggle to reflect the qualitative nature of trust across different political systems, cannot easily match. The second limitation concerns the possibility of reverse causation. The assertion that declining trust leads to lower turnout faces a valid counter-argument: declining turnout may also damage trust by creating governments with weak mandates and questionable legitimacy, which in turn depresses future participation. This circularity exists, yet it does not weaken the argument; instead, it enhances it. The negative cycle shown in the Austrian case supports trust's importance. Conversely, the Australian case serves as a near-natural experiment in the opposite direction—compulsory voting interrupts the downward trend by ensuring widespread participation regardless of temporary fluctuations in trust. This stabilizes the conditions under which trust is built. This imbalance supports the view that trust is the initial factor, even if the relationship is ultimately cyclical.

Several avenues for future research arise from these findings. First, scholars should look into the specific ways that certain institutional actions interact with trust to either maintain or restore participation. Citizens' assemblies, deliberative mini-publics, ranked-choice voting, and participatory budgeting are all innovations that aim to make democratic processes seem more legitimate and responsive. Their long-term effects on trust, and how that trust affects turnout, need thorough investigation. Second, the growing research on digital media and political communication includes a less-explored area. Social media platforms are now significant players in shaping political trust. They can both engage new participants and deepen cynicism through the algorithmic spread of distrust and outrage. It is crucial to understand how information environments influence the trust-turnout link in today's digital world. Third, the connection between corporate influence and democratic participation needs more focused theoretical and empirical study. If rising market concentration accounts for a quarter of the global decline in democracy, as the evidence suggests, then managing private economic power also presents a challenge for democratic participation—something the voter turnout research has yet to fully address.

Democracy has never been a self-sustaining system. It relies on the belief that ordinary people will return to vote, giving their trust to imperfect institutions. When that belief falters—when citizens view their vote as merely a pointless gesture in a rigged system—democracy doesn’t fail dramatically. It gradually erodes, mandate by mandate, election by election. The turnout crisis, in this context, isn’t just about civic engagement; it’s a crisis of faith. Once lost, restoring faith is incredibly challenging.

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Author 100194

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Reviewer Final Recommendation: Accept with minor revisions

Reviewer Feedback: Overall, the paper is well written with a clear goal of assessing various tissues for genes associated with histone modification and alternative splicing events to inform upon mechanistic insights potentially involved in a variety of pathologies (i.e., cancer, cardiomyopathy, autoimmune disorders). These mechanistic insights can elucidate potential avenues for therapeutic intervention. Results from this study begin to address gaps in the literature pertaining to the role of histone modification genes and alternative splicing in neural and non-neural tissues. The author provides a great overview of the literature to set the stage for the experimental design that investigated H3K36me3 histone modification and Bin1, Mef2a, Cltb, and Pkm alternative splicing events. The author does a fantastic job discussing the literature and relating the literature back to the study findings clearly and concisely with ample detail to clearly define the gaps that the study addresses. Minor revisions are included below to bolster the manuscript for acceptance:

- In the abstract, line 12 should read "They also influence splicing **outcomes…"** instead of "…outcome…". If you are referring to one splicing outcome, then use the singular versus plural form.
- In the abstract, line 21 should read "…we analyzed the differences in H3K36me3 **signals…"** instead of "…signal…".
- In the abstract, line 33 should read "… splicing **outcomes…"** instead of "…outcome…". If you are referring to one splicing outcome, then use the singular versus plural form.
- For citations throughout the paper, each sentence that is not your own should be cited so please read through the manuscript and add additional citations as needed. Typically, this pertains to the Introduction and Discussion sections.
- In the Introduction, line 78, please spell out 4 as four as anything between 0-9 should be spelled out and not numerated.
- Figure 1 and Figure 2 are generated well and convey the main takeaways beautifully. The figure legends are well written as well. Great work!
- In the Methodology, line 95 please spell out 4 as noted above in previous feedback.

- Throughout the manuscript please double check tense for the various sections. Anything describing the study typically should be in past tense (Methods, Results) as well as when you are discussing previous literature findings.
- Please spell out all contractions (i.e., can't, don't) throughout the manuscript.
- Please ensure that the text formatting is aligned with the journal's guidelines (i.e., font size, style, spacing) when doing a final read through of the manuscript.
- All figures in the Results are clear, concise and report the main findings well.
- In the Discussion, line 250 should read "…in **regard** to…" instead of "regards".
- In the Discussion, lines 254-255 I would recommend restructuring this sentence and making it a bit clearer (the sentence beginning with "However, the study was..").
- Your Discussion section focuses mostly on limitations although there are strong findings to highlight from your study. I would recommend bolstering the interpretation and importance of your results and what they add to the current literature. Strengthening your discussion of your main findings and linking the importance will help keep the reader interested. You do a great job with this in the final paragraph of the Discussion.
- In your Conclusion section, I would recommend strengthening how your results impact the current literature and highlight how they provide mechanistic insight that can be used for future development of therapeutics for the pathologies you outline in your introduction (and discussion).

Investigation of histone modification and alternative splicing association in neural and non-neural tissues

Comments:

This study investigates the correlation between the H3K36me3 histone modification and alternative splicing across neural and non-neural tissues, with a specific focus on its roles in tissue-specific and developmental regulation. This is a hypothesis-driven computational study investigating the association between H3K36me3 and alternative splicing across neural and non-neural tissues. The topic itself presents an interesting approach toward understanding epigenetic regulatory mechanisms; however, the overall study lacks sufficient academic rigor and logical coherence. In particular, the conclusions are drawn primarily from qualitative visual observations and statistical summaries, and the rationale for selecting the target genes and histone mark is not adequately justified in the Introduction section. In its current form, the manuscript reads more like a descriptive compilation of public datasets than a fully developed scientific study. Substantial structural revision and deeper analytical interpretation are required for the work to meet the standards of a professional research paper.

Major Issues

1. Insufficient Rationale for Gene and Histone Mark Selection

Although the manuscript attempts to explain why the four genes (Bin1, Mef2a, Cltb, and Pkm) were specifically selected for analysis and why H3K36me3 was prioritized over other histone modifications, the author needs to elaborate further. The biological and conceptual rationale for these choices is inadequately developed in the Introduction. The manuscript might want to establish the biological relevance of these genes in the context of autism, neurodevelopment, disease pathology, or developmental regulation. Importantly, the current explanation for gene selection appears in the Methods section, although it functions more appropriately as conceptual background information or as part of the rationale motivating the study. This content should therefore be relocated to the Introduction section to strengthen the logical foundation of the work.

2. Descriptive Results Without Integrative Interpretation

The Results section primarily consists of sequential descriptions of browser-based observations (plus statistical summary) for individual genes. While descriptive reporting is informative, the manuscript lacks the integrative interpretation and analytical synthesis expected in an academic research article. (If possible), The author should attempt to identify broader regulatory patterns linking H3K36me3 to alternative splicing across multiple genes. Conversely, cases that do not conform to the proposed hypothesis should also be critically discussed to provide mechanistic insight. Such interpretation should be explicitly integrated into the Discussion section rather than remaining at the level of isolated observations.

Minor Issues:

1. RNAP II (RNA Polymerase II) -> The author needs to mention the full name first followed by the abbreviation.

2. Line 43: DNA is the genetic material which universally codes for the proteins of almost all organisms. A singular diploid cell in the human body contains 6 billion base pairs, spanning 2 meters wide end-to-end. To compact it for packaging within the 10 micrometre nucleus, the DNA is wrapped around an octamer of positively charged proteins known as histones, which, when combined with associated RNAs, form chromatin(ADD REFERENCES).

-> In addition to this sentence, the author needs to update more references for the entire draft

3. Could you clarify which statistical software or program was used to perform the unpaired two-tailed t-tests?

4. The final sentence of the Introduction should describe the goal and scope of the study in a more detailed and formal manner.

Final Recommendation

- Accept with major revisions (acceptance conditional on satisfactory major revisions)

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Investigation of histone modification and alternative splicing association in neural and non-neural tissues

[redacted]

[redacted], Nishinomiya-city, Japan

Keywords:
Epigenetics, Chromatin, H3K36me3 RNA Splicing,

Abstract

Pre-mRNA splicing is a complex and vital process utilized in eukaryotes. Histone modifications are known to affect splicing through recruiting factors to the nascent pre-mRNA. They also influence splicing outcomes through modulating the elongation rate of RNA Polymerase II by altering the degree of chromatin compaction. We hypothesized that the H3K36me3 histone modification in genes known to be neurally regulated would be enriched compared to non-neural tissues, as a potential contributor to tissue-specific splicing. Using publicly available ChIP-Seq and RNA-Seq data from embryonic and postnatal mouse tissues, we analyzed the differences in H3K36me3 signals between brain and non-neural tissues for select genes known to play an important role in brain function. We found that exon inclusion was associated with H3K36me3 across tissues in the Bin1 gene, although the correlation was less pronounced in other genes. Furthermore, we observed that changes in H3K36me3 during brain development were associated with changes in alternative splicing patterns at the same developmental time point. By identifying these associations, we postulate that H3K36me3 could serve as a key determinant of alternative splicing outcomes.

1. Introduction

DNA is the genetic material which universally codes for the proteins of almost all organisms. A singular diploid cell in the human body contains six billion base pairs, spanning two meters wide end-to-end (Piovesan et al., 2019). To compact it for packaging within the 10 micrometer nucleus, the DNA is wrapped around an octamer of positively charged proteins known as histones, which, when combined with associated RNAs, form chromatin (Georgopoulos, 2017). Chromatin can be modified in various ways, such as DNA methylation and histone modifications. Histone modifications are known to affect

key properties of the chromatin, such as DNA accessibility, altering the extent to which proteins and transcription factors must compete with histones for DNA binding and utilization (Pfluger & Wagner, 2007). For example, accessibility was decreased by H3K27 methylation, and increased by acetylation or H3K36 methylation (Bannister & Kouzarides, 2011; Greer & Shi, 2012).

Alternative splicing is the process of producing multiple distinct mRNAs (isoforms) from the same pre-mRNA via joining exons (coding regions) in different combinations, used by higher eukaryotes for increased transcriptomic and proteomic diversity (Wright et al., 2022). It is mediated by splicing factors that guide the spliceosome, a complex assembled along the introns of small ribonuclear proteins, to the splice sites to cut out introns (non-coding regions) (Marasco & Kornblihtt, 2023). Alternative splicing preferentially occurs at suboptimal splice sites, giving rise to varying proportions of the variants, but splicing factors can bind to certain sequences to influence which splice site is used (Marasco & Kornblihtt, 2023). This process is vital to eukaryotes, and its misregulation was implicated in cancers and neurological disorders (Marasco & Kornblihtt, 2023).

Recent studies illuminated strong connections between histone modifications and alternative splicing (Yustis et al., 2024). The research in post-translational modifications, chromatin state, and elongation speed's ability to directly influence splicing has arisen, due to evidence that splicing occured simultaneously to transcription, in other words, co-transcriptionally (Leung et al., 2019)(Figure 1A)

Two models are prominent as the current consensus of how the chromatin landscape shapes splicing outcome: the recruitment and the kinetic model. The recruitment model described chromatin and its modifications' interactions with splicing factors during co-transcriptional splicing (Luco et al., 2011; Yustis et al., 2024). The kinetic model proposed that the elongation rate of RNA Polymerase II (RNAP II) altered splice site selection and spliceosome assembly by modulating the availability of splice sites (Yustis et al., 2024). Histone modifications that induce chromatin compaction could also affect the elongation rate, leading to changes in splicing outcomes (de la Mata et al., 2003). Mechanistic models for particular modifications, such as H3K36me3, have been found, where they can regulate splicing through a chromatin reader protein, such as MRG15, which can then recruit splicing factors, such as polypyrimidine tract-binding protein (Figure 1B) (Luco et al., 2010). While the associations between histone modifications and alternative splicing in the brain are well studied, as the organ with a high frequency of alternative splicing in higher eukaryotes, the connections and comparisons of the same relationships in non-neural organs are not explored well.

H3K36me3 was chosen in this study because of multiple criteria. Firstly, it had an experimentally proven role in influencing RNA splicing and an outlined mechanism (Luco et al., 2010). Although other modifications had demonstrated enrichment in spliced exon regions and associations with alternative splicing, a computational study investigating histone modifications between IMR90 and H1 cells found H3K36me3 and H3K79me1 to be the only modifications clearly correlated to alternative splicing (Shindo et al., 2013). Lastly, as H3K36me3, in particular, was also strongly associated with half the alternatively spliced events that change upon human embryonic stem cell differentiation, H3K36me3 was chosen so that tissue-specific and developmental correlations would be more grounded (Xu et al., 2018).

The genes analyzed in this study were manually selected, based on their involvement in neurological conditions, such that all four genes chosen were known to contain neurally regulated splicing events. This was verified by referring to the list provided in previous literature (Irimia et al., 2014). Moreover, the genes were selected so that they can be compared against their histone modifications and their influence on alternative splicing in the brain. Specifically, Bridging Integrator 1 (Bin1, NM_001083334) was a neurally regulated gene with functions including regulating endocytosis, which has

isoforms relevant to Alzheimer's disease, as well as being affected by DNA methylation (De Jager et al., 2014). Bin1 was also a tumour suppressor that was inactivated through attenuation or mis-splicing in human cancers. Its inactivation could particularly increase the likelihood of lung cancer (Chang et al., 2007). Myocyte enhancer factor 2A (Mef2a, NM_001291192) was a transcription factor and an important regulator of cardiomyocyte hypertrophy downstream of Ca/CaM that was typically expressed in the heart and neural cells, which could be affected by certain histone modifications, such as acetylation (Passier et al., 2000; Sharma et al., 2014). The aberrant S-nitrosylation (SNO)-MEF2A/TLX cascade negatively regulated adult neurogenesis in Alzheimer's disease (Okamoto et al., 2014), additionally a systematic mutational screening of the MEF2A gene identified a deletion mutation in patients with an autosomal dominant form of coronary artery disease, adCAD1 (Wang et al., 2003).

Clathrin light chain B (Cltb, NM_028870) was shown to connect endocytic machinery to the cytoskeleton by binding Hip1/R, which in turn binds to actin. It is predicted to be involved in clathrin-dependent endocytosis and could similarly be affected by histone modification profiles (Das et al., 2021a; Forn et al., 2013). Cltb was abnormally distributed in the hippocampi of patients with Alzheimer's and concentrated at neurofibrillary tangles and was upregulated in mouse hippocampi models with Alzheimer's (Nakamura, 1994). It was also present in increased amounts in the neuronal perikarya of Pick's disease patients compared to healthy patients (Nakamura et al., 1994). Pyruvate kinase, muscle (Pkm, NM_011099), was involved in canonical glycolysis and was shown to be influenced by H3K36me3 (Luco et al., 2010). Splicing preference for the downstream mutually exclusive exon forms the isoform Pkm2 that was found in elevated levels in postmortem Alzheimer's Disease mice brains and has been implicated in its pathology (Zhang et al., 2025). It could also promote tumour growth (Lin et al., 2021). The selection criteria were designed to ensure that the genes are alternatively spliced before comparing them across tissues. Furthermore, it could increase the probability that H3K36me3 can directly or indirectly regulate gene splicing, rather than merely correlating with it, although it will still naturally require wet lab experimentation to test any associations.

In this study, we aimed to investigate the extent to which the histone modification H3K36me3 acted as a tissue-specific splicing regulator, examining its level of presence in exons that are distinctly spliced across tissues. An additional objective was to determine if H3K36me3 could be significant for developmental differences in exon inclusion percentages to assess its potential impacts on development.

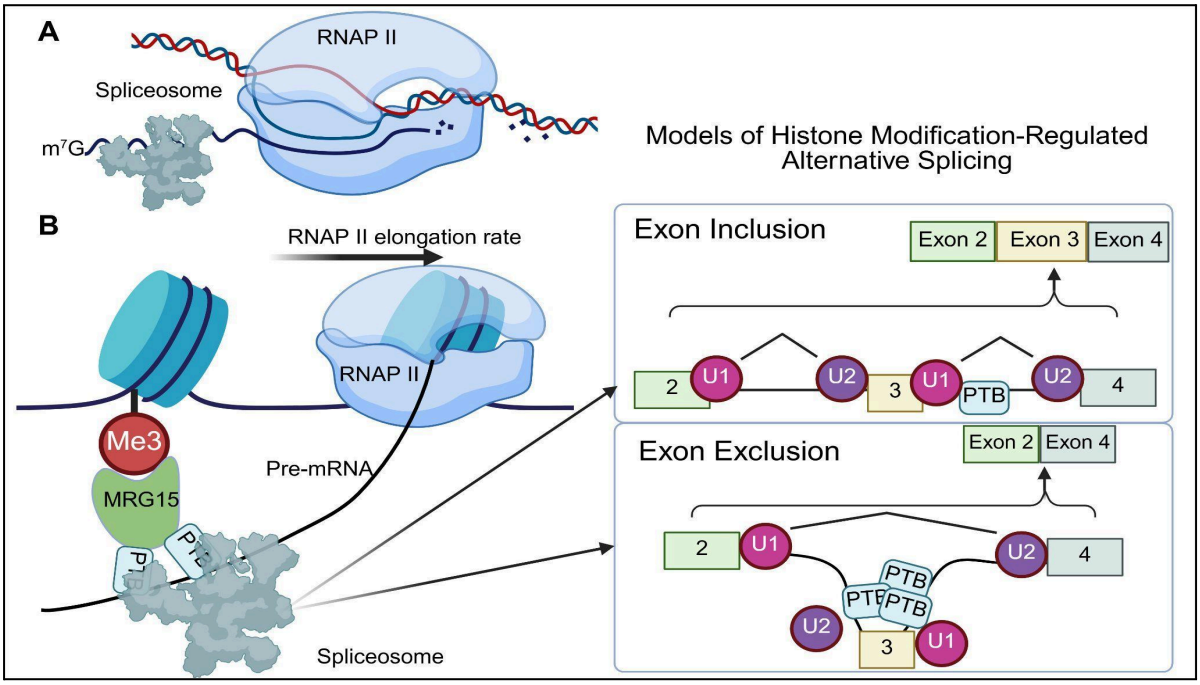

Figure 1: Co-transcriptional splicing and models for histone modification-regulated alternative splicing. (A) A schematic figure illustrating how the RNA Polymerase II (RNAP II) undergoes elongation simultaneously to splicing by the spliceosome, after the m^7G cap has been bound. **(B)** A schematic figure depicting the effects of chromatin states on differential splicing outcome through affecting RNAP II elongation speed and chromatin adaptors. H3K36me3 is shown here to be interacting with the chromatin adaptor protein MORF-related gene 15 (MRG15), which then recruits the polypyrimidine tract binding protein (PTB), adapted from (Luco et al., 2010). Subsequently, its location-dependent splicing outcomes are illustrated on the right, with the potential mechanisms. Adapted from (Chou et al., 2000). Figure generated using BioRender.

2. Methodology

Epigenome browser visualisation of Tabula Muris and ENCODE data

UCSC Genome Browser was used to visualise the publicly available Tabula Muris single-cell (Fluorescence-Activated Cell Sorting (FACS) performed cells sequenced with SmartSeq2 protocol) RNA-Seq data and ENCODE 3 project's Histone Modification ChIP-Seq data in Mus musculus genome (December 2011 (GRCm38/mm10), GCA_000001635.2) (ENCODE Project Consortium, 2012; Perez et al., 2025; Schaum et al., 2018). Although many other modifications, such as H3K9ac and H3K27me3, had previously demonstrated effects on alternative splicing, they were not focused on here due to the scope of this study. (Agirre et al., 2021).

The H3K36me3 data from mice of age postnatal 0 (P0), the oldest available (out of E10.5, E11.5, E12.,5 E13.5, E14.5, E15.5,

E16.5, and P0), were used to understand the relationship between histone modifications and alternative splicing during development. Additionally, the signal from embryonic 14 (E14) mice was also quantified to complement the Cortexa data discussed later. We analyzed the H3K36me3 signal ±200 bp from the intron-exon boundaries, which we decided upon from past literature (Agirre et al., 2021; Shindo et al., 2013). The tissues that the neuronal cell population was compared against were manually selected by observing if their expression levels were comparable to those of the neuron. Specifically, tissues were used if their flanking exons had an expression signature value of at least 10 % of the neuron's expression, to ensure that any differences in the splicing could not simply be attributed to the difference in expression. However, some tissues with RNA-Seq data could not be utilized for analysis due to the ENCODE dataset employed only containing 12 tissues.

Measuring developmental exon inclusion percentages

Cortexa database was used to analzse the changes in exon inclusion percentage in the cerebral cortex in mice with age (Weißbach et al., 2024). We searched the gene names "Bin1", "Pkm", "Cltb", and "Mef2a", which had the splicing events recorded. Although the coordinates of the splicing events in Cortexa did not exactly match those in the GENCODE VM23 Transcript set used in the UCSC Browser, the corresponding events were found as those with equivalent length of the relevantly spliced exons.

Statistical analysis

To quantify the RNA-Seq and ChIP-Seq data, the exact coordinates of the desired region were found using UCSC's Table Browser, with the following configurations: Clade: Mammal, Genome: Mouse, Assembly: Dec. 2011 (GRCm38/mm10), Group: Expression and Regulation, Track: Histone Modifications, Table: H3K36me3 [Selected Organ] [Selected Age]. From the Table Browser, we obtained the summary statistics that included the mean and the standard deviation. Mean and standard deviation values were used to generate graphs on BioRender. We then performed unpaired two-tailed t-tests with alpha< 0.05, variances of the samples being homogenous, and normal distribution using BioRender Graph.

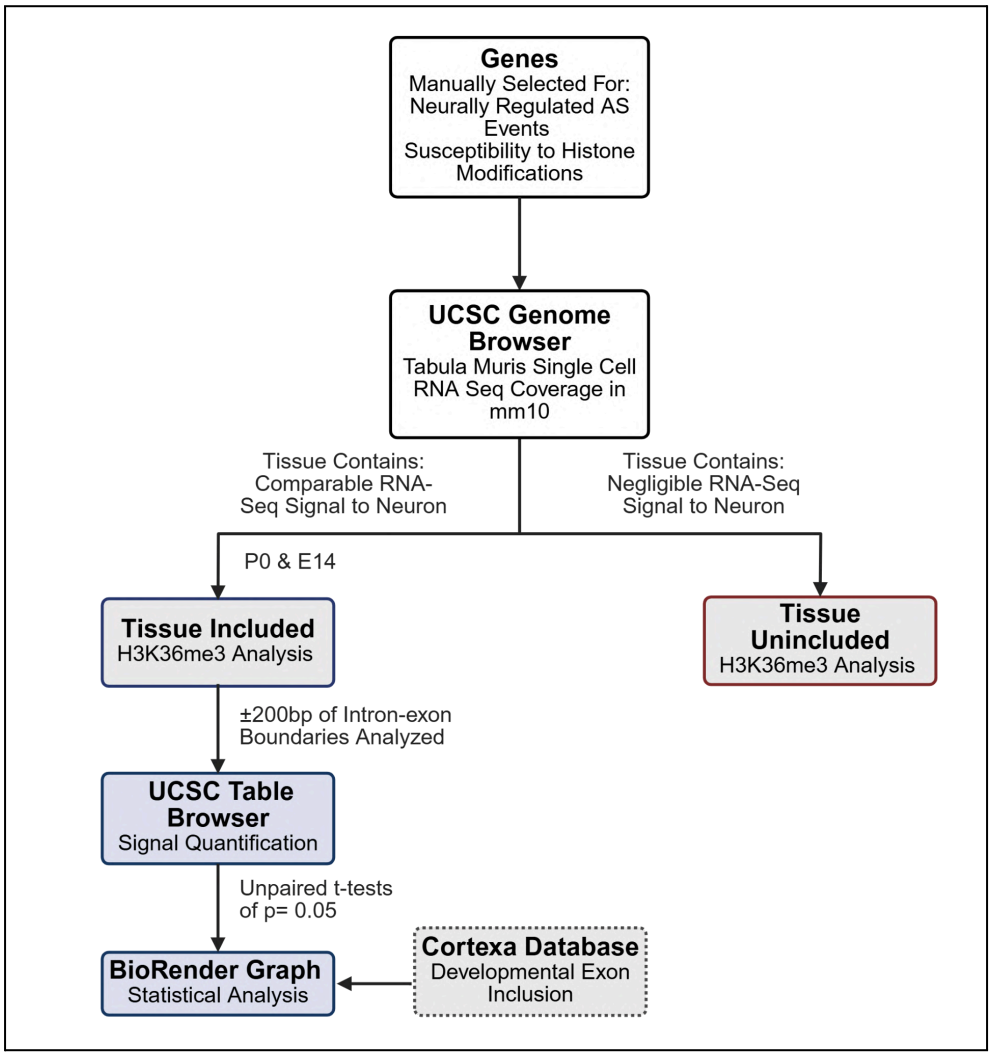

Figure 2: Methodological workflow of this study. Flowchart depicting the gene selection process, data visualization and analysis methods. The genes were selected based on their susceptibility to alternative splicing in neurons. The tissues were chosen if they had sufficient flanking exon expression signal relative to neurons. The UCSC Table Browser was used to obtain summary statistics, which were analyzed and plotted using the BioRender Graph tool. The Cortexa database was used to analyze the changes in developmental exon inclusion percentages with changes in H3K36me3.

3. Results

Exon 7 of Bin1 is differentially spliced in neurons

Analysis of Exon 7 of Bin1 revealed differential splicing between neurons and lung endothelia. Exon 7 encodes part of the BAR domain that has been implicated in dynamin regulation and regulation of membrane targeting specificity (Spooner & Dixon, 2025). Both flanking exons were expressed to comparable levels in both neurons and the lung endothelia, while neurons exclusively had exon 7 included. The percentage spliced in (PSI) relative to average RNA-Seq of its flanking exons for exon 7 were 88.5% and 1.15%, respectively (to 3 significant figures). This was in concordance with findings suggesting a functional role of exon 7 in controlling the efficiency of neural cell endocytosis by promoting Dnm2 interactions (Ellis et al., 2012). Analyzing the corresponding methylation pattern showed H3K36me3 signatures in the lung near the exon, which were outside of the exon region (quantified in the red box, Fig. 3A), while the midbrain had peaks both near and inside the region. Further quantification of H3K36me3 signal in the region displayed significant differences in H3K36me3 signal between the lung and all three areas of the fore, mid and hindbrain for Bin1, where the signal appeared greater in all areas of the brain. However, among the genes analyzed in this study, Bin1 is the only gene to display significant organ-specific H3K36me3 signals (Fig. 3B). We next asked if Exon 7 is developmentally regulated in the mouse brain. The inclusion level of the exon in the mouse brain increases significantly from E14 to E16 (78.6% to 93.8%, respectively), but not from E16 to P0 (Fig. 3C); however, no significant differences were found between the H3K36me3 signals of the brain samples aged E14.5 to P0 for all three brain regions (Fig. 3D).

There was a limitation to this data, which was that the H3K36me3 data were derived from a bulk assay of many cell types within the tissue, while the RNA-Seq data were cell-specific. Thus, we have included the RNA-Seq data of all the types of brain cells for which the data are accessible in the Tabula Muris tracklist in part E, including the neuron, astrocyte, OPC, microglia, and oligodendrocytes. The splicing patterns were distinct, where neurons had a high degree of inclusion of exon 7, while OPC, astrocytes, and oligodendrocytes had much lower inclusion, and microglia had no observable inclusion. The expression of the gene itself varied widely, where the microglia and oligodendrocytes had much greater RNA-Seq signal than the rest. (Fig. 3E) This was in concordance with past literature on Bin1 (Dourlen et al., 2025).

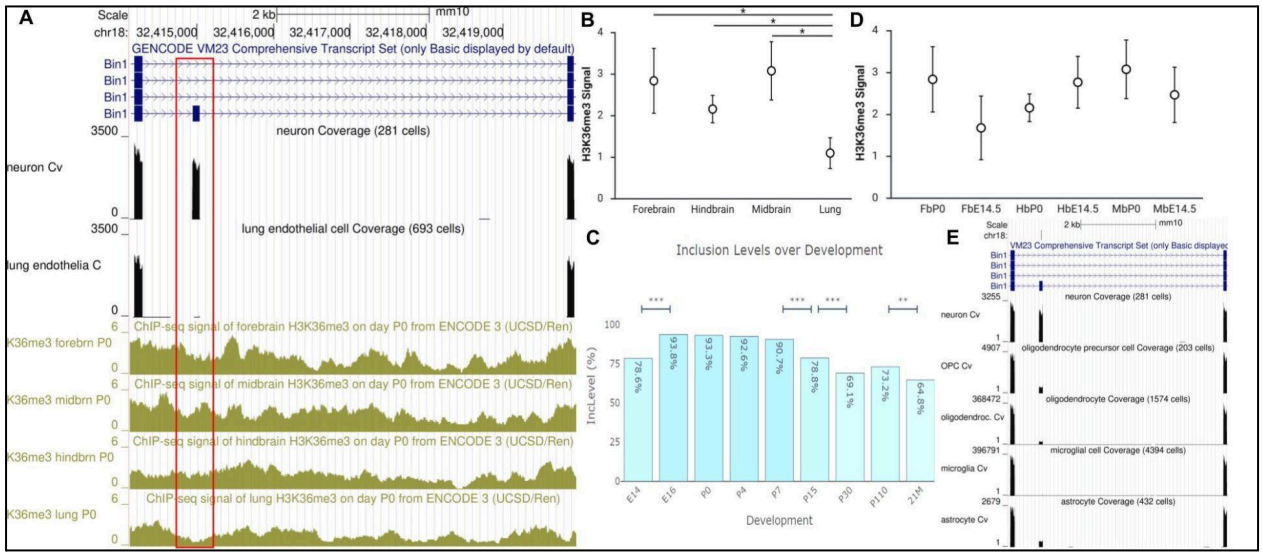

Figure 3: mRNA and H3K36me3 and alternative splicing analyses of Bin1 Exon 7. (A) The RNA-Seq data of neurons and lung endothelia visualized along with the associated tissues' H3K36me3 signals. **(B)** A graph where the circle represents

the mean and the tails/error bars represent the standard deviation (SD) of the H3K36me3 signal of the four different tissues. P values: 0.0251 (Forebrain - Lung), 0.0123 (Midbrain - Lung), 0.0208 (Hindbrain - Lung). **(C)** The changes in inclusion levels over development, adapted from Cortexa (Weißbach et al., 2024). **(D)** Graph of H3K36me3 signal of the three brain regions (Forebrain - Fb, Midbrain - Mb, Hindbrain - Hb) from Age E14.5 to P0, where the circle represents the mean, and the error bars represent the SD (B and D, quantified region: chr18:32,414,746-32,415,240 | NM_001083334). **(E)** RNA-Seq data of the neuron, OPC, oligodendrocyte, microglia, and astrocyte. Their y-axes are not normalized at the microglia despite its 100-fold greater expression to emphasize the neuron-specific splicing pattern. P-values: * < 0.05, ** < 0.01, *** < 0.001. Note: Graphs without indicated statistical significance indicate a lack of significant differences.

Skipping of exon 9 of Mef2a is differentially regulated in neurons

Similar to Bin1, Exon 9 of Mef2a also displayed differential splicing between the neuron and the lung endothelia, where the lung exclusively excluded it (PSIs were 67.5% to 2.11%, respectively), while the cardiac muscle was spliced similarly to the neuron (PSI 84.7%). The cardiac muscle is shown for comparison with the neuron. Exon 9's function is unknown, but it was more skipped in patient's hearts with Type 2 diabetes (Nutter et al., 2016). The flanking exons of all the neuron, heart, and lung were comparably expressed. All quantified regions had small peaks within them, with generally similar H3K36me3 signature patterns across the tissues in the visible areas. (Fig. 4A) Quantification of the regions indicated no significant differences between the H3K36me3 signals of any tissues, where all five tissues had very similar mean signatures. (Fig. 4B) We then asked whether Exon 9 was developmentally regulated in the mouse brain. Although there appeared to be a general trend of increasing exon inclusion, there were no significant differences between E14 and P0, the ages studied. (Fig. 4C) Similarly, we found no significant differences between the H3K36me3 signatures in all three brain regions. (Fig. 4D)

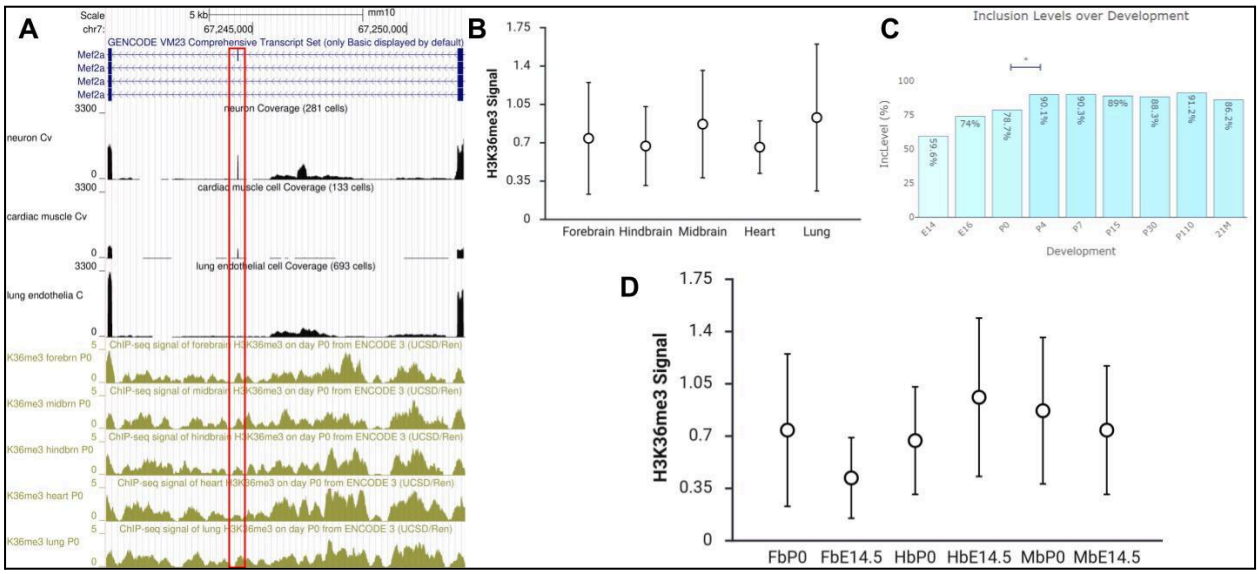

Figure 4: mRNA and H3K36me3 and alternative splicing analyses in Mef2a Exon 9. (A) The RNA-Seq data of neuron,

cardiac muscle, and lung endothelia visualized along with the associated tissues' H3K36me3 signals. **(B)** A graph where the circle represents the mean and the tails/error bars represent the standard deviation (SD) of the H3K36me3 signal of the five different tissues. **(C)** The changes in inclusion levels over development, adapted from Cortexa (Weißbach et al., 2024). **(D)** Graph of H3K36me3 signal of the three brain regions from Age E14.5 to P0, where the circle represents the mean, and the error bars represent the SD (B and D, quantified region: chr7:67,244,319-67,244,743 | NM_001291192). P-values: * < 0.05, ** < 0.01, *** < 0.001. Note: Graphs without indicated significance indicate a lack of significant differences.

Exon 5 of Cltb is differentially spliced in neurons and developmentally regulated

Next, Exon 5 of Cltb also exhibited differential splicing, where neurons exclusively included it, which was concordant with the exon 5 containing isoform being neuron-specific in mice (Das et al., 2021b). Specifically, the PSIs were 87.8% in the neuron, 0.0680% in the hepatocytes, and 0.794% in the lung endothelia. Both flanking exons are comparably expressed in all tissues. Peaks were noticeable in the quantified regions of the hindbrain, liver, and lung. (Fig. 5A) Further quantification evinced the lack of significant differences between the tissues' trimethylation signatures. Forebrain and midbrain had markedly smaller standard deviations, which suggested that they did not contain peaks in the region. (Fig. 5B) When we next asked if Exon 5 was developmentally regulated, we found two significant differences in inclusion in the analyzed timeframe of E14 to P0, increasing from 17.5% to 30.7% from E14 to E16 and from 30.7% to 38.5% from E16 to P0. (Fig. 5C) Subsequent quantification demonstrated a significant difference between the H3K36me3 signature of E14.5 and P0 Forebrain, an increase with age, while the hind and midbrain showed no significance. (Fig. 5D)

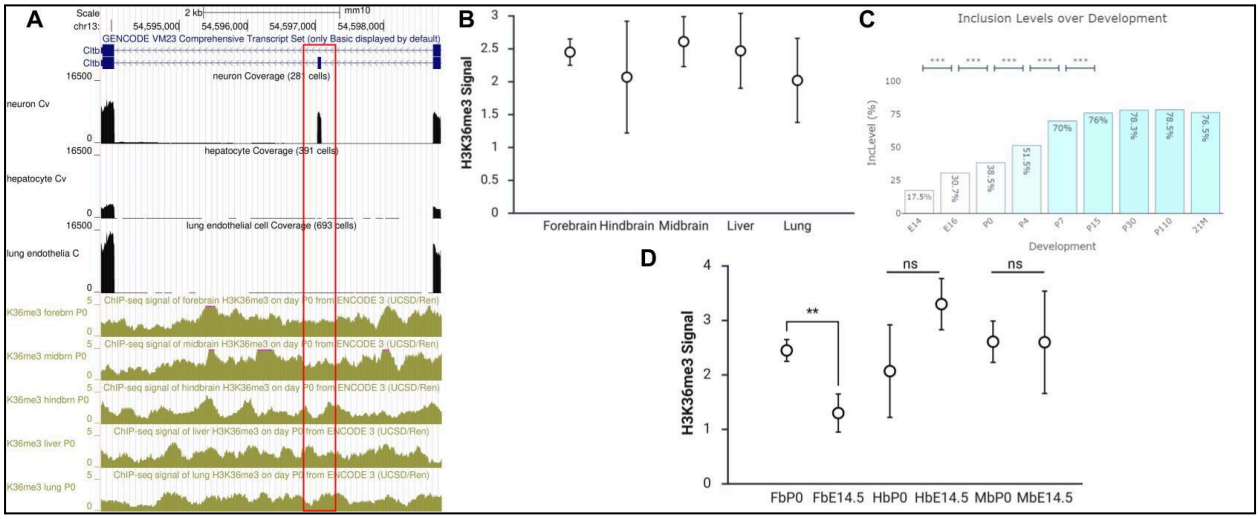

Figure 5: mRNA and H3K36me3 and alternative splicing analyses in Cltb Exon 5. (A) The RNA-Seq data of neuron, hepatocyte, and lung endothelia visualized along with the associated tissues' H3K36me3 signals. **(B)** A graph where the circle represents the mean and the tails/error bars represent the standard deviation (SD) of the H3K36me3 signal of the five different tissues. **(C)** The changes in inclusion levels over development, adapted from Cortexa (Weißbach et al., 2024). **(D)** Graph of H3K36me3 signal of the three brain regions from Age E14.5 to P0, where the circle represents the mean, and the error bars represent the SD (B and D, quantified region: chr13:54,596,823-54,597,278 | NM_028870). P value: 0.00781 (FbP0 - FbE14.5). P-values: * < 0.05, ** < 0.01, *** < 0.001. Note: Graphs without indicated significance indicate a lack of

significant differences.

Mutually exclusive exons 9 and 10 of Pkm are differentially spliced in neurons

Exons 9 and 10 of Pkm (Pkm1 and 2, respectively) were mutually exclusive and exhibited differential splicing, where the neuron and cardiac muscle tended to include Exon 9 preferentially (PSIs expressed as (Exon 9 PSI, Exon 10 PSI) were (74.7%, 13.1%) and (84.1% vs 4.23%), respectively), while the lung endothelia appeared to prefer inclusion of Exon 10 (PSI (14.3% vs 62.7%)). The sole inclusion of exon 9 forms the isoform Pkm1, which was more commonly found in the brain and heart (Li et al., 2024), compared to the counterpart Pkm2 that exclusively includes exon 10 that enhances tumorigenesis, which has been found elevated in lung adenocarcinoma (Zhu et al., 2021). The trimethylation tracks display larger peaks in the quantified region of Exon 9 than in Exon 10. (Fig. 6A) In analyzing both exons, we found no significant differences in either, although the lung appeared to have a smaller signal than both the neuron or cardiac muscle. (Fig. 6B & 6C) Following this, we examined whether these exons were developmentally regulated. The event saw two significant changes in preference for inclusion with age from E14 to P0. Specifically, Exon 9 inclusion was increasingly preferred with age (34.5% to 67.5% to 81.6% from E14 to E16 to P0), while Exon 10 inclusion preference decreased by equal proportions. (Fig. 6D) Additional quantification of the trimethylation signature indicated the absence of significant differences in the region of Exon 9, but also a significant difference and decrease in H3K36me3 from E14 to P0 Midbrain. The other regions did not signify any significant changes in Exon 10, however. (Fig. 6E & 6F)

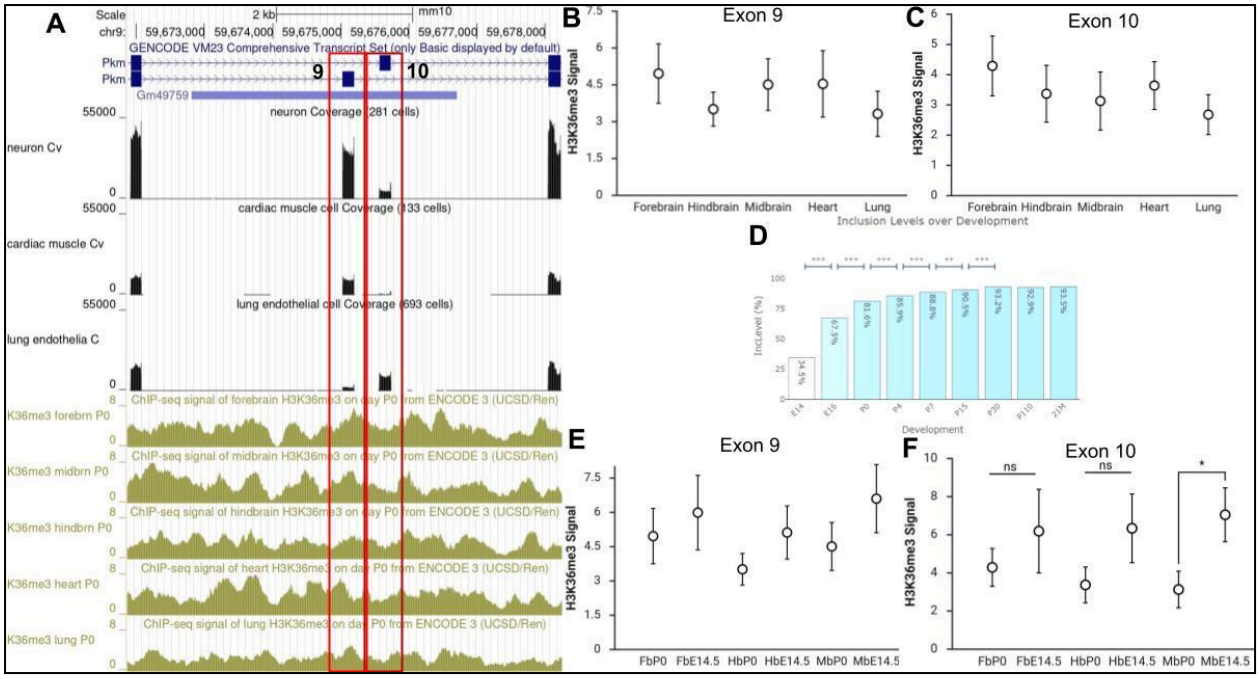

Figure 6: mRNA and H3K36me3 and alternative splicing analyses in Pkm Exons 9 and 10. (A) The RNA-Seq data of neuron, cardiac muscle, and lung endothelia visualized along with the associated tissues' H3K36me3 signals. **(B and C)** Graphs where the circle represents the mean and the tails/error bars represent the standard deviation (SD) of the H3K36me3 signal of the five different tissues. **(D)** The changes in inclusion levels over development, adapted from Cortexa

(Weißbach et al., 2024). In this instance, the percentages are calculated such that the percentage inclusion of exon 9 (shown above) and the percentage inclusion of exon 10 add up to 100%. **(E and F)** Graph of H3K36me3 signal of the three brain regions from Age E14.5 to P0, where the circle represents the mean, and the error bars represent the SD (B, C, E and F, Quantified regions: Exon 9: chr9:59,674,826-59,675,395, Exon 10: chr9:59,675,364-59,675,930 | NM_011099). P value: 0.0164 (MbP0 - MbE14.5). P-values: * < 0.05, ** < 0.01, *** < 0.001. Note: Graphs without indicated significance indicate a lack of significant differences.

4. Discussion

To understand the co-regulation of H3K36me3 and alternative splicing, we investigated four genes. Our findings suggest that differences in splicing across tissues for neurally regulated alternative splicing events may be and in part, regulated by H3K36me3 in Bin1, but less so in other genes. Tissue-specific splicing of Bin1 being correlated to H3K36me3 and not Mef2a or Cltb, was consistent with the literature (Forn et al., 2013; Sharma et al., 2014; Shindo et al., 2013). This tissue-specific difference in H3K36me3 presence could potentially lead to further analysis of non-neural regulation of alternative splicing to uncover targets to modulate to influence and reduce aberrant splicing in Alzheimer's brain tissues.

Pkm2 was known to be affected by H3K36me3 in PNT2 cells compared to hMSCs, yet it did not appear to be a major factor for tissue-specific splicing (Luco et al., 2010). This discrepancy may be due to how we quantified the H3K36me3 signals for the region containing the mutually exclusively spliced exons, while they compared the signal for upstream exons and introns as well. Otherwise, different factors, such as nucleosome occupancy, histone variants, and chromatin remodelling complexes, could be more significant components that affect splicing outcome across organs. However, this study only indicated correlations; thus, perturbation experiments are recommended to further understand H3K36me3's role in alternative splicing in interesting cases such as Bin1, as well as its potential functions in inter-tissue communication in regard to splicing.

There was no evidence of H3K36me3 being correlated with changes in developmental splicing outcomes in Bin1 and Mef2a, although it was in Cltb and Pkm. However, Cltb and Pkm both had greater developmental changes in splicing then Bin1 and Mef2a (increase of 21% and 47.1% against 14.7% and 19.1%, respectively) and significantly increased from E14 to E16 and from E16 to P0, while Bin1 and Mef2a did not. Thus, we hypothesize that changes in H3K36me3 may be associated with a threshold for changes in splicing outcome, which could be a potential analysis in future investigations. . The observation in Cltb was supported, as Cltb was known to be regulated by alternative splicing in a developmental stage-specific manner (Blue et al., 2018). The correlation was also corroborated by previous literature in mouse tissue (Hu et al., 2020). However, this study was also based on predictions and data analysis; thus, we once again recommend experiments be carried out to examine this relationship. Furthermore, as the Cortexa database used only covers neural alternative splicing events, similar research in other tissues and organs may also be fruitful. However, splicing, being known to affect the chromatin landscape in bilateral communications, could complicate the directionality of the relationship (Yustis et al., 2024).

A limitation of this research was that, although H3K36me3 was typically known to enhance exon inclusion, there are modifications that were known to silence exons (Shindo et al., 2013; Zhou et al., 2014). These were not able to be accounted for due to time and technological constraints; however, this could have also been affecting the splicing outcome by being increased or removed between tissues instead of H3K36me3 or through various combinations of them that were known to affect differential splicing (Chen et al., 2019). For example, Mef2a is a primary downstream target of

the calcium-CaMKII/PKD-HDAC pathway, which can phosphorylate histones and cause hyperacetylation that could possibly be affecting Mef2a splicing instead of H3K36me3 (Sharma et al., 2014). Similarly, Cltb regulation across normal tissue to adenomas was associated with a change in H3K4me3 and H3K27me3 (Forn et al., 2013), and H2BK12ac, H4K5ac, and H3K4me3 was enriched in Bin1 exon regions in IMR90 cells (Shindo et al., 2013).

The transcriptional elongation speed of RNAP II could have also affected the splicing patterns, but they could not be accounted for in this study (Zhou et al., 2014). Lastly, the methylation data were not cell-specific, so perturbation experiments could be difficult to accurately perform.

The research on two cases of correlation between developmental alternative splicing and H3K36me3 may hopefully drive experimental studies. This could then illuminate H3K36me3 as a potential target of aberrant alternative splicing during development. Specifically, it could potentially address errors in embryo growth, as it is known that embryo growth retardation is likely caused by Pkm1 increase and Pkm2 decrease, as Pkm2 was highly expressed in embryos and essential for embryo development (Lin et al., 2021).

5. Conclusions

This study added evidence for H3K36me3's potential for altering differential splicing across tissues in Bin1, while suggesting that other factors, in part, act as key contributors for future investigations, and uncovered two cases where differences in H3K36me3 were associated with differences in developmental splicing in this investigation of histone modifications' effects on differential splicing. Although there were certain constraints, such as counter-balancing modifications and elongation speed of RNAP II, perturbation experiments could fully uncover the connections between H3K36me3, tissue-specific and developmental splicing that were previously underexplored, which could drive new perspectives. In future studies, H3K36me3 could potentially be a therapeutic target for embryo growth retardation and avenue for resolving aberrant splicing in Alzheimer's.

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Data & Code Availability Statement

TabulaMurisSingleCellData:
https://genome.ucsc.edu/cgi-bin/hgTrackUi?hgsid=2303618686_hFf8vDaGo1RWvlEehhPpGvHhSniv&db=mm10&c=chr2&g=tabulaMuris

ChIP-Seq:
https://www.encodeproject.org/chip-seq/histone-encode3/

Cortexa:
https://cortexa-01.zdv.uni-mainz.de/

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Author Biography

The author is a 16 year-old student researcher of chromatin biology at Crimson Global Academy in Japan. Inspired by the immense compaction in DNA packaging, he enjoys investigating the mechanisms of chromatin regulation through epigenetics. Additionally, he hopes to apply his theoretical research to improving the lives of the rare disease community, and runs an educational YouTube channel about rare diseases. He hopes to major in molecular biology in university.

Mentor Contribution Statement

The mentor for this research project was Dr Kif Liakath-Ali, Lecturer at the University of Southampton, UK. He served as the sole supervisor throughout the work. He met with the author weekly via Zoom to provide guidance, discuss progress, and offer critical feedback. He recommended relevant databases for analysis, which the author independently explored and interrogated. Dr Liakath-Ali reviewed the data analysis and read multiple drafts of the manuscript to identify issues of logic, clarity, or sophistication; however, all analyses, interpretations, and written materials were prepared entirely by the author.

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Investigation of histone modification and alternative splicing association in neural and non-neural tissues

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Abstract

Pre-mRNA splicing is a complex and vital process utilized in eukaryotes. Histone modifications are known to affect splicing through recruiting factors to the nascent pre-mRNA. They also influence splicing outcome through modulating the elongation rate of by altering the degree of chromatin compaction. We hypothesized that the H3K36me3 histone modification in genes known to be neurally regulated would be enriched compared to non-neural tissues, as a potential contributor to tissue-specific splicing. Using publicly available ChIP-Seq and RNA-Seq data from embryonic and postnatal mouse tissues, we analyzed the differences in H3K36me3 signal between brain and non-neural tissues for select genes known to play an important role in brain function. We found that exon inclusion was associated with H3K36me3 across tissues in the Bin1 gene, although the correlation was less pronounced in other genes. Furthermore, we observed that changes in H3K36me3 during brain development were associated with changes in alternative splicing patterns at the same developmental time point. By identifying these associations, we postulate that H3K36me3 could serve as a key determinant of alternative splicing outcome.

1. Introduction

DNA is the genetic material which universally codes for the proteins of almost all organisms. A singular diploid cell in the human body contains six billion base pairs, spanning two meters wide end-to-end (Piovesan et al., 2019). To compact it for packaging within the 10 micrometer nucleus, the DNA is wrapped around an octamer of positively charged proteins known as histones, which, when combined with associated RNAs, form chromatin (Georgopoulos, 2017). Chromatin can be modified in various ways, such as DNA methylation and histone modifications. Histone modifications are known to affect

key properties of the chromatin, such as DNA accessibility, altering the extent to which proteins and transcription factors must compete with histones for DNA binding and utilization (Pfluger & Wagner, 2007). For example, accessibility was decreased by H3K27 methylation, and increased by acetylation or H3K36 methylation (Bannister & Kouzarides, 2011; Greer & Shi, 2012).

Alternative splicing is the process of producing multiple distinct mRNAs (isoforms) from the same pre-mRNA via joining exons (coding regions) in different combinations, used by higher eukaryotes for increased transcriptomic and proteomic diversity (Wright et al., 2022). It is mediated by splicing factors that guide the spliceosome, a complex assembled along the introns of small ribonuclear proteins, to the splice sites to cut out introns (non-coding regions) (Marasco & Kornblihtt, 2023). Alternative splicing preferentially occurs at suboptimal splice sites, giving rise to varying proportions of the variants, but splicing factors can bind to certain sequences to influence which splice site is used (Marasco & Kornblihtt, 2023). This process is vital to eukaryotes, and its misregulation was implicated in cancers and neurological disorders (Marasco & Kornblihtt, 2023).

Recent studies illuminated strong connections between histone modifications and alternative splicing (Yustis et al., 2024). The research in post-translational modifications, chromatin state, and elongation speed's ability to directly influence splicing has arisen, due to evidence that splicing occured simultaneously to transcription, in other words, co-transcriptionally (Leung et al., 2019)(Figure 1A)

Two models are prominent as the current consensus of how the chromatin landscape shapes splicing outcome: the recruitment and the kinetic model. The recruitment model described chromatin and its modifications' interactions with splicing factors during co-transcriptional splicing (Luco et al., 2011; Yustis et al., 2024). The kinetic model proposed that the elongation rate of ~~RNAP II~~ (RNA Polymerase II (RNAP II)) altered splice site selection and spliceosome assembly by modulating the availability of splice sites (Yustis et al., 2024). Histone modifications that induce chromatin compaction could also affect the elongation rate, leading to changes in splicing outcomes (de la Mata et al., 2003). Mechanistic models for particular modifications, such as H3K36me3, have been found, where they can regulate splicing through a chromatin reader protein, such as MRG15, which can then recruit splicing factors, such as polypyrimidine tract-binding protein (Figure 1B) (Luco et al., 2010). While the associations between histone modifications and alternative splicing in the brain are well studied, as the organ with a high frequency of alternative splicing in higher eukaryotes, the connections and comparisons of the same relationships in non-neural organs are not explored well.

H3K36me3 was chosen in this study because of multiple criteria. Firstly, it had an experimentally proven role in influencing RNA splicing and an outlined mechanism (Luco et al., 2010). Although other modifications had demonstrated enrichment in spliced exon regions and associations with alternative splicing, a computational study investigating histone modifications between IMR90 and H1 cells found H3K36me3 and H3K79me1 to be the only modifications clearly correlated to alternative splicing (Shindo et al., 2013). Lastly, as H3K36me3, in particular, was also strongly associated with half the alternatively spliced events that change upon human embryonic stem cell differentiation, H3K36me3 was chosen so that tissue-specific and developmental correlations would be more grounded (Xu et al., 2018).

The genes analyzed in this study were manually selected, ~~based~~s on their involvement in neurological conditions, such that all four genes chosen were known to contain neurally regulated splicing events. This was verified by referring to the list provided in previous literature (Irimia et al., 2014). Moreover, the genes were selected so that they can be compared against their histone modifications and their influence on alternative splicing in the brain. Specifically, Bridging Integrator 1 (Bin1, NM_001083334) was a neurally regulated gene with functions including regulating endocytosis, which has

isoforms relevant to Alzheimer's disease, as well as being affected by DNA methylation (De Jager et al., 2014). Bin1 was also a tumour suppressor that was inactivated through attenuation or mis-splicing in human cancers. Its inactivation could particularly increase the likelihood of lung cancer (Chang et al., 2007). Myocyte enhancer factor 2A (Mef2a, NM_001291192) was a transcription factor and an important regulator of cardiomyocyte hypertrophy downstream of Ca/CaM that was typically expressed in the heart and neural cells, which could be affected by certain histone modifications, such as acetylation (Passier et al., 2000; Sharma et al., 2014). The aberrant S-nitrosylation (SNO)-MEF2A/TLX cascade negatively regulated adult neurogenesis in Alzheimer's disease (Okamoto et al., 2014), additionally a systematic mutational screening of the MEF2A gene identified a deletion mutation in patients with an autosomal dominant form of coronary artery disease, adCAD1 (Wang et al., 2003).

Clathrin light chain B (Cltb, NM_028870) was shown to connect endocytic machinery to the cytoskeleton by binding Hip1/R, which in turn binds to actin. It is predicted to be involved in clathrin-dependent endocytosis and could similarly be affected by histone modification profiles (Das et al., 2021a; Forn et al., 2013). Cltb was abnormally distributed in the hippocampi of patients with Alzheimer's and concentrated at neurofibrillary tangles and was upregulated in mouse hippocampi models with Alzheimer's (Nakamura, 1994). It was also present in increased amounts in the neuronal perikarya of Pick's disease patients compared to healthy patients (Nakamura et al., 1994). Pyruvate kinase, muscle (Pkm, NM_011099), was involved in canonical glycolysis and was shown to be influenced by H3K36me3 (Luco et al., 2010). Splicing preference for the downstream mutually exclusive exon forms the isoform Pkm2 that was found in elevated levels in postmortem Alzheimer's Disease mice brains and has been implicated in its pathology (Zhang et al., 2025). It could also promote tumour growth (Lin et al., 2021). The selection criteria were designed to ensure that the genes are alternatively spliced before comparing them across tissues. Furthermore, it could increase the probability that H3K36me3 can directly or indirectly regulate gene splicing, rather than merely correlating with it, although it will still naturally require wet lab experimentation to test any associations.

In this study, we aimed to investigate the extent to which the histone modification H3K36me3 acted as a tissue-specific splicing regulator, examining its level of presence in exons that are distinctly spliced across tissues. An additional objective was to determine if H3K36me3 could be significant for developmental differences in exon inclusion percentages to assess its potential impacts on development.

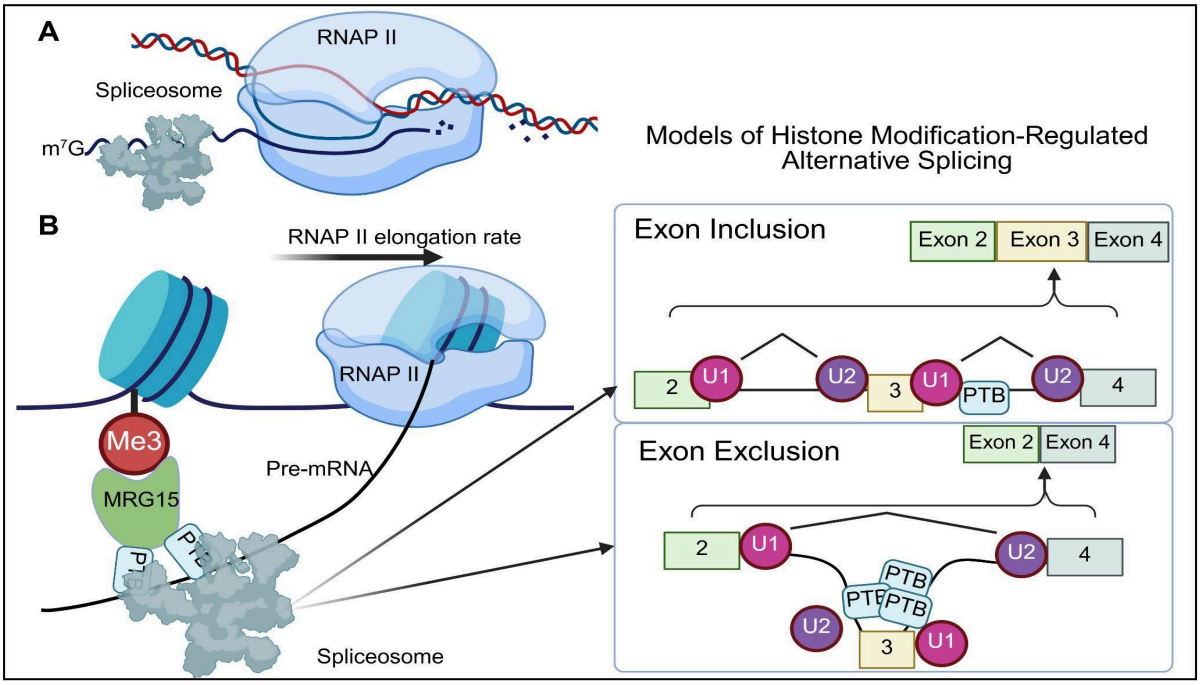

Figure 1: Co-transcriptional splicing and models for histone modification-regulated alternative splicing. (A) A schematic figure illustrating how the RNA Polymerase II (RNAP II) undergoes elongation simultaneously to splicing by the spliceosome, after the m^7G cap has been bound. **(B)** A schematic figure depicting the effects of chromatin states on differential splicing outcome through affecting RNAP II elongation speed and chromatin adaptors. H3K36me3 is shown here to be interacting with the chromatin adaptor protein MORF-related gene 15 (MRG15), which then recruits the polypyrimidine tract binding protein (PTB), adapted from (Luco et al., 2010). Subsequently, its location-dependent splicing outcomes are illustrated on the right, with the potential mechanisms. Adapted from (Chou et al., 2000). Figure generated using BioRender.

2. Methodology

Epigenome browser visualisation of Tabula Muris and ENCODE data

UCSC Genome Browser was used to visualise the publicly available Tabula Muris single-cell (Fluorescence-Activated Cell Sorting (FACS) performed cells sequenced with SmartSeq2 protocol) RNA-Seq data and ENCODE 3 project's Histone Modification ChIP-Seq data in Mus musculus genome (December 2011 (GRCm38/mm10), GCA_000001635.2) (ENCODE Project Consortium, 2012; Perez et al., 2025; Schaum et al., 2018). Although many other modifications, such as H3K9ac and H3K27me3, had previously demonstrated effects on alternative splicing, they were not focused on here due to the scope of this study. (Agirre et al., 2021).

¶

The H3K36me3 data from mice of age postnatal 0 (P0), the oldest available (out of E10.5, E11.5, E12.,5 E13.5, E14.5, E15.5, E16.5, and P0), were used to understand the relationship between histone modifications and alternative splicing during development. Additionally, the signal from embryonic 14 (E14) mice was also quantified to complement the Cortexa data discussed later. We analyzed the H3K36me3 signal ±200 bp from the intron-exon boundaries, which we decided upon from past literature (Agirre et al., 2021; Shindo et al., 2013). The tissues that the neuronal cell population was compared against were manually selected by observing if their expression levels were comparable to those of the neuron. Specifically, tissues were used if their flanking exons had an expression signature value of at least 10 % of the neuron's expression, to ensure that any differences in the splicing could not simply be attributed to the difference in expression. However, some tissues with RNA-Seq data could not be utilized for analysis due to the ENCODE dataset employed only containing 12 tissues.

Measuring developmental exon inclusion percentages

Cortexa database was used to analzse the changes in exon inclusion percentage in the cerebral cortex in mice with age (Weißbach et al., 2024). We searched the gene names "Bin1", "Pkm", "Cltb", and "Mef2a", which had the splicing events recorded. Although the coordinates of the splicing events in Cortexa did not exactly match those in the GENCODE VM23 Transcript set used in the UCSC Browser, the corresponding events were found as those with equivalent length of the relevantly spliced exons.

Statistical analysis

To quantify the RNA-Seq and ChIP-Seq data, the exact coordinates of the desired region were found using UCSC's Table Browser, with the following configurations: Clade: Mammal, Genome: Mouse, Assembly: Dec. 2011 (GRCm38/mm10), Group: Expression and Regulation, Track: Histone Modifications, Table: H3K36me3 [Selected Organ] [Selected Age]. From the Table Browser, we obtained the summary statistics that included the mean and the standard deviation. Mean and standard deviation values were used to generate graphs on BioRender. We then performed unpaired two-tailed t-tests with alpha< 0.05, variances of the samples being homogenous, and normal distribution using BioRender Graph.

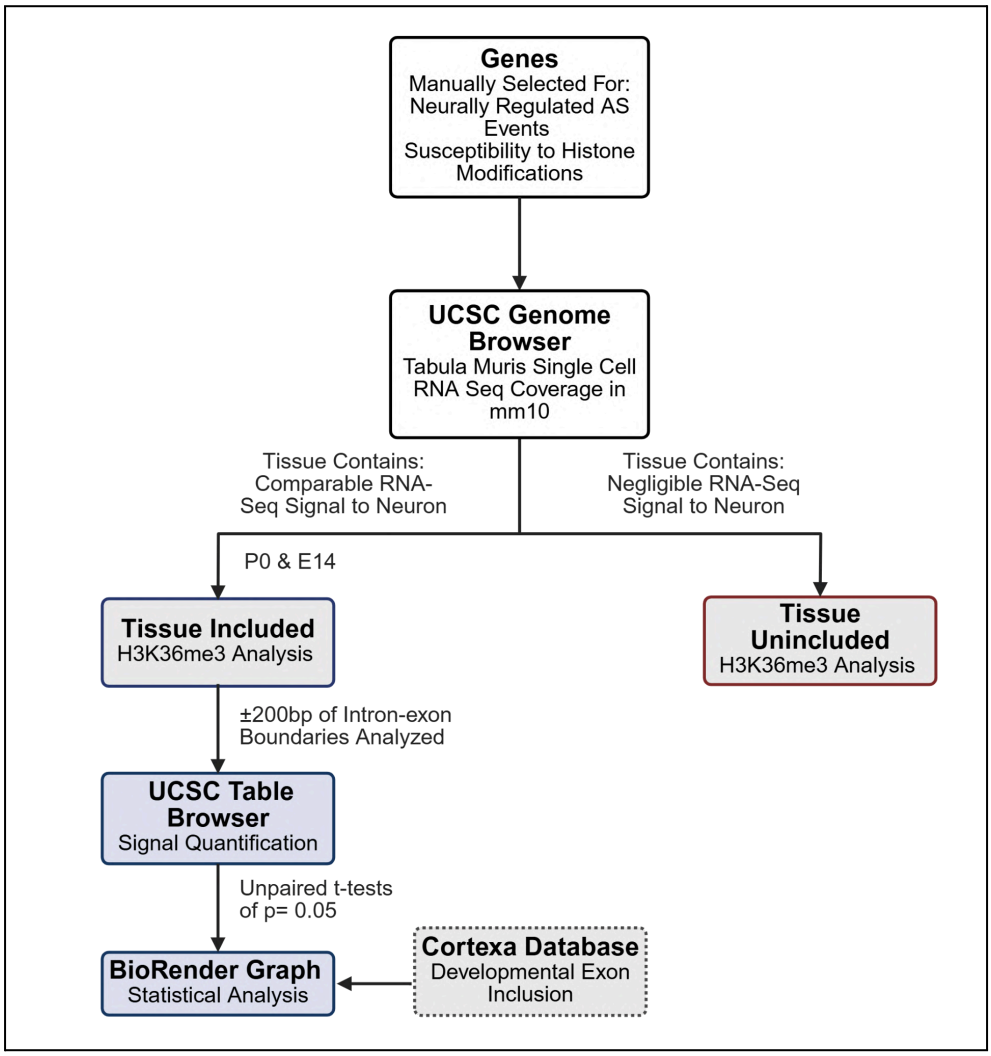

Figure 2: Methodological workflow of this study. Flowchart depicting the gene selection process, data visualization and analysis methods. The genes were selected based on their susceptibility to alternative splicing in neurons. The tissues were chosen if they had sufficient flanking exon expression signal relative to neurons. The UCSC Table Browser was used to obtain summary statistics, which were analyzed and plotted using the BioRender Graph tool. The Cortexa database was used to analyze the changes in developmental exon inclusion percentages with changes in H3K36me3.

3. Results

Exon 7 of Bin1 is differentially spliced in neurons

Analysis of Exon 7 of Bin1 revealed differential splicing between neurons and lung endothelia. Exon 7 encodes part of the BAR domain that has been implicated in dynamin regulation and regulation of membrane targeting specificity (Spooner & Dixon, 2025). Both flanking exons were expressed to comparable levels in both neurons and the lung endothelia, while neurons exclusively had exon 7 included. The percentage spliced in (PSI) relative to average RNA-Seq of its flanking exons for exon 7 were 88.5% and 1.15%, respectively (to 3 significant figures). This was in concordance with findings suggesting a functional role of exon 7 in controlling the efficiency of neural cell endocytosis by promoting Dnm2 interactions (Ellis et al., 2012). Analyzing the corresponding methylation pattern showed H3K36me3 signatures in the lung near the exon, which were outside of the exon region (quantified in the red box, Fig. 3A), while the midbrain had peaks both near and inside the region. Further quantification of H3K36me3 signal in the region displayed significant differences in H3K36me3 signal between the lung and all three areas of the fore, mid and hindbrain for Bin1, where the signal appeared greater in all areas of the brain. However, among the genes analyzed in this study, Bin1 is the only gene to display significant organ-specific H3K36me3 signals (Fig. 3B). We next asked if Exon 7 is developmentally regulated in the mouse brain. The inclusion level of the exon in the mouse brain increases significantly from E14 to E16 (78.6% to 93.8%, respectively), but not from E16 to P0 (Fig. 3C); however, no significant differences were found between the H3K36me3 signals of the brain samples aged E14.5 to P0 for all three brain regions (Fig. 3D).

There was a limitation to this data, which was that the H3K36me3 data were derived from a bulk assay of many cell types within the tissue, while the RNA-Seq data were cell-specific. Thus, we have included the RNA-Seq data of all the types of brain cells for which the data are accessible in the Tabula Muris tracklist in part E, including the neuron, astrocyte, OPC, microglia, and oligodendrocytes. The splicing patterns were distinct, where neurons had a high degree of inclusion of exon 7, while OPC, astrocytes, and oligodendrocytes had much lower inclusion, and microglia had no observable inclusion. The expression of the gene itself varied widely, where the microglia and oligodendrocytes had much greater RNA-Seq signal than the rest. (Fig. 3E) This was in concordance with past literature on Bin1 (Dourlen et al., 2025).

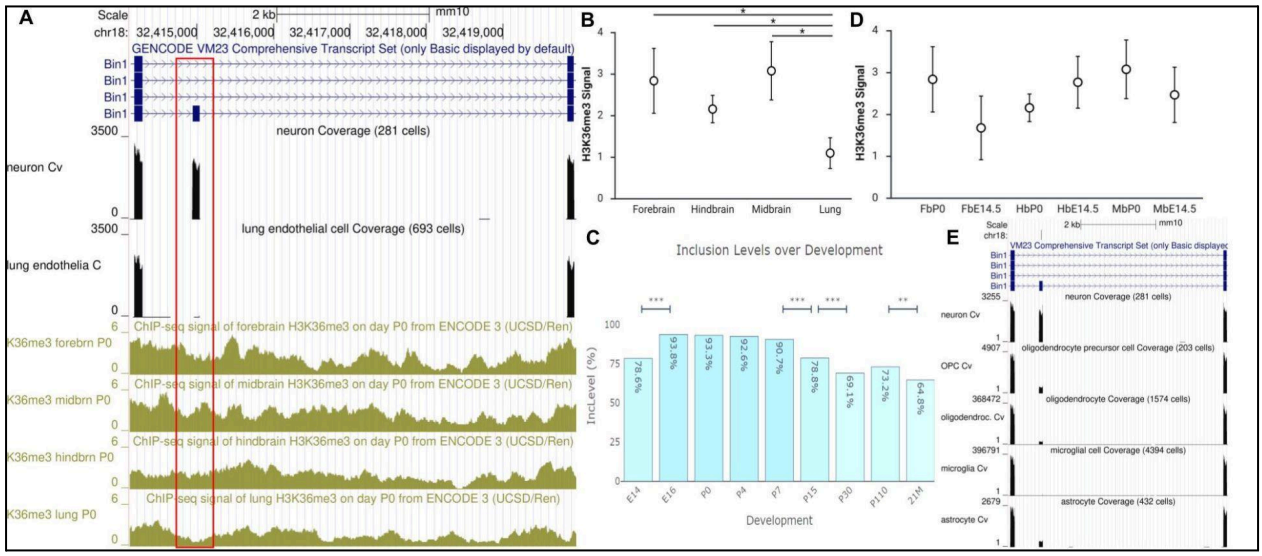

Figure 3: mRNA and H3K36me3 and alternative splicing analyses of Bin1 Exon 7. (A) The RNA-Seq data of neurons and lung endothelia visualized along with the associated tissues' H3K36me3 signals. **(B)** A graph where the circle represents

the mean and the tails/error bars represent the standard deviation (SD) of the H3K36me3 signal of the four different tissues. P values: 0.0251 (Forebrain - Lung), 0.0123 (Midbrain - Lung), 0.0208 (Hindbrain - Lung). **(C)** The changes in inclusion levels over development, adapted from Cortexa (Weißbach et al., 2024). **(D)** Graph of H3K36me3 signal of the three brain regions (Forebrain - Fb, Midbrain - Mb, Hindbrain - Hb) from Age E14.5 to P0, where the circle represents the mean, and the error bars represent the SD (B and D, quantified region: chr18:32,414,746-32,415,240 | NM_001083334). **(E)** RNA-Seq data of the neuron, OPC, oligodendrocyte, microglia, and astrocyte. Their y-axes are not normalized at the microglia despite its 100-fold greater expression to emphasize the neuron-specific splicing pattern. P-values: * < 0.05, ** < 0.01, *** < 0.001. Note: Graphs without indicated statistical significance indicate a lack of significant differences.

Skipping of exon 9 of Mef2a is differentially regulated in neurons

Similar to Bin1, Exon 9 of Mef2a also displayed differential splicing between the neuron and the lung endothelia, where~~with~~ the lung exclusively excluded it (PSIs were 67.5% to 2.11%, respectively)~~ing it~~, while the cardiac muscle was spliced similarly to the neuron (PSI 84.7%). The cardiac muscle is shown for comparison with the neuron. Exon 9's function is unknown, but it was more skipped in patient's hearts with Type 2 diabetes (Nutter et al., 2016). The flanking exons of all the neuron, heart, and lung were comparably expressed. All quantified regions had small peaks within them, with generally similar H3K36me3 signature patterns across the tissues in the visible areas. (Fig. 4A) Quantification of the regions indicated no significant differences between the H3K36me3 signals of any tissues, where all five tissues had very similar mean signatures. (Fig. 4B) We then asked whether Exon 9 was developmentally regulated in the mouse brain. Although there appeared to be a general trend of increasing exon inclusion, there were no significant differences between E14 and P0, the ages studied. (Fig. 4C) Similarly, we found no significant differences between the H3K36me3 signatures in all three brain regions. (Fig. 4D)

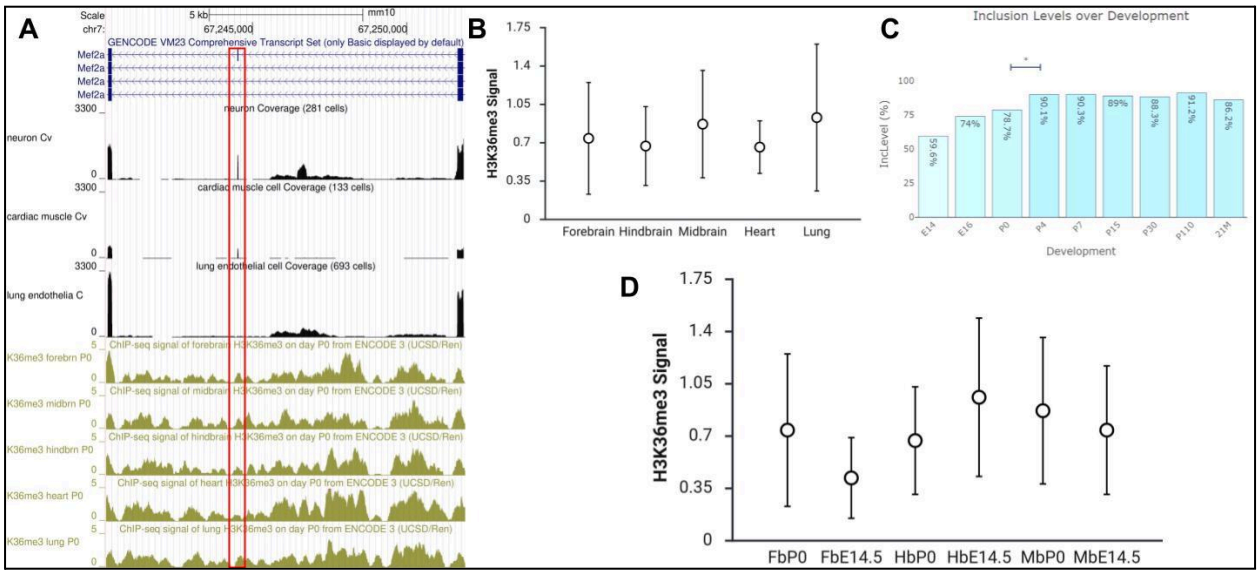

Figure 4: mRNA and H3K36me3 and alternative splicing analyses in Mef2a Exon 9. (A) The RNA-Seq data of neuron,

cardiac muscle, and lung endothelia visualized along with the associated tissues' H3K36me3 signals. **(B)** A graph where the circle represents the mean and the tails/error bars represent the standard deviation (SD) of the H3K36me3 signal of the five different tissues. **(C)** The changes in inclusion levels over development, adapted from Cortexa (Weißbach et al., 2024). **(D)** Graph of H3K36me3 signal of the three brain regions from Age E14.5 to P0, where the circle represents the mean, and the error bars represent the SD (B and D, quantified region: chr7:67,244,319-67,244,743 | NM_001291192). P-values: * < 0.05, ** < 0.01, *** < 0.001. Note: Graphs without indicated significance indicate a lack of significant differences.

Exon 5 of Cltb is differentially spliced in neurons and developmentally regulated

Next, Exon 5 of Cltb also exhibited differential splicing, where neurons exclusively included it, which was concordant with the exon 5 containing isoform being neuron-specific in mice (Das et al., 2021b). Specifically, the PSIs were 87.8% in the neuron, 0.0680% in the hepatocytes, and 0.794% in the lung endothelia. Both flanking exons are comparably expressed in all tissues. Peaks were noticeable in the quantified regions of the hindbrain, liver, and lung. (Fig. 5A) Further quantification evinced the lack of significant differences between the tissues' trimethylation signatures. Forebrain and midbrain had markedly smaller standard deviations, which suggested that they did not contain peaks in the region. (Fig. 5B) When we next asked if Exon 5 was developmentally regulated, we found two significant differences in inclusion in the analyzed timeframe of E14 to P0, increasing from 17.5% to 30.7% from E14 to E16 and from 30.7% to 38.5% from E16 to P0 ~~respectively~~. (Fig. 5C) Subsequent quantification demonstrated a significant difference between the H3K36me3 signature of E14.5 and P0 Forebrain, an increase with age, while the hind and midbrain showed no significance. (Fig. 5D)

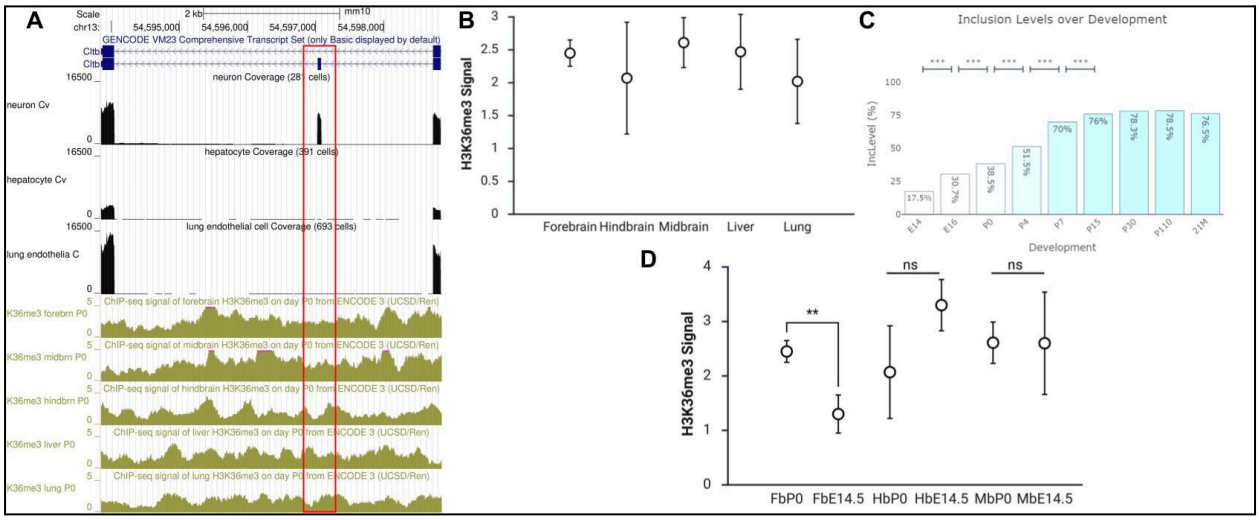

Figure 5: mRNA and H3K36me3 and alternative splicing analyses in Cltb Exon 5. (A) The RNA-Seq data of neuron, hepatocyte, and lung endothelia visualized along with the associated tissues' H3K36me3 signals. **(B)** A graph where the circle represents the mean and the tails/error bars represent the standard deviation (SD) of the H3K36me3 signal of the five different tissues. **(C)** The changes in inclusion levels over development, adapted from Cortexa (Weißbach et al., 2024). **(D)** Graph of H3K36me3 signal of the three brain regions from Age E14.5 to P0, where the circle represents the mean, and the error bars represent the SD (B and D, quantified region: chr13:54,596,823-54,597,278 | NM_028870). P value: 0.00781 (FbP0 - FbE14.5). P-values: * < 0.05, ** < 0.01, *** < 0.001. Note: Graphs without indicated significance indicate a lack of

significant differences.

Mutually exclusive exons 9 and 10 of Pkm are differentially spliced in neurons

Exons 9 and 10 of Pkm (Pkm1 and 2, respectively) were mutually exclusive and exhibited differential splicing, where the neuron and cardiac muscle tended to include Exon 9 preferentially (PSIs expressed as (Exon 9 PSI, Exon 10 PSI) were (74.7%, 13.1%) and (84.1% vs 4.23%), respectively), while the lung endothelia appeared to prefer inclusion of Exon 10 (PSI (14.3% vs 62.7%)).▪ The sole inclusion of exon 9 forms the isoform Pkm1, which was more commonly found in the brain and heart (Li et al., 2024), compared to the counterpart Pkm2 that exclusively includes exon 10 that enhances tumorigenesis, which has been found elevated in lung adenocarcinoma (Zhu et al., 2021). The trimethylation tracks display larger peaks in the quantified region of Exon 9 than in Exon 10. (Fig. 6A) In analyzing both exons, we found no significant differences in either, although the lung appeared to have a smaller signal than both the neuron or cardiac muscle. (Fig. 6B & 6C) Following this, we examined whether these exons were developmentally regulated. The event saw two significant changes in preference for inclusion with age from E14 to P0. Specifically, Exon 9 inclusion was increasingly preferred with age (34.5% to 67.5% to 81.6% from E14 to E16 to P0), while Exon 10 inclusion preference decreased by equal proportions. (Fig. 6D) Additional quantification of the trimethylation signature indicated the absence of significant differences in the region of Exon 9, but also a significant difference and decrease in H3K36me3 from E14 to P0 Midbrain. The other regions did not signify any significant changes in Exon 10, however. (Fig. 6E & 6F)

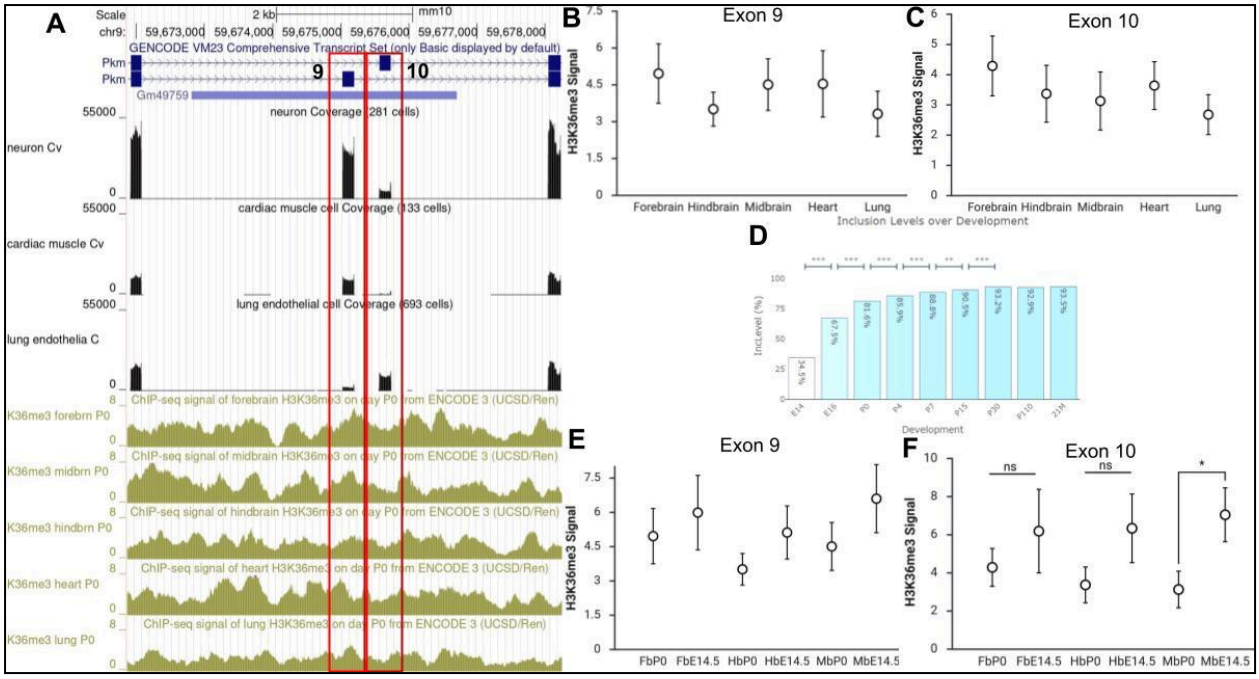

Figure 6: mRNA and H3K36me3 and alternative splicing analyses in Pkm Exons 9 and 10. (A) The RNA-Seq data of neuron, cardiac muscle, and lung endothelia visualized along with the associated tissues' H3K36me3 signals. **(B and C)** Graphs where the circle represents the mean and the tails/error bars represent the standard deviation (SD) of the H3K36me3 signal of the five different tissues. **(D)** The changes in inclusion levels over development, adapted from Cortexa

(Weißbach et al., 2024). In this instance, the percentages are calculated such that the percentage inclusion of exon 9 (shown above) and the percentage inclusion of exon 10 add up to 100%. **(E and F)** Graph of H3K36me3 signal of the three brain regions from Age E14.5 to P0, where the circle represents the mean, and the error bars represent the SD (B, C, E and F, Quantified regions: Exon 9: chr9:59,674,826-59,675,395, Exon 10: chr9:59,675,364-59,675,930 | NM_011099). P value: 0.0164 (MbP0 - MbE14.5). P-values: * < 0.05, ** < 0.01, *** < 0.001. Note: Graphs without indicated significance indicate a lack of significant differences.

4. Discussion

To understand the co-regulation of H3K36me3 and alternative splicing, we investigated four genes. Our findings suggest that differences in splicing across tissues for neurally regulated alternative splicing events may be and in part, regulated by H3K36me3 in Bin1, but less so in other genes. Tissue-specific splicing of Bin1 being correlated to H3K36me3 and not Mef2a or Cltb, was consistent with the literature (Forn et al., 2013; Sharma et al., 2014; Shindo et al., 2013). This tissue-specific difference in H3K36me3 presence could potentially lead to further analysis of non-neural regulation of alternative splicing to uncover targets to modulate to influence and reduce aberrant splicing in Alzheimer's brain tissues.

Pkm2 was known to be affected by H3K36me3 in PNT2 cells compared to hMSCs, yet it did~~es~~ not appear to be a major factor for tissue-specific splicing (Luco et al., 2010). This discrepancy may be due to how we quantified the H3K36me3 signals for the region containing the mutually exclusively spliced exons, while they compared the signal for upstream exons and introns as well. Otherwise, different~~Other~~ factors, such as nucleosome occupancy, histone variants, and chromatin remodelling complexes, could be more significant components that affect splicing outcome across organs. However, this study only indicated correlations; thus, perturbation experiments are recommended to further understand H3K36me3's role in alternative splicing in interesting cases such as Bin1, as well as its potential functions in inter-tissue communication in regards to splicing.

There was no evidence of H3K36me3 being correlated with changes in developmental splicing outcomes in Bin1 and Mef2a, ~~but~~ although it was ~~did so~~ in Cltb and Pkm. However, Cltb and Pkm both had greater developmental changes in splicing then Bin1 and Mef2a (increase of 21% and 47.1% against 14.7% and 19.1%, respectively) and significantly increased from E14 to E16 and from E16 to P0, while Bin1 and Mef2a did not. Thus, we hypothesize that changes in H3K36me3 may be associated with a threshold for changes in splicing outcome, which could be a potential analysis in future investigations. ~~although we have not tested this further~~. The observation in Cltb was supported, as Cltb was known to be regulated by alternative splicing in a developmental stage-specific manner (Blue et al., 2018). The correlation was also corroborated by previous literature in mouse tissue (Hu et al., 2020). However, this study was also based on predictions and data analysis; thus, we once again recommend experiments be carried out to examine this relationship. Furthermore, as the Cortexa database used only covers neural alternative splicing events, similar research in other tissues and organs may also be fruitful. However, splicing, being known to affect the chromatin landscape in bilateral communications, could complicate the directionality of the relationship (Yustis et al., 2024).

A limitation of this research was that, although H3K36me3 was typically known to enhance exon inclusion, there are modifications that were known to silence exons (Shindo et al., 2013; Zhou et al., 2014). These were not able to be accounted for due to time and technological constraints; however, this could have also been affecting the splicing outcome by being increased or removed between tissues instead of H3K36me3 or through various combinations of them that were known to affect differential splicing (Chen et al., 2019). For example, Mef2a is a primary downstream target of

the calcium-CaMKII/PKD-HDAC pathway, which can phosphorylate histones and cause hyperacetylation that could possibly be affecting Mef2a splicing instead of H3K36me3 (Sharma et al., 2014). Similarly, Cltb regulation across normal tissue to adenomas was associated with a change in H3K4me3 and H3K27me3 (Forn et al., 2013), and H2BK12ac, H4K5ac, and H3K4me3 was enriched in Bin1 exon regions in IMR90 cells (Shindo et al., 2013).

The transcriptional elongation speed of RNAP II could have also affected the splicing patterns, but they could not be accounted for in this study (Zhou et al., 2014). Lastly, the methylation data were not cell-specific, so perturbation experiments could be difficult to accurately perform.

The research on two cases of correlation between developmental alternative splicing and H3K36me3 may hopefully drive experimental studies. This could then illuminate H3K36me3 as a potential target of aberrant alternative splicing during development. Specifically, it could potentially address errors in embryo growth, as it is known that embryo growth retardation is likely caused by Pkm1 increase and Pkm2 decrease, as Pkm2 was highly expressed in embryos and essential for embryo development (Lin et al., 2021).

5. Conclusions

This study added evidence for H3K36me3's potential for altering differential splicing across tissues in Bin1, while suggesting that other factors, in part, act as key contributors for future investigations, and uncovered two cases where differences in H3K36me3 were associated with differences in developmental splicing in this investigation of histone modifications' effects on differential splicing. Although there were certain constraints, such as counter-balancing modifications and elongation speed of RNAP II, perturbation experiments could fully uncover the connections between H3K36me3, tissue-specific and ~~and~~ developmental splicing that were previously underexplored,~~,~~ which could drive new perspectives. In~~to~~ future studies, H3K36me3 could potentially be a therapeutic target for embryo growth retardation and avenue for resolving aberrant splicing in~~.~~ Alzheimer's.

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https://doi.org/10.1186/s13046-021-01858-1

Data & Code Availability Statement

TabulaMurisSingleCellData:
https://genome.ucsc.edu/cgi-bin/hgTrackUi?hgsid=2303618686_hFf8vDaGo1RWvlEehhPpGvHhSniv&db=mm10&c=chr2&g=tabulaMuris

ChIP-Seq:
https://www.encodeproject.org/chip-seq/histone-encode3/

Cortexa:
https://cortexa-01.zdv.uni-mainz.de/

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All figures in this manuscript were made with the help of BioRender.

Author Biography

The author is a 16 year-old student researcher of chromatin biology at Crimson Global Academy ininin Japan. Inspired by the immense compaction in DNA packaging, hHe enjoys investigating the mechanisms of chromatin regulation through epigenetics. Additionally, he hopes to apply his theoretical research to improving the lives of the rare disease community, and runs an educational YouTube channel about rare diseases. He hopes to major in molecular biology in university.

Mentor Contribution Statement

~~[You must send us a detailed mentor contribution statement of at most 300 words describing your mentor's explicit role(s) and contribution(s) in the writing of your manuscript. There should be one statement per mentor, and each statement should be written in third-person.]~~¶

¶

~~[redacted]My mentor is a mentor at Indigo Research and a University of Southampton Lecturersupervised my project. He was the sole supervisor of this project. Specifically, he met with the author weekly over Zoom to provide advice and~~

~~feedback on the author's work. He suggested the names of suitable databases, which the author then analyzed and asked questions about. He verified the data analysis and proofread the manuscript multiple times for logical or sophistication errors, but these were prepared wholly by the author themselves.~~

The mentor for this research project was Dr Kif Liakath-Ali, Lecturer at the University of Southampton, UK. He served as the sole supervisor throughout the work. He met with the author weekly via Zoom to provide guidance, discuss progress, and offer critical feedback. He recommended relevant databases for analysis, which the author independently explored and interrogated. Dr Liakath-Ali reviewed the data analysis and read multiple drafts of the manuscript to identify issues of logic, clarity, or sophistication; however, all analyses, interpretations, and written materials were prepared entirely by the author.

¶

~~he suggested suitable databases, reviewed my data analysis, and proofread my manuscript and figures.~~

Ryota Watanabe (RW): I thank the reviewer 1 for their time and helpful comments.

Reviewer 1:

Reviewer Final Recommendation: Accept with minor revisions
Reviewer Feedback: Overall, the paper is well written with a clear goal of assessing various tissues for genes associated with histone modification and alternative splicing events to inform upon mechanistic insights potentially involved in a variety of pathologies (i.e., cancer, cardiomyopathy, autoimmune disorders). These mechanistic insights can elucidate potential avenues for therapeutic intervention. Results from this study begin to address gaps in the literature pertaining to the role of histone modification genes and alternative splicing in neural and non-neural tissues. The author provides a great overview of the literature to set the stage for the experimental design that investigated H3K36me3 histone modification and Bin1, Mef2a, Cltb, and Pkm alternative splicing events. The author does a fantastic job discussing the literature and relating the literature back to the study findings clearly and concisely with ample detail to clearly define the gaps that the study addresses. Minor revisions are included below to bolster the manuscript for Acceptance:

• In the abstract, line 12 should read “They also influence splicing outcomes...” instead of “...outcome...”. If you are referring to one splicing outcome, then use the singular versus plural form.
• In the abstract, line 21 should read “...we analyzed the differences in H3K36me3 signals...” instead of “...signal...”.
• In the abstract, line 33 should read “... splicing outcomes...” instead of “...outcome...”. If you are referring to one splicing outcome, then use the singular versus plural form.

RW: All three of the above have been fixed.

• In the Introduction, line 78, please spell out 4 as four as anything between 0-9 should be spelled out and not numerated.
• In the Methodology, line 95 please spell out 4 as noted above in previous feedback.

RW: All instances of this error have now been fixed.

• Figure 1 and Figure 2 are generated well and convey the main takeaways beautifully. The figure legends are well written as well. Great work!
• All figures in the Results are clear, concise and report the main findings well.

RW: Thank you very much.

• Please spell out all contractions (i.e., can’t, don’t) throughout the manuscript.

RW: They have now been fixed.

• In the Discussion, line 250 should read “...in regard to...” instead of “regards”.

RW: This has been fixed now.

• In the Discussion, lines 254-255 I would recommend restructuring this sentence and making it a bit clearer (the sentence beginning with “However, the study was..”).

RW: Thank you for your input. I have now restructured the sentence.

• Please ensure that the text formatting is aligned with the journal’s guidelines (i.e., font size, style, spacing) when doing a final read through of the manuscript.

RW: The formatting of the “Measuring developmental exon inclusion percentages” section has been fixed. The line spacing throughout has also been adjusted to 1.15.

• Throughout the manuscript please double check tense for the various sections. Anything describing the study typically should be in past tense (Methods, Results) as well as when you are discussing previous literature findings.

RW: I’ve now reviewed the tense throughout the text. For example: “Bin1 was also a tumour suppressor that was inactivated through attenuation or mis-splicing in human cancers. Its inactivation could particularly increase the likelihood of lung cancer (Chang et al., 2007).

• For citations throughout the paper, each sentence that is not your own should be cited so please read through the manuscript and add additional citations as needed. Typically, this pertains to the Introduction and Discussion sections.

RW: More citations have been added where necessary. For example, “A singular diploid cell in the human body contains six billion base pairs, spanning two meters wide end-to-end (Piovesan et al., 2019).”

• Your Discussion section focuses mostly on limitations although there are strong findings to highlight from your study. I would recommend bolstering the interpretation and importance of your results and what they add to the current literature. Strengthening your discussion of your main findings and linking the importance will help keep the reader interested. You do a great job with this in the final paragraph of the Discussion.

RW: I’ve added a couple of sentences in the discussion to add more value to the research.

“Additionally, this tissue-specific difference in H3K36me3 presence could potentially lead to further analysis of non-neural regulation of alternative splicing to uncover targets to modulate to influence and reduce aberrant splicing in Alzheimer's brain tissues”

• In your Conclusion section, I would recommend strengthening how your results impact the current literature and highlight how they provide mechanistic insight that can be used for future development of therapeutics for the pathologies you outline in your introduction (and discussion).

RW: I’ve modified the conclusion by writing: “In future studies, H3K36me3 could potentially be a therapeutic target for embryo growth retardation and avenue for resolving aberrant splicing in Alzheimer’s” to add in areas for therapeutic development in the future.

RW: I thank Reviewer 2 for their time and extensive feedback on the study, which greatly helped to improve the manuscript.

Reviewer 2:

Comments:
This study investigates the correlation between the H3K36me3 histone modification and alternative splicing across neural and non-neural tissues, with a specific focus on its roles in tissue-specific and developmental regulation. This is a hypothesis-driven computational study investigating the association between H3K36me3 and alternative splicing across neural and non-neural tissues. The topic itself presents an interesting approach toward understanding epigenetic regulatory mechanisms; however, the overall study lacks sufficient academic rigor and logical coherence. In particular, the conclusions are drawn primarily from qualitative visual observations and statistical summaries, and the rationale for selecting the target genes and histone mark is not adequately justified in the Introduction section. In its current form, the manuscript reads more like a descriptive compilation of public datasets than a fully developed scientific study. Substantial structural revision and deeper analytical interpretation are required for the work to meet the standards of a professional research paper.

Major Issues
1. Insufficient Rationale for Gene and Histone Mark Selection
Although the manuscript attempts to explain why the four genes (Bin1, Mef2a, Cltb, and Pkm) were specifically selected for analysis and why H3K36me3 was prioritized over other histone modifications, the author needs to elaborate further. The biological and conceptual rationale for these choices is inadequately developed in the Introduction. The manuscript might want to establish the biological relevance of these genes in the context of autism, neurodevelopment, disease pathology, or developmental regulation.

Importantly, the current explanation for gene selection appears in the Methods section, although it functions more appropriately as conceptual background information or as part of the rationale motivating the study. This content should therefore be relocated to the Introduction section to strengthen the logical foundation of the work.

RW: The rationale has been relocated to the Introduction and further rationale has been added. For example, “Cltb was abnormally distributed in the hippocampi of patients with Alzheimer’s and concentrated at neurofibrillary tangles and was upregulated in mouse hippocampi models with Alzheimer’s (Nakamura, 1994).”

2. Descriptive Results Without Integrative Interpretation
The Results section primarily consists of sequential descriptions of browser-based observations (plus statistical summary) for individual genes. While descriptive reporting is informative, the manuscript lacks the integrative interpretation and analytical synthesis expected in an academic research article.

RW: The Results section now contains more integrative interpretations. For example, “The percentage spliced in (PSI) relative to average RNA-Seq of its flanking exons for exon 7 were 88.5% and 1.15%, respectively (to 3 significant figures). This was in concordance with findings suggesting a functional role of exon 7 in controlling the efficiency of neural cell endocytosis by promoting Dnm2 interactions (Ellis et al., 2012).”

[continuation of 2nd comment]
(If possible), The author should attempt to identify broader regulatory patterns linking H3K36me3 to alternative splicing across multiple genes.

RW: I agree that defining broader regulatory patterns linking H3K36me3 to alternative splicing across many genes would be highly informative, especially given emerging evidence that H3K36me3 can coordinate exon choice and splice-factor recruitment across gene networks (Hu et al. 2020). In this current study, however, we aimed to establish a link using four well-known candidate genes that provide a system to test the association. These results form the necessary foundation for scaling up: the approaches and insights developed here will directly enable future work examining multi-gene or genome-wide patterns to determine whether shared chromatin–splicing signatures emerge.

Hu, Qiwen, Casey S. Greene, and Elizabeth A. Heller. 2020. ‘Specific Histone Modifications Associate with Alternative Exon Selection during Mammalian Development’. *Nucleic Acids Research* 48 (9): 4709–24. https://doi.org/10.1093/nar/gkaa248.

Conversely, cases that do not conform to the proposed hypothesis should also be critically discussed to provide mechanistic insight. Such interpretation should be explicitly integrated into the Discussion section rather than remaining at the level of isolated observations.

RW: This has been revised. For example, I have added: “Pkm2 was known to be affected by H3K36me3 in PNT2 cells compared to hMSCs, yet it did not appear to be a major factor for tissue-specific splicing (Luco et al., 2010). This discrepancy may be due to how we quantified the H3K36me3 signals for the region containing the mutually exclusively spliced exons, while they compared the signal for upstream exons and introns as well.

Minor Issues:
1. RNAP II (RNA Polymerase II) -> The author needs to mention the full name first followed by the abbreviation.

RW: This has been fixed.

2. Line 43: DNA is the genetic material which universally codes for the proteins of almost all organisms. A singular diploid cell in the human body contains 6 billion base pairs, spanning 2 meters wide end-to-end. To compact it for packaging within the 10 micrometre nucleus, the DNA is wrapped around an octamer of positively charged proteins known as histones, which, when combined with associated RNAs, form chromatin(ADD REFERENCES).

-> In addition to this sentence, the author needs to update more references for the entire draft

RW: More citations have been added throughout the manuscript. For example, “A singular diploid cell in the human body contains six billion base pairs, spanning two meters wide end-to-end (Piovesan et al., 2019).”

3. Could you clarify which statistical software or program was used to perform the unpaired two-tailed t-tests?

RW: BioRender Graph, the statistical software within BioRender, was used. This information has now been clarified in the manuscript.

4. The final sentence of the Introduction should describe the goal and scope of the study in a more detailed and formal manner.

RW: The final sentence has been modified to improve focus and detail.

Final Recommendation

● Accept with major revisions (acceptance conditional on satisfactory major revisions)

Overall, the manuscript has been significantly improved and is much clearer and better supported than the previous version. However, a few issues remain that need to be addressed to further strengthen the study's clarity and rigor.

Introduction

The Introduction has clearly improved, especially in providing stronger biological justification for the relevance of the selected genes, making the study more convincing than before. However, a few points still need refinement. The section is still somewhat weighted toward detailed gene-by-gene descriptions and disease associations, which can make the paper's main focus feel a bit scattered. The rationale for choosing H3K36me3 could also be strengthened by emphasizing its well-established mechanistic role in co-transcriptional splicing, rather than focusing primarily on neurological contexts. In addition, the gene selection criteria should be stated more clearly and concisely, and some of the disease-related details could be shortened. Finally, the research gap, (e.g.,especially regarding neural vs. non-neural tissues and developmental context, could be made more explicit to better highlight the study's significance.

Figure2 (and method)

One point that may need clarification is the statistical workflow. The current description is somewhat unclear regarding where the unpaired t-tests were actually performed, and BioRender is primarily a visualization tool rather than a statistical analysis platform. It would be helpful to clearly specify the dedicated software used for statistical testing to ensure reproducibility and avoid ambiguity.

Final Recommendation

● Accept with minor revisions (acceptance conditional on satisfactory minor revisions)

6/1/2026

Submission 100183: *Investigation of histone modification and alternative splicing association in neural and non-neural tissues*

Post-Revision Critical Review: The authors have done a wonderful job addressing the reviewer comments and with the revisions the updated manuscript has a stronger logical flow and concrete throughline. Outlined in this paragraph are minor revisions to strengthen the manuscript further. The addition of the conceptual rationale for the histone markers assessed in this paper in the Introduction section demonstrates a stronger foundational knowledge for determining a rigorous experimental design for the study. In the Introduction section lines 45-46 should be clarified as the current sentence has grammatical errors and the main point of the sentence gets lost. Try to reframe the sentence and make it more straightforward to state why H3K36me3 was chosen for this study. In the Method section line 379 there is a typo that should be corrected to “analyze”. In the Methods section and potentially in the figure legends for all figures representing data findings (i.e., Figures 3-6) I recommend adding in the sample sizes/n’s per group for the reader to understand the group sizes to interpret the findings more translationally. For all statistics mentioned in the Results section, I recommend adding in the t-test findings with the respective p values in correct manuscript reporting format. Adding in the statistical outputs for the t-tests for all significant findings and noting non-significant findings in parentheses with $p>0.05$ or the exact p values would strengthen the results section and allow for a deeper statistical interpretation with statistical transparency. In the Discussion section line 525 please remove the additional period in the sentence. New additions in the Discussion section aid in explaining how results from the current studies can be used to interpret the findings and advance the field. In the Conclusions section line 563 the sentence ends abruptly stating that the results can drive new perspectives. I recommend expanding on this and adding in specific directions to clarify this statement. Overall well done!

Final Decision: Accept with minor revisions

A Journal for Young Researchers

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Investigation of histone modification and alternative splicing association in neural and non-neural tissues

[redacted]

[redacted], Nishinomiya-city, Japan

Keywords:
Epigenetics,
H3K36me3
Chromatin,
RNA Splicing,

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Abstract

Pre-mRNA splicing is a complex and vital process utilized in eukaryotes. Histone modifications are known to affect splicing through recruiting factors to the nascent pre-mRNA. They also influence splicing outcomes through modulating the elongation rate of RNA Polymerase IIRNAPH by altering the degree of chromatin compaction. We hypothesized that the H3K36me3 histone modification in genes known to be neurally regulated would be enriched compared to non-neural tissues, as a potential contributor to tissue-specific splicing. Using publicly available ChIP-Seq and RNA-Seq data from embryonic and postnatal mouse tissues, we analyzed the differences in H3K36me3 signals between brain and non-neural tissues for select genes known to play an important role in brain function. We found that exon inclusion was associated with H3K36me3 across tissues in the Bin1 gene, although the correlation was less pronounced in other genes. Furthermore, we observed that changes in H3K36me3 during brain development were associated with changes in alternative splicing patterns at the same developmental time point. By identifying these associations, we postulate that H3K36me3 could serve as a key determinant of alternative splicing outcomes.

1. Introduction

DNA is the genetic material which universally codes for the proteins of almost all organisms. A singular diploid cell in the human body contains six billion base pairs, spanning two meters wide end-to-end (Piovesan et al., 2019). To compact it for packaging within the 10 micrometer nucleus, the DNA is wrapped around an octamer of positively charged proteins known as histones, which, when combined with associated RNAs, form chromatin (Georgopoulos, 2017). Chromatin can be

modified in various ways, such as DNA methylation and histone modifications. Histone modifications are known to affect key properties of the chromatin, such as DNA accessibility, altering the extent to which proteins and transcription factors must compete with histones for DNA binding and utilization (Pfluger & Wagner, 2007). For example, accessibility was decreased by H3K27 methylation, and increased by acetylation or H3K36 methylation (Bannister & Kouzarides, 2011; Greer & Shi, 2012).

Alternative splicing is the process of producing multiple distinct mRNAs (isoforms) from the same pre-mRNA via joining exons (coding regions) in different combinations, used by higher eukaryotes for increased transcriptomic and proteomic diversity (Wright et al., 2022). It is mediated by splicing factors that guide the spliceosome, a complex assembled along the introns of small ribonuclear proteins, to the splice sites to cut out introns (non-coding regions) (Marasco & Kornblihtt, 2023). Alternative splicing preferentially occurs at suboptimal splice sites, giving rise to varying proportions of the variants, but splicing factors can bind to certain sequences to influence which splice site is used (Marasco & Kornblihtt, 2023). This process is vital to eukaryotes, and its misregulation was implicated in cancers and neurological disorders (Marasco & Kornblihtt, 2023).

Recent studies illuminated strong connections between histone modifications and alternative splicing (Yustis et al., 2024). The research in post-translational modifications, chromatin state, and elongation speed's ability to directly influence splicing has arisen, due to evidence that splicing occured simultaneously to transcription, in other words, co-transcriptionally (Leung et al., 2019a) (Figure 1A)

Two models are prominent as the current consensus of how the chromatin landscape shapes splicing outcome: the recruitment and the kinetic model. The recruitment model described chromatin and its modifications' interactions with splicing factors during co-transcriptional splicing (Luco et al., 2011; Yustis et al., 2024). The kinetic model proposed that the elongation rate of ~~RNAP II~~ (RNA Polymerase II (RNAP II)) altered splice site selection and spliceosome assembly by modulating the availability of splice sites (Yustis et al., 2024). Histone modifications that induce chromatin compaction could also affect the elongation rate, leading to changes in splicing outcomes (de la Mata et al., 2003). Mechanistic models for particular modifications, such as H3K36me3, have been found, where they can regulate splicing through a chromatin reader protein, such as MRG15, which can then recruit splicing factors, such as polypyrimidine tract-binding protein (Figure 1B) (Luco et al., 2010). While the associations between histone modifications and alternative splicing in the brain are well ~~studied~~characterized, as the organ with a high frequency of alternative splicing in higher eukaryotes, the significance to specific gene splicing of histone modifications and their changes across~~the connections and comparisons of the same relationships in~~ non-neural tissues and developmental phases ~~organs is~~are underexplored~~not explored well~~.

H3K36me3 was chosen in this study because it had an experimentally proven role in influencing RNA splicing and an outlined mechanism (Luco et al., 2010). Additionally, the chromodomain protein Eaf3 has been established to link H3K36me3 and the spliceosome for co-transcriptional splicing (Leung et al., 2019b). Although other modifications had demonstrated enrichment in spliced exon regions and associations with alternative splicing, a computational study investigating histone modifications between IMR90 and H1 cells found H3K36me3 and H3K79me1 to be the only modifications clearly correlated to alternative splicing (Shindo et al., 2013). Moreover, as H3K36me3, in particular, was also strongly associated with half the alternatively spliced events that change upon human embryonic stem cell differentiation, H3K36me3 was chosen so that tissue-specific and developmental correlations would be more grounded (Xu et al., 2018).

The genes analyzed in this study were manually selected, ~~bases on their involvement in neurological conditions,~~ such that

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all four genes chosen ~~were known to contain~~had known neurally regulated splicing events, involvement in neurological conditions, and susceptibility to epigenetic modification. This was verified by referring to the list provided in previous literature (Irimia et al., 2014). ~~Moreover, the genes were selected so that they can be compared against their histone modifications and their influence on alternative splicing in the brain.~~ Specifically, Bridging Integrator 1 (Bin1, NM_001083334) was a neurally regulated gene with functions including regulating endocytosis, which has isoforms relevant to Alzheimer's disease, as well as being affected by DNA methylation (De Jager et al., 2014). Bin1 was also a tumour suppressor that was inactivated through attenuation or mis-splicing in human cancers. Its inactivation could particularly increase the likelihood of lung cancer (Chang et al., 2007). Myocyte enhancer factor 2A (Mef2a, NM_001291192) was a transcription factor that was typically expressed in the heart and neural cells, which could be affected by certain histone modifications, such as acetylation (Passier et al., 2000; Sharma et al., 2014). The aberrant S-nitrosylation (SNO)-MEF2A/TLX cascade was reported to negatively regulate adult neurogenesis in Alzheimer's disease (Okamoto et al., 2014).

Clathrin light chain B (Cltb, NM_028870) was shown to connect endocytic machinery to the cytoskeleton by binding Hip1/R, which in turn binds to actin. It is predicted to be involved in clathrin-dependent endocytosis and could similarly be affected by histone modification profiles (Das et al., 2021a; Forn et al., 2013). Cltb was abnormally distributed in the hippocampi of patients with Alzheimer's and was upregulated in mouse hippocampi models with Alzheimer's (Nakamura, 1994). It was also present in increased amounts in the neuronal perikarya of Pick's disease patients compared to healthy patients (Nakamura et al., 1994). Pyruvate kinase, muscle (Pkm, NM_011099), was involved in canonical glycolysis and was shown to be influenced by H3K36me3 (Luco et al., 2010). Splicing preference for the downstream mutually exclusive exon forms the isoform Pkm2 that was found in elevated levels in postmortem Alzheimer's Disease mice brains and has been implicated in its pathology (Zhang et al., 2025). It could also promote tumour growth (Lin et al., 2021). The selection criteria were designed to ensure that the genes are alternatively spliced before comparing them across tissues. Furthermore, it could increase the probability that H3K36me3 can directly or indirectly regulate gene splicing, rather than merely correlating with it, although it will still naturally require wet lab experimentation to test any associations.

In this study, we aimed to investigate the extent to which the histone modification H3K36me3 acted as a tissue-specific splicing regulator, examining its level of presence in exons that are distinctly spliced across tissues. An additional objective was to determine if H3K36me3 could be significant for developmental differences in exon inclusion percentages to assess its potential impacts on development.

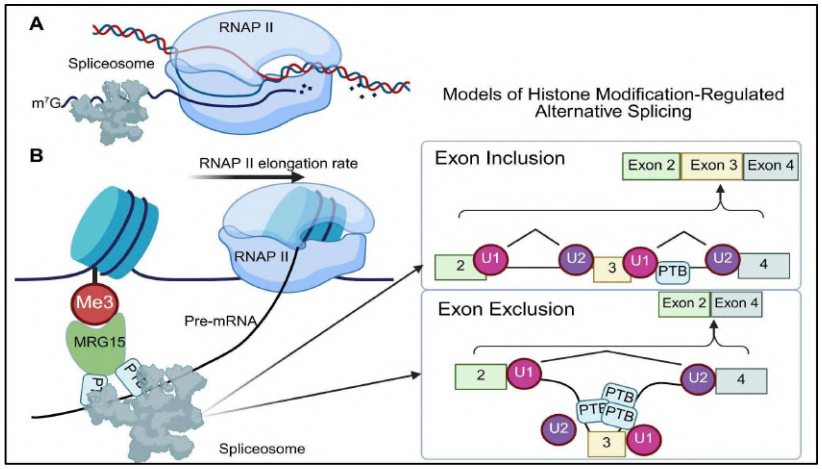

Figure 1: Co-transcriptional splicing and models for histone modification-regulated alternative splicing. (A) A schematic figure illustrating how the RNA Polymerase II (RNAP II) undergoes elongation simultaneously to splicing by the spliceosome, after the m^7G cap has been bound. **(B)** A schematic figure depicting the effects of chromatin states on differential splicing outcome through affecting RNAP II elongation speed and chromatin adaptors. H3K36me3 is shown here to be interacting with the chromatin adaptor protein MORF-related gene 15 (MRG15), which then recruits the polypyrimidine tract binding protein (PTB), adapted from (Luco et al., 2010). Subsequently, its location-dependent splicing outcomes are illustrated on the right, with the potential mechanisms. Adapted from (Chou et al., 2000). Figure generated using BioRender.

2. Methodology

Epigenome browser visualisation of Tabula Muris and ENCODE data

UCSC Genome Browser was used to visualise the publicly available Tabula Muris single-cell (Fluorescence-Activated Cell Sorting (FACS) performed cells sequenced with SmartSeq2 protocol) RNA-Seq data and ENCODE 3 project's Histone Modification ChIP-Seq data from two biological replicates generated by the ENCODE consortium in Mus musculus genome (December 2011 (GRCm38/mm10), GCA_000001635.2) (ENCODE Project Consortium, 2012; Perez et al., 2025; Schaum et al., 2018). Although many other modifications, such as H3K9ac and H3K27me3, had previously demonstrated effects on alternative splicing, they were not focused on here due to the scope of this study. (Agirre et al., 2021).

The H3K36me3 data from mice of age postnatal 0 (P0), the oldest available (out of E10.5, E11.5, E12.,5 E13.5, E14.5, E15.5,

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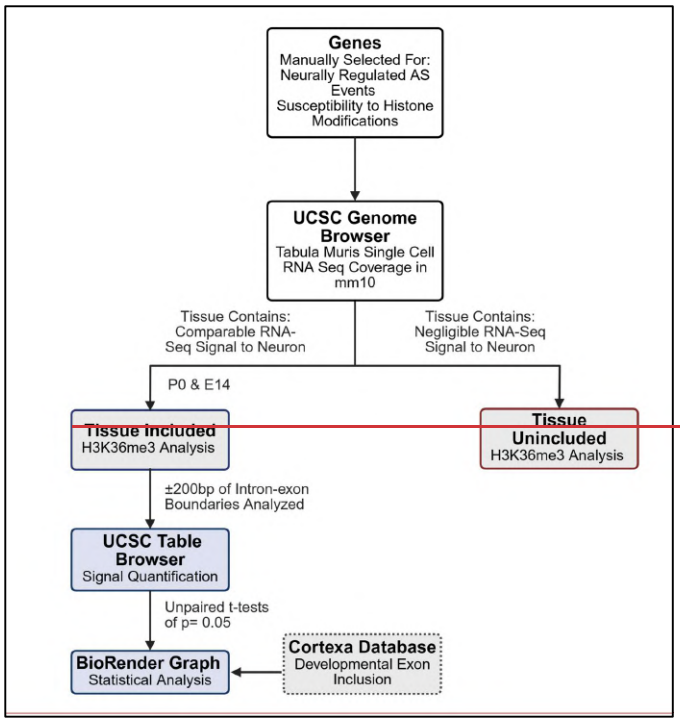
E16.5, and P0), were used to understand the relationship between histone modifications and alternative splicing during development. Additionally, the signal from embryonic 14 (E14) mice was also quantified to complement the Cortexa data discussed later. We analyzed the H3K36me3 signal ±200 bp from the intron-exon boundaries, which we decided upon from past literature (Agirre et al., 2021; Shindo et al., 2013). The tissues that the neuronal cell population was compared against were manually selected by observing if their expression levels were comparable to those of the neuron. Specifically, tissues were used if their flanking exons had an expression signature value of at least 10 % of the neuron's expression, to ensure that any differences in the splicing could not simply be attributed to the difference in expression. However, some tissues with RNA-Seq data could not be utilized for analysis due to the ENCODE dataset employed only containing 12 tissues.

Measuring developmental exon inclusion percentages

Cortexa database was used to ~~analzse~~analyze the changes in exon inclusion percentage in the cerebral cortex in mice with age (Weißbach et al., 2024). We searched the gene names "Bin1", "Pkm", "Cltb", and "Mef2a", which had the splicing events recorded. Although the coordinates of the splicing events in Cortexa did not exactly match those in the GENCODE VM23 Transcript set used in the UCSC Browser, the corresponding events were found as those with equivalent length of the relevantly spliced exons.

Statistical analysis

To quantify the RNA-Seq and ChIP-Seq data, the exact coordinates of the desired region were found using UCSC's Table Browser, with the following configurations: Clade: Mammal, Genome: Mouse, Assembly: Dec. 2011 (GRCm38/mm10), Group: Expression and Regulation, Track: Histone Modifications, Table: H3K36me3 [Selected Organ] [Selected Age]. From the Table Browser, we obtained the summary statistics that included the mean and the standard deviation. Mean and standard deviation values were used to generate graphs on BioRender Graphing (a newly added function to the visualization tool). We then performed unpaired two-tailed t-tests with alpha< 0.05, variances of the samples being homogenous, and normal distribution using BioRender Graphing.


Genes
Manually Selected For:
Neurally Regulated AS Events
Susceptibility to Histone Modifications
UCSC Genome Browser
Tabula Muris Single Cell RNA Seq Coverage in mm10
Tissue Contains: Comparable RNA-Seq Signal to Neuron
Tissue Contains: Negligible RNA-Seq Signal to Neuron
P0 & E14
Tissue Included
H3K36me3 Analysis
Tissue Unincluded
H3K36me3 Analysis
±200bp of Intron-exon Boundaries Analyzed
UCSC Table Browser
Signal Quantification
Unpaired t-tests of p= 0.05
BioRender Graph
Statistical Analysis
Cortexa Database
Developmental Exon Inclusion

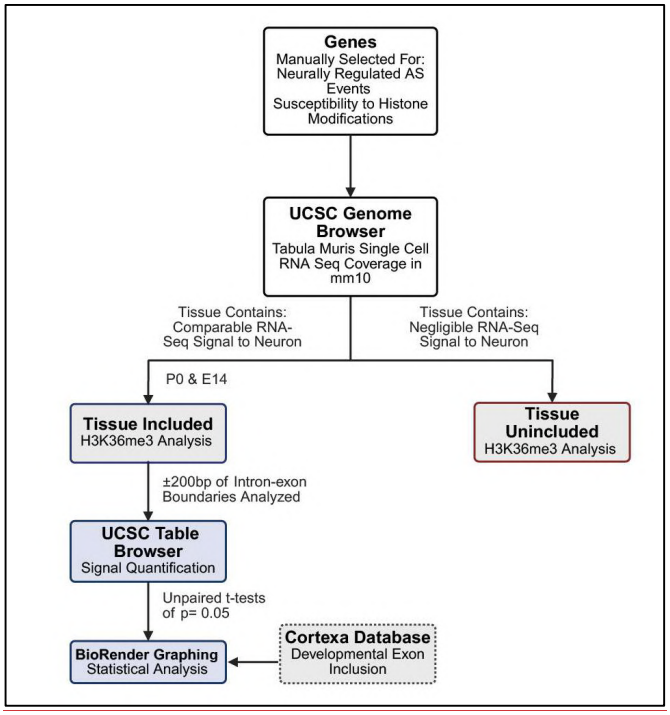


Figure 2: Methodological workflow of this study. Flowchart depicting the gene selection process, data visualization and analysis methods. The genes were selected based on their susceptibility to alternative splicing in neurons. The tissues were chosen if they had sufficient flanking exon expression signal relative to neurons. The UCSC Table Browser was used to obtain summary statistics, which were analyzed and plotted using the BioRender Graphing tool. The Cortexa database was used to analyze the changes in developmental exon inclusion percentages with changes in H3K36me3.

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3. Results

Exon 7 of Bin1 is differentially spliced in neurons

Analysis of Exon 7 of Bin1 revealed differential splicing between neurons and lung endothelia. Exon 7 encodes part of the BAR domain that has been implicated in dynamin regulation and regulation of membrane targeting specificity (Spooner & Dixon, 2025). Both flanking exons were expressed to comparable levels in both neurons and the lung endothelia, while neurons exclusively had exon 7 included. The percentage spliced in (PSI) relative to average RNA-Seq of its flanking exons for exon 7 were 88.5% and 1.15%, respectively (to 3 significant figures). This was in concordance with findings suggesting a functional role of exon 7 in controlling the efficiency of neural cell endocytosis by promoting Dnm2 interactions (Ellis et al., 2012). Analyzing the corresponding methylation pattern showed H3K36me3 signatures in the lung near the exon, which were outside of the exon region (quantified in the red box, Fig. 3A), while the midbrain had peaks both near and inside the region. Further quantification of H3K36me3 signal in the region displayed significant differences in H3K36me3 signal between the lung and all three areas of the fore, mid and hindbrain for Bin1, where the signal appeared greater in all areas of the brain. However, among the genes analyzed in this study, Bin1 is the only gene to display significant organ-specific H3K36me3 signals (Fig. 3B). We next asked if Exon 7 is developmentally regulated in the mouse brain. The inclusion level of the exon in the mouse brain increases significantly from E14 to E16 (78.6% to 93.8%, respectively), but not from E16 to P0 (Fig. 3C); however, no significant differences were found between the H3K36me3 signals of the brain samples aged E14.5 to P0 for all three brain regions (Fig. 3D).

There was a limitation to this data, which was that the H3K36me3 data were derived from a bulk assay of many cell types within the tissue, while the RNA-Seq data were cell-specific. Thus, we have included the RNA-Seq data of all the types of brain cells for which the data are accessible in the Tabula Muris tracklist in part E, including the neuron, astrocyte, OPC, microglia, and oligodendrocytes. The splicing patterns were distinct, where neurons had a high degree of inclusion of exon 7, while OPC, astrocytes, and oligodendrocytes had much lower inclusion, and microglia had no observable inclusion. The expression of the gene itself varied widely, where the microglia and oligodendrocytes had much greater RNA-Seq signal than the rest. (Fig. 3E) This was in concordance with past literature on Bin1 (Dourlen et al., 2025).

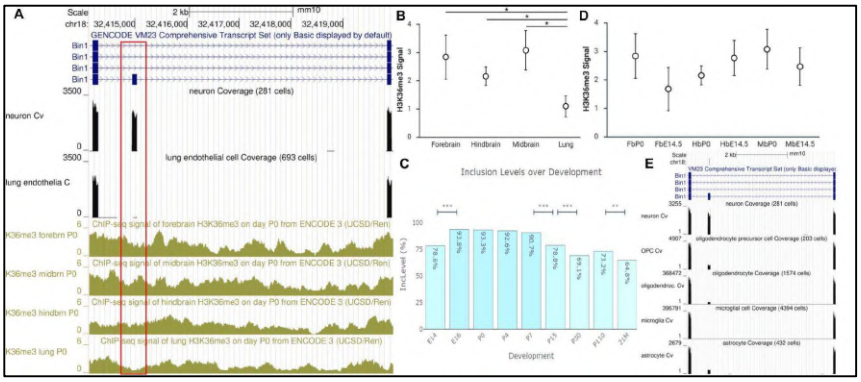

Figure 3: mRNA and H3K36me3 and alternative splicing analyses of Bin1 Exon 7. (A) The RNA-Seq data of neurons (281 cells) and lung endothelia (693 cells) visualized along with the associated tissues' H3K36me3 signals. **(B)** A graph where the

circle represents the mean and the tails/error bars represent the standard deviation (SD) of the H3K36me3 signal of the four different tissues. P values: 0.0251 (Forebrain - Lung), 0.0123 (Midbrain - Lung), 0.0208 (Hindbrain - Lung). 495 bases analyzed in (B) and (D). **(C)** The changes in inclusion levels over development, adapted from Cortexa (Weißbach et al., 2024). **(D)** Graph of H3K36me3 signal of the three brain regions (Forebrain - Fb, Midbrain - Mb, Hindbrain - Hb) from Age E14.5 to P0, where the circle represents the mean, and the error bars represent the SD (B and D, quantified region: chr18:32,414,746-32,415,240 | NM_001083334). (FbP0 – FbE14.5: p>0,05), (HbP0 – HbE14.5: p>0,05), (MbP0 – MbE14.5: p>0,05). **(E)** RNA-Seq data of the neuron, OPC, oligodendrocyte, microglia, and astrocyte. Their y-axes are not normalized at the microglia despite its 100-fold greater expression to emphasize the neuron-specific splicing pattern. P-values: * < 0.05, ** < 0.01, *** < 0.001. Note: Graphs without indicated statistical significance indicate a lack of significant differences.

Skipping of exon 9 of Mef2a is differentially regulated in neurons

Similar to Bin1, Exon 9 of Mef2a also displayed differential splicing between the neuron and the lung endothelia, where~~with~~ the lung exclusively excluded it (PSIs were 67.5% to 2.11%, respectively)~~ing it~~, while the cardiac muscle was spliced similarly to the neuron (PSI 84.7%). The cardiac muscle is shown for comparison with the neuron. Exon 9's function is unknown, but it was more skipped in patient's hearts with Type 2 diabetes (Nutter et al., 2016). The flanking exons of all the neuron, heart, and lung were comparably expressed. All quantified regions had small peaks within them, with generally similar H3K36me3 signature patterns across the tissues in the visible areas. (Fig. 4A) Quantification of the regions indicated no significant differences between the H3K36me3 signals of any tissues, where all five tissues had very similar mean signatures. (Fig. 4B) We then asked whether Exon 9 was developmentally regulated in the mouse brain. Although there appeared to be a general trend of increasing exon inclusion, there were no significant differences between E14 and P0, the ages studied. (Fig. 4C) Similarly, we found no significant differences between the H3K36me3 signatures in all three brain regions. (Fig. 4D)

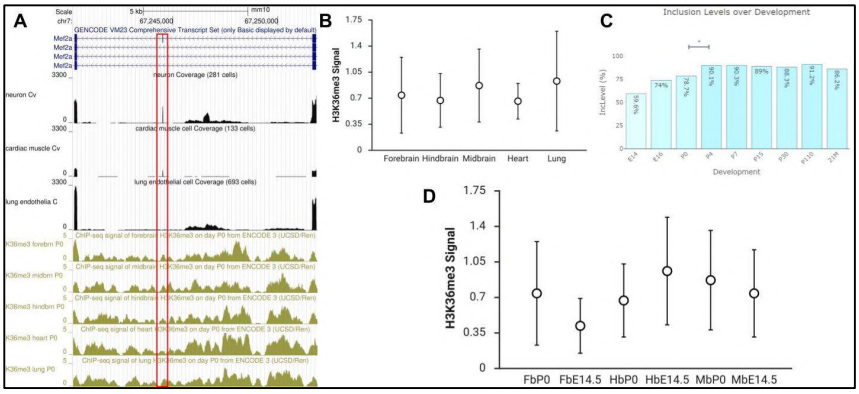

Figure 4: mRNA and H3K36me3 and alternative splicing analyses in Mef2a Exon 9. (A) The RNA-Seq data of neuron (281 cells), cardiac muscle (133 cells), and lung endothelia (693 cells) visualized along with the associated tissues' H3K36me3 signals. **(B)** A graph where the circle represents the mean and the tails/error bars represent the standard deviation (SD) of the H3K36me3 signal of the five different tissues. (Forebrain – Heart, Lung: p>0,05), (Hindbrain – Heart, Lung: p>0,05), (Midbrain – Heart, Lung: p>0,05). 425 bases analyzed in (B) and (D). **(C)** The changes in inclusion levels over development, adapted from Cortexa (Weißbach et al., 2024). **(D)** Graph of H3K36me3 signal of the three brain regions from Age E14.5 to P0, where the circle represents the mean, and the error bars represent the SD (B and D, quantified region: chr7:67,244,319-67,244,743 | NM_001291192). (FbP0 – FbE14.5: p>0,05), (HbP0 – HbE14.5: p>0,05), (MbP0 – MbE14.5: p>0,05). P-values: * < 0.05, ** < 0.01, *** < 0.001. Note: Graphs without indicated significance indicate a lack of significant differences.

Exon 5 of Cltb is differentially spliced in neurons and developmentally regulated

Next, Exon 5 of Cltb also exhibited differential splicing, where neurons exclusively included it, which was concordant with the exon 5 containing isoform being neuron-specific in mice (Das et al., 2021b). Specifically, the PSIs were 87.8% in the neuron, 0.0680% in the hepatocytes, and 0.794% in the lung endothelia. Both flanking exons are comparably expressed in all tissues. Peaks were noticeable in the quantified regions of the hindbrain, liver, and lung. (Fig. 5A) Further quantification evinced the lack of significant differences between the tissues' trimethylation signatures. Forebrain and midbrain had markedly smaller standard deviations, which suggested that they did not contain peaks in the region. (Fig. 5B) When we next asked if Exon 5 was developmentally regulated, we found two significant differences in inclusion in the analyzed timeframe of E14 to P0, increasing from 17.5% to 30.7% from E14 to E16 and from 30.7% to 38.5% from E16 to P0 ~~respectively~~. (Fig. 5C) Subsequent quantification demonstrated a significant difference between the H3K36me3 signature of E14.5 and P0 Forebrain, an increase with age, while the hind and midbrain showed no significance. (Fig. 5D)

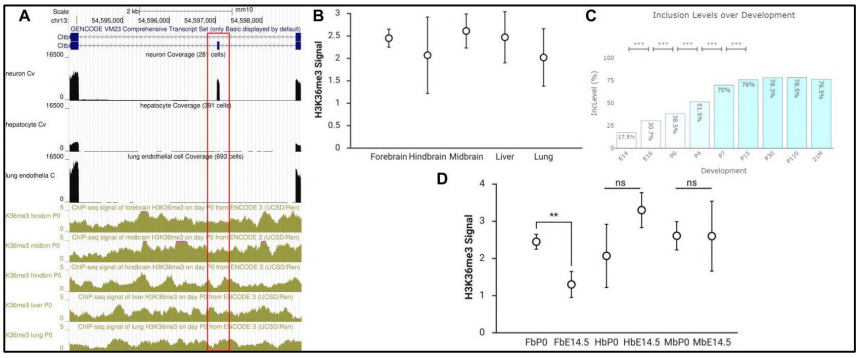

Figure 5: mRNA and H3K36me3 and alternative splicing analyses in Cltb Exon 5. (A) The RNA-Seq data of neuron (28 cells), hepatocyte (391 cells), and lung endothelia (693 cells) visualized along with the associated tissues' H3K36me3 signals. **(B)** A graph where the circle represents the mean and the tails/error bars represent the standard deviation (SD) of

the H3K36me3 signal of the five different tissues. (Forebrain – Heart, Lung: p>0.05), (Hindbrain – Heart, Lung: p>0.05), (Midbrain – Heart, Lung: p>0.05). 456 bases analyzed in (B) and (D). **(C)** The changes in inclusion levels over development, adapted from Cortexa (Weißbach et al., 2024). **(D)** Graph of H3K36me3 signal of the three brain regions from Age E14.5 to P0, where the circle represents the mean, and the error bars represent the SD (B and D, quantified region: chr13:54,596,823-54,597,278 | NM_028870). P value: 0.00781 (FbP0 - FbE14.5). (HbP0 – HbE14.5: p>0.05), (MbP0 – MbE14.5: p>0.05). P-values: * < 0.05, ** < 0.01, *** < 0.001. Note: Graphs without indicated significance indicate a lack of significant differences.

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Mutually exclusive exons 9 and 10 of Pkm are differentially spliced in neurons

Exons 9 and 10 of Pkm (Pkm1 and 2, respectively) were mutually exclusive and exhibited differential splicing, where the neuron and cardiac muscle tended to include Exon 9 preferentially (PSIs expressed as (Exon 9 PSI, Exon 10 PSI) were (74.7%, 13.1%) and (84.1% vs 4.23%), respectively), while the lung endothelia appeared to prefer inclusion of Exon 10 (PSI (14.3% vs 62.7%)). The sole inclusion of exon 9 forms the isoform Pkm1, which was more commonly found in the brain and heart (Li et al., 2024), compared to the counterpart Pkm2 that exclusively includes exon 10 that enhances tumorigenesis, which has been found elevated in lung adenocarcinoma (Zhu et al., 2021). The trimethylation tracks display larger peaks in the quantified region of Exon 9 than in Exon 10. (Fig. 6A) In analyzing both exons, we found no significant differences in either, although the lung appeared to have a smaller signal than both the neuron or cardiac muscle. (Fig. 6B & 6C) Following this, we examined whether these exons were developmentally regulated. The event saw two significant changes in preference for inclusion with age from E14 to P0. Specifically, Exon 9 inclusion was increasingly preferred with age (34.5% to 67.5% to 81.6% from E14 to E16 to P0), while Exon 10 inclusion preference decreased by equal proportions. (Fig. 6D) Additional quantification of the trimethylation signature indicated the absence of significant differences in the region of Exon 9, but also a significant difference and decrease in H3K36me3 from E14 to P0 Midbrain. The other regions did not signify any significant changes in Exon 10, however. (Fig. 6E & 6F)

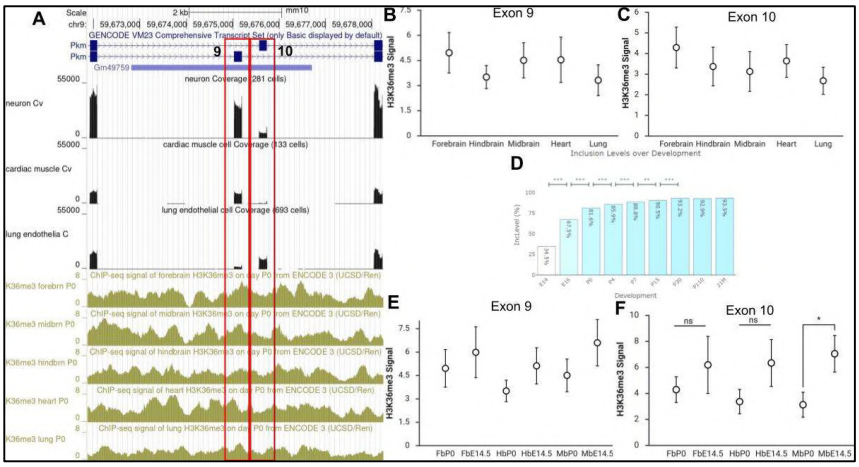

Figure 6: mRNA and H3K36me3 and alternative splicing analyses in Pkm Exons 9 and 10. (A) The RNA-Seq data of neuron (281 cells), cardiac muscle (133 cells), and lung endothelia (693 cells) visualized along with the associated tissues' H3K36me3 signals. **(B and C)** Graphs where the circle represents the mean and the tails/error bars represent the standard deviation (SD) of the H3K36me3 signal of the five different tissues. (Forebrain – Heart, Lung: p>0,05), (Hindbrain – Heart, Lung: p>0,05), (Midbrain – Heart, Lung: p>0,05). 570 bases analyzed in (B, C) and (E, F). **(D)** The changes in inclusion levels over development, adapted from Cortexa (Weißbach et al., 2024). In this instance, the percentages are calculated such that the percentage inclusion of exon 9 (shown above) and the percentage inclusion of exon 10 add up to 100%. **(E and F)** Graph of H3K36me3 signal of the three brain regions from Age E14.5 to P0, where the circle represents the mean, and the error bars represent the SD (B, C, E and F, Quantified regions: Exon 9: chr9:59,674,826-59,675,395, Exon 10: chr9:59,675,364-59,675,930 | NM_011099). P value: 0.0164 (MbP0 - MbE14.5). (FbP0 – FbE14.5: p>0,05), (HbP0 – HbE14.5: p>0,05), (MbP0 – MbE14.5: p>0,05). -P-values: * < 0.05, ** < 0.01, *** < 0.001. Note: Graphs without indicated significance indicate a lack of significant differences.

4. Discussion

To understand the co-regulation of H3K36me3 and alternative splicing, we investigated four genes. Our findings suggest that differences in splicing across tissues for neurally regulated alternative splicing events may be and in part, regulated by H3K36me3 in Bin1, but less so in other genes. Tissue-specific splicing of Bin1 being correlated to H3K36me3 and not Mef2a or Cltb, was consistent with the literature (Forn et al., 2013; Sharma et al., 2014; Shindo et al., 2013). T-his tissue-specific difference in H3K36me3 presence could potentially lead to further analysis of non-neural regulation of alternative splicing to uncover targets to modulate to influence and reduce aberrant splicing in Alzheimer's brain tissues.

Pkm2 was known to be affected by H3K36me3 in PNT2 cells compared to hMSCs, yet it dido~~es~~ not appear to be a major

factor for tissue-specific splicing (Luco et al., 2010). This discrepancy may be due to how we quantified the H3K36me3 signals for the region containing the mutually exclusively spliced exons, while they compared the signal for upstream exons and introns as well. Otherwise, different~~Other~~ factors, such as nucleosome occupancy, histone variants, and chromatin remodelling complexes, could be more significant components that affect splicing outcome across organs. However, this study only indicated correlations; thus, perturbation experiments are recommended to further understand H3K36me3's role in alternative splicing in interesting cases such as Bin1, as well as its potential functions in inter-tissue communication in regard~~s~~ to splicing.

There was no evidence of H3K36me3 being correlated with changes in developmental splicing outcomes in Bin1 and Mef2a, ~~but~~ although it was ~~did so~~ in Cltb and Pkm. However, Cltb and Pkm both had greater developmental changes in splicing then Bin1 and Mef2a (increase of 21% and 47.1% against 14.7% and 19.1%, respectively) and significantly increased from E14 to E16 and from E16 to P0, while Bin1 and Mef2a did not. Thus, we hypothesize that changes in H3K36me3 may be associated with a threshold for changes in splicing outcome, which could be a potential analysis in future investigations. ~~although we have not tested this further.~~ The observation in Cltb was supported, as Cltb was known to be regulated by alternative splicing in a developmental stage-specific manner (Blue et al., 2018). The correlation was also corroborated by previous literature in mouse tissue (Hu et al., 2020). However, this study was also based on predictions and data analysis; thus, we once again recommend experiments be carried out to examine this relationship. Furthermore, as the Cortexa database used only covers neural alternative splicing events, similar research in other tissues and organs may also be fruitful. However, splicing, being known to affect the chromatin landscape in bilateral communications, could complicate the directionality of the relationship (Yustis et al., 2024).

A limitation of this research was that, although H3K36me3 was typically known to enhance exon inclusion, there are modifications that were known to silence exons (Shindo et al., 2013; Zhou et al., 2014). These were not able to be accounted for due to time and technological constraints; however, this could have also been affecting the splicing outcome by being increased or removed between tissues instead of H3K36me3 or through various combinations of them that were known to affect differential splicing (Chen et al., 2019). For example, Mef2a is a primary downstream target of the calcium-CaMKII/PKD-HDAC pathway, which can phosphorylate histones and cause hyperacetylation that could possibly be affecting Mef2a splicing instead of H3K36me3 (Sharma et al., 2014). Similarly, Cltb regulation across normal tissue to adenomas was associated with a change in H3K4me3 and H3K27me3 (Forn et al., 2013), and H2BK12ac, H4K5ac, and H3K4me3 was enriched in Bin1 exon regions in IMR90 cells (Shindo et al., 2013).

The transcriptional elongation speed of RNAP II could have also affected the splicing patterns, but they could not be accounted for in this study (Zhou et al., 2014). Lastly, the methylation data were not cell-specific, so perturbation experiments could be difficult to accurately perform.

The research on two cases of correlation between developmental alternative splicing and H3K36me3 may hopefully drive experimental studies. This could then illuminate H3K36me3 as a potential target of aberrant alternative splicing during development. Specifically, it could potentially address errors in embryo growth, as it is known that embryo growth retardation is likely caused by Pkm1 increase and Pkm2 decrease, as Pkm2 was highly expressed in embryos and essential for embryo development (Lin et al., 2021).

5. Conclusions

This study added evidence for H3K36me3's potential for altering differential splicing across tissues in Bin1, while

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suggesting that other factors, in part, act as key contributors for future investigations, and uncovered two cases where differences in H3K36me3 were associated with differences in developmental splicing in this investigation of histone modifications' effects on differential splicing. Although there were certain constraints, such as counter-balancing modifications and elongation speed of RNAP II, perturbation experiments could fully uncover the connections between H3K36me3, tissue-specific and ~~and~~ developmental splicing that were previously underexplored, ~~,~~ which could drive further investigation of the epigenetic regulation of tissue-specific and developmental conditions~~new perspectives~~. ~~Into~~ future studies, H3K36me3 could potentially be a therapeutic target for embryo growth retardation and avenue for resolving aberrant splicing in ~~-~~ Alzheimer's.

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Data & Code Availability Statement

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Following publicly-available databases used in this study.

TabulaMurisSingleCellData:
https://genome.ucsc.edu/cgi-bin/hgTrackUi?hgsid=2303618686_hFf8vDaGo1RWvlEehhPpGvHhSniv&db=mm10&c=chr2&g=tabulaMuris

ChIP-Seq:
https://www.encodeproject.org/chip-seq/histone-encode3/

Bin1:
https://genome.ucsc.edu/s/RWucscgb/mm10_H3K36me3_RNA%2DSeq_UCSC_Bin1_AllOrgans_FigureV1

Mef2a:
https://genome.ucsc.edu/s/RWucscgb/mm10_H3K36me3_RNA%2DSeq_UCSC_Mef2a_AllOrgans_FigureV1

Cltb:
https://genome.ucsc.edu/s/RWucscgb/mm10_H3K36me3_RNA%2DSeq_UCSC_Cltb_AllOrgans_FigureV1

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Cortexa:
https://cortexa-01.zdv.uni-mainz.de/

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All figures in this manuscript were made with the help of BioRender.

Author Biography

~~The author~~Ryota Watanabe is a 16 year-old student researcher of chromatin biology at Crimson Global Academy in~~in~~ Japan. Inspired by the immense compaction in DNA packaging, h~~H~~e enjoys investigating the mechanisms of chromatin regulation through epigenetics. Additionally, he hopes to apply his theoretical research to improving the lives of the rare disease community, and runs an educational YouTube channel about rare diseases. He hopes to major in molecular biology in university.

Mentor Contribution Statement

~~[You must send us a detailed mentor contribution statement of at most 300 words describing your mentor's explicit role(s) and contribution(s) in the writing of your manuscript. There should be one statement per mentor, and each statement should be written in third-person.]~~

~~[redacted]My mentor is a mentor at Indigo Research and a University of Southampton Lecturersupervised my project. He was the sole supervisor of this project. Specifically, he met with the author weekly over Zoom to provide advice and feedback on the author's work. He suggested the names of suitable databases, which the author then analyzed and asked questions about. He verified the data analysis and proofread the manuscript multiple times for logical or sophistication errors, but these were prepared wholly by the author themselves.~~

The mentor for this research project was Dr Kif Liakath-Ali, ~~[Lecturer at the University of Southampton, UK.]~~ He served as the sole supervisor throughout the work. He met with the author weekly via Zoom to provide guidance, discuss progress, and offer critical feedback. He recommended relevant databases for analysis, which the author independently explored and interrogated. Dr Liakath-Ali reviewed the data analysis and read multiple drafts of the manuscript to identify issues of logic, clarity, or sophistication; however, all analyses, interpretations, and written materials were prepared entirely by the author.

~~he suggested suitable databases, reviewed my data analysis, and proofread my manuscript and figures.~~

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