Generating CRISPR tools to investigate epigenetic changes during brain organoid development

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Abstract

This project focuses on utilizing molecular biology techniques to investigate epigenetic changes in brain organoids by targeting specific genes and proteins. The study involves the construction of key genetic constructs, including **TIGRE-dCas9-GCN4** and **pVLX_KDM5A_scFV-gRNA**, to modulate gene expression. Through the design of guide RNAs targeting *NEUROD2*, and using the demethylase *KDM5A*, the project aims to elucidate the impact of epigenetic modifications on gene regulation and chromatin remodeling processes. The flexibility of the protocol enables seamless targeting of other regions and epigenetics modifications. The inclusion of control constructs and the utilization of GFP as a reporter gene will enhance the robustness and specificity of the results.

1. Theoretical background

1.1. Epigenetic marks

In eukaryotes, DNA wraps around histone octamers to form nucleosomes, which can be modified chemically. These modifications on histone tails, like methylation and acetylation, affect chromatin structure and gene accessibility without changing DNA sequence. Understanding the impact of these modifications on gene expression requires inducing their gain or loss in neutral regions to assess causality. A specific modification, ***H3K4me3***, is associated with active gene promoters, while *H3K9me3* and *H3K27me3* are linked to transcriptional regression (Policarpi et al., 2022). The correlation between *H3K4me3* and transcription exists, but to investigate causality, experiments inducing loss of *H3K4me3* by a histone demethylase are necessary to determine if transcription is downregulated there.

1.2. Molecular tools

To achieve our goal of precisely modifying specific epigenetic marks, we will utilize the **CRISPRoff** system with a catalytically dead Cas9 (**dCas9**) to target genomic loci without cutting DNA (Nuñez et al., 2021). The guide RNA (**gRNA**) will direct the dCas9 to the desired locus, enabling us to modify epigenetic marks without altering the genetic sequence. Additionally, we plan to introduce the demethylase enzyme **KDM5A**, known for its ability to remove methyl groups from (*H3K4me3*), to induce the desired epigenetic changes (Torres et al., 2015). The employment of the **GCN4** tag will facilitate the targeted recruitment of *KDM5A* to specific genomic loci, via **GCN4-scFV**.

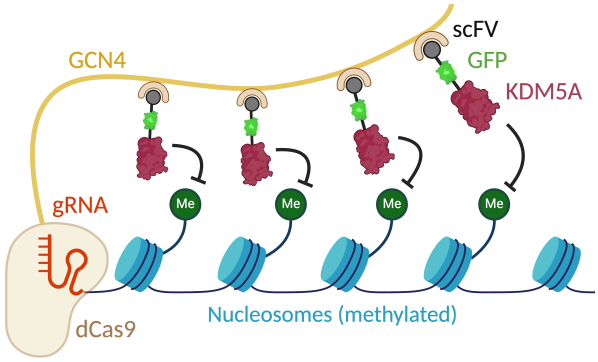

Figure 1: Schematic of the experimental setup

Moreover, we will incorporate the fusion protein **T2A-EGFP**, consisting of a self-cleaving peptide T2A derived from a virus and the enhanced green fluorescent protein (EGFP). This fusion protein serves as a powerful tool for visualizing and tracking cells that have successfully expressed the modified proteins, allowing us to monitor the efficacy of our setup.

2. Applied experiments

2.1. Protocols

The first step of the experimental design is to create primers for the fusions and sequencing, and synthesize gRNAs targeting *NEUROD2*. Two key constructs are then designed: separating *dCas9* and *KDM5A* in the experimental design allows flexibility and optimizes functionality with different controls.

The *TIGRE-dCas9-GCN4* plasmid is assembled through **Gibson Assembly** with a backbone and 2 inserts. The backbone, extracted from *pMM1* via restriction digest, contains the **CAGGS promoter** for transcription and **homology arms** for precise integration of the *dCas9* construct. Gene components, *dCas9-GCN4* and *BSD* resistance, are obtained through PCR amplification, confirmed on agarose gel, and purified for integration.

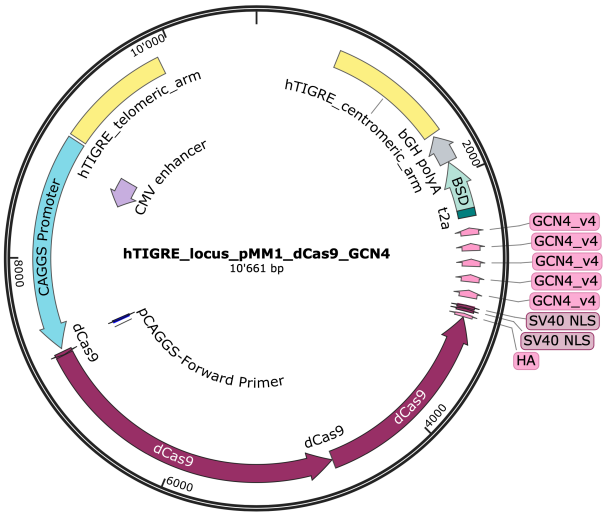

Figure 2: *TIGRE-dCas9-GCN4* plasmid map

The second construct requires growing multiple intermediate products that lead to the *KDM5A-scFV-T2A-EGFP* fusion protein. Initial steps involve PCR amplification of *GCN4-scFV* and *T2A-EGFP*, followed by **fusion PCR** assembly leveraging primer overhangs for homology regions. Then the product was purified, cloned into **pJet** for blunt-end insertion, verificatied through colony PCR and restriction analysis, and integrated with *NEUROD2 gRNA*. The most intricate phase entails cloning *KDM5A* into the plasmid, as **cDNA** amplification yield may vary based on RNA purity and PCR efficiency.

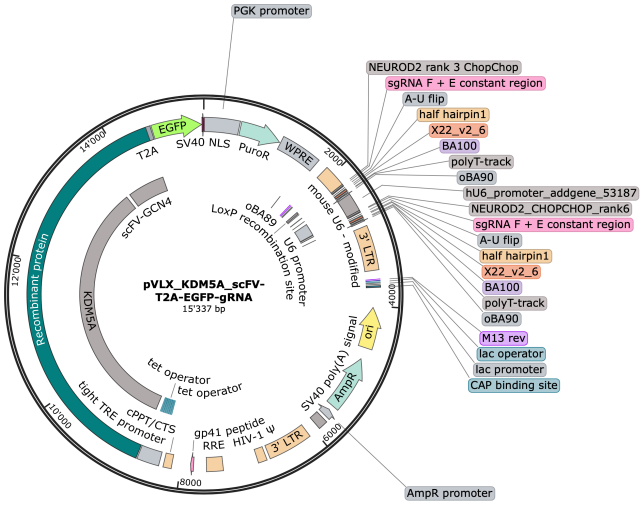

Figure 3: *pVLX_KDM5A_scFV-gRNA* plasmid map

Once both constructs have been obtained, the next step is to transfect these constructs into brain organoids, and using GFP fluorescence as a readout for successful expression and localization.

2.2. Results

Initially, the gene amplification process was smooth, but challenges arose during Gibson assembly. Despite successful isolation and purification of *dCas9-GCN4*, *BSD*, and *pMM1* backbone products, combining them into a plasmid failed to yield colonies post-transfection. Various strategies like adjusting incubation times, altering molar ratios, and attempting fusion PCR before ligation were employed without success.

The fusion PCR yielded contradictory outcomes. While the isolated and purified *scFV-EGFP* PCR fusion product matched the expected size, discrepancies emerged during ligation into pJet and sequencing. Specifically, a 200bp section was **absent** in the *scFV-GCN4* region of the fused product in two instances. To address this issue, isolating the *scFV* in pJet before fusion may be necessary as the problem appears to stem primarily from this component rather than *EGFP*.

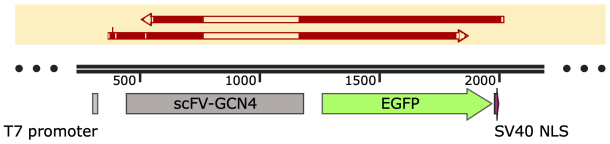

Figure 4: Missing region in *pJet-scFV-T2A-EGFP*

Despite fusion PCR setbacks, *KDM5A* amplification from cDNA was attempted. However, the amplification process resulted in multiple bands on the gel, none matching the expected size. This suggests the need to **reisolate cDNA** from a purer RNA source. To achieve this, RNA integrity from various sources is quantified by denaturing fragments with *formaldehyde* and running a gel to visualize 18S and 28S bands. Following that, cDNA is synthesised using reverse transcription, and gene halves are amplified for fusion using sequencing primers and restriction enzymes. Although successful with the second gene half, achieving the desired size for the first half of *KDM5A* remains difficult.

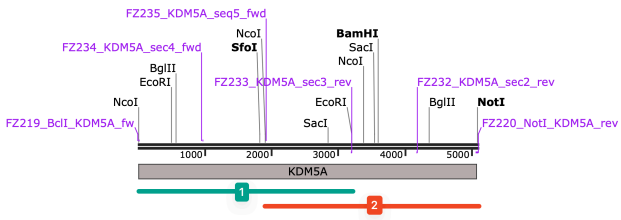

Figure 5: *KDM5A* partial amplification map with sequencing primers

3. Further experimentations

The next phase of the project involves **co-transfecting** both *TIGRE-dCas9-GCN4* and *pVLX_KD-M5A_scFV-gRNA* constructs into brain organoids. By utilizing GFP fluorescence as a readout for successful expression and localization, the presence of the modified proteins within the organoids can be visually confirmed. Subsequently, the impact of demethylation can be assessed and **quantified** by measuring changes in gene expression levels using quantitative real-time polymerase chain reaction (**qRT-PCR**). This analytical approach enables a precise quantitative evaluation of gene expression alterations induced by demethylation, facilitating a comparative analysis of gene expression profiles before and after the demethylation process.

Additionally, to further investigate the epigenetic modifications induced by *KDM5A* and their effects on gene expression, chromatin immunoprecipitation (**ChIP**) assays can be employed. This technique involves using specific antibodies to target proteins associated with DNA, enabling the identification of protein-DNA interactions. By performing ChIP with antibodies specific to histone modifications affected by *KDM5A*, such as *H3K4me3*, the changes in chromatin structure and gene expression can be elucidated.

3.1. Controls

To validate the specificity and efficacy of the experimental approach, several controls could be included:

- Empty vector controls: no colonies post-transfection due to the lack of antibiotic resistance
- Non-targeting gRNA constructs to assess off-target effects: to put aside any non-specific effect on gene expression induced by the transfection itself
- Constructs lacking the *KDM5A* or *GCN4* components to determine their contributions to observed changes: to specifically verify the *dCas9* loci targetting using GFP (but without demethylase)
- GFP-only constructs: to monitor transfection efficiency and organoid viability.

3.2. Flexibility and adaptability

The experimental setup allows for the **substitution** of the methylase component, like *KDM5A*, with demethylases such as *KDM6B* or *LSD1*, offering adaptability to explore varied effects on histone methylation and gene expression in brain organoids.

This flexibility, described in step 7 of the protocol and in (Policarpi, Munafò, Tsagkris, Carlini, & Hackett, 2022), enables the investigation of **different epigenetic modifiers** and their influence on gene regulation. This comparative analysis enhances understanding of epigenetic mechanisms in neural development and disease conditions, providing insights into the complex processes governing neural development and associated pathologies.

4. Conclusion

The project's focus on utilizing CRISPR tools to investigate epigenetic changes during brain organoid development has significant implications for advancing our understanding of epigenetic regulation in neural development. Creating a flexible mechanism to quickly induce epigenetic modifications at specific loci will enhance the knowledge of epigenome characterisation. By further exploring diverse epigenetic modifiers, this research has the potential to shed light on the complex relationship between epigenetic modifications and gene expression in brain organoids.

References

Figure 1 is adapted from (Policarpi et al., 2022, fig. 1A) and has been created using BioRender.com.

Figure 2-5 have been created using the SnapGene software (www.snapgene.com).

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