

Examining the effect of repeated cocaine exposure on epigenetic modifications within the nucleus accumbens

Amanda Chow¹, Ramesh Chandra², Kara Cover², Michel Engeln², T. Chase Francis², Shweta Das², Mary Kay Lobo²

¹University of Maryland, College Park, MD 20742, ²Department of Anatomy and Neurobiology, University of Maryland School of Medicine, Baltimore, MD 21201

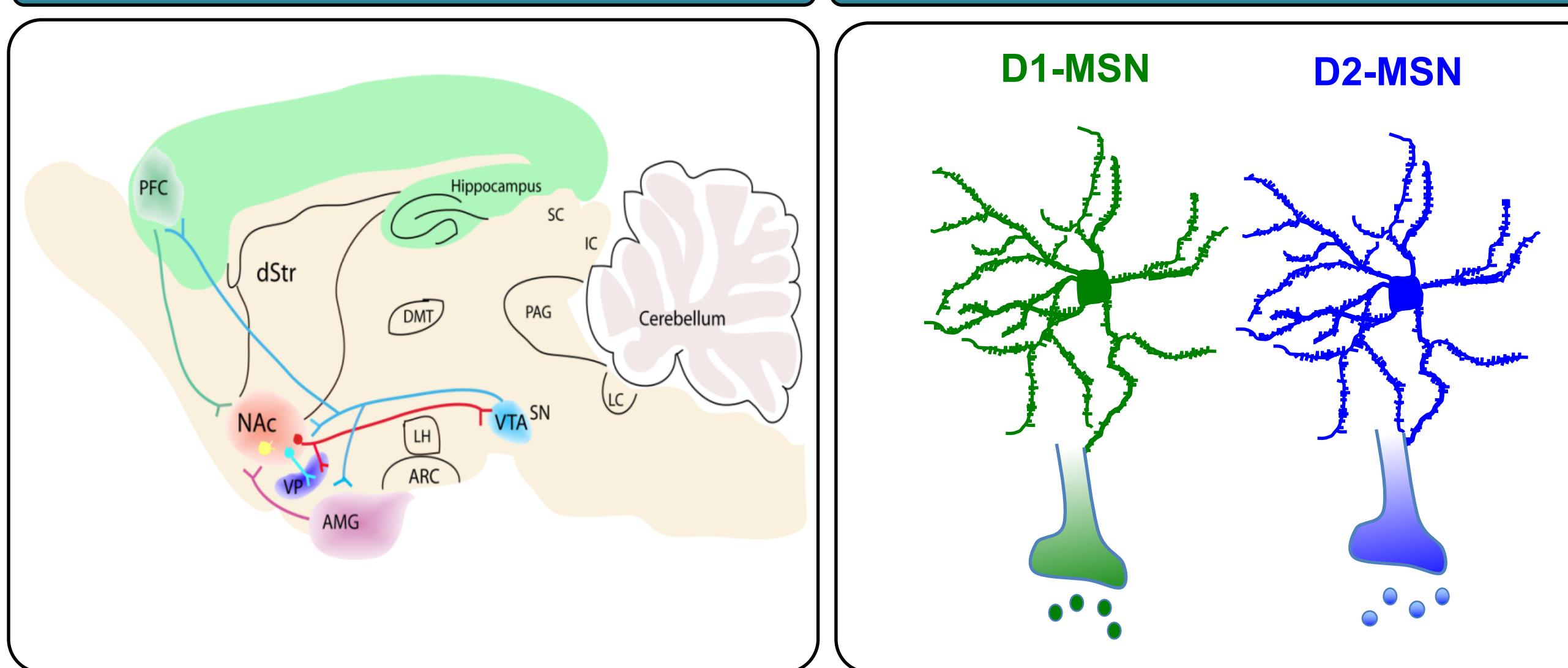
Introduction

The nucleus accumbens (NAc) is a brain region known for its pivotal role in the reward circuit, which mediates motivation for seeking rewards. Repeated exposure to addictive drugs such as cocaine can modify the reward pathway in the NAc. Previous studies have demonstrated that the transcriptional regulator, Egr3, is essential to modifying the transcription of many genes involved in cocaine-mediated plasticity; however, there is little information about the mechanism by which Egr3 itself is epigenetically regulated. To provide more information on this, we examined the enrichment of specific epigenetic markers on the promoter of Egr3 and on the promoter of its co-repressor, Nab2. These epigenetic markers have previously been shown to be involved in drug addiction. Mice were administered intraperitoneal injections of cocaine (20mg/kg) or saline once a day for seven days. Then, they underwent a 24-hour period of withdrawal before NAc tissue was collected. We performed chromatin immunoprecipitation (ChIP) to examine enrichment of H3K4me3, H3K27me3, H3K9me2, and KDM1A across Egr3 and Nab2 promoters after cocaine administration versus after saline administration. The lysine modifications are able to either activate or repress gene expression, while KDM1A is a lysine demethylating enzyme (KDM) that demethylates di- or mono-methylated H3K4 or H3K9. From these data and our previous data demonstrating that Egr3 and Nab2 bidirectionally regulate dopamine 1 receptor enriched medium spiny neurons (D1-MSNs) or dopamine 2 receptor enriched MSNs (D2-MSNs), we were able to infer in which MSN subtype these epigenetic marks occur. Following this, we used the CRISPR-Cas9 system to specifically demethylate certain epigenetic markers. To do this, we will create (1) a mutant of Cas9 fused to CIBN, and (2) a mutant of CRY2 fused to KDM1A. Under blue light, these two mutants will bind, and KDM1A will be able to demethylate at a targeted place on the Egr3 promoter. Currently, we are screening guide RNAs to target the Cas9-CIBN complex to specific locations on the Egr3 promoter. Overall, this will allow us to examine the exact mechanism and epigenetic modifications involved in cocaine-mediated plasticity.

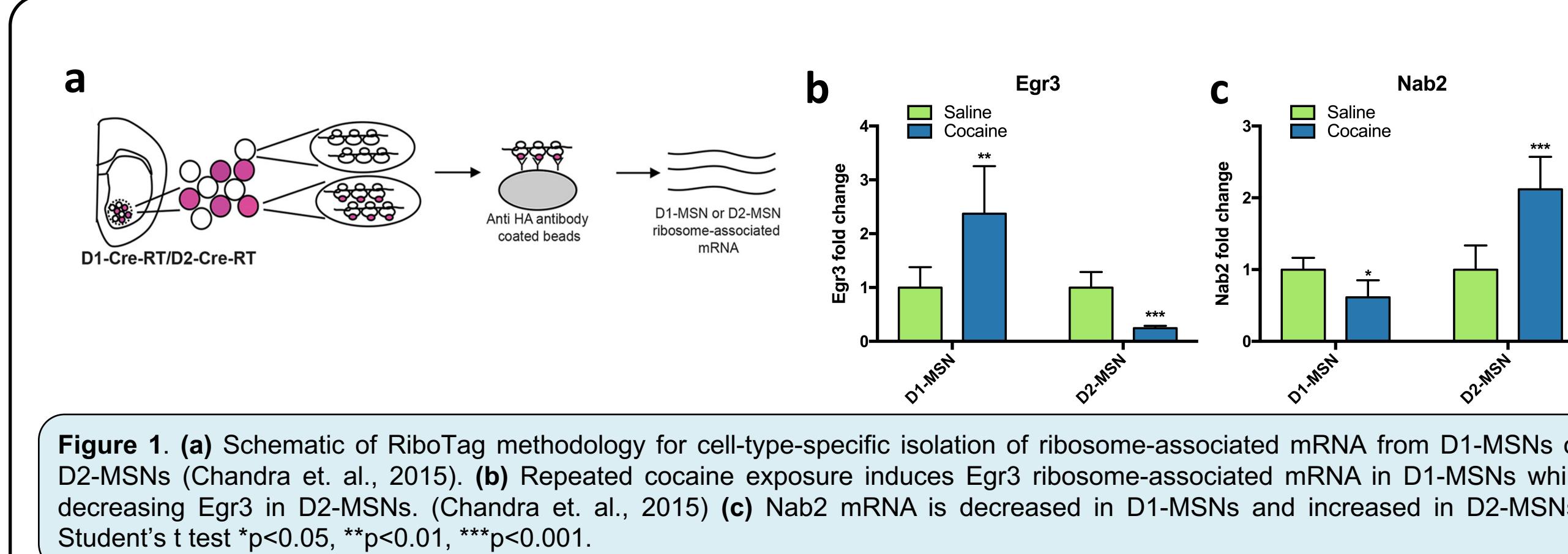
Aims

- Examine enrichment of histone marks H3K4me3, K3K27me3, H3K9me2 on Egr3 and Nab2 promoters in NAc tissue after 7 days of cocaine injection (20mg/kg).
- Examine binding of histone demethylase enzyme KDM1A on Egr3 and Nab2 promoters in NAc tissue after 7 days of cocaine injection (20mg/kg).
- Design a CRISPR strategy to alter lysine methylation at specific loci, the Egr3 promoter, by cloning guide RNAs and cloning Cre-dependent constructs CRY2:KDM1A and dCas9:CIBN.

Mesolimbic reward pathway



NAc cell-type specific ribosome-associated mRNA analysis from D1-Cre-RT and D2-Cre-RT mice



Feedback mechanism for Egr3 transcription

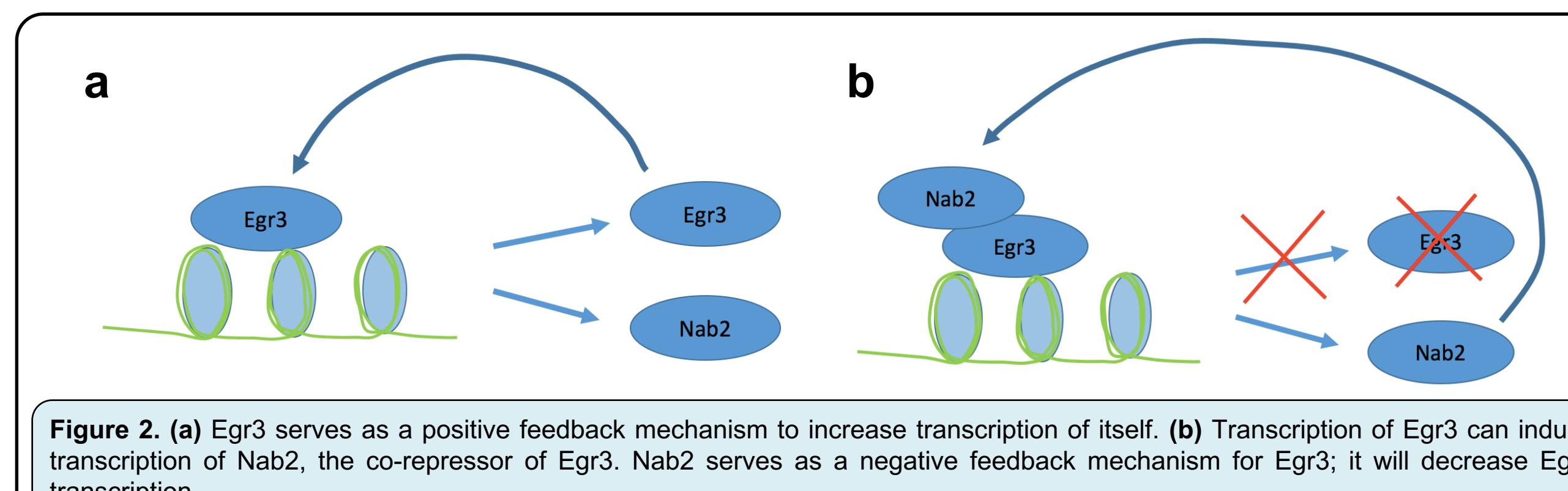


Figure 2. (a) Egr3 serves as a positive feedback mechanism to increase transcription of itself. (b) Transcription of Egr3 can induce transcription of Nab2, the co-repressor of Egr3. Nab2 serves as a negative feedback mechanism for Egr3; it will decrease Egr3 transcription.

Epigenetic markers of transcription activation and repression

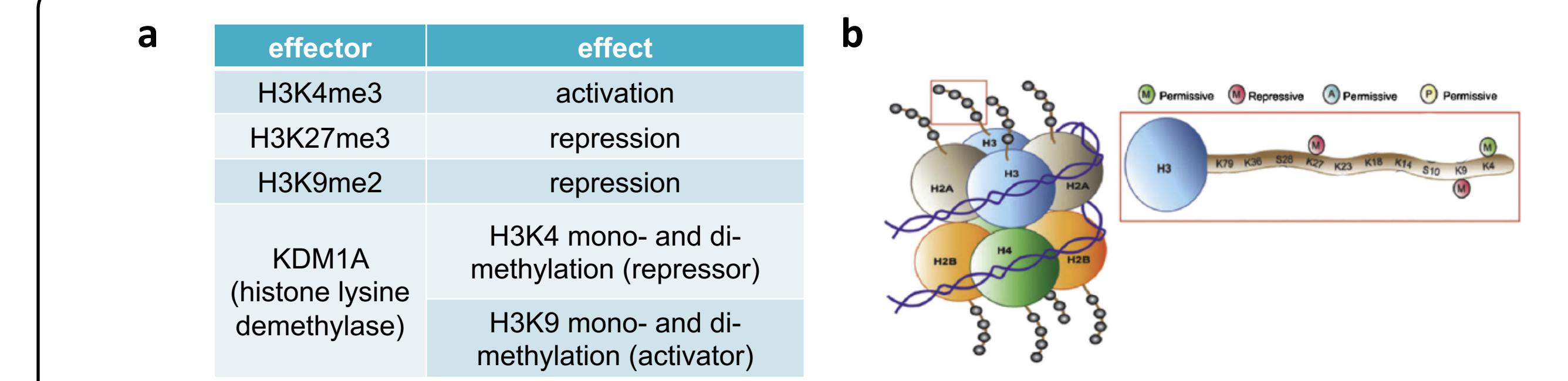


Figure 3. (a) Summary of histone lysine methylation markers that have a role in cocaine-mediated plasticity (b) Location of histone lysine methylation markers on the H3 histone. (modified from Nestler, 2014)

H3K4me3 and H3K27me3 enrichment on Egr3 and Nab2 promoters in NAc after cocaine exposure

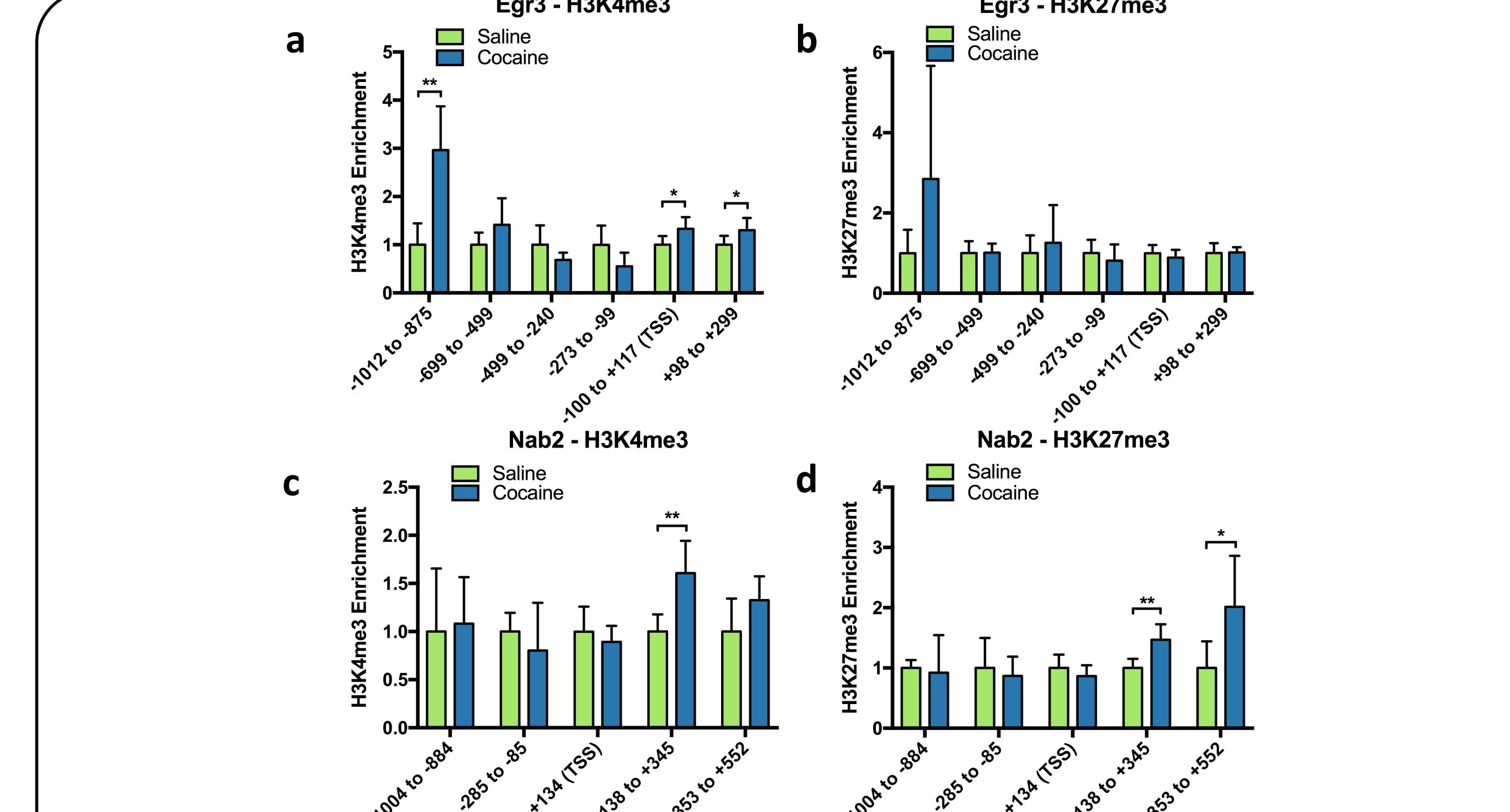


Figure 4. (a) H3K4me3 is enriched in several locations along the Egr3 promoter of the cocaine group. (b) H3K27me3 does not significantly change in the Egr3 promoter after cocaine exposure. (c) H3K4me3 is significantly enriched at the +172 bp region of the Nab2 in the cocaine group. (d) H3K27me3 is enriched after the TSS region of the Nab2 promoter after cocaine exposure. Student's t test *p<0.05, **p<0.01.

H3K9me2 and KDM1A enrichment on Egr3 and Nab2 promoters in NAc after cocaine exposure

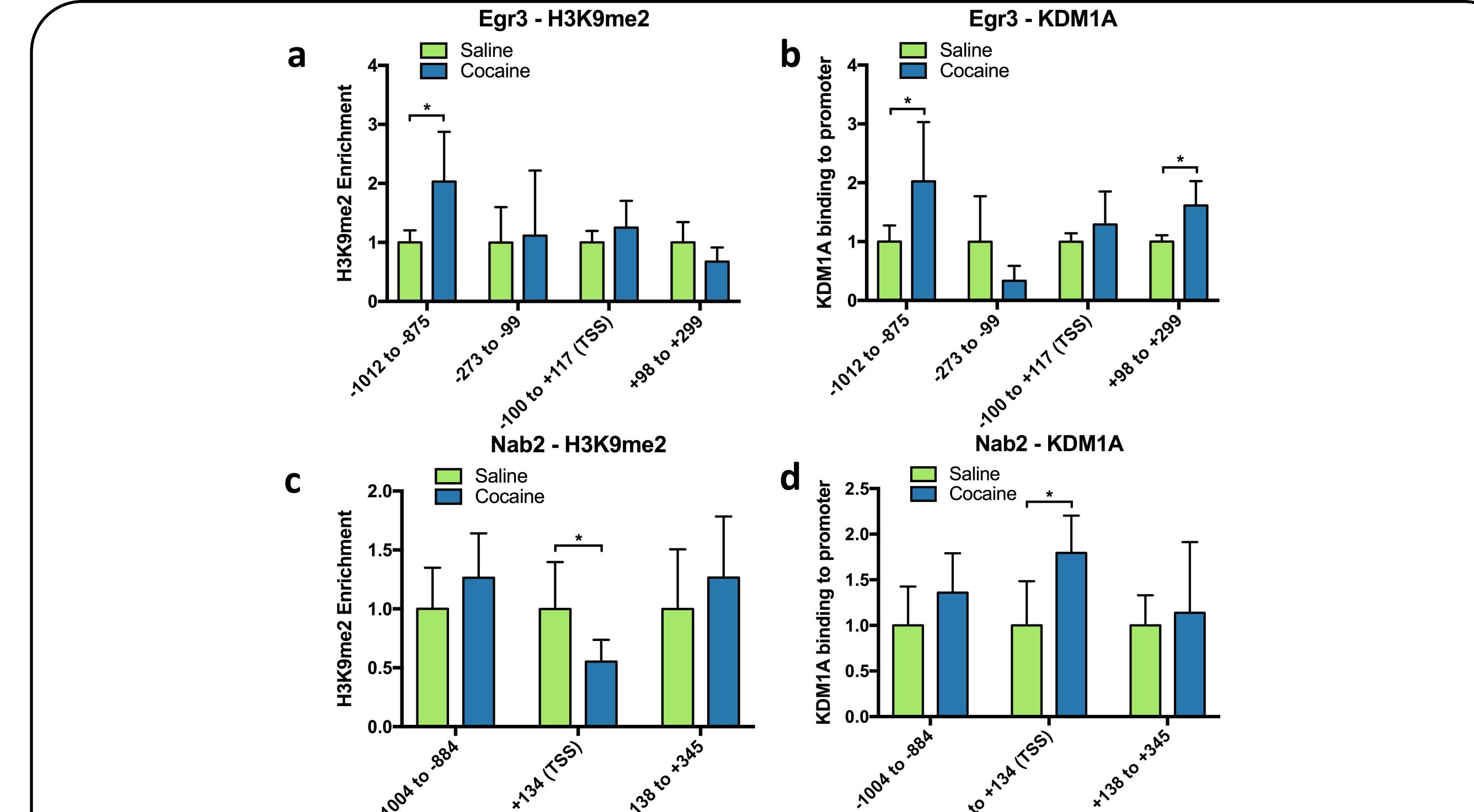


Figure 5. (a) H3K9me2 is enriched at the -1012 to -875 bp upstream of the transcription start site of the Egr3 promoter. (b) KDM1A binding to the Egr3 promoter is increased -1072 to -875 bp upstream of and +98 to +299 bp downstream of TSS. (c) H3K9me2 is decreased at the TSS of Nab2. (d) KDM1A binding to the Nab2 promoter is increased at the TSS of Nab2. Student's t test *p<0.05.

Conclusion

- The above data suggests that H3K4me3 is possibly associated with increased Egr3 transcription. This suggestion is consistent with increased Egr3 mRNA as in D1-MSNs after chronic cocaine injection. H3K9me2 is possibly associated with decreased Egr3 transcription and is consistent with decreased Egr3 mRNA as in D2-MSNs.
- H3K4me3 enrichment, decreased H3K9me2 enrichment and increased KDM1A binding with the Nab2 promoter suggests an increase in Nab2 transcription and this is consistent with increased Nab2 mRNA in D2-MSNs after chronic cocaine injection. Similarly, H3K27me3 enrichment may suggest decreased Nab2 transcription and is consistent with decreased Nab2 mRNA as in D1-MSNs.

Transcriptional Regulation of Egr3 using CRISPR

The CRISPR-Cas9 system is a bacterial-derived mechanism that has been exploited for genetic modification. Traditionally, it has been used for complete gene knockout, using Cas9's endonuclease activity. The CRISPR-Cas9 system can also be used to activate or repress gene transcription using dCas9, a form of the Cas9 protein with mutations at D10A and N580A which removes endonuclease activity. We are using a light-activated system with dCas9 to modify transcription of our target gene Egr3. In this system, the CIBN domain in dCas9 binds to fused CRY2:KDM1A under the presence of blue light. This allows for targeted KDM1A function, and therefore, site-directed histone lysine demethylation. This will allow us to specifically modify histone methylation sites that may be involved in Egr3 transcription in D1-MSNs and D2-MSNs.

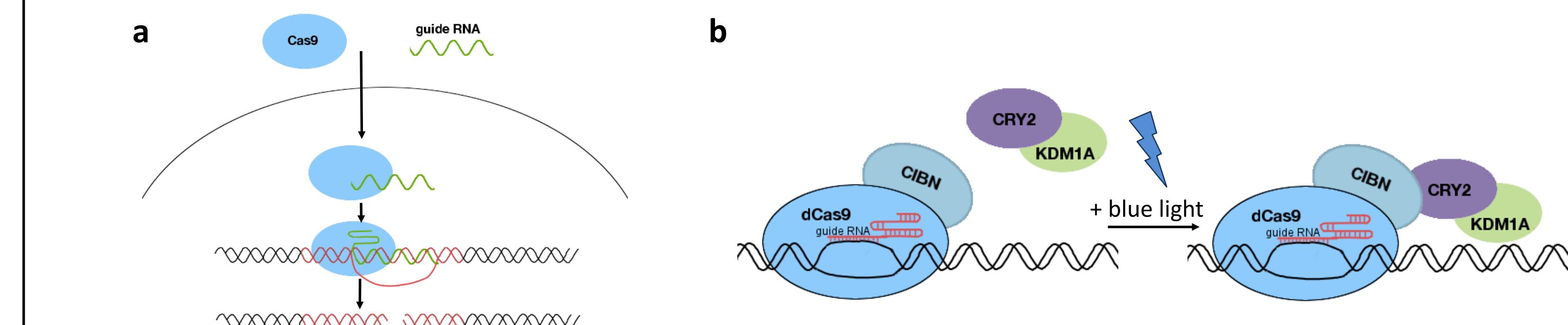
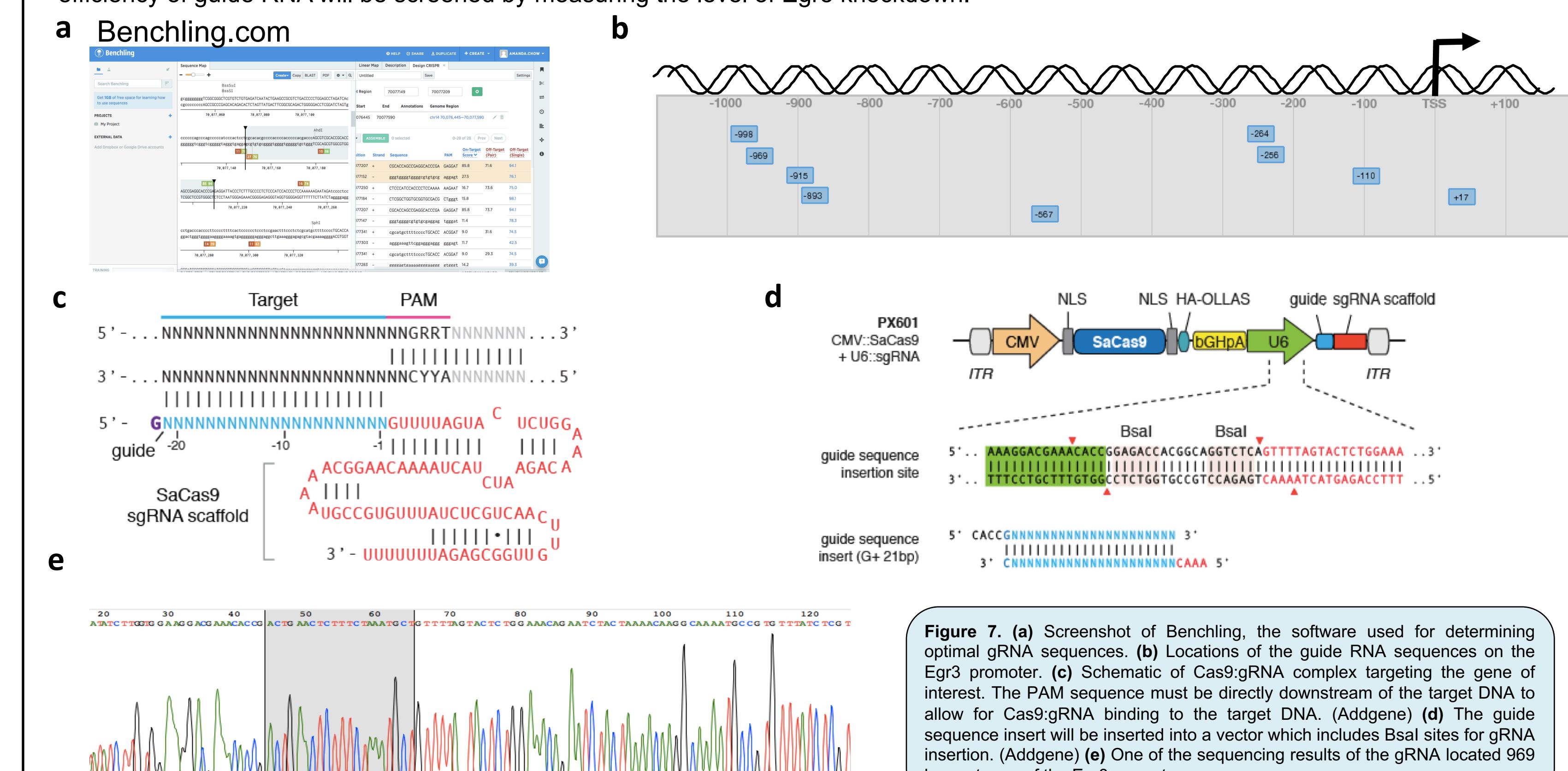


Figure 6. (a) Mechanism of the CRISPR-Cas9 system being used for gene knockout. (b) The CRISPR-Cas9 system can be used for epigenetic modification using a blue photo activation. In the presence of blue light, CRY2 undergoes a conformational change that enables heterodimerization with CIBN, which causes binding of CRY2:KDM1A to the targeted DNA sequence and transcriptional alteration of the downstream gene. (Polstein and Gersbach, 2015).

Guide RNA design and screening

We have designed guide RNAs using <http://www.benchling.com>. The gRNA contains 21 bp which binds to the target gene, and the gRNA must be upstream of a protospacer adjacent motif (PAM) sequence that is specific to the Cas9 protein of each bacterial species. For Sa-Cas9 (from *Staphylococcus aureus*), the required PAM sequence is NNGRRT. We have cloned the guide RNA under the U6 promoter into a vector that also expresses the Sa-Cas9 protein. We have successfully cloned nine gRNAs targeting the Egr3 promoter, and this has been confirmed by sequencing. Currently, we are transfecting the plasmid carrying Sa-Cas9 and the guide RNA vector into N2A cells. The efficiency of guide RNA will be screened by measuring the level of Egr3 knockdown.



Cloning Strategy and Future Directions

Finally, we will be cloning dCas9:CIBN and CRY2:KDM1A Cre-dependent constructs. The most efficient guide RNA will be cloned in a vector carrying luciferase and fluorescence protein GFP. Luciferase produces the wavelength of light needed to cause interaction between the CRY2 and CIBN domain.

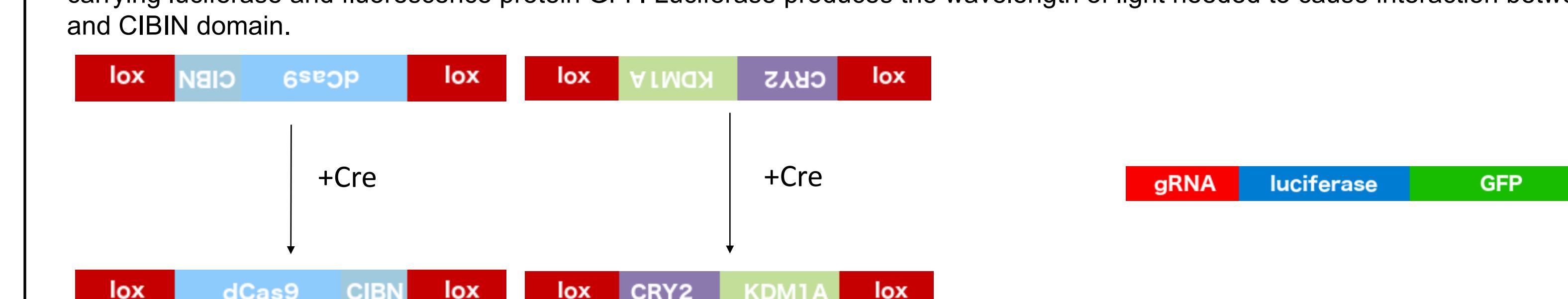


Figure 8. The Cre-dependent dCas9:CIBN construct and the CRY2:KDM1A constructs will be cloned so that they can be expressed using the Cre-lox system in D1-Cre mice or D2-Cre mice in a cell-subtype specific manner. We hope to clone guide RNA containing luciferase and GFP to produce blue light to target our CRISPR system.

References

- Maze et al., Ann. NY Acad. Sci. (1216):99-113 (2011) The epigenetic landscape of addiction.
- Kurnik et al., Curr. Opin. Neurobiol. (30):112-121 (2015) Regulation of chromatin states by drugs of abuse.
- Kumbrink et al., J. Cell. Biochem. (111):207-217 (2010) EGR1, EGR2, and EGR3 activate the expression of their coregulator NAB2 establishing a negative feedback loop in cells of neuroectodermal and epithelial origin.
- Chandra et al., J. Neurosci. (35):7927-7937 (2015) Opposing role for Egr3 in nucleus accumbens cell subtypes in cocaine action.
- Nestler, Neuropharmacol. (76):259-268 (2014) Epigenetic mechanisms of drug addiction.
- Polstein et al., Nat. Chem. Biol. (11):198-200 (2015) A light inducible CRISPR-Cas9 system for control of endogenous gene activation.
- Special thanks to all members of the Lobo Lab: Mary Kay Lobo, Ramesh Chandra, Michel Engeln, Hyungwoo Nam, Chase Francis, Lace Riggs, Shweta Das, Brianna Evans, Julia Bazzini, Alison Gaynor, Miranda Fennell
- This study was supported by NIDA R01DA038613. This research was supported in part by the University of Maryland Scholars Summer Research Program, an initiative of the University of Maryland: MPowering the State.