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Nicholas Heliotis

University of Connecticut - Storrs, nah986@hotmail.com

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Pharmacological Inhibition of histone methyltransferase G9a affects expression of Citron kinase target genes in neural stem cells

Nicholas Heliotis

Department of Physiology and Neurobiology, University of Connecticut

Abstract

Regulated repression of gene expression by post-translational modification of histones is required for normal development. The histone methyltransferase G9a is essential for embryonic development, and we have shown that phosphorylation of G9a by the CitK, a gene required for normal CNS development, gates gene repression and dimethylation of histone H3 at lysine 9 (H3K9me2) in neural progenitors. CitK and G9a co-localize to promoter regions of genes up-regulated in CitK null cells. CitK mutant progenitors lack H3K9me2 at promoter regions of up-regulated genes, and re-expression of CitK restores both repression of gene expression and H3K9me2 occupancy. In this thesis we examine the role of the G9a inhibitor Bix-01294 effect on gene expression in neural stem cells. Interestingly, we found that the addition of Bix-01294 causes changes in gene expression that are consistent with changes observed in the null CitK cells. These data support a connection between CitK and its effect on G9a as a means by which neural stem cells regulate gene expression.

Introduction

In eukaryotic organisms, histone modifications are essential for normal cellular function. These covalent alterations to histones occur directly to the N-terminal tails of specific amino acids post-translationally. Such modifications thus lead to significant impacts on transcription, which in turn provide the information the cells require to perform their allotted tasks. Among the variety of histone modifications that arise, phosphorylation, acetylation, and methylation can occur (Koch, et al. 2007). Any combination of these can occur to each of the individual amino acids comprising the N-terminal tail of a single histone, providing many outcomes and possible mutations as well. For example, serine-10 in histone H3 can be phosphorylated while lysine-9 in the same histone can be methylated. The vast majority of these modifications are responsible for regulating the expression of specific genes. As some can have up-regulating effects, others can

lead to the repression of gene expression (Boulias, et al. 2004). Normal eukaryotic development relies heavily on the repression of genes through these post-translational histone modifications. Past studies have suggested that the repressive effects allow for specific genes to be transcriptionally silent when the protein they produce is required, otherwise they remain dormant when not necessary. DNA is packaged with the nucleus of the cell into what is known as chromatin. Chromatin is made up of octamers of histones wrapping up to 147 nucleic acids around them. The post translational modification of the histone tail affects the transcription of the gene packaged in the chromatin (Bassi, et al. 2011).

There are a variety of chromatin modifiers. Current literature has identified at least eight types of modifications, including these mentioned previously and ubiquitylation, deimination, etc. Among the enzymes responsible for modifications, methyltransferases and kinases are the most well studied and their activities are the best understood. The overall action of these chromatin modifiers is to provide a means of unwinding or winding the tightly bound chromatin through mediating the interactions between nucleosomes, or to guide other proteins to specific sites within the chromatin based on DNA sequence. Epigenetic information can be carried out by methylation of the H3 and H4 tails (Tachibana, et al. 2002). These tails are approximately 100 amino acids in length and any lysine and arginine can be methylated. For example, the methylation taking place at lysine-9 of histone H3 (H3K9) is involved in the repression of transcription of the genes (Roopra, et al. 2004).

Histone H3 lysine 9 is di-methylated by G9a, the eukaryotic histone methyltransferase EHMT2. The function of G9a is extremely important in embryonic development; specifically in the formation of centromeres and telomeres during mitosis and meiosis and in silencing euchromatic regions of the genome (Sampath, et al. 2007). The centromere is the region of the

chromosome where two sister chromatids are attached to one another. Its role is essential in the proper segregation of the sister chromatids during the aforementioned cellular processes. The telomeres are located at either end of each chromatid and contain unimportant repetitive sequences of nucleotides, whose duty is to protect the genes nearest the ends from degradation. These two structures mentioned are made up of heterochromatin, a subclass of chromatin. Heterochromatin is bound very tightly and is believed to be involved in the gene repression and is composed of H3K9me2 and H3K9me3 (Song, et al. 2011). This is due to the strands of DNA located within these chromatin molecules being inaccessible as a result of its solidly-packed nature. The importance of G9a has also been identified in euchromatic regions of the genome. Euchromatin is a type of chromatin that is loosely packed and heavily concentrated with genes often undergoing transcription. The greater part of the mammalian genome consists of euchromatin. As in other studies, it was found that the loss of G9a led to the non-existence of H3K9me2 in euchromatin. Current literature suggests that the methylation of H3K9 mediated by G9a is involved in creating the chromatin architecture in these regions, forming inhibitive states that essentially prohibit gene transcription. Past data studying the expression of a number of genes indicate that the HMTase activity of G9a upon H3K9 leads to a significant decline in transcription (Roopra, et al. 2004). Additionally, it was discovered that the methylation of H3K4 is in fact associated with an increase in gene expression, the opposite effect of methylation of H3K9. Thus, it was proposed that the G9a-mediated methylation of H3K9 down-regulates the activity of H3K4, suggesting that the function G9a to silence gene expression is done through either up-regulating H3K9me2 or by negatively regulating H3K4 methylation.

Citron Kinase is a protein required for proper development of the central nervous system. Like all kinases its activity via phosphorylation. Found in neural stem cells, CitK is activated by

the GTPase *RhoA*, a type of signaling G-protein found biochemically further upstream from CitK. Current literature shows that RhoA is involved in regulating the formation of the actin cytoskeleton, although it has a number of different targets (Eda, et al. 2001). The enzymatic activity of this GTPase is to hydrolyze GTP, freeing a phosphate ion with which CitK can phosphorylate. CitK has also been discovered to have a vital role in cytokinesis, the last part of cell division when two daughter cells are formed from the splitting of a single parent cell. During this cellular process, RhoA and CitK are present at the cleavage furrow, where this complex is then able to perform its tasks. Interestingly, CitK plays a significant part in the activation of G9a. CitK is responsible for phosphorylating G9a at Serine-70 (S70). Together, CitK and G9a co-localize in the promoter regions of up-regulated genes in wild type neural progenitor cells, indicating that they function cooperatively in repressing the expression of these genes (Chen, et al. 2011).

Originally only thought to be found in the cytoplasm of neural progenitor cells, it has recently been discovered that the CitK protein is also found in the nucleus. This finding has helped in the understanding of the CitK-G9a interaction and how they work together in gene expression/repression. They co-localize at specific regions of genes which demonstrates not only their cooperative relationship, but also their biochemical interaction. This was identified by co-immunoprecipitation of CitK and G9a. In the absence of CitK, methylation of H3K9 by G9a does not occur. H3K9 and G9a co-localize at specific promoters with CitK at targeted genes. The vast majority of these target genes of CitK are involved in neural stem cell self-renewal and the further development of the central nervous system. A phosphomimetic variant of G9a (mimicking phosphorylation at Serine-70) was able to recover proper levels of H3K9me2 at the promoter sites in cells and rescues transcription levels (Girgenti, et al. 2012).

This thesis investigates three questions that were proposed after reviewing all of this data. First we asked whether or not the recovery of normal H3K9me2 levels done by CitK can be blocked by the addition of a G9a inhibitor. The action of the G9a inhibitor *Bix-01294* on the rescue of H3K9me2 levels was measured and found to in fact block rescue. Additionally, this thesis examines what the outcome would be of introducing the G9a inhibitor into CitK^{WT/WT} cells. It was found that the gene expression levels of the CitK targets were nearly identical to those of the mutant cells which lacked CitK all together. Lastly, we investigated the effects of a Rho inhibitor by itself on gene expression, as well as a Rho inhibitor plus the G9a inhibitor. Together, these results suggest a strong interplay between CitK and G9a in regulatory gene expression in neural stem cells.

Materials and Methods

Cell Culture

E11 rat neocortex was dissociated using mechanical disruption followed by trypsin treatment for neural progenitor culture. Neural progenitors were then plated on poly-DLysing(0.1mg/mL) and Laminin(1:200) coated coverslips. Neural progenitors were maintained in DMEM supplemented with 1% penicillin and streptomycin, L-Glutamine, Sodium Pyruvate, N2 supplement, B27 supplement, bFGF (20ng/mL) and EGF(20ng/mL). Cells were grown at 37oC humidified incubator with 5% CO₂. Cell were treated with the G9a inhibitor BIX-01294 (10uM), the Rho-inhibitor Y-27632 (15uM) or their vehicle DMSO for control. Cells were treated for 24 hours and RNA was isolated after this period.

RNA Isolation

RNA was isolated from a n = 3 of flathead and CitK^{WT/WT} forebrain and n = 2 flathead and CitK^{WT/WT} progenitor culture, using the RNAqueous kit (Ambion). 500 µl of Lysis / Binding

Solution was added to the samples in order to release RNA from tissue. The samples were sonicated to further this goal. Sonication was followed by centrifugation for 3 minutes to remove any debris. 500 μ l of 64% Ethanol was added to each sample lysate and the mixture was drawn through a filter cartridge. Lysates were centrifuged and any flow-through was discarded. Filter cartridges were subsequently washed with 700 μ l of Wash Solution #1 and two 500 μ l aliquots of Wash Solution #2/3. Flow-through was discarded all three times, and the samples were then centrifuged for 3 minutes in order to remove any excess wash solution. After transferring to a fresh collection tube, bound RNA was eluted twice via preheated (95° C) Elution Solution. A 30-second centrifugation after each aliquot was used to collect the eluate. RNA concentration was measured for each sample through the use of a Nanodrop© spectrophotometer set to read at 260 nanometers, and the results recorded. Isolated RNA was stored at -20° C for future use.

cDNA Synthesis

cDNA synthesis was conducted using Invitrogen SuperScript III (Invitrogen). Briefly, 1 μ g of isolated RNA from each sample (n = 3 tissue RNA or n = 2 progenitor culture RNA) was pipetted into a tube containing 1 μ l 50 μ M oligo(dT)₂₀ primer or random hexamers. Another 1 μ l of 10 mM dNTP mix was added, and the total volume was brought up to 10 μ l using DEPC-treated water. After 5 minutes of incubation at 65° C and 1 minute on ice, 10 μ l cDNA Synthesis Mix was added to each tube. Synthesis Mix contained 2 μ l 10X RT buffer, 4 μ l 25 mM MgCl₂, 2 μ l 0.1M DTT, 1 μ l RNaseOUT and 1 μ l SuperScript III RT. Following centrifugation, the mixtures were incubated for 50 minutes at 50° C, and then for 5 minutes at 85° C to terminate the reaction. Samples were centrifuged and 1 μ l RNase H was pipetted into each tube to remove any RNA. After a final incubation of 20 minutes at 37° C, synthesized cDNA was stored at -20° C for future experiments.

Primer Design

Coding sequences for all genes (see TABLE 1) were obtained through the NCBI website. These sequences were then used in Primer3 (<http://frodo.wi.mit.edu/>) for primer design. The product size range was restricted to between 105 and 175 base pairs for optimal qRT-PCR amplification. The amplicon also contained sequence from the RNA-sequencing reads. Primer GC percentage was kept within a range of 20 to 80 and a GC clamp was added to promote stronger binding. Primer T_m was optimized to 65° C, with a 60° C to 70° C range, and primer size was limited to 18-22 base pairs (see Figure 1 for primer sequences). The primers were then BLASTed to ensure that they were specific for each gene. Only primers that contained 100% homology to a known *Rattus norvegicus* gene and an E value of less than 0.1 were selected.

Quantitative Real-time PCR and Analysis

qRT-PCR was performed using an ABI 7500 instrument (Applied Biosystems) running the following parameters for 40 cycles: 94° C for 2 s, 60° C for 30 s and 72° C for 30 s. The Fast SYBR Green Master Mix (ABI) was used with reaction volumes of 20 µL. An initial dilution series was conducted with 1, 2 and 4 µl of each cDNA sample to determine primer efficiency. Relative gene concentrations were normalized against Gapdh, a housekeeper gene that is not linked to citron kinase and should have similar expression levels in both CitK^{FH/FH} and CitK^{WT/WT}. Gene-specific primers were tested for specificity through melt curve analysis.

Results

Wild-type cells treated with the G9a inhibitor mimic expression levels of CitK target genes in mutant cells lacking CitK

The G9a inhibitor Bix-01294 was added to CitK^{WT/WT} neural stem cells and the gene expression levels of seven CitK target genes were analyzed. These expression levels were

compared to mutant neural stem cells that lacked the CitK enzyme (CitK^{FH/FH} cells). There were no significant differences in gene expression between these two treatments. Additionally, these expression levels were then compared to CitK^{WT/WT} and flat head cells treated only with DMSO to act as controls. The CitK^{FH/FH} control cells exhibited the highest levels of gene expression, while the CitK^{WT/WT} control cells had the least amount of expression. The Bix treated cells (both WT and Fh) showed intermediate expression compared to the control group. (Figure 1A)

Non-CitK targets are regulated by G9a inhibition

The gene expression levels of seven non-regulated (non-CitK target) genes were compared in several different treatments. Mutant cells lacking CitK (CitK^{FH/FH} cells) were treated with DMSO for the control group or Bix-01294 inhibiting G9a. The same was done to CitK^{WT/WT} cells, and gene transcription activity was examined. The CitK^{WT/WT} and CitK^{FH/FH} control groups had very similar expression levels, all of which were between 1.0 and 2.0 times normal levels. The CitK^{FH/FH} and CitK^{WT/WT} cells in the presence of the G9a inhibitor exhibited significantly increased gene expression levels, nearly matching those of CitK target genes. Expression levels between CitK^{WT/WT} and CitK^{FH/FH} cells with Bix are also similar to each other, with the exception of Taf6 and Fezf1 which showed higher levels in CitK^{WT/WT} cells over CitK^{FH/FH} cells. (Figure 2B)

CitK target genes are upregulated in the presence of the G9a inhibitor

Rat neural progenitors were treated with the G9a inhibitor Bix-01294. The gene expression of twenty-four genes targeted by CitK was then analyzed using quantitative polymerase chain reaction. In the presence of this inhibitor, we saw a significant upregulation of the expression of these genes. Almost half of these target genes experienced at least a five-fold increase in expression, as seen with p21, H2AZ, Notch1, Ncam, Chmp1a, and others. Spt7 had the

greatest increase in expression, at 8.3 times compared to wild type expression levels. (Figure 2A)

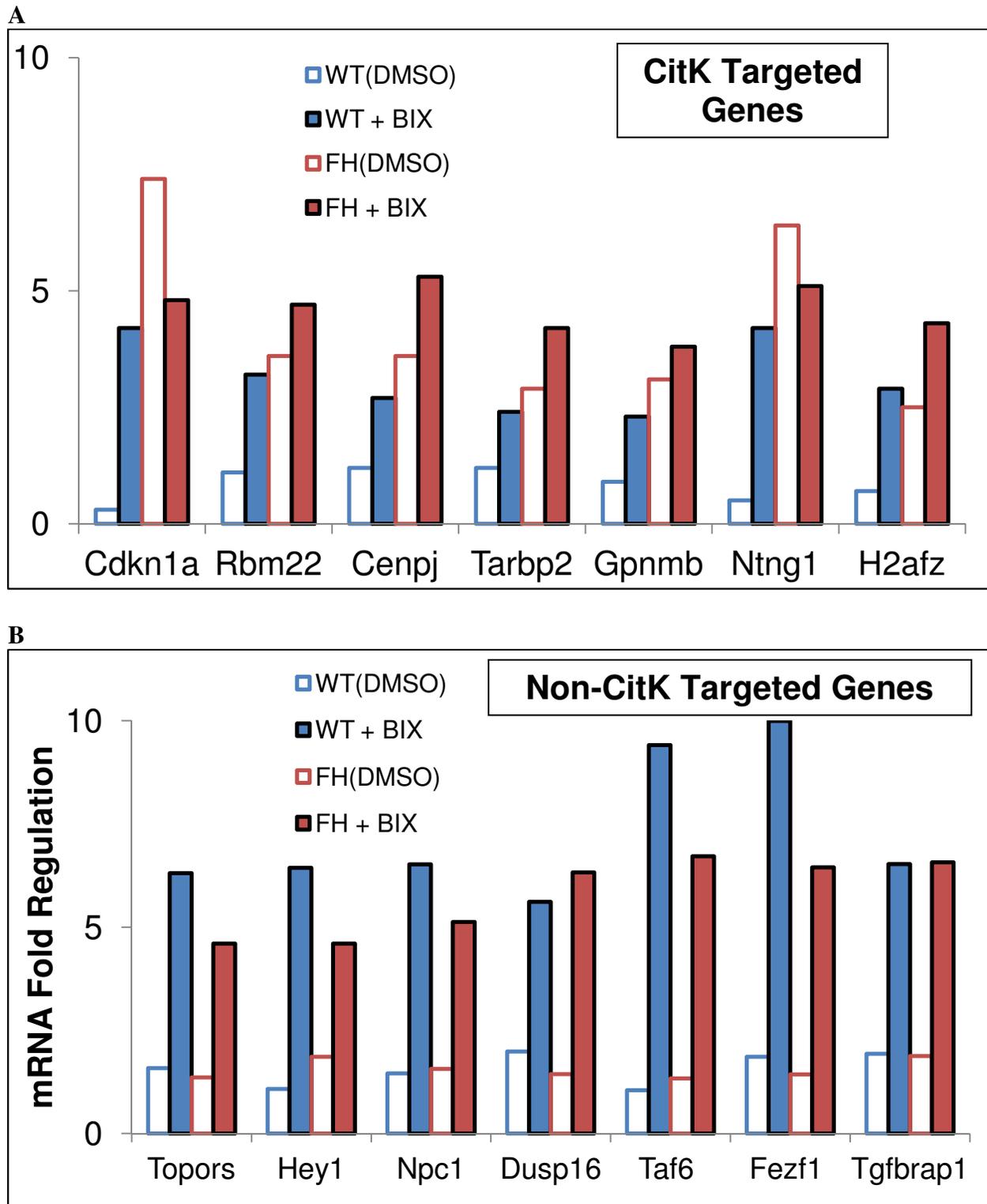


Figure 1. Comparison of gene expression levels of wild-type cells and mutant cells lacking CitK in the presence and absence of the G9a inhibitor Bix-01294. (A) WT CitK target genes treated with Bix mimic FH expression levels. (B) WT non-CitK targets treated with Bix mimic CitK target gene expression levels

Rho inhibitor has no effect on gene expression of CitK target genes

The addition of a Rho inhibitor was thought to decrease gene expression of the target genes by preventing the activation of CitK. However, there was no significant effect on expression levels compared with that of wild type cells. The same twenty-four genes were examined as with the G9a inhibitor. The vast majority of these genes had expression levels of between 1.0 and 1.5 times the levels of normal neural stem cells, suggesting that the Rho inhibitor did not affect any of the processes involved in gene expression. There were a few genes that did have a slightly higher expression level than their CitK^{WT/WT} counterparts, including AurKB which had a 1.80 times increase in gene expression. (Figure 2A)

G9a inhibitor plus Rho inhibitor shows varying effects on CitK target gene expression levels

Again analyzing the gene expression levels for the same genes as with the previous two experiments, we treated the cells with a combination of the G9a inhibitor plus the Rho inhibitor. All of the target genes exhibited an increase in expression levels, however all showed lower levels than with the G9a inhibitor alone. The majority of the genes examined showed at least a four-fold increase in expression, with four genes with over six times the CitK^{WT/WT} level. p21 had the greatest increase in gene expression with 7.52x higher expression levels than CitK^{WT/WT} cells, being the only one with an over seven-fold increase. (Figure 2B)

Non-CitK target genes treated with Rho and G9a inhibitors exhibit down-regulation of expression levels

We tested what effect the three inhibitor treatments would have on the three non-CitK targeted genes TCFP2B, BMI1, and Foxg1. The Rho inhibitor alone had no significant effect on the expression levels of these genes, showing only a slight increase for each (1.90, 1.40, and 1.30, respectively) over CitK levels. In the presence of the G9a inhibitor alone, these three

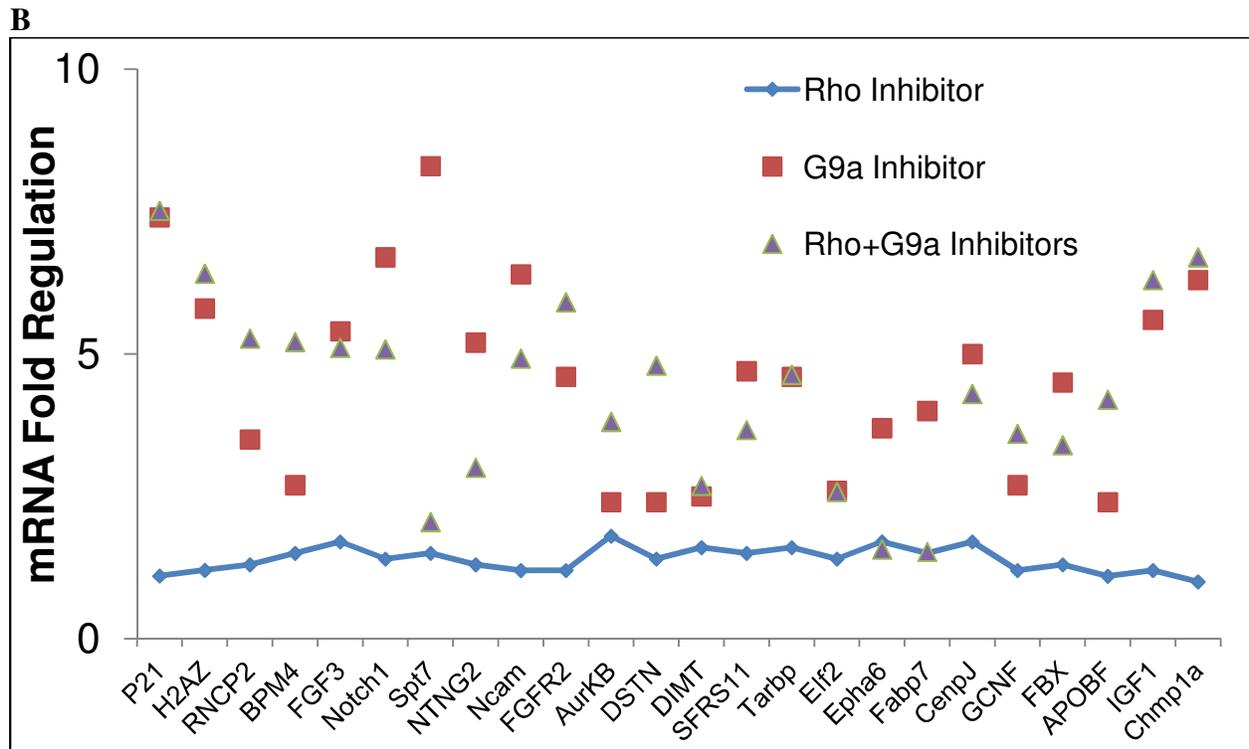
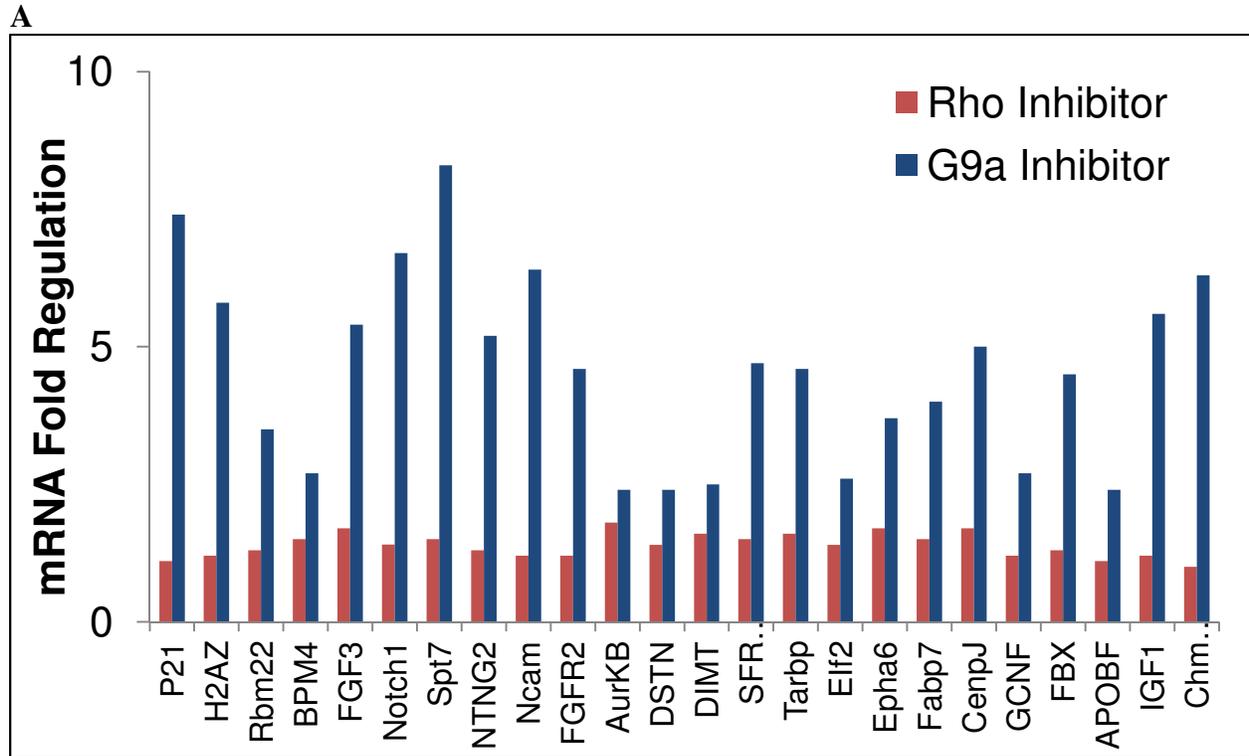


Figure 2. Gene expression levels of 24 CitK target genes. (A) Expression levels of target genes in tissues treated with the G9a inhibitor Bix-01294. (B) Comparison of gene expressions of the same genes in the presence of a Rho inhibitor, G9a inhibitor, and levels of gene expression in tissues treated with a combination of G9a inhibitor + Rho inhibitor.

genes showed varying levels of down-regulation. TCFP2B exhibited an 8.7x decrease in expression, while Foxg1 showed a modest down-regulation of 2.1x. The cells treated with G9a+Rho inhibitors had nearly identical expression levels, although BMI1 had over double the amount of down-regulation as compared to only G9a inhibition. (Figure 3)

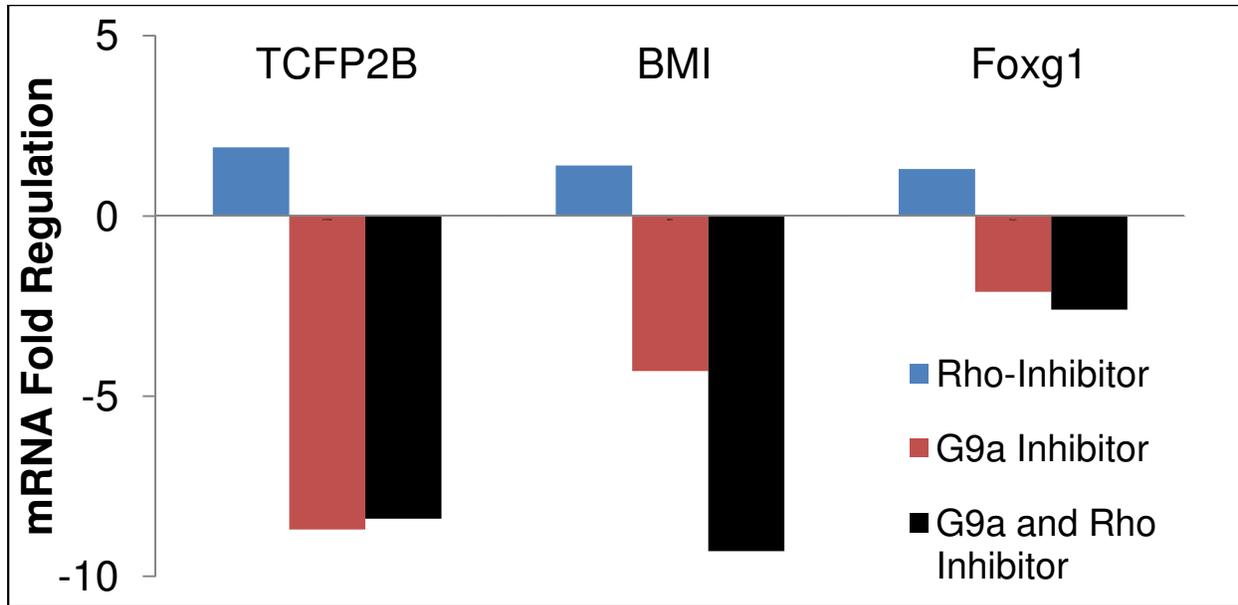


Figure 3. Three non-CitK target genes are down-regulating in the presence of the G9a inhibitor Bix-01294 alone and in the presence of the G9a inhibitor + Rho inhibitor. The Rho inhibitor alone exhibits no significant effect on gene expression levels.

Discussion

By exploring the role of CitK in neural stem cells, we were led into investigating what the importance of the two enzymes G9a and RhoA and the extent of their involvement in proper division of these cells. We first tested the effects of the G9a inhibitor Bix-01294 on gene expression in CitK^{WT/WT} neuronal stem cells on both CitK targeted and non-CitK targeted genes, which was then compared to gene expression levels on the same genes but in *flathead* mutant cells lacking CitK. Additionally, we analyzed the role of a Rho inhibitor on CitK targeted genes,

as well as a combination of Rho and G9a inhibitors and compared their relative expression levels.

The enzyme G9a is responsible for gene repression in neural stem cells and must first be activated by CitK through phosphorylation in order for its effects to be seen. By allowing CitK to function normally in these cells but blocking the action of G9a, we can understand G9a's specific role in gene repression of these CitK target genes. First, in comparing expression levels of CitK target genes in the presence of the G9a inhibitor against those in CitK^{WT/WT} cells and mutants lacking CitK, we see that they mimic the mutant cells. There is a significant increase in gene expression in the cells with the inhibitor, as also in the case with the same treatments being done with non-CitK targets as well. The gene expression levels on an additional twenty-four CitK targets were also measured in the presence of this inhibitor, and again we saw the same upregulation in expression as with the other two experiments. Even though G9a is a repressive marker of gene expression, G9a inhibition also caused the down regulation of some genes, specifically those which were found to be down regulated after loss of CitK. These data suggest a strong interaction between G9a and CitK in regulating neural progenitor gene expression.

Next, we were curious in the effect that RhoA had on gene expression in these same CitK target genes. Because RhoA is required in order to activate CitK, we believed that by adding a Rho inhibitor into CitK^{WT/WT} neural stem cells, we would also see an increase in gene expression because CitK would not be activated, thus not initiating G9a and ultimately stopping gene repression. We saw almost no change in expression levels on the same twenty-four genes we examined with the G9a inhibitor. Expression levels all hovered around 1.0 over wild type (or no change), indicating that the amount of transcript produced is about the same as in CitK^{WT/WT} cells. Therefore, inhibiting Rho has no effect on gene expression and it is not entirely essential

in activating CitK. With this data though, we can infer that perhaps there are different levels of regulation; some genes can possibly be more susceptible to Rho inactivation than others.

Finally, we were also interested in seeing how adding the G9a inhibitor and the Rho inhibitor in the CitK^{WT/WT} neuronal stem cells would affect the gene expression of the same twenty-four CitK targets. We thought that it would be interesting if there could possibly be an additive effect with these two inhibitors; perhaps gene expression would be even further upregulated than with just the G9a inhibitor alone. However, upon gathering our data with this combination of inhibitors, we saw something quite strange. There seemed to be no correlation between the expression levels of cells treated with this and that of the cells treated with only the G9a inhibitor. Although very few genes in both experiments had very similar expression levels, such as p21, DIMT, Tarbp, and Chmp1, the vast majority had very different values. The gene DSTN saw a two-fold increase in expression in the presence of both inhibitors, which is what we might have expected to see. Nonetheless, we saw many genes with the opposite results; Fabp7 had a 2.5-fold decrease in expression in the presence of both inhibitors, and Spt7 had a rather significant four-fold decrease in expression with the same treatment, as compared to that with just the G9a inhibitor. This suggests that Rho may have a different effect at different promoters regulated by CitK and G9a.

Because the results with this treatment were unexpected, we believe there must be more regulatory enzymes and domains within these cells that also play even a minor role in gene expression. Perhaps RhoA is working in another pathway that detours and avoids the CitK and G9a co-localization and activates something else further downstream, providing these results. As such, the inhibition of G9a is thus null. There is nothing in current literature that specifically looks into this, though. With this new-found knowledge, we can propose future experiments to

further develop an understanding of these mechanisms. There is a possibility that a CitK inhibitor may play a role in regulating gene expression. For example the addition of a Rac activator and seeing if there is any significant effect on gene expression is one potential future experiment. With that being said though, it is clear that the enzyme G9a is responsible for gene expression in neural stem cells and that inhibiting it creates cells that mimic CitK loss in CitK^{WT/WT} cells. Additionally, we can conclusively say that a Rho inhibitor has no effect on gene expression, even though it plays a role in activating CitK.

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