Determining the Genomic Localization and Binding Partners of Zinc Finger Protein 410

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Acceptance of Senior Honors Thesis

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Abstract

The results of a folate deficiency study affecting cognition in mice suggested the altered genes may be controlled by a transcription factor known as Zinc Finger Protein 410 (Zfp410). Due to a lack of literature on Zfp410's interacting proteins and DNA-binding location, our study aims to further elucidate the role Zfp410 plays in affecting cognition. A custom antibody was used to determine the Zfp410 isoforms present in mouse and rat brains. Moreover, the antibody was used to determine the binding partners of Zfp410 in the brain and locate specific genomic regions/sequences with which it associates in vivo. These results may further elucidate the molecular pathways of Zfp410 in relation to cognition and learning.

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Background

Vitamin B9, or folic acid, has been recognized as an important factor that contributes to increasing DNA stability and is the limiting reagent for the formation of the primary methylating agent in the S-adenosylmethionisne (SAM) cycle (Bailey & Gregory, 1999; Abbasi et al., 2018; Crider et al., 2012). Previous studies have identified folic acid to be a crucial cofactor in amino acid metabolism. B vitamins serve as a cofactor for homocysteine and aid in the conversion of homocysteine to the amino acid methionine, which perpetuates the SAM cycle (Bailey & Gregory, 1999). Studies in humans have found that folate deficiencies led to increased levels of homocysteine, resulting in cognitive deficits related to memory, learning, and visual spatial pathways (Fenech, 2012; Kim et al, 2010). Folic acid has been also identified to be heavily involved in altering gene expression, specifically by gene methylation (Fenech, 2012; Milman, 2012; Crider et al., 2012).

Gene methylation is associated with the repression of a gene, preventing transcription and the subsequent proteins from being made. This mechanism is done by the act of DNA methyltransferase enzymes, and it occurs at cytosine residues (Salbaum & Kappen, 2012). The targeted cytosines are typically followed by a guanosine, known as CpG sites, and regions within the genome that contains large stretches of these bases are named CpG islands. Promoters for housekeeping genes may be typically found in these CpG islands, which typically remain unmethylated (Moore et al, 2013). DNA methylation inhibits promoters from recruiting transcription factors which leads to gene silencing. While this may be important for regulating diseases; several studies have found DNA methylation to be crucial during development and cell differentiation (Salbaum & Kappen, 2012). In addition, DNA methylation may also be the cause

of diseases, such as cancer, if oncogenes can be transcribed in an unregulated manner. In contrast, hypermethylation of tumor suppressor genes also produce cancer by inhibiting the regulators of the oncogenes (Mahmoud & Ali, 2019).

With this knowledge in mind, the aim of the previous study in our lab was to determine if folic acid altered gene expression in mice (Lawton et al., 2020). It was observed that diets lacking folic acid led to cognitive defects in mice with corresponding altered gene expression (Lawton et al., 2020). The genes with altered expression were analyzed using bioinformatics analysis and suggested two transcription factors, proteins that regulate gene expression, may control the expression of these altered genes (**Figure 1**). The two candidates were MafB and Zinc Finger Protein 410 (Zfp410), and bioinformatics analysis provided a putative binding motif for each transcription factor (**Figure 1**). While MafB is a well-known transcription factor that plays a role in regulating the anti-inflammatory and proliferation response in microglia, Zfp410 may play a more prominent role in neuronal cells. Zfp410 has been identified as a transcription factor, but the literature lacks what function it has in the brain or which genes it may regulate (Vinjamur et al., 2021; Lee & Young, 2013; Tanigawa et al., 2019). Previous studies have shown that Zfp410 has been linked with cognitive deficits in mice and young adults but have not reported where it can be found on DNA or which proteins it may interact with (Lawton et al., 2020). Although in our previous study, the expression of Zfp410 was revealed to not be regulated by folic acid, the data suggests that folic acid plays a role in regulating transcription of these genes in a diet-dependent manner (Lawton et al., 2020; Salbaum & Kappen, 2012; Ulrich, 2005). Zfp410, although not expressed in a folate-dependent manner might still be regulating gene expression in a folate-dependent mechanism that affects its ability to form complexes with other proteins or DNA.

Figure 1

Position weight matrix showing putative binding site of murine Zfp410 and MafB

Motif	Position Weight Matrix	Type	*p*-Value	% Target	% BG	Fold	Match / Detail
1.		*de novo*	1e-12	5.54	0.31	17.9	MafB Homer (0.591)
2.		*de novo*	1e-12	6.27	0.49	12.8	Zfp410 Jaspar (0.643)

Note. Through bioinformatics analysis tools a putative binding site for Zfp410 and MafB were identified using the mouse genome. Used with permission from Lawton et al. (2020). Folate-Dependent Cognitive Impairment Associated With Specific Gene Networks in the Adult Mouse Hippocampus. *Frontiers in Nutrition* 7, 574730. https://doi:10.3389/fnut.2020.574730.

Since the function of Zfp410 and the mechanisms as to how it might control gene expression has not been identified, the aim of this study was to determine which proteins Zfp410 interacts with and identify where the transcription factor binds to DNA in the brain. To study the role Zfp410 plays in cognitive pathways, we decided to switch models from folate deficient mice to methotrexate injected rats to observe the cognitive deficient behavior in the interest of time and a more cost-efficient manner. Methotrexate acts as a folate inhibitor of an enzyme in the SAM cycle and prevents the conversion of homocysteine to methionine (McGrattan et al., 2018). This process makes a product known as S-adenosylmethionine, which is involved in methylation of proteins, DNA, and small molecules in the brain. While DNA methylation may be important for the regulation of genes, methylation of neurotransmitters allows for transport and conversion of chemicals to other neurotransmitters.

Like folate deficient diets, methotrexate injection (folate inhibition) results in a cognitive deficiency in rat models (Lawton et al., 2020; Fardell et al., 2010). A study on rheumatoid arthritis patients where a low dosage of methotrexate was used as a treatment has correlated elevated levels of homocysteine with reduced cognitive function, commonly referred to as “brain fog" after rheumatoid arthritis treatment or chemotherapy (van Ede et al., 2002).

While Zfp410 has been implicated to be involved in the process of cognition and learning, it is not clear how this transcription factor influences the outcomes of cognitive deficiency and what pathways its involved in. Our study is centered around the interacting proteins and the DNA binding sites of this transcription factor within the rat genome. The identification of these regions may further describe molecular pathways involved in cognition and learning as well as implicate other protein factors that play important roles in cognitive pathways.

Aim 1: Characterization of the Custom Polyclonal Zfp410 Antibody

Overview

Since Zfp410 lacks an abundance of commercially available antibodies, previous work on this project included purchasing the coding DNA of the 39 kDa mouse isoform of Zfp410 to overexpress and purify Zfp410 from *E. coli* for antibody production. Once a sufficient amount of recombinant protein had been purified, it was sent to the Pocono Rabbit Farm and Laboratory to make a custom polyclonal antibody by injecting some of the purified protein into a rabbit. A pre-immune serum sample from the rabbit was received for future experimentation and was later used as a negative control. After 5 injections, the custom antibody serum from the rabbit was received. Once obtained, the antibody needed to be characterized using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting to determine if

specific antibodies had been made. SDS-PAGE gels are used for separating proteins according to their molecular weight with a range of 5-250 kDa and utilizing polyacrylamide as the gel matrix. Sodium dodecyl sulfate (SDS) is an ionic detergent that disrupts cell membranes and is used to denature proteins for separation. Once separated, the proteins are transferred to a membrane which allows for binding of the antibody. To prevent non-specific binding, the membrane is blocked using non-fat dry milk, allowing the milk proteins to cover non-specific regions of the membrane. The western blot technique allows a specific protein to be identified using an antibody that is specific to the protein of interest, such as Zfp410. Once the Zfp40 antibody was characterized and we confirmed the presence of Zfp410 in various extracts, our antibody could be used to accomplish the aims of the study.

Methods: Extract Preparation and Western Blot

About 150mg of frozen (-80°C) female rat brain tissue was placed in a chilled 15mL Potter-Elvehjem homogenizer and dounced 20 times on ice in 1mL of lysis buffer (25mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA, 1% NP-40, 5% glycerol, and 1 protease inhibitor tablet per 10 mLs added at the time of use). After 15 minutes of centrifugation in a chilled centrifuge at 15,000 rpm, the supernatant was moved to a fresh 1.5 mL tube and the pellet was discarded. The supernatant was stored in a -80°C freezer for future use.

A 6% SDS-PAGE gel was prepared using 6% polyacrylamide 1:38, 2mLs of 4X lower buffer (1.5M Tris Base, 0.4% sodium dodecyl sulfate, pH adjusted to 8.8) brought to 8mLs with reverse osmosis (RO) water. After several inversions of the tube, 45µL of 10% ammonium persulfate (APS) and 12µL of TEMED (tetramethylethylenediamine) were added to the tube. After mixing quickly, the solution was added to the gel apparatus using a 1 mL disposable transfer pipette. Any bubbles at the top of the interface were removed by adding a few drops of

water-saturated butanol. While solidifying, the upper gel consisted of 3% polyacrylamide (1:38 ratio of bis:acrylamide), 2mLs of upper buffer (500mM Tris Base, 0.4% SDS, adjusted pH to 6.8) brought to 5mLs with RO water. After pouring off the butanol once the lower gel was solidified, 30µL of 10% APS and 8µL of TEMED were added to the upper gel tube and mixed quickly. A clean 1mL disposable transfer pipette was used to add the upper gel solution on top of the solidified lower gel, filling the gel apparatus until it reached the top. A 10-well comb was added immediately after, and the solution was allowed to solidify. Once the gel was fully solidified, the comb was removed, and the gel was prepared to run. The gels were removed and placed in the SDS-PAGE running apparatus. After 200mLs of 1X SDS running buffer (192mM glycine, 25mMTris base, 0.1% SDS) was added to the middle chamber of the running apparatus, 500mLs of the running buffer was added to the outer chamber of the system. The rat brain extract was prepared for SDS-PAGE by centrifugation in a chilled centrifuge for 10 minutes at 15,000 rpm. After 10µL of the supernatant was removed to a new tube, 10µL of 2x Laemmli sample buffer was added, and boiled for 4 minutes in a heat block at 100°C. This process was repeated for the HeLa cytoplasmic extract, along with the mouse brain extracts previously prepared as mentioned above. After 2µL of protein ladder was added to the first well, 10µL of each sample was loaded into separate wells adjacent to one another, and the power system turned on. The gel ran at 23mA for 30 minutes, followed by 35mA for 45 minutes. After the gel finished running, it was transferred to the western blot apparatus. A transfer sandwich was constructed with the gel and nitrocellulose membrane oriented properly while soaking in ~600mLs of 1x transfer buffer (25mM Tris base, 192mM glycine, and 20% methanol). Once completed, the transfer sandwich was placed in the western blot transfer apparatus and ran for 1 hour at 400mA. After the transfer was completed, the membrane was incubated in 3% dry milk

blocking solution (3g non-fat dry milk in 100mLs of Tris-buffered saline with Tween 20[TBS-T]) overnight while rocking at room temperature. After pouring out the blocking solution, the membrane was rinsed with ~50mLs of TBS-T (20mM Tris, 150mM NaCl, 0.05% Tween 20) for 3 minutes and a total of 3 washes. The membrane was incubated with the primary antibody (1:2,000 dilution in a 1% blocking solution) for 2 hours at 4°C while rocking. After 3 washes with TBS-T for 3 minutes, the membrane was incubated with the secondary antibody (1:20,000 dilution in a 1% blocking solution) for 1 hour at room temperature. After 3 more subsequent washes with TBS-T for 3 minutes, the membrane was visualized at 700 nm using the *Li-COR* UV spectrometer.

Results

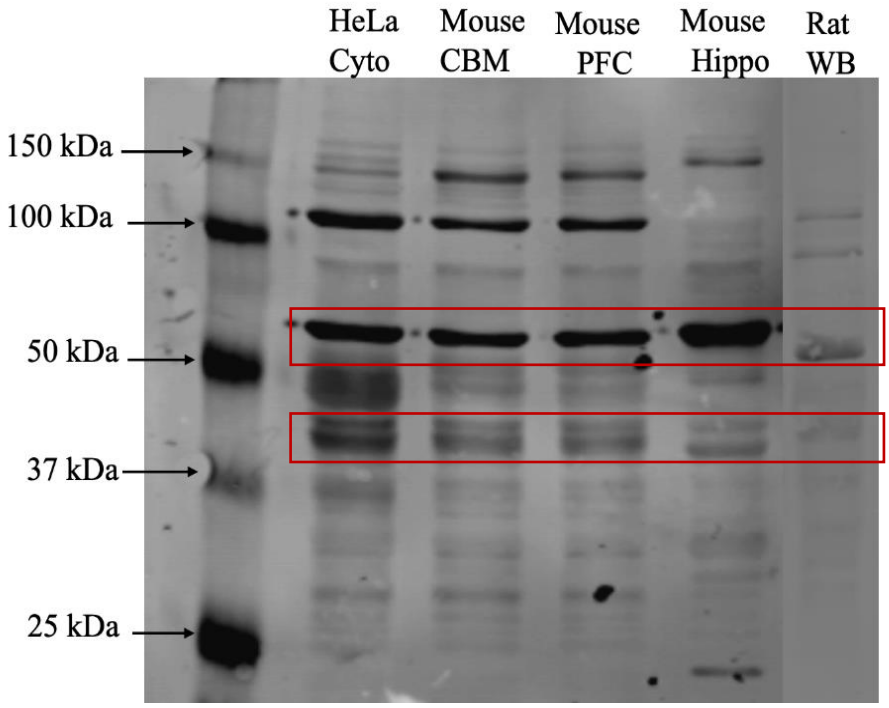
The resulting image from the western blot demonstrated Zfp410 was detected by the custom antibody in several extracts previously prepared from cancer cells (HeLa cytoplasmic extract), mouse cerebellum, prefrontal cortex, hippocampus, and rat brain (**Figure 2**). This also demonstrates our polyclonal antibody can be used to identify Zfp410 in multiple species, even though the antibody was generated against a purified mouse isoform of Zfp410. Several extracts were tested to observe which tissues Zfp410 was in, notably the whole rat brain which was used in future experimentation. Bands above 37 kDa suggested the antibody detected other isoforms of Zfp410 or Zfp410-like proteins (52 and 100 kDa respectively). The 52 kDa isoform of Zfp410 has been reported to be found in both human and rats, but not previously reported in mice. While the protein ladder shows an approximate molecular weight, mass spectrometry may be necessary to determine if the band a little higher than 50 kDa is truly the 52 kDa isoform of Zfp410.

Throughout all extracts, a band can be seen at ~75 kDa though no isoform of Zfp410 has been identified at this molecular weight. A study reported that Zfp410 may be able to be

sumyolated, which is a chemical modification that has been identified to increase the half-life of proteins or may aid in the repair of damaged DNA (Benanti et al., 2002). Proteins that have been sumoylated have been reported to run about 20-25 kDa higher, adding to their molecular weight. If the 52 kDa isoform of Zfp410 is sumoylated, it would be reasonable to suggest that the 75 kDa band could be a sumoylated version of Zfp410. While this was not part of the aims of the study, if Zfp410 can be modified in such a way this may further elucidate the role it plays in cognitive and molecular pathways.

Figure 2

Western blot showing Zfp410 in various extracts



Note. A western blot was performed using extracts from a human cancer cell line, brain tissue obtained from mice and rats. Figure by Mariko Locke.

Aim 2: Identification of Potential Zfp410 Binding Partners

Overview

After the polyclonal antibody had been characterized, it was ready to be used in identifying Zfp410 interacting proteins from rat brain extracts. The co-immunoprecipitation (Co-IP) assay utilizes an agarose resin that is specific to the antibody species, in this case Protein A agarose beads were used to bind antibodies generated from a rabbit (Bergmann-Leitner et al., 2008). Using our antibody, we formed a protein complex that binds to Zfp410 and pulls any interacting proteins attached to it out of solution. After a series of washes to remove non-specific proteins bound to the resin, the protein complex containing Zfp410 and its binding partners were separated from the beads using a high salt buffer containing dithiothreitol (DTT), a reducing agent to sever the linkage to the resin (Sousa et al., 2011). Once the protein complex was isolated, SDS-PAGE was used in tandem with the western blot assay and silver stain analysis to visualize and identify potential binding partners of Zfp410. Western blots identify a specific protein, in this case all isoforms of Zfp410 were observed. Conversely, silver stain analysis is a highly sensitive technique that provides a way to visually analyze all protein bands that were pulled out of solution (Hempelmann & Krafts, 2017). Together these techniques allowed us to get a visual representation of the isolated Zfp410 complex and determine which proteins could be potential Zfp410 interacting proteins. After looking over the images, we chose a band we thought might be an interacting protein. We then utilized tandem mass spectrometry, a technique used to determine the identity of an unknown protein using mass spectrometry, along with a compound library that matched the fragmentation patterns to known proteins. From this data, we were able to purchase commercially available antibodies for each respective candidate and confirmed them via western blot.

Methods: Co-Immunoprecipitation, Silver Stain, and Western Blot Analysis

The ethanol-preserved Protein A agarose resin was prepared by taking 100µL and spinning at 3,000 rpm for 2 minutes. The ethanol was removed carefully by pipetting and 50µL of Co-IP wash buffer (20mM Tris pH 7.5, 150mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 10% glycerol, 0.2% NP-40) was added to resuspend the resin. This step was repeated twice, centrifuging at 3,000 rpm between each addition of wash buffer to ensure ethanol was removed from the resin. 10µL of 10mg/mL bovine serum albumin (BSA) was added to the resuspended resin and allowed to equilibrate on a rocker for 1 hour at 4°C. After blocking, the resin was rinsed with 1mL of wash buffer and spun at 3,000 rpm for 2 minutes. The supernatant was discarded, and the resin was rinsed a total of 2 times before resuspending with 50µL of wash buffer. Once a 50/50 slurry was made, 40µL of resin was transferred to each tube. 32µL of the appropriate antibody was added to the correspondingly labeled tube. Once the antibodies were added, the tubes were collected and incubated at 4°C for 4 hours while rocking. After incubation, the resin was rinsed with 1mL wash buffer followed by centrifugation for 2 minutes at 3,000 rpm. A total of two washes were completed for each tube. An extract mixture was prepared by mixing 210µL of the previously prepared rat brain extract with 420µL of wash buffer to dilute the extract. 300µL of the extract mixture was put into each tube, and overnight incubation at 4°C allowed sufficient time for binding interactions to occur between the antibody and Zfp410 complex. After incubation, the resin was rinsed 2 times with 1mL of wash buffer and centrifugation at 3,000 rpm for 2 minutes between each wash. After the final wash, the supernatant was discarded and 30µL of elution buffer (20mM Tris pH 7.5, 500mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 20% glycerol, and 50mM DTT added at time of use) was added to each tube. After a 5 minute incubation followed by mixing with gentle flicking, the resin was

centrifuged at 15,000 rpm for 2 minutes. The supernatant from each tube was collected and moved to a fresh, appropriately labeled tube and 10uL of 4x sample buffer (250mM Tris pH 7.5, 40% glycerol, 0.04% bromophenol blue, 4% SDS, and 588mM β-mercaptoethanol) was added. After mixing, the tubes were stored at -20°C for future use.

An SDS-PAGE gel was prepared as mentioned previously, and once solidified, 2μL of protein ladder was loaded. Next to it, 15μL of each sample was loaded into separate wells of the gel. Electrophoresis began at 23mA for 30 minutes, then increased to 35mA for 45 minutes until the dye front reached the bottom of the gel. Prior to the electrophoresis run finishing, 100mLs of 10% SDS was added to a clean Pyrex dish and sat for 10 minutes before rinsing the container with copious amounts of running water. After electrophoresis was finished, one gel was transferred to the clean glass container and 300mLs of 50% methanol was added. The other gel was used for western blot and the western blot protocol was followed as listed previously. The glass container was placed on a rocker for 30 minutes at room temperature. After rocking, the solution was discarded and 300mLs of 5% methanol was added to the container and placed on the rocker. After 10 minutes, the solution was discarded, and the gel was rinsed with 50mLs of RO water 3 times. A solution containing 5μM DTT in 100mLs RO water was added to the container and placed on a rocker for 20 minutes. After the solution was discarded, 100mLs of 0.1% $AgNO_3$ was added to the container and placed on the rocker for 20 minutes. A developing solution was prepared by mixing 200mLs of sodium carbonate and 89μL of 37% formalin. After the solution in the container was discarded, the gel was rinsed with 100mLs of RO water and discarded. This was followed by two washes (50mLs each) with the developing solution. The solution from the container was discarded in an appropriately labeled container for safe disposal at a later time. The remaining 100mLs of developing solution was transferred to the container

and placed on the rocker. After ~15 minutes, dark bands appeared on the gel and 5g of citric acid was added to the container to stop the reaction. Aluminum foil was used to cover the container for 10 minutes, before discarding the solution into an appropriate container. De-ionized (DI) water was added to the container until imaging by a Chemi-Doc imager.

After a band was selected for tandem mass spectrometry analysis, the gel was cut using a sterile razor blade and the gel slice placed in a clean 1.5 mL tube. The sample was sent to Duke Proteomics for tandem mass spectrometry analysis. Confirmation assays were completed using the methodology above using commercial antibodies at a 1:1,000 dilution and purchased from ThermoFisher.

Results

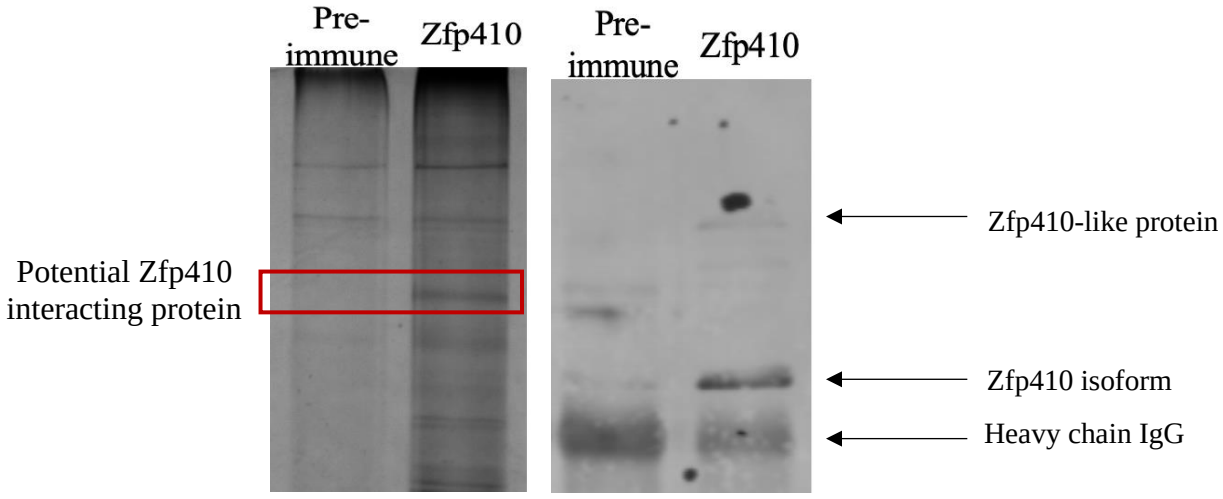
The silver stain analysis method took several weeks to adjust the protocol, with many long nights spent looking at various silver stain protocols and switching every reagent that was used. After 2 months, a working silver stain protocol was verified and used to complete the analysis portion of the Co-IP assay. Once the Co-IP conditions were empirically determined, silver stain analysis showed several bands at sizes ranging from 37-250 kDa. The western blot showed Zfp410 as well as other isoforms (52 kDa) and a potential Zfp410-like protein (100 kDa). Using both methods of visualization, we were able to confirm Zfp410 was pulled down and identify a possible binding partner. The pre-immune sample served as a negative control, which contained serum from the rabbit before immunization against Zfp410. This also showed some background proteins that may have bound non-specifically to the Protein A resin and was useful in determining which bands were potential interacting proteins. This sample was compared to the Zfp410 antibody sample. The criteria for selecting potential Zfp410 interacting proteins were bands that appeared in the Zfp410 sample but absent in the pre-immune sample

from the silver stain. The western blot was used to determine if a band was present at that weight, suggesting a potential isoform of Zfp410 or a similar protein in structure. Using the previously described criteria, a band at ~180 kDa was selected as a potential binding partner of Zfp410 (**Figure 3**).

Once analysis was complete and data was received from Duke Proteomics, a list of the top 5 candidates were selected by scoring of percentage sequence coverage and molecular weight (**Table 1**). The top 3 proteins in **Table 1** are involved in metabolic functions of the cell, while the proteins encoded by the genes Smarca2 and Smarca4 are involved in chromatin remodeling complexes. If Zfp410 was confirmed to be associated with chromatin remodeling factors, such as these two proteins, more research could be done on the role Zfp410 plays in such pathways. Commercial antibodies were ordered for all 5 proteins and the presence of Zfp410 was confirmed by western blotting.

Figure 3

Zfp410 co-immunoprecipitation followed by silver stain and western blot



Note. A Zfp410 co-immunoprecipitation assay was performed followed by silver stain (left) and western blot (right) analysis. Figure by Mariko Locke.

Table 1

Mass Spectrometry Data of Potential Zfp410 Interacting Proteins

Name of identified protein	Gene name	Molecular weight (kDa)	Protein identification probability	Percentage sequence coverage
Intersectin-1	Itsn1	194	100%	29.70%
Kinesin family member 21A	Kif21a	185	100%	23.20%
Ser/Thr-protein kinase MRCK beta	Cdc42bpb	182	100%	25.00%
SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, type 2	Smarca2	182	100%	9.14%
Transcription activator BRG1	Smarca4	181	100%	16.20%

Note: Table of potential interacting proteins with Zfp410, table by Mariko Locke.

Aim 3: Determining Locations Where Zfp410 is Bound to DNA in the Brain

Overview

After completion of the Co-IP assay, our custom antibody made against Zfp410 was further characterized and confirmed the antibody was suitable for immunoprecipitation assays. With this information, we were able to use this antibody for our other goal: determination of where Zfp410 binds in the brain and if methotrexate displacement of Zfp410 results in a cognitive deficiency. Male rats were obtained and tested after methotrexate injection by the novel object test at one and two months post-injection. After behavioral data was collected, the hippocampus of each rat was obtained for chromatin immunoprecipitation followed by qualitative polymerase chain reaction (qPCR) analysis. Chromatin immunoprecipitation (ChIP) is similar to the Co-IP assay as described in previously, however instead of studying protein-protein interactions, the ChIP assay is used to identify protein-DNA interactions. To do this,

crosslinking the protein/DNA complex in formaldehyde is vital for ChIP to succeed. Once the complex is formed, our Zfp410 antibody pulls the complex out of solution and the DNA can be purified for polymerase chain reaction analysis (PCR). This technique is used for confirmation since a small amount of DNA is required and by the end of PCR, the small amount of DNA is amplified multiple times. For sequencing, PCR analysis allowed us to determine if DNA was present in our sample and provided signals to adjust the protocol if needed.

Due to the lack of literature on where Zfp410 binds to DNA, we sent an initial sample containing DNA pulled down using our Zfp410 antibody for ChIP-Seq analysis. The sequencing results show all the regions in the genome where Zfp410 was found on DNA, and these regions were used to find a suitable positive control for Zfp410. An input sample containing the original genetic material was also purified and sent as a comparison, further refining our search for Zfp410 specific regions. After reviewing the data, 4 primers were ordered at different locations and a total of 6 ChIPs (3 methotrexate treated rats and 3 control rats) were sent off for ChIP-Seq analysis.

Methods: ChIP and qPCR Analysis

Fresh rat hippocampus tissue was obtained and crosslinked in 1.5% formalin/phosphate buffered saline (PBS) solution (10mM sodium phosphate pH 7.4 and 150mM NaCl) for 15 minutes at 37°C, followed by quenching the reaction with 500μL of 2.5M glycine and incubation on ice for at least 10 minutes. The tubes were spun at 720 rpm for 5 minutes in a chilled centrifuge. After the supernatant was discarded, the tissue was rinsed with 2mLs of cold PBS. The tissue was spun at 720 rpm for 5 minutes and the supernatant discarded. The cross-linked tissue was moved to fresh tubes and frozen at -80°C for later use. About 120mgs of tissue was weighed out and kept on ice until placed in a 1.5 mL homogenizer with 500μL of cold PBS that

contained protease inhibitors. After the tissue was dounced 15 times on ice, 26µL of 20% SDS was added to the solution and gently mixed. The tube was left at room temperature for 30 minutes. After the incubation period, the Branson Digital Sonifier was used at 27% amplitude for 10 seconds to sonicate the homogenized material 9 times with 1 minute rests on ice between each 10 second burst. After sonication, the material was spun in a chilled centrifuge at 15,000 rpm for 10 minutes. The supernatant was removed to a fresh tube and the pellet discarded. Then, 20µL of the supernatant was transferred to a fresh 1.5 mL tube as an input sample. Each reaction used 100µL of the supernatant with 900µL of the ChIP dilution buffer (0.5% Triton X-100, 2mM EDTA, 20mM Tris buffer pH 8.0, 150mM NaCl, 1mM DTT, 1 protease inhibitor tablet at time of use, and 2µg of sonicated salmon sperm DNA). After 100µL of Protein A agarose beads were placed into a new tube and spun down at 3,000 rpm in a centrifuge for 2 minutes, the supernatant was removed and 50µL of 10mM TE buffer (10mM Tris-HCl pH 8.0, 0.1mM EDTA) was used to resuspend the resin, followed by an additional centrifugation. Next, 50µL of 10mM TE buffer was added to the beads and mixed before 20µL of resin was aliquoted to each ChIP reaction. The tubes containing the diluted ChIP material and resin were placed on a rocker for 2 hours at 4°C. After incubation, the tubes were spun down in a chilled centrifuge at 15,000 rpm for 1 minute. The supernatants were transferred to appropriately labeled tubes. To the tube labeled “H3”, 3µg of purified histone H3 antibody was added. The pre-immune antibody sample had 16µL of non-immunized rabbit serum added, while the Zfp410 tube had 16µL of Zfp410 antibody rabbit serum added. All tubes were placed on a rocker overnight at 4°C. 200µL of Protein A agarose resin was transferred to a 1.5 mL tube, spun down at 3,000 rpm for 2 minutes and the supernatant discarded. 100µL of 10mM TE buffer was added to the resin, spun at 3,000 rpm for 2 minutes and the supernatant discarded. 100µL of 10mM TE buffer was added to the resin to prepare a

50/50 slurry before 20µL of 10mg/mL BSA was added to the tube. 20µL of 10mg/mL sonicated salmon sperm DNA was added to the tube, which was incubated overnight at 4°C while rocking. The blocked resin was spun in a chilled centrifuge at 3,000 rpm for 2 minutes. After the supernatant was discarded, the resin was washed twice with 1 mL of 10mM TE. After the supernatant was discarded from the second wash, the resin was resuspended in 100µL of 10mM TE buffer. The tubes containing the diluted ChIP material were placed on ice and 40µL of the blocked resin was added to each tube. The tubes were then incubated for 2 hours at 4°C while rocking. After incubation, the tubes were obtained and spun at 3,000 rpm in a chilled centrifuge for 3 minutes. The supernatant was discarded, and the resin was rinsed 4 times with 1mL of ChIP wash buffer (0.25% NP-40, 0.05% SDS, 2mM EDTA, 20mM Tris pH 8.0, 250mM NaCl, 0.1µL/mL Leupeptin, and 0.1µL/mL Aprotinin) with centrifugation as noted above between each wash. After the last wash with ChIP wash buffer, 1mL of 10mM TE buffer was used to wash the resin a final time. After centrifugation, the supernatant was discarded and 100µL of ChIP elution buffer (100mM $NaHCO_3$, 1% SDS) was added to the resin. 80µL of ChIP elution buffer was added to the input sample and all tubes were incubated overnight in a 65°C water bath. After incubation, the tubes were spun at 15,000 rpm for 2 minutes. The supernatant was collected and moved to fresh tubes and 2.5µL of 20mg/mL proteinase K was added to all tubes which were incubated at 65°C for 1 hour. After incubation, 97.5µL of 10mM TE buffer was added to all tubes before adding 200µL of the bottom layer of phenol:chloroform:isoamyl alcohol (PCIAA). Each tube was mixed by vortexing thoroughly, followed by centrifugation at 15,000 rpm for 5 minutes. The aqueous layer was moved to a fresh tube and the rest discarded. The PCIAA step was repeated and followed by centrifugation. After the supernatant was removed to a new tube, 200µL of chloroform was added and vortexed thoroughly. The tubes were centrifuged at 15,000

rpm for 5 minutes and the top layer was moved to a fresh tube. A solution containing 20µL of 3M NaOAc, 3 volumes of 100% ethanol, 1.5µL of 10mg/mL glycogen was added to each tube and incubated at -80°C overnight. The tubes were spun at 15,000 rpm for 20 minutes in a chilled centrifuge. After the supernatant was removed, 300µL of 70% ethanol was used to rinse the pellets. The tubes were quickly vortexed, then spun at 15,000 rpm for 5 minutes. The supernatant was removed, and the pellets were allowed to air dry before resuspending in 50µL of molecular grade water.

After resuspending the DNA in 50µL of molecular grade water, the master mix solutions for qPCR were prepared. The primer set designed around the gene of interest with a potential putative binding site (Hpn) had been ordered from IDT (**Table 2**). Conditions taken into consideration during primer design were: optimal Tm of 60°C, the PCR product to be between 80-140 bases, GC clamp, GC content between 40-60%, and the length of the PCR primers were between 18-22 bases. A 100mM primer stock was prepared for each primer set. Each PCR reaction contained 2µL of 100mM primer stock, 6µL of molecular grade water, 10µL of 2X Sybr-Green PCR master mix, and 2µL of DNA for a final volume of 20µL. PCR ran according to the following scheme: 98°C for 3 minutes, 98°C for 10 seconds, 55°C for 30 seconds, 65°C for 5 seconds, then 95 °C for 5 minutes. A total of 50 amplification cycles were completed.

Results

After the behavioral tests were completed and scored, the rat hippocampus tissue was collected for ChIP. The methotrexate treated rats showed cognitive decline in comparison to the controls, as expected. Since ChIP assays are typically done on a monolayer of cells and our model utilizes hippocampus tissue from rats, the protocol required further development to maximize the efficiency of the assay. One of the steps taken to refine our protocol was

determining how many sonications were necessary for our immunoprecipitation to work. Sonication applies a high frequency that ruptures cell membranes and shears DNA into smaller pieces. This process is vital to ensure the chromatin is an appropriate size prior to purification and analysis by qPCR. After several weeks of experimentation, it was empirically determined that 9 sonications were required to obtain DNA that ranges from 200-1000 bases in size. DNA that is too large may be more difficult to replicate during PCR, and DNA extracted that is less than 200 bases may form primer dimers.

Table 2

Primer sequences designed to identify Zfp410

	Genes	Forward Sequence	Reverse Sequence
Positive controls			
	Hpn	CATAGGCAGGCACTGACAC	TGGAAGAGCAGCTAATACCC
	Chr1	GCTATGCTGTGCTGTTCCAG	ACAGTGGGGTGGAGAAAGAC
	Chr7	GCTCCAACTTAACGCTACGC	AGGTGGACCAACAAGAGCAC
	Chr9	GAATACACGACTGGGTGCAG	CTGGGGGAGGGAATGTAAAC
Negative controls			
	Hpn	CCTTGGCTTCCCGAATG	GCAAAACCTGGGAGATGG
	Chr1	AAGAGGAGCCAAGGATAGGC	CCTGTGACCAGTGTGGAATG

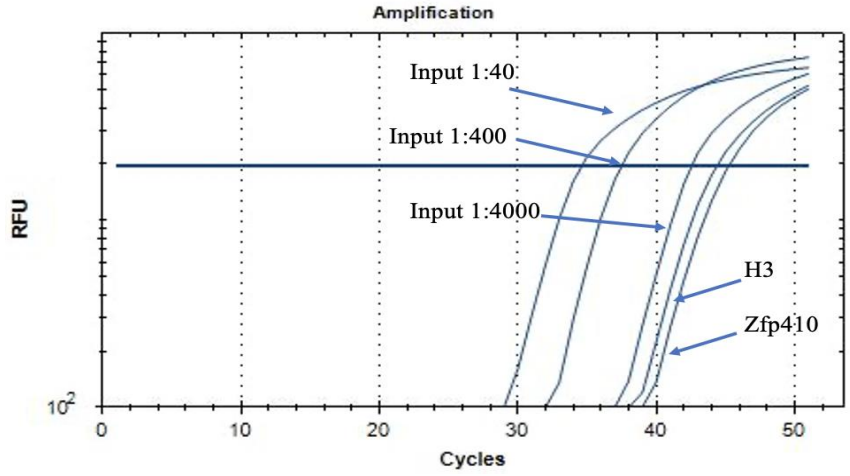
Note: Table of PCR primers designed, table by Mariko Locke.

For the initial part of this assay, a histone H3 antibody was used as a positive control due to the abundance of histones within the genome (Duan et al., 2019). The negative control contained no antibody, while the pre-immune serum from the rabbit showed the background signal during qPCR. After bioinformatics analysis and searching for a homologue of the genes affected by folate deficiency, a putative Zfp410 binding site was determined at the Hpn gene located on chromosome 1 in rats (Lawton et al., 2020). Once the binding site was determined

using the putative motif for Zfp410, a forward and reverse primer set was designed around this region. A negative control primer set was designed to confirm the specificity of the binding site, and an absence of Zfp410 was predicted at this region in the genome. A series of input dilutions were used to compare the efficiency of the immunoprecipitation to 5%, 0.5%, 0.05%, and 0.005% of the DNA in the original sample. The results included an abundant signal of the positive control (histone H3) at the region away from the Zfp410 binding site, and low Zfp410 signal. In contrast, at the Zfp410 binding region of Hpn an abundant histone H3 signal was observed along with a signal from the Zfp410 immunoprecipitation. In addition, the Zfp410 signal appeared to be similar to the histone H3 signal (**Figure 4**). This suggests that Zfp410 is abundant at this region when compared to H3.

Figure 4

PCR analysis of H3 and Zfp410 ChIP assay



Note. PCR analysis of H3 and Zfp410 ChIP assay in chromosome 1 in rats was performed for ChIP-Seq analysis to identify more regions where Zfp410 is found in the rat genome. Figure by Mariko Locke.

Since the literature lacks confirmed locations where Zfp410 binds to DNA in the brain, the ChIP material obtained from using our Zfp410 antibody was sent off for sequencing (ChIP-Seq analysis) to identify more regions in the genome that may have Zfp410 bound more frequently than at Hpn. The initial data gave us 77,986 regions where Zfp410 was found to be bound to DNA after sequencing and subsequent bioinformatics analysis. Of the regions that were identified, there were 8,742 out of 77,986 that were found to be near a promoter region. This supports the finding that Zfp410 is a transcription factor, due to its location on DNA relative to the transcription pathway. Not all regions at a promoter will be used to confirm the location of Zfp410 since the data needs to be reviewed for reproducibility. Shown in **Figure 5**, 3 of the 77,986 peaks were chosen as positive controls. These were chosen based on fold enrichment, which compares Zfp410 to the input (background signal). In **Figure 5A-C**, the peaks represent the amount of DNA bound to Zfp410 at localized regions and confirm our antibody successfully pulled down the Zfp410-DNA complex (note the y axis in **Figure 5A-C**). Additionally, these peaks show that the correct number of sonications were used to shear DNA down to the appropriate size. We obtained good resolution, largely in part due to the appropriate rounds of sonication done. The bar at the top of **Figure 5A-C** shows 1,000 bases and the peaks are well within that range as we expected the DNA to be about 200-1,000 bases long. From a bioinformatics standpoint, if the range of the peak is short there will be less DNA from which to search for a binding sequence for Zfp410.

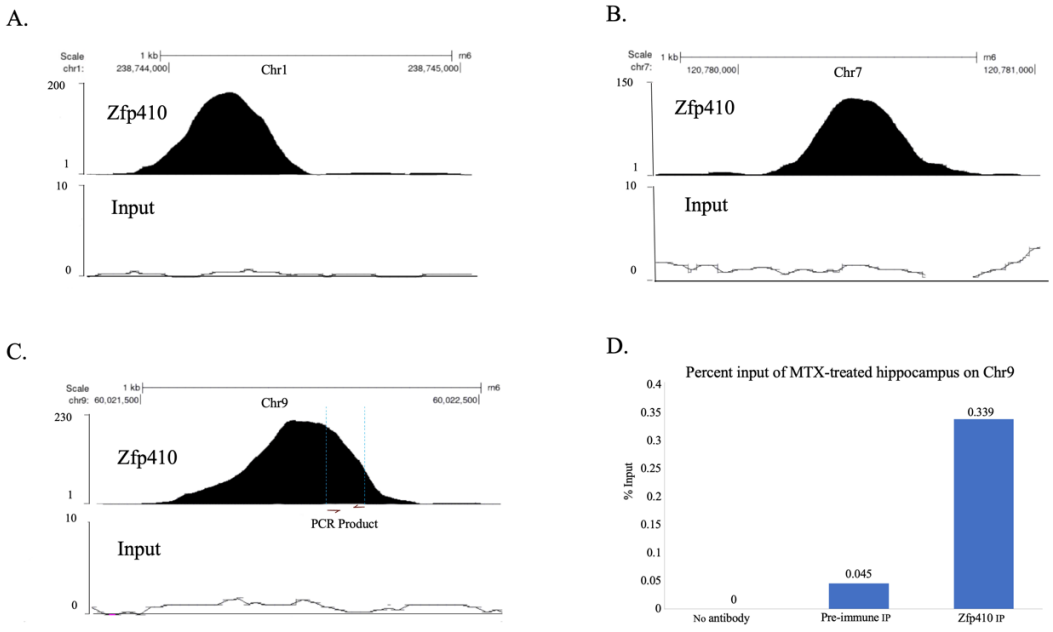
A total of 4 sets of primers were designed for these regions (3 positive controls and 1 negative control) and ordered. **Figure 5D** shows the percent input of the Zfp410 immunoprecipitation (IP) sample, the non-specific pre-immune IP, and the no antibody IP. This compares the amount of DNA bound to Zfp410 to all of the DNA present in the original sample.

Additionally, the fold enrichment between the Zfp410 IP and pre-immune IP was 7.42, showing the signal of our Zfp410 antibody relative to a non-specific antibody.

The next step was to complete 6 ChIP assays (3 methotrexate treated rats, and 3 control rats) and the material was sent for ChIP-Seq analysis to determine if Zfp410 was displaced by the methotrexate injections. qPCR analysis was done to look at the efficiency of the following ChIP assays for the 3 chosen positive control regions. The primers for chromosome 9 gave the best qPCR data as evaluated by our qPCR criteria and is being used as the positive control to test the reproducibility of our 6 ChIPs that were sent for sequencing (**Figure 5D,** note difference between Zfp410 IP vs Pre-immune IP).

Figure 5

Initial ChIP-Seq data with PCR analysis of Chr9



Note. After the initial ChIP-Seq data was reviewed, three positive control regions were chosen for PCR and a qualitative analysis of a MTX-treated ChIP was performed. Figure by Mariko Locke.

Conclusion

While transcription factors are vital in regulating cellular functions, they can also be targeted to treat diseases. Many cancer treatments target the interactions between transcription factor complexes to prevent oncogene expression, which prevents tumor growth (Lambert et al., 2018). Future medical research may include targeting the interactions between Zfp410 complexes to negate the detrimental effects of methotrexate on cognition.

A paper published in 2021 demonstrated the role Zfp410 plays in repressing fetal hemoglobin genes in mature red blood cells (Vinjamur et al., 2021). Although this study was quite limited in the results found, it does illustrate how this transcription factor is under current investigation by other groups and the significance it could have to further elucidating these pathways.

A potential limit to our study was only using male rats to observe how methotrexate influences cognition; however, female rats were also purchased. After injection and novel object tests were completed, behavioral data demonstrated that methotrexate treated females did not experience as much of a cognitive deficit as male rats did. This could suggest that female rats are more resilient to combatting the effects of methotrexate and that these pathways may be important for those studying brain fog caused by methotrexate.

The aims of the project have been completed since several potential Zfp410 interacting proteins and regions within the rat genome where Zfp410 is bound have been identified. The results obtained from this project sets the foundation for further investigation on Zfp410 and its functions. Additionally, more work in the lab can be done to redo experiments to demonstrate the data is reproducible and determine if Zfp410 is found at the same location in 3-6 different rats by qPCR analysis. Further bioinformatics refinement can also be done to narrow the number of

peaks chosen for confirmation assays as well as compile a gene ontology report to determine the cellular locations, biological processes, and specifically the molecular functions that are impacted by Zfp410.

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