NOTICE:
The copyright law of the United States (Title 17, United States Code) governs the making of reproductions of copyrighted material. One specified condition is that the reproduction is not to be "used for any purpose other than private study, scholarship, or research." If a user makes a request for, or later uses a reproduction for purposes in excess of "fair use," that user may be liable for copyright infringement.

RESTRICTIONS:
This student work may be read, quoted from, cited, and reproduced for purposes of research. It may not be published in full except by permission by the author.

Albright College Gingrich Library

Rescuing of long-distance DNA interactions required for CSR by YY1

Justin Hoffman

Candidate for the degree

Bachelor of Sciences

Submitted in partial fulfilment of the requirements for

College Honors

Departmental Distinction in Chemistry & Biochemistry

Albright College Gingrich Library

Karen Campbell, Ph.D.

Adam Hersperger, Ph.D.

Alberto Cacicedo, Ph.D.

F. Wilbur Gingrich Library
Special Collections Department
Albright College

Release of Senior Thesis

I hereby grant to the Special Collections Department of the F. Wilbur Gingrich Library at Albright College the nonexclusive right and privilege to reproduce, disseminate, or otherwise preserve the Senior Honors Thesis described below in any noncommercial manner that furthers the educational, research or public service purposes of Albright College. Copyright privileges remain with me, the author.

Title: Rescuing of long-distance DNA interactions required for CSR by YY1

Signature of Author: _____ Date: 4/17/18

Printed Name of Author: Justin Hoffman

Street Address: 801 W. County Line Road

City, State, Zip Code: Hatboro, PA, 19040

Albright College Gingrich Library

Justin Hoffman

Senior Honors Thesis

Rescuing of long-distance DNA interactions required for CSR by YY1

Abstract:

Ying Yang1 (YY1) is a ubiquitously expressed transcription factor, regulating a dazzling list of genes of fundamental biologic processes. Along with cellular proliferation, differentiation, embryogenesis, replication and apoptosis, one of the crucial functions of YY1 is to aid in class-switch recombination (CSR) during B cell development. YY1 conditional knockout in activated splenic B cells interferes with CSR. YY1 is known to perform this process by mediating the long-distance DNA interactions that govern B cell maturation. DNA-bound YY1 provides a platform for recruitment of various protein complexes (directly or indirectly) to mediate long-distance DNA interactions. The different functional domains of YY1 involved in the long-distance DNA loops have been identified; however, the partner proteins are yet to be identified. The current study was aimed at elucidating the functional domain of YY1 that dictates long-distance DNA interactions required for CSR. The speculated domain of YY1 responsible for this process was successfully isolated and amplified, however, due to low infectivity of splenic B cells, this project warrants future research.

Albright College Gingrich Library

Introduction/Background:

Yin Yang 1 (YY1) is a ubiquitously expressed protein with various known functions, especially to B cells (1). In 1985, *in vitro* studies with B cells demonstrated the existence of a protein that bound to the Eμ1 site of the immunoglobulin heavy chain Eμ enhancer. Through further experimentation, YY1 was identified as the protein binding the μE1 site (1). Other regions

along the immunoglobulin heavy chain locus were identified as sites of interactions with YY1, including the 3' regulatory region (3'RR) located at the 3' end of the locus (2). YY1 was later isolated and purified in 1991 by three separate labs which collectively agreed on naming the protein YY1 (1). Since the isolation of YY1, various functions have been described over the years. YY1 was shown to be crucial for embryonic development as for the lack of YY1 results in lethality. YY1 was also shown to aid in cell growth and differentiation. YY1 is associated with many cancers including B cell lymphomas. Most recently, YY1 has been associated with mechanisms such as X-chromosome inactivation, imprinting, and viral gene expression (1).

Since its isolation, YY1 has been extensively studied determining several functional domains contained within the protein as well as the various functions of the protein. All proteins, including YY1, are comprised of amino acids or residues, the basic structural unit of a protein with each amino acid containing a carboxyl group (C) and an amino group (N). Together, a chain of amino acids contains both a C-terminus and an N-terminus and is functionally referred to as a protein. Starting at the N-terminus, the first 100 amino acids of YY1 encode several key features. Amino acids 16-29 display the potential to form a helical structure because of the sequence of amphipathic and negatively charged residues, and this region is referred to as the acid stretch. 43-53 contains 11 acidic residues (acid stretch) and 70-80 consists of eleven positively charged histidine residues (histidine stretch). The region separating these two segments is rich in neutral glycine residues. 80-100 is rich in proline and glutamine (Figure 1).

Albright College Gingrich Library

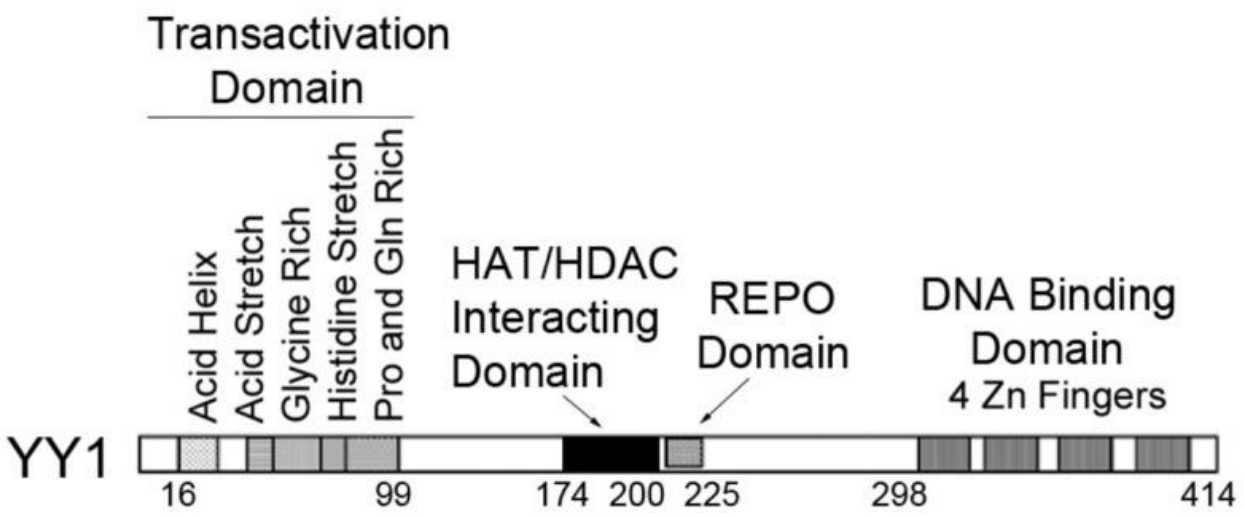


Figure 1. YY1 domains and associated functions

(1)

The domain 174-200 has known interactions with histone acetyltransferases (HAT) and histone deacetylases (HDAC). Because of this domain, YY1 is partially thought to enhance and repress gene expression because of its association with HAT and HDAC respectively (1, 3). 201-226 and 296-414 displayed sequence homology to Polycomb Group (PcG) protein which consists of a family of proteins involved in several processes: epigenetic chromosomal condensation, stable transcriptional repression, control of cell proliferation, hematopoietic development, in addition to stem cell self-renewal (1, 4). Experimentation revealed the functionality of YY1 to act as a PcG protein (1). The functionality of YY1 as a PcG protein was demonstrated in B cell development offering up new information about the potential mechanisms governing B cell proliferation. 201-226 was functionally named the REPO domain because of its ability to Recruit Polycomb. While the YY1 mutant lacking the REPO domain can perform most YY1 tasks, it is unable to perform many of the PcG tasks. The domain 298-414 was functionally named the DNA binding domain due to the four zinc finger motifs (1).

Albright College Gingrich Library

B cells are one of the various immune cells found in the mammalian body that serve as a host defense system to various pathogens, including bacteria, viruses and fungi. The relevance of B cells, with regards to YY1, is that YY1 helps in B cell development. These types of cells are created in primary lymphoid organs including the bone marrow and later mature in secondary lymphoid organs including the lymph nodes and spleen. The mechanism by which B cells protect the host is through the production of antibodies, also known as immunoglobulins (Ig) (5).

Immunoglobulins start as proteins comprised of four subunits or polypeptide chains. There are two heavy chains and two light chains (Figure 2). The terms heavy and light refer to the number of amino acids or residues, the smallest structural unit of a protein, with heavy implying a longer chain of amino acids. Together, the four subunits are held together by disulfide bonds between cysteine residues of each subunit. The combination of the subunits forms a “Y” shaped protein (Figure 2). While the immunoglobulin is comprised of several subunits, together, the various regions of the “Y” shaped protein can be classified by the functionality of each region. At the end of each N-termini arm of the immunoglobulin, there are antigen-binding sites that are contained within what is referred to as the variable region. The remaining region of the immunoglobulin heading towards to C-termini is referred to as the constant region (Figure 2).

Albright College Gingrich Library

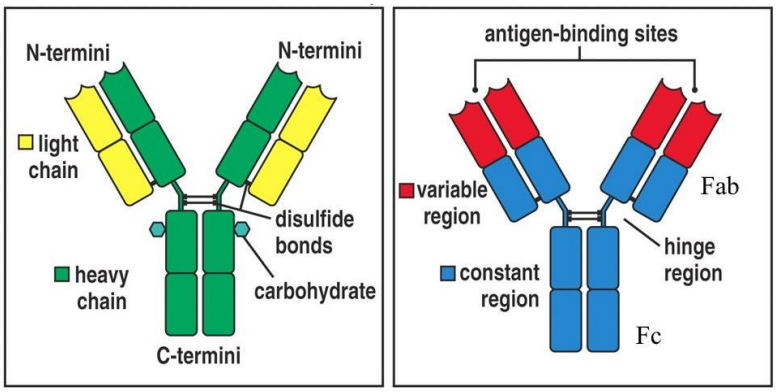


Figure 2. Basic antibody structure

(5)

The variable region, generally speaking, is given its name due to the nature of its ability to vary from one immunoglobulin to the next. The variation allows for the recognition of different types of antigens. One classification of an antigen is a molecule or a fragment of a molecule that can bind to an immunoglobulin at the antigen-binding site (5). These molecules can either be derived from the host or from a foreign invading pathogen. Those molecules that are derived from a foreign pathogen are typically recognized as foreign due to the high variability of the antigen-binding site located upon the variable region. Upon recognition of foreign antigens, a signaling event warns the cell to increase production of immunoglobulins as well as other immune cells to help rid the host of the foreign invader (5). Likewise, the constant region is given its name due to the low variability within an immunoglobulin class.

Albright College Gingrich Library

B cells undergo several developmental stages between the time of synthesis and the time of release from the bone marrow. The stages leading up to the release of B cells from the bone marrow are: pro-B, pre-B, immature B, and mature B (1). While the physical B cell is derived from a stem cell precursor, there are particular loci that ultimately code for the different units of the later produced immunoglobulins. Both the heavy and light chain loci are comprised of multiple genes.

The heavy chain has only one locus which has 38-46 Variable (V) genes, 23 Diversity (D) genes, 6 Joining (J) genes, and 9 Constant (C) genes. The light chain has two loci, the kappa (κ) locus and the lambda (λ) locus. The κ locus has 34-38 V genes, 5 J genes, and 1 C gene, while the λ locus has 29-33 V genes, 4-5 J genes, and 4-5 C genes (5, Figure 3).

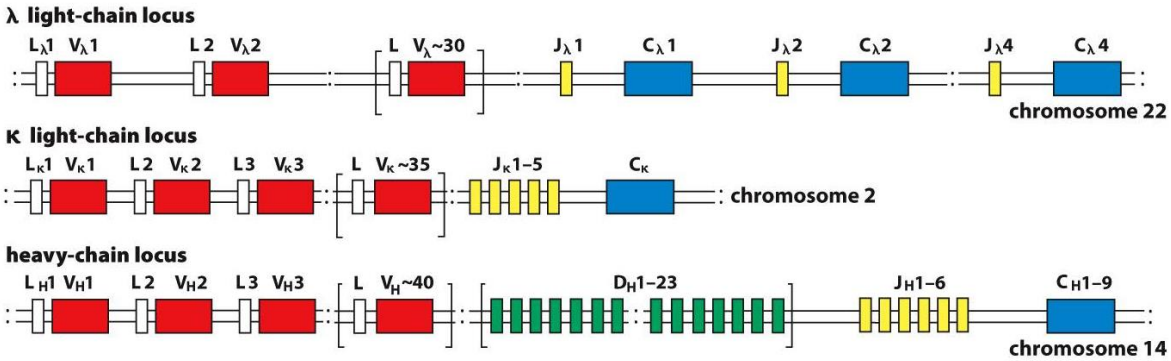


Figure 3. Immunoglobulin heavy and light chain loci

(5)

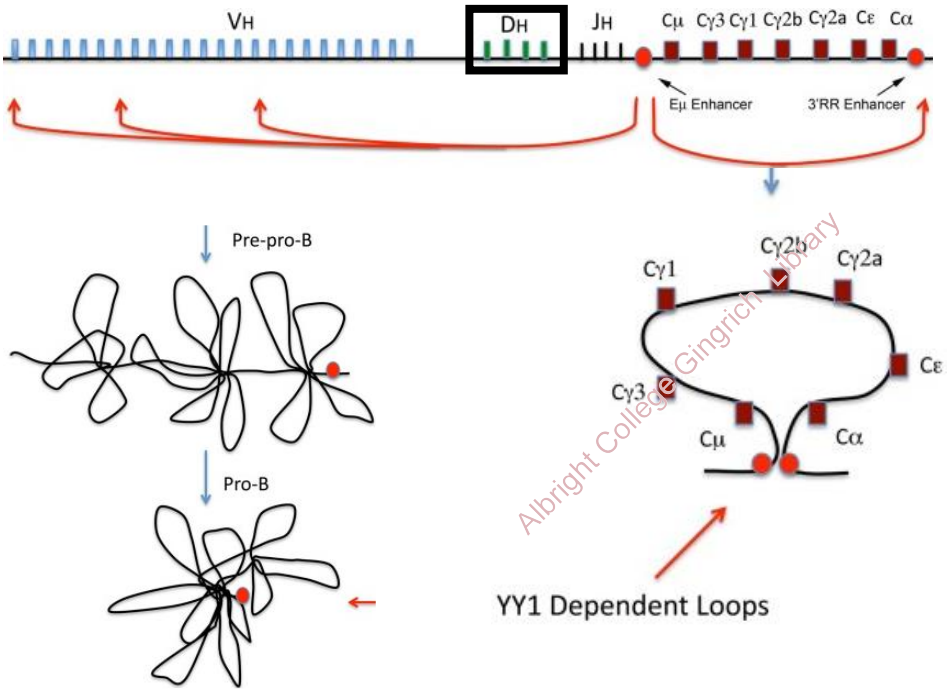
Unlike the heavy chain locus, neither the κ locus or the λ locus has D genes because they are not required for the formation of their respective gene products of light chain subunits. Immunoglobulin heavy chain genes and light chain genes each giving rise to the heavy and the light chain subunits of the immunoglobulin structure.

Albright College Gingrich Library

Each gene is separated by a large distance as the immunoglobulin loci are 2.4-3.2 Mb in length. In order for transcriptional activity to occur, the loci must undergo somatic rearrangement through long-distance DNA interactions resulting in the contraction of the loci bringing the various genes in close proximity to produce and link V, (D), J, and C segments to produce functionally active heavy and light chain gene products. The contracted formation is known to take on various shapes including rosette-like structures as well as loops (1). This step in immunoglobulin heavy chain synthesis is referred to as the pro-B cell stage where the heavy chain locus is contracted and

accessible for recombination (5). YY1 was shown to play a role in somatic recombination of the heavy chain due to the slowing, and even arrest in some cases, of immunoglobulin production at the pro-B cell stage after YY1 knockout (1). YY1 has ultimately been shown to associate with several enhancer elements along immunoglobulin loci. Without YY1, there is a decrease in immunoglobulin contraction and the rearrangement process (1).

Somatic recombination of the light chain loci occurs at the pre-B cell stage where similar rosette structures allow for light chain synthesis. This process, similar to heavy chain synthesis, is partly governed by the presence of YY1 that helps in the folding process (1). This was determined experimentally by introducing pre-rearranged heavy chain gene to YY1 knockout B cells. The introduction of the pre-rearranged product only partially rescued immunoglobulin synthesis with B cell development still being affected by the YY1 knockout (1, Figure 4).



Albright College Gingrich Library

Figure 4. Immunoglobulin heavy and light chain loci (omit boxed DNA for light chain loci)

(1)

The maturation process of the B cell continues to become an immature B cell after the arrest of light chain loci rearrangement. The mature B cell is then defined as a cell capable of binding to and responding to a specific antigen (5). At the time of maturation, the B cell is then able to be released from the bone marrow into circulation with IgM, the first class of immunoglobulin, bound to the cell surface (5).

There are five different immunoglobulin classes: IgM, IgG, IgD, IgA, and IgE. Each of the five classes is defined by the structural differences that arise in the constant regions of each immunoglobulin (5, Figure 5).

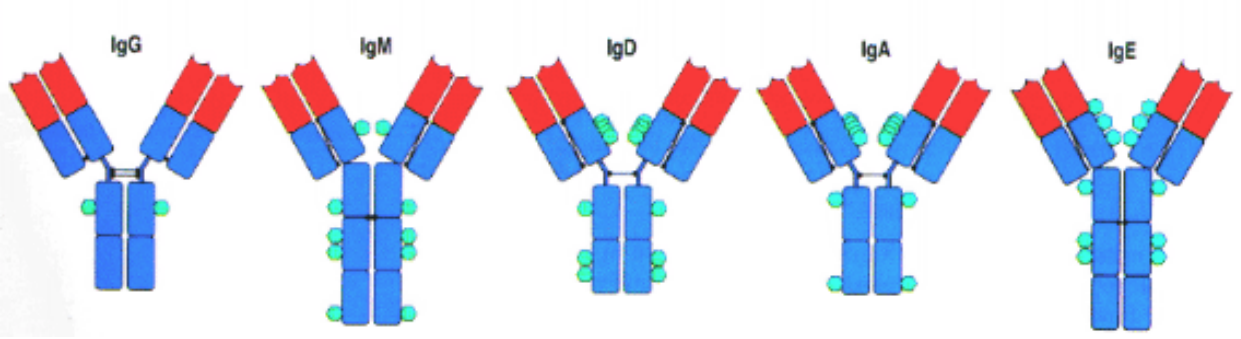


Figure 5. Structures of different immunoglobulin classes

(5)

Albright College Gingrich Library

Different structural features include the length of the constant region as well as the addition of various carbohydrates, or sugars, to the constant region. Ultimately, it is these structural features that result in distinctive functions of each class because of the quaternary structures each class of immunoglobulin can form (5). Provided a mature B cell expresses only IgM after leaving the bone marrow, there is a process referred to as class-switch recombination (CSR) that must be employed in order to alter the constant region of the immunoglobulin to ultimately change to IgM, IgG, IgD, IgA, or IgE (5).

A mature B cell expressing IgM on its surface remains IgM until the immunoglobulin binds with the antigen it is specific for. Upon recognizing and binding the correct antigen, the B cell begins to form a germinal center in the lymph node. This germinal center is a site of rapid expansion and proliferation of B cells that are specific to the antigen recently detected. During this germinal center reaction, two different processes take place simultaneously (1). The first process is referred to as somatic hypermutation. Through this process, the specificity of the antigen-binding site for the encountered antigen increases allowing for more efficient recognition of the foreign pathogen throughout the host (1). The other process that occurs during the germinal center reaction is the aforementioned CSR, also known as isotype switching (1).

While membrane bound IgM is the first class of immunoglobulin produced, the other classes can be formed through the process of CSR at which point the B cell is considered to be a plasma cell. During this process, one of five immunoglobulin classes is produced with each serving a different effector function: IgM, IgG, IgD, IgA, or IgE. From one germinal center, each one of these types may by created, but each individual cell in the germinal center expresses one class, however, all have the same specificity for the same foreign antigen (5). It is through this process that various immunoglobulins are able to be synthesized and are sent throughout the body to find the foreign pathogen.

Albright College Gingrich Library

During CSR, the C genes of the heavy locus are rearranged to ultimately offer up a new constant region. Of the nine different C genes present on the heavy chain locus, one of them is expressed and replaces the previous constant region. Through this process, the constant region is altered without affecting the specificity of the antigen-binding site (5). Over the years, this process has been studied extensively resulting in the discovery of several potential key players that allow

CSR to occur. While there are many proteins that control DNA interactions, many of them are poorly understood (2).

Activation-induced DNA deaminase (AID) was one of the first proteins to be shown to play a significant role in the process of CSR. AID initiates the process of CSR by introducing double-stranded breaks in the DNA at switch sequences (6). Switch sequences are the target sequences that help in the processes of a new constant region synthesis. The previously synthesized V, D, and J exons repair the double-stranded break in the DNA sequence by linking up with the newly switched C gene. This linkage is facilitated through the formation of a loop over a 200-kb region between nearby sequences including the Eμ enhancer and the 3' regulatory region (3'RR) located at the 3' end of the immunoglobulin heavy chain locus. This loop formation is formally referred to as the Eμ-3'RR DNA loop (Figure 6).

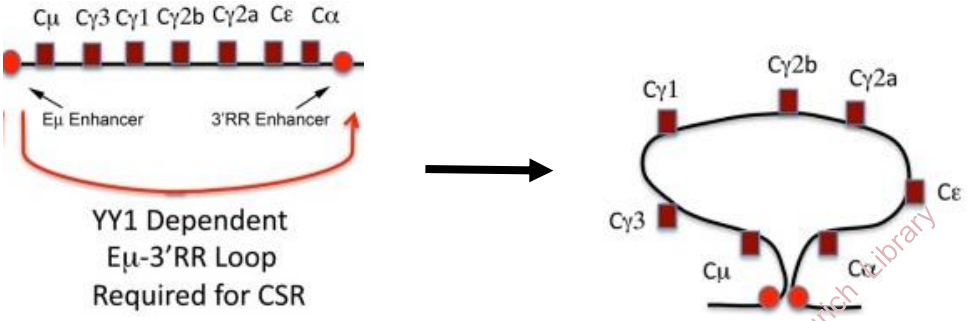


Figure 6. Eμ-3'RR DNA loop formation

(1)

Albright College Gingrich Library

While AID is necessary for the double-stranded break, experimental results suggest that YY1 physically interacts with AID to regulate its accumulation. While the conditional knockout YY1 showed some amount of class-switching, the results did show a significant decrease in the amount of CSR (2). Using a technique referred to as 3D-FISH, the Eμ and 3'RR sites were probed

to detect their interactions. In addition to the decrease in CSR, there was also a decrease in Eμ-3'RR DNA loop formation (2). Provided there are various domains of distinct features throughout YY1, the researchers went to creating various constructs of YY1 to determine which were able to rescue class-switching and/or Eμ-3'RR DNA loop formation (Figure 7).

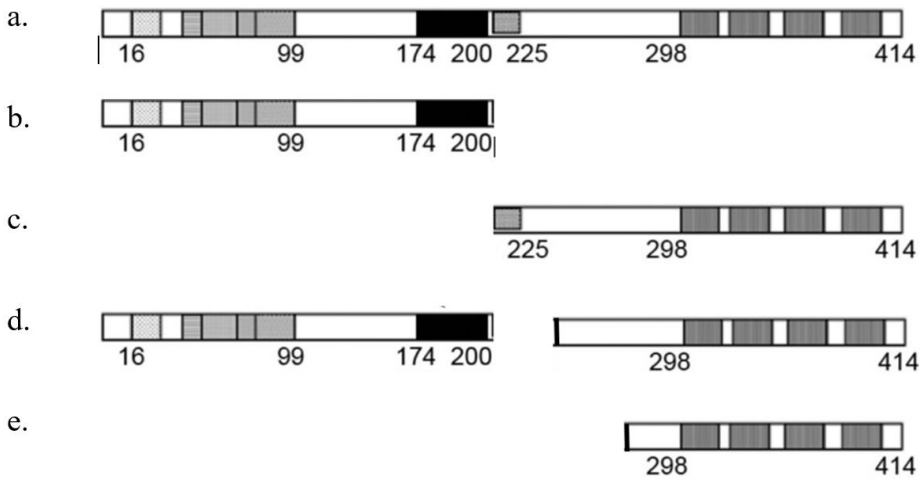


Figure 7. Previously studied YY1 constructs: (a.) wild-type (1-414) (b.) 1-200 mutant (c.) 201-414 mutant (d.) YY1ΔREPO mutant and (e.) 288-414 mutant.

(1)

Albright College Gingrich Library

The wild-type YY1 and 201-414 mutant demonstrated the ability to restore Eμ-3'RR DNA loop formation while the 1-200 mutant was unable to rescue loop formation. In parallel with these results, the wild-type YY1, 201-414 mutant, and YY1ΔREPO mutant demonstrated the ability to rescue class-switching while the 1-200 mutant and 288-414 mutant were unable to rescue class-switching. In both experiments, the C terminal half of YY1 was capable of restoring both Eμ-3'RR DNA loop formation and class-switching (2). The overall findings of this study helped to demonstrate the structural support YY1 provides B cells during class-switching allowing for isotype switching mediated by long-distance DNA interactions (2).

While this study helped to define the general region(s) of YY1 necessary for class-switching and Eμ-3'RR DNA loop formation, the purpose of the current study was to isolate the minimum sequence of amino acids in YY1 that was capable of rescuing both class-switching and Eμ-3'RR DNA loop formation. The mutant constructed in this study was 226-290. Successful synthesis of this construct would ultimately allow for the determination of the regions of YY1 necessary to govern vital processes such as immunoglobulin locus contraction during B cell development, provided so little is known about the mechanisms of these events. The mechanisms of these long-distance DNA interactions have only been proposed and this research could provide further insight to previously proposed immunoglobulin contraction mechanisms. These mechanisms as well as others are particularly important in regulatory immune functions and provide insight to other long-distance DNA interactions outside of B cell development. Additionally, the underlying mechanisms of immunoglobulin synthesis may allow for future immunotherapies and advanced diagnosis with regards to a more translational and applicable research.

Materials and Methods:

Primer Design:

Albright College Gingrich Library

The mouse YY1 from 226-290 amino acids was cloned into the pMX vector at the EcoRI-XhoI sites with the Flag peptide inserted at the N terminus for sub sequential antibody probing.

Forward Primer	5'-ATT AAT **GAA TTC** ***ATG GAC TAC AAA GAC GAT GAC GAC AAG*** TCG GAT GAA AAA AAA GAT ATT- 3'
Reverse Primer	5'-CGA TGG **CTC GAG** TCA ATC TTC TTT AAT TTT TCT TGG C-3'

Table 1. Sequence of the cloning primer used for the study (Integrated DNA Technologies)

In Table 1, the sequence of the forward and reverse primers are mentioned. The restriction site sequences are kept in bold and the Flag tag sequence is italicized. The primers were dissolved to a 100 µM stock concentration. For a working concentration, the primers were diluted 10 fold.

YY1 226-290 PCR:

Reagent	**Volume**	**Final Concentration**
5X PCR buffer	5 µl	1X
Deoxynucleotide mix	0.5 µl	200 µM of each dNTP
Forward Primer	0.75 µl	400 nM
Reverse Primer	0.75 µl	400 nM
High Fidelity Taq enzyme	0.5 µl	2 Unit/reaction
Template (plasmid DNA)	0.5 µl	20 ng
PCR grade water	Add up to 25 µl	

Table 2. PCR reaction components and final concentration

For the PCR reaction, full lengthYY1 cloned in pMX plasmid was used as the template. In order to optimize the PCR reaction, four separate PCR reactions were set up using different 5x buffers. Buffers A, B, C, and D contained 7.5, 10, 12.5, and 17.5 mM $MgCl_2$ at pH 8.5, respectively. All other reagents used are mentioned in Table 2. The PCR cycling conditions were followed as mentioned in Table 3.

Step	**Temperature**	**Time**	**Number of cycles**
Initial denaturation	94°C	2 minutes	1
Denaturation	94°C	1 minute	34 cycles
Annealing	55°C	1 minute	
Extension	72°C	1 minute	
Final Extension	72°C	7 minutes	1
Cooling	4°C	Infinite hold	

Table 3. PCR cycling conditions

Albright College Gingrich Library

For further downstream steps, the PCR products were purified to remove all the salts from the PCR reactions. For purification, a QIAQuick PCR Purification Kit was used.

Restriction Digestion, Ligation, Ligation Transformation, and Amplification:

For restriction digestion, two separate reaction mixtures were prepared for both vector and insert. The first contained 10 µl (2 µg) of pMX plasmid, 6 µL of 10x Cutsmart buffer, 2 µL of EcoRI , 2 µL of XhoI, and 40 µL of nuclease free water for a total of 60 µL. The second contained 50 µL (4.5 µg) of the purified *YY1* 226-290 insert, 6 µL of 10x Cutsmart buffer, 2 µL of EcoRI, and 2 µL of XhoI for a total volume of 60 µL. After adding all components, each reaction mixture was mixed by tapping and spun briefly followed by incubating at 37°C for 1.5 hours. After incubation, each reaction mixture was mixed with 12 µL of 6x Gel Loading Dye and run alongside a 2-log ladder on a 1% TBE-agarose gel containing 2 µL of ethidium bromide for 15 minutes at 120V. The gel was placed on a UV transilluminator and crisp bands of correct size were chopped out of the gel using a scalpel. The gel pieces were taken in microcentrifuge tubes, weighed and purified following the protocol of QIAQuick Gel Extraction Kit. The concentration of both the digested pMX plasmid and the YY1 226-290 insert were measured using the spectrophotomer for the downstream steps.

Ligation protocol with T4 DNA (MO202) Ligase was following according to New England Biolabs (Inc.) using 10x T4 DNA Ligase Buffer, T4 DNA Ligase, digested pMX(1) and digested *YY1* 226-290 insert, and nuclease free water. Two different ligation ratios were performed: 3:1 and 5:1 (*YY1* insert:pMX(1)). In addition, a pMX plasmid control was given no digested *yy1* 226-290 insert. After addition of all reagents, each mixture was spun down and incubated at 16°C overnight.

Albright College Gingrich Library

The next day, 5 µL of each ligation product was added to separate 50 µL aliquots of competent DH5α cells (Invitrogen, Catalog #18265017). The cells were kept on ice for 30 minutes followed by heat-shock treatment at 42°C for 1 minute and subsequent snap chill on ice for 5 minutes. 1 mL of SOC broth was added to the cells and incubated in the shaker for 1 hour at 37°C.

After incubation, the DH5α cells were centrifuged at 5000 rpm for 5 minutes. 75% of the supernatant was removed and the remaining amount was used to resuspend the bacterial pellet. After resuspension of the bacterial pellet, cells were plated on 100 μg/mL ampicillin plates and incubated for 16 hours. The following day, five individual colonies from each culture plate were placed in 4 mL solutions of LB broth containing 100 μg/mL of ampicillin in polystyrene tubes. Each culture was incubated in the shaker at 37°C for 16 hours with 100 μg/mL ampicillin. The next day, the overnight bacterial cultures were used to isolate the plasmids using QIAprep Plasmid Miniprep Kit.

The same procedure was followed for the transformation and amplification of Ecohelper plasmid in subsequent methods.

5 μL of each pMX-*YY1* (226-290) plasmid was added to 10 separate reaction mixtures with each containing 0.5 μL of EcoRI, 0.5 μL of XhoI, 3 μL of 10x Cutsmart buffer and 21 μL of nuclease free water to digest each plasmid to confirm correct *YY1* 226-290 insert length. A positive pMX control was also digested containing no *YY1* insert. Each reaction was incubated at 37°C for 1.5 hours.

Albright College Gingrich Library

Culture of Cell lines:

HEK293 or 3T3 cell lines were maintained in T-25 flasks of complete DMEM media containing Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS), 1% Pen Strip, and 1% Non-essential amino-acids (NEAA). Cells were maintained in 37°C incubators at 5% CO_2. All maintenance and sub culturing took place in a BSC-2 laminar flow hood using sterilized reagents. Cell cultures were periodically sub cultured to maintain the cell line. Sub culturing occurred by removing DMEM media using a vacuum pipette followed by cleaning of the

cells using PBS. PBS is removed using a vacuum pipette and 0.5mL of 0.05% Trypsin-EDTA (1x) is added to peel the cells. The flask was incubated in 37°C for 2 minutes. After 2 minutes, 2 mL of complete DMEM media was added to the flask to halt digestion followed by centrifugation at 1000 rpm for 5 minutes and resuspension in complete DMEM.

Plat-E cells (Platinum E packaging cell line) were maintained using the previously prescribed methods with exception to the complete media used. Complete DMEM media contained DMEM, 10% FBS, 1% Pen Strip, 1% NEAA, 10 µL of puromycin, and 10 µL of blastisidin.

Cell Counting:

Cell counting was conducted by gently mixing 100 µL of cells with 400 µL of Trypan Blue. 100 µL of each cell solution was pipetted under the slide of the hemocytometer. Cells were counted using a light microscope at 10x magnification. Four of the nine mm^2 squares on the hemocytometer were counted to determine the cell concentration. The four mm^2 were averaged and multiplied by the dilution factor of 5 and 10^4 to determine the cells per mL to provide concentration for plating.

Lipofectamine Transfection:

Albright College Gingrich Library

HEK293 cells were transfected at 70-80% confluency. HEK293 cells were seeded in a 35 mm dish a day before transfection and cultured in complete DMEM without antibiotics. On the day of transfection, in two -1.5 mL microfuge tubes, 200 µL of OptiMEM Media was added for each plasmid. To one tube, 8 µL of lipofectamine 2000 was added slowly and directly to the media and pipetted/spun to ensure proper mixture. To the other tube, 4 µg of plasmid weas added and pipetted to ensure proper mixture. Each tube was incubated at room temperature for 5 minutes to equilibrate. After incubation, the separate reaction mixtures were combined and pipetted, followed by 20 minutes of incubation at room temperature. Prior to transfection, HEK293 cells were washed

once with PBS. After washing HEK293 cells, 1 mL of DMEM media was added to each well. The lipofectamine/plasmid mixture was then added dropwise to the well. After addition of the lipofectamine/plasmid mixture, wells were mixed gently and incubated 37°C for 5 hours. After incubation, the DMEM media is removed followed by the addition of 2 mL of complete DMEM media and incubation at 37°C for 48 hours to express the protein.

Cellular YY1 226-290 Quantification:

The transfected HEK293 cells were visualized under a fluorescent microscope to verify the percentage of GFP expression as the pMX plasmid has a GFP cloned in a different ORF. The plates of transfected HEK293 cells containing the YY1 226-290 construct were kept on ice. Media was removed using vacuum pipettes followed by washing of the cells with ice cold PBS and finally they were scrapped in PBS. Cells were transferred to pre-chilled 1.5mL microfuge tubes and centrifuged at 5000 rpm for 5 minutes in 4°C. After centrifugation, the supernatant was removed using a vacuum pipette. 100 µL of chilled detergent containing RIPA lysis buffer with 1 µL of protease inhibitor was added to each microfuge tube. The cell pellet was resuspended by pipetting a few times while avoiding bubbles. Using a set of BSA standards (0.25, 0.5, 1, 2, 4, and 8 µL/mL), a standard protein curve was created to measure the concentration of protein in each sample. All measurements were performed using UV-Vis spectrometer at 595 nm.

Albright College Gingrich Library

SDS-PAGE and Western Blotting:

20 µg of total protein for each sample was mixed with equal volumes of 2x Laemmli Buffer. Samples were placed on a 95°C heating block for 10 minutes. After 10 minutes, samples were spun down and loaded in the wells of a 15% SDS-PAGE gel alongside 5 µL of PageRuler Plus

protein standard ladder. The gel was run at 30mAmps until the bromophenol blue dye reached the very end of the gel.

After running the gel, the proteins were transferred to a nitrocellulose membrane through wet transfer method. The transfer was done at 200 mAmps for 50 minutes.

After transfer, the membrane was blocked using 5% Milk in 1x TBST for 30 minutes at room temperature on the shaker. Following blocking, the membrane was rinsed with 1x TBST. The membrane was then stained with primary antibody in 5% BSA and 1x TBST (Flag M2 Antibody from Sigma) in the 4°C rocker overnight. The next day, the membrane was washed three times for five-minute intervals on the rocker using 1x TBST. The membrane was incubated in secondary antibody (Anti-mouse Horseradish peroxidase) in a 10 mL solution of 5% milk in 1x TBST and placed on the shaker for one hour. The membrane was again washed three times for five-minute intervals on the rocker using 1x TBST.

After washing, the membrane is placed in Chemiluminescent Substrate (1:1 solution containing Luminol and H_2O_2) and kept in the dark for a minute. Then the membrane was placed in saran wrap and placed in an autoradiography box. Autoradiographs were placed in autoradiograph box and visualized using a developer machine in the dark room.

Albright College Gingrich Library

YY1 226-290 retrovirus production using Plat-E cells and Ecohelper plasmid:

Plat-E cells were transfected at 70-80% confluency. 1.5 mL of OptiMEM media was added to two-15 mL conical tubes, for each plasmid. To one tube, 60 µL of lipofectamine 2000 was added directly to the media and pipetted to ensure proper mixture. To the other tube, 18 µg of pMX-YY1 (226-290) and 6 µg of Ecohelper plasmid were added and pipetted well. Each tube was incubated at room temperature for 5 minutes and then mixed together After 20 minutes of incubation at room

temperature, the mix was added to 3 mL of DMEM. Cells were mixed gently and incubated. After 5 hours of incubation, the media was removed followed by the addition of 4 mL of complete DMEM media and incubation at 37°C for 48 hours. Virus particles were collected by centrifuging the supernatant at 2000 rpm for 10 minutes at 4°C. YY1 226-290 viral construct titers were measured using 3T3 cells. Viral particles were added to 3T3 cells in a 12 well plate and incubated at 37°C for 16 hours. After incubation, the media was removed and 500 µL of fresh complete DMEM media was added. Wells were pipetted, and the contents were transferred to Flow-Cytometry test tubes (Data not shown).

YY1 226-290 virus infection of splenic B cells:

Follicular B cells were purified from mouse spleen with anti-CD23-biotin (Biolegend) and streptavidin microbeads (MACS; Miltenyi Biotec) followed by activation with LPS and IL-4. After 24 hours, cells were transduced with retrovirus supernatant containing YY1 226-290-pMX constructs. After 48 hours, cells were harvested for co-immunoprecipitation assays. An aliquot of live cells was saved to check the percentage of infection.

Flow Cytometry Assay:

Albright College Gingrich Library

Cells were transferred to round bottom polystyrene tubes and centrifuged at 1500 rpm for 5 minutes. Following centrifugation, the supernatant was decanted off and the cells were washed with FACS buffer (0.5% BSA in 1X PBS, 2mM EDTA). After centrifugation, the supernatant was decanted off and the cells were resuspended in 300 µL of MACS buffer with 1µg/ml DAPI.

YY1 226-290 nuclear extraction in splenic B cells/co-Immunoprecipitation (co-IP) Assay:

Infected splenic B cells were centrifuged at 5000 rpm for 5 minutes. The supernatant was decanted, and the cells were resuspended in 2 mL of PBS buffer and pipetted into two-1.5 mL

microfuge tubes. After centrifugation, the supernatant was decanted, and the wet mass of the cell pellet was measured to determine the appropriate amount of Buffer A and 10% NP40 detergent. After the addition of buffer A, the cells were vortexed on the highest setting for 15 seconds and then placed on ice for 10 minutes. After incubation, the NP40 was added and the cells were vortexed on the highest setting for 5 seconds and then placed on ice for 1 minute. The tube was centrifuged at 16,000 g for 5 minutes. The supernatant containing the cytoplasmic protein was kept in a fresh tube and the nuclear pellet was resuspended in 1 mL of IP lysis buffer. Every 15 minutes for 1.25 hours, the nuclear pellet was pipetted with a large orifice tip to enhance the extraction of nuclear protein. Finally, the solution was centrifuged at 16000g for 5 minutes and the supernatant was saved as NE. Extracted proteins were quantified using the Bradford Assay, standardizing with BSA (Data not shown).

The co-IP assay was performed according to the protocol prescribed in the FLAG Immunoprecipitation Kit from Sigma-Aldrich. co-IP sample products were subsequently used in the western blotting procedures previously described.

Albright College Gingrich Library

Results:

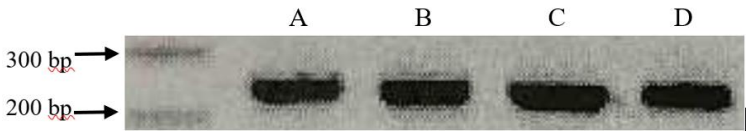


Figure 8. **MigR1 *YY1* PCR**. MigR1 *YY1* PCR using EcoRI and XhoI of various buffers (A, B, C, and D). 5 µL of each PCR reaction was mixed with 1 µL of 6x Gel Loading Dye and run alongside a 2-log ladder on a 1.5% TBE-agarose gel containing 2 µL of ethidium bromide for 30 minutes at 120V. All lanes show positive results with a band between 200 and 300 bp. This bp range accounts for the 64 amino acids of the desired region of YY1 in addition to the added FLAG Tag and GFP. All lanes were combined and used in subsequent restriction digestion.

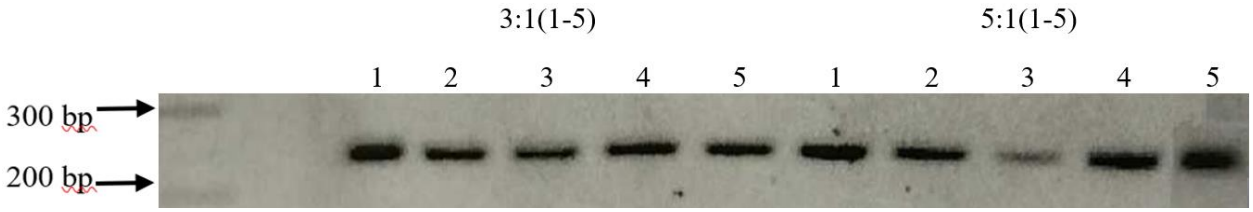


Figure 9. **Restriction digestion of pMX-*YY1* 226-290 insert**. Each reaction mixture was mixed with 5 µL of 6x Gel Loading Dye and run alongside a 2-log ladder on a 1.5% TBE-agarose gel containing 2 µL of ethidium bromide for 45 minutes at 180V. All lanes showed positive results with a band between 200 and 300 bp. Because each reaction yielded positive results, one sample of each ligation ratio (3:1(1), 5:1(1)) was sent to be sequenced and was verified as the correct *YY1* 226-290 sequence using NCBI Blast (Data not shown).

Albright College Gingrich Library

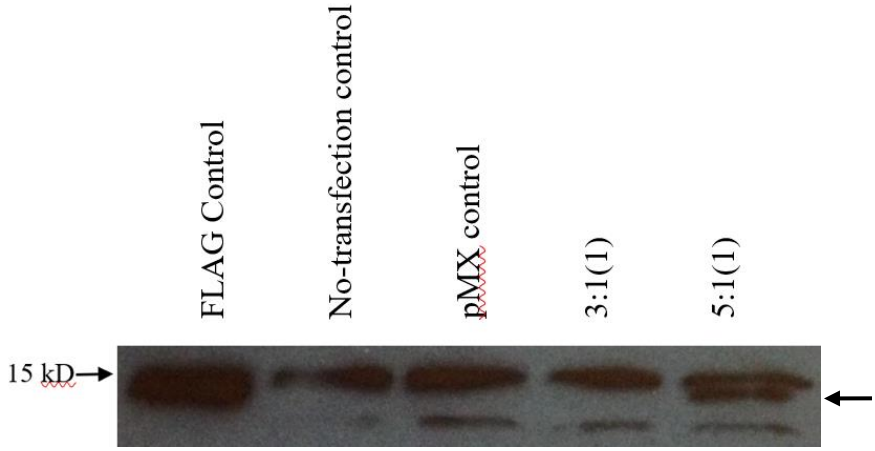


Figure 10. **Western blot of YY1 constructs.** 20 µg of total protein for each sample was mixed with an equal volume of 2x Laemmli Buffer. Samples were heated at 95°C for 10 minutes. Protein samples were run on a 15% SDS-PAGE gel at 30mAmps. Proteins were transferred to a nitrocellulose membrane at 200mAmps for 50 minutes using wet transfer methods. Membrane was blocked with 5% milk in 1x TBST. Primary antibody was 5% BSA and 1x TBST (Flag M2 Antibody from Sigma). Secondary antibody was 5% milk in 1x TBST (Anti-mouse Horseradish peroxidase). The membrane was placed in Chemiluminescent Substrate (1:1 solution containing Luminol and H_2O_2) and kept in dark for a minute. Then the membrane was placed in saran wrap and placed in an autoradiography box. This autoradiograph was exposed for 5 minutes and placed in an autoradiograph box and visualized using a developer machine in the dark room. 5:1(1) shows a unique nonspecific band just below the 15 kD band. Expected kD of the YY1 226-290 construct was 8.43 kD. The protein lysate was sent to proteomics to confirm the expression of the YY1 226-290 construct in 5:1(1) (Data not shown). Successful synthesis of YY1 226-290 construct prompted retrovirus synthesis for infection of splenic B cells.

Albright College Gingrich Library

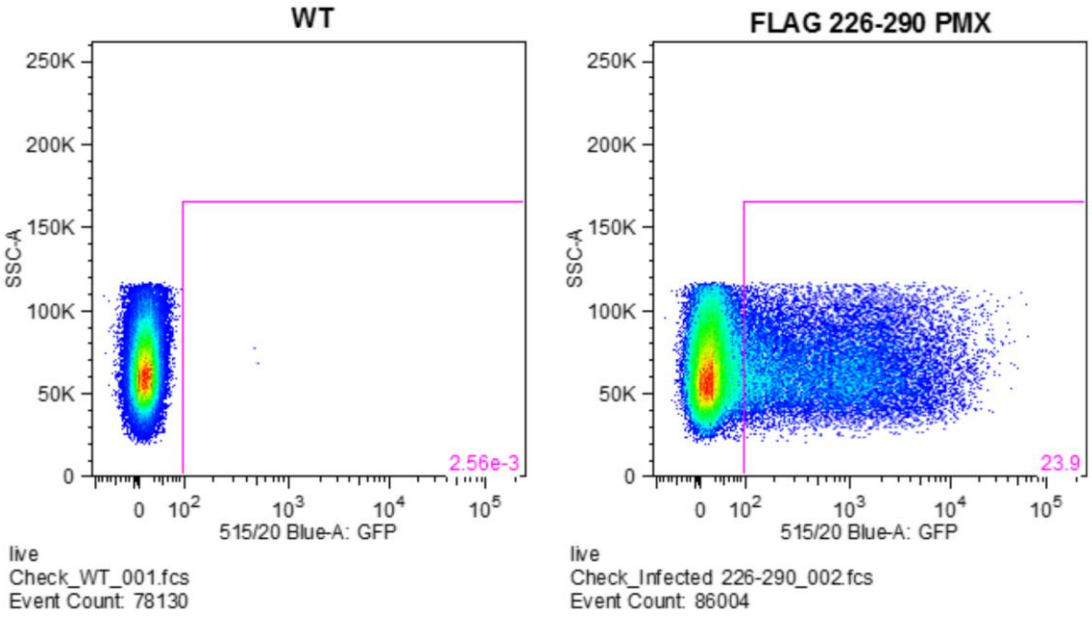


Figure 11. **YY1 226-290 retrovirus infection of splenic B cells.** Flow cytometry assay of splenic B cells infected with WT pMX plasmid and FLAG conjugated 226-290 pMX-YY1 plasmid construct. Follicular B cells were purified from mouse spleen with anti-CD23-biotin (Biolegend) and streptavidin microbeads (MACS; Miltenyi Biotec) followed by activation with LPS and IL-4. After 24 hours, cells were transduced with retrovirus supernatant containing YY1 226-290-pMX constructs. The samples were run on a FACS machine followed by compensating for GFP and DAPI channels. Infected splenic B cell percentage is indicated in pink as shown by the GFP gated axis. There was a 2.56 e-3% infection rate of WT pMX and 23.9% infection rate of pMX-*yy1* 226-290. Infection rate confirmed infectivity of splenic B cells to be used in co-IP assay.

Albright College Gingrich Library

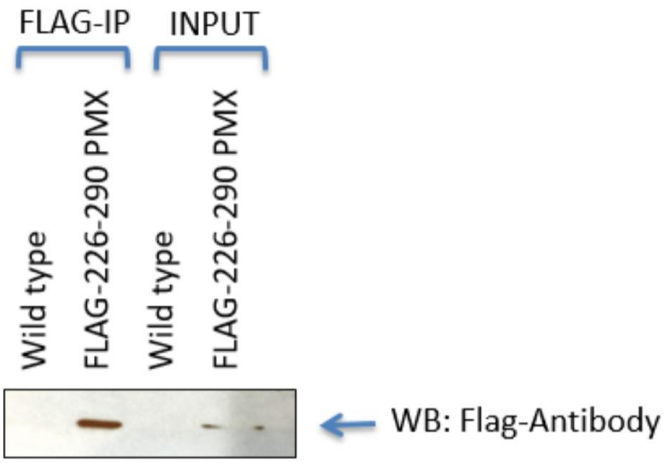


Figure 12. **YY1 226-290 co-IP of splenic B nuclear extract.** Co-IP of splenic B cell nuclear extract using Flag Antibody. FLAG-IP was 500 µL and INPUT was 20 µL of nuclear extract. The co-IP assay was performed according to the protocol prescribed in the FLAG Immunoprecipitation Kit from Sigma-Aldrich. Both showed weakly positive results with light banding patterns. Associated proteins could not be quantified and identified due to poor yield. Long-distance DNA interacting proteins associated with CSR could not be identified.

Discussion:

Since its discovery and isolation through the mid-80's to early 90's, YY1 has been identified as a key player in various processes, including those involved in B cell development. Most recently, studies have implicated the importance of YY1 in processes including long-distance DNA interactions, such as those required in class-switch recombination (CSR) (2). The purpose of this study was to augment previous research in the field to ultimately help determine the necessary regions or domains of YY1 required to rescue Eµ-3'RR DNA loop formation and ultimately its associated functions in CSR.

Albright College Gingrich Library

The early weeks of this study were spent isolating and amplifying the *YY1* 226-290 insert. After successful transfection of the pMX-*YY1* 226-290 vector, western blotting was used to

determine expression of the YY1 226-290 construct set out to synthesize. After weeks of western blotting, the YY1 226-290 construct was isolated and characterized. The expected size of the construct was 8.43 kD provided the construct is 64 amino acids in length in addition to the 8 amino acid FLAG Tag. The YY1 construct was only seen in the 5:1(1) ligation ratio mixture as seen in Figure 10 as a "ghost" band just below the band at 15 kD that is not seen in any other lane. There was initial skepticism of the band, but repeated attempts using various percentage SDS-PAGE gels yielded the same result with a band only showing up in the 5:1(1) lane. Mass spectrometry results helped to confirm structure of the supposedly synthesized construct (Data not shown). WT YY1 is known to run at a size greater than expected, therefore it does not come as a great surprise that the 226-290 construct too ran at a size nearly double of what was predicted.

After confirming the success of constructing the 226-290 YY1 mutant, pMX-*YY1* 226-290 virus was made to infect splenic B cells to ultimately determine the interactions this construct would potentially have with the immunoglobulin heavy chain locus. Due to the low infectivity of splenic B cells, they were infected twice with the pMX-*YY1* 226-290 virus to potentially maximize infectivity to increase the overall yield in subsequent co-IP experiments.

Albright College Gingrich Library

Flow-cytometry of the infected splenic B cells shows the infection rate of the splenic B cells with both a negative control pMX plasmid virus and the pMX-*YY1* 226-290 virus. The percent infection of the negative control was low as expected at 2.56 e-3% while the pMX-*YY1* 226-290 virus showed an infection rate of 23.9% (Figure 11).

After determination of infection percentage, co-IP of the splenic B nuclear extract was performed to elucidate the 226-290 YY1 mutant and any associated molecules including AID, the protein known to be required for CSR to occur (Figure 12). While the western blot did show a more intense FLAG band in the IPed sample compared to the INPUT, the overall infectivity of the

splenic B cells as seen from the flow-cytometry assay was fairly low. The intent following the co-IP was to send the nuclear isolate containing the 226-290 YY1 construct and any associated proteins to the proteomics department to determine any and all associated proteins, including potentially AID. That being said, the co-IP products were set to be sent to the proteomics department for analysis, however, poor product yield, likely due to the low splenic B cell infectivity, hindered 226-290 YY1 mutant construct experimentation.

Due to immediate time constraints, further experimentation could not be completed at the time. While, the current project is currently paused, research continues around YY1 and its various functions. As part of the future studies, further efforts will go into maximizing infectivity of the splenic B cells using various *YY1* virus constructs. Potentially increasing the size of the plates used during infection to overall increase the number of cells capable of being infected. In addition, infecting even more than two times may be found to serve as beneficial.

Albright College Gingrich Library

References:

1. Atchison, ML. Function of YY1 in long-distance DNA interactions. *Fimmu*. 2014; 5(45): 1-11.
2. Mehra P, Gerasimova T, Basu A, Jha V, Banerjee A, Sindhava V, Gray F, Berry CT, Sen R, Atchison M. YY1 controls Eμ-3'RR DNA loop formation and immunoglobulin heavy chain class switch recombination. *Blood Advances*. 2016; 1(1): 15-20.
3. Park K, Atchison ML. Isolation of a candidate repressor/activator, NF-E1 (YY-1, δ), that binds to the immunoglobulin κ 3' enhancer and the immunoglobulin heavy-chain μE1 site. *Proc. Natl. Acad. Sci.* 1991; 88(1991): 9804-9808.
4. Golbabapour S, Majid NA, Hassandarvish P, Hajrezaie M, Abdulla MA, Hadi AHA. Gene Silencing and Polycomb Group Proteins: An Overview of their Structure, Mechanisms and Phylogenetics. *A Journal of Integrative Biology*. 2013; 17(6): 283-296).
5. Parham, P. (2015). *The Immune System* (4th ed.). New York City, NY: Garland Science.
6. Chua KF, Alt FW, Manis JP. The Function of AID in Somatic Mutation and Class Switch Recombination: Upstream or Downstream of DNA Breaks. *J. Exp. Med.* 2002; 195(9): F37-F41.

Albright College Gingrich Library