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UNIVERSITY OF CALIFORNIA SAN DIEGO

Production and purification of Fructose 2,6-Bisphosphate and assaying its activity as the cofactor
of Polynucleotide Kinase 3'-Phosphatase

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Chemistry

by

Wyatt Miller

Committee in charge:

Professor Gourisankar Ghosh, Chair
Professor Patricia A. Jennings
Professor Sushil K. Mahata

2023

The Thesis of Wyatt Miller is approved, and it is acceptable in quality
and form for publication on microfilm and electronically.

University of California San Diego

2023

Dedication

I dedicate this thesis to my mom, who has always supported me through challenging times.

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ABSTRACT OF THE THESIS

Investigations in the role of PFKFB3 in DNA repair machinery and neurological disease.

By

Wyatt Miller

Master of Science in Chemistry

University of California San Diego, 2023

Professor Gourisankar Ghosh, Chair

The maintenance of the genome is one of the foremost functions of all living organisms. The genome undergoes constant assault by environmental hazards, leaving behind different types of DNA alterations. Unrepaired DNA damage has been linked to several neurological diseases. Among many proteins involved in DNA damage repair, polynucleotide kinase 3' phosphatase (PNKP) is a key regulator of DNA repair. PNKP removes 3'-phosphate at the DNA breaks. Our

collaborator has reported that PNKP levels are unchanged in poly-glutamine neurological diseases, it is inactive. Another kinase phosphatase, phosphofructokinase fructose-2,6-bisphosphatase 3 (PFKFB3), is linked to DNA repair. My thesis addresses the question if the product of PFKFB3 supports PNKP activity. Here I document that PNKP interacts with fructose-2,6-bisphosphate produced by PFKFB3. Furthermore, I describe the enzymatic production and purification of fructose-2,6-bisphosphate in bulk quantities. PNKP 3' Phosphatase activity levels are significantly lower in the nuclear extracts of HD and SCA3 patients' brains. Exogenous F2,6BP rescued PNKP 3' phosphatase activity in the brain nuclear extracts of those samples. Moreover, F2,6BP association with PNKP was indicated by bio-layer interferometry and protein stability assays. We thus postulate that F2,6BP serves in vivo as a cofactor for the proper 3' phosphatase of PNKP and, brain health.

I.   Introduction

DNA Damage Repair

Genome maintenance is arguably the most important function of an organism. The genome undergoes continual stress and damage from a multitude of endogenous and exogenous factors. Damage in the genome can result from DNA replication errors, mutagenic agents, oxidative stress, physical damage, and biological agents. Multiple systems are in place to prevent the accumulation of DNA damage and to preserve the genome. Some of these systems include Base Excision Repair, Nucleotide Excision Repair, Mismatch Repair, Homologous Recombination, Non-Homologous End Joining, and Translesion Synthesis (Chatterjee and Walker).

Among the DNA damages, single-stranded and double-stranded DNA breaks (SSBs and DSBs) are considered to be lethal if remain unrepaired. These breaks are mainly caused by replication errors and ionizing radiation. Failure to repair DNA breaks are known to accumulate in multiple neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), and Amyotrophic Lateral Sclerosis (ALS) (Ranganathan, Sapozhnikov, Ni, Li, and Song).

DSBs are repaired either via error-free homologous recombination (HR) whch use the homologous segment of the sister chromatid generated during replication, or the non-homologous end-joining (NHEJ) pathway, which is generally considered to be error-prone (Rodgers and McVey). The majority of neuronal cells in adult human brains are in the non-proliferating phase. Therefore, DSB repair cannot occur via the HR pathway and cells must utilize the NHEJ pathway. NHEJ is of two types: classical or C-NHEJ, involving Ku, DNA-PK, 53BP1 (p53 binding protein 1) and DNA Lig IV-XRCC4; and alternative end-joining, or Alt-EJ, which involves PARP1, Lig IIIα/XRCC1 or Lig I. Alt-EJ, inherently error-prone, which occurs only when the standard repair process fails, primarily as a backup for both C-NHEJ and HR

(Chakraborty, Tapryal, Venkova, Horikoshi, Pandita, Sarker, Sarkar, Pandita, and Hazra).

It was recently shown that DSBs in the transcribed genome are repaired via the error-free C-NHEJ pathway in post-mitotic, non-cycling cells like neurons where nascent homologous RNA provides the template for restoring the missing information (Chakraborty, Tapryal, Venkova, Horikoshi, Pandita, Sarker, Sarkar, Pandita, and Hazra).

Polynucleotide Kinase 3'-Phosphatase

Both SSB and DSB leave 3'-end breakpoint with a phosphate attached and often the 5'-end is devoid of a phosphate. The 3'-phosphate must be removed for strand extension by the DNA pol and the free 5'-end must be phosphorylated for the DNA ligation to occur. One of the critical enzymes in both DNA break repair (DRB) is polynucleotide kinase 3'-phosphatase (PNKP). Thus, PNKP is a multifunctional enzyme that plays a critical role in initiating the DNA repair complex for DRB (Weinfeld, Mani, Abdou, Aceytuno, and Glover). PNKP performs two essential functions: phosphorylating the 5' hydroxyl terminus of a broken DNA strand and removing 3'-phosphate groups from DNA termini (Weinfeld, Mani, Abdou, Aceytuno, and Glover). Both ends are necessary for the DNA repair machinery to carry out the subsequent functions of ligation and elongation.

PNKP has three domains: a kinase domain, a phosphatase domain, and an N-terminal non-catalytic domain known as Forkhead-Associated (FHA). The C-terminal kinase domain is responsible for attachment of a phosphate at the 5'-end of the DNA, while the phosphatase domain catalyzes the 3'-phosphate group removal. It should be noted that these domains are non-functional in isolation suggesting both these domains communicate with each other for their activity. The FHA domain aids in recruiting PNKP to the sites of DNA damage (Tsukada, Shimada, Imamura, Saikawa, Ishiai, and Matsumoto). PNKP also has a domain known as the

Oligonucleotide/Oligosaccharide-Binding (OB) Fold which is involved in the binding of PNKP to DNA and RNA (Li and Yu). PNKP is an essential protein and deletion of the gene encoding PNKP is embryonically lethal due to defective neurogenesis (Shin, Alpaugh, Hallihan, Sinha, Crowther, Martin, Scheidl-Yee, Yang, Yoon, Goldsmith, Berger, De Almeida, Dufour, Dobrinski, Weinfeld, Jirik, and Biernaskie). Several mutations within the FHA and phosphatase domain result in different neurological diseases.

Neurological disease and DNA Damage

Two well known poly-glutamine (poly-Q) expansion diseases are also linked to unrepaired DNA damages (Pradhan, Gao, Bush, Zhang, Wairkar, and Sarkar). One of them, Huntington's disease (HD) is an extremely damaging neurological condition where nerve cells in the brain break down over time. The disease impacts 30,000 individuals in the United States, with an additional 200,000 individuals at risk of developing the condition. The mortality rate associated with Huntington's disease differs among individuals and is influenced by a range of factors, including the age at which the condition begins and the progression of the disease. In HD the number of polyQ expanded from around 16 and 35 in normal population to >41 in HD patients in a large protein called Hungtinton (HTT) protein. The greater the length of polyQ, more severe is the disease (Yushchenko, Deuerling, and Hauser).

Spinocerebral atexia 3 (SCA 3) is another polyQ neurodegenerative disorder caused by the polyQ expansion of ATXN3 protein (McLoughlin, Moore, and Paulson). SCA3 disease is characterized by progressive movement problems. Currently, there is no cure for SCA3, and only treatments exist to help manage the symptoms. SCA3 is a relatively rare disease, affecting

around 5 out of every 100,000 individuals worldwide. Both HTT and ATXN3 are involved in

DBR. However, the disease mutants are defective in repair resulting in neuronal death.
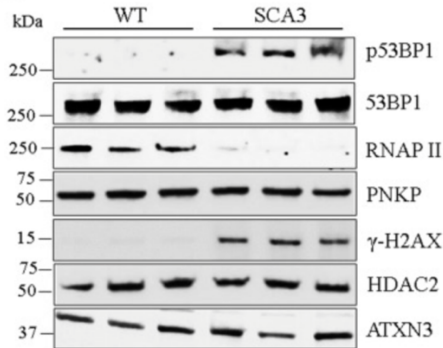
Figure 1. Western Blot of Healthy and SCA3 disease patient samples. Samples are taken from the cortex of multiple individuals. (Chakraborty, Tapryal, Venkova, Mitra, Vasquez, Sarker, Duarte-Silva, Huai, Ashizawa, Ghosh, Maciel, Sarkar, Hegde, Chen, and Hazra)

Figure 1 shows the effect of the DNA repair complex in brain tissue of SCA3 patients (Chakraborty, Tapryal, Venkova, Mitra, Vasquez, Sarker, Duarte-Silva, Huai, Ashizawa, Ghosh, Maciel, Sarkar, Hegde, Chen, and Hazra). In tissue samples from WT non-disease group showed repair of damage as marked by no association between PNKP and γH2AX and phosphorylated 53BP1 (P53BP1). In the disease group they remain associated with PNKP suggesting lack of repair. Also interestingly, RNAPII is completely degraded in SCA3 IP further confirming DNA damage repair defect (Chakraborty, Tapryal, Venkova, Mitra, Vasquez, Sarker, Duarte-Silva, Huai, Ashizawa, Ghosh, Maciel, Sarkar, Hegde, Chen, and Hazra).

Figure 2 shows that the amount of PNKP is the same across the diseases (Chakraborty, Tapryal, Venkova, Mitra, Vasquez, Sarker, Duarte-Silva, Huai, Ashizawa, Ghosh, Maciel, Sarkar, Hegde, Chen, and Hazra). From this we can conclude that something eles is causing the PNKP to be less active and that the relative ratio of protein does not change from healthy to disease state.
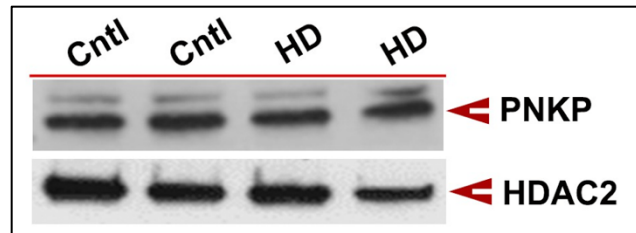
Figure 2. Western Blot of PNKP present in Huntington Disease tissue extract. Lanes 1 and 2 are healthy cortex tissue lysate. Lanes 3 and 4 are Huntington disease cortex tissue lysate. (Chakraborty, Tapryal, Venkova, Mitra, Vasquez, Sarker, Duarte-Silva, Huai, Ashizawa, Ghosh, Maciel, Sarkar, Hegde, Chen, and Hazra)

IKK and NF-κB

NF-κB is a transcription factor that plays an important role in the regulation of the immune response, inflammation, and cell survival (Oeckinghaus and Ghosh). NF-κB controls the expression of genes that involved in inflammatory response (Liu, Zhang, Joo, and Sun). NF-κB is inactive when it is bound to the protein IκBα. . NF-κB is activated when the protein IκBα is phosphorylayed by IKK. Upon Phosphoryation of IκBα by IKK, NF-κB is translocated to the nucleus and transcription of target genes is started (Oeckinghaus and Ghosh).

IKK is a kinase which its main known role is the phosphorylation of IκBα to induce the translocation of NF-κB to the nucleus and initiate transcription (Oeckinghaus and Ghosh). IKK consists of multiple forms as there is IKK1 homodimer, IKK2 homodimer and IKK1/2 Heterodimer. IKK is known to be activated by cytokines such as TNF-α, and IL-1 (Liu, Zhang, Joo, and Sun).

NF-kB is involved in DNA repair pathway by regulating the expression of some DNA proteins. IKK is also involved in DNA repair independent of its NF-kB activation function. For example, IKK2 activates and phosphoryate ATM kinase, an important regulator of DNA damage repair.

PFKFB3 is a critical regulator of DBR

The Hazra group at UT Galveston found the DBR defect was at least in part due to the defect in PNKP activity. That is, PNKP was unable to remove 3'-phosphate from the damaged DNA. Although the PNKP protein levels remained the same in both non-disease and disease brian tissue samples (Figure 2). As shown in Figure 3, (Chakraborty, Miller, Huai, Biswas, Mandal,

Figure 3. Radioactive Phosphate Removal by PNKP in Huntington Disease tissue extract. Lanes 1 and 2 are cortex lysate from healthy humans. Lanes 3 and 4 are cortex lysate from humans with Huntington disease. Lanes 5 is only substrate and no protein added. Lane 6 is substrate with pure PNKP. (Chakraborty, Miller, Huai, Biswas, Mandal, Bosca, Ghosh, and Hazra)
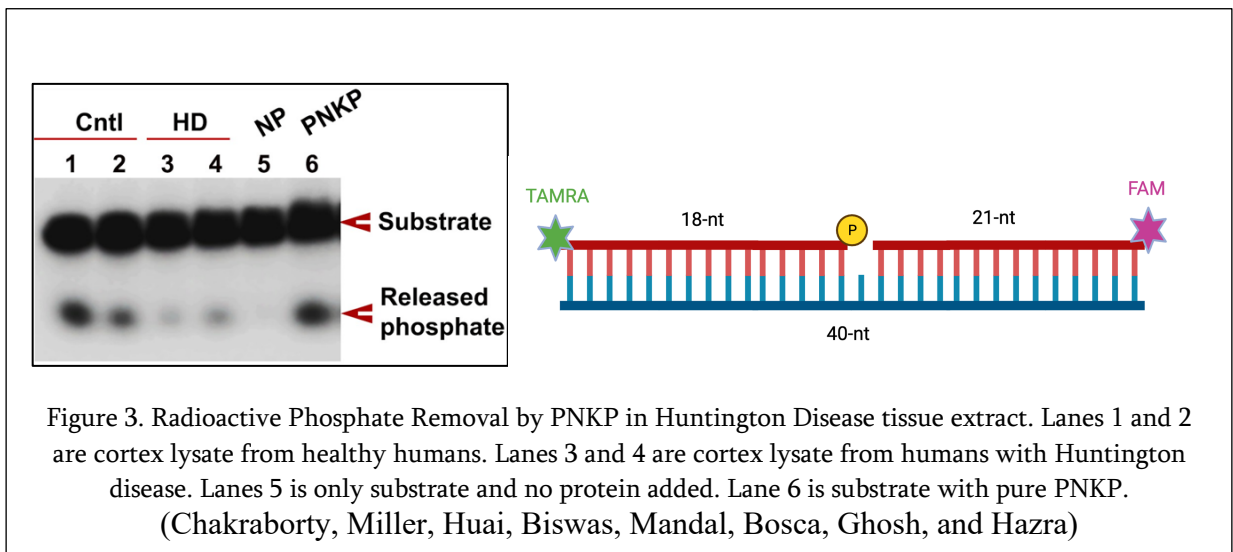
Bosca, Ghosh, and Hazra) demonstrated that PNKP exhibited significantly reduced 3' phosphatase activity in in the same samples as shown in figure 2. In this assay which developed in their lab a gapped DNA duplex is used with radiolabeld 3'-phosphate. WT extract or recombinant PNKP in the presence of ATP can remove the 3'-phosphate which migrates faster in the gel (Figure 3). They calculated PNKP's activity to be only 20% of that in a healthy non-disease individual Figure 4 (Chakraborty, Miller, Huai, Biswas, Mandal, Bosca, Ghosh, and Hazra).
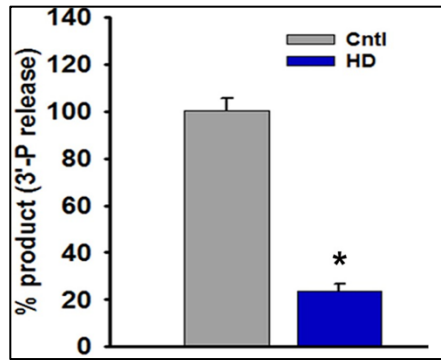
Figure 4. Quantified PNKP phosphate removal in Huntington Disease tissue extract. Quantified phosphate release (bottom band) (Chakraborty, Miller, Huai, Biswas, Mandal, Bosca, Ghosh, and Hazra)

Altogether, these results confirmed that the loss of PNKP activity is not due to the loss of protein levels, but for other reasons. That is, under disease conditions PNKP lose its activity due to some other factors. This conclusion is drawn as PNKP is the sole known enzyme responsible for removing a 3' Phosphate at a break in a DNA strand. They proposed that some cofactor must be missing in the nuclear extracts which is responsible for PNKP activity.

The Hazra group subsequently identified PFKFB3 is the enzyme that is also associated with PNKP in the DBR complex, Figure 5 (Chakraborty, Miller, Huai, Biswas, Mandal, Bosca, Ghosh, and Hazra).
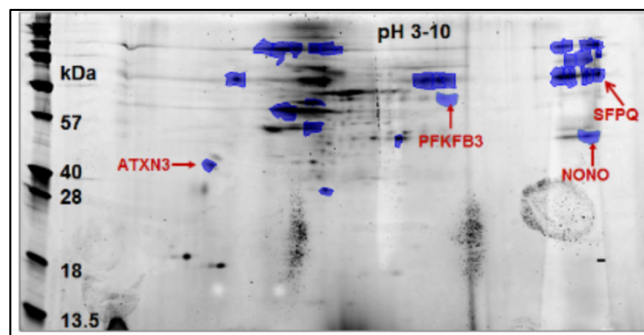
Figure 5. Elution of Proteins associated with PNKP. Proteins that associated from PNKP were eluted off using a pH gradient, starting from 3 in the number 2 lane and ending with pH 10 in lane 10. (Chakraborty, Miller, Huai, Biswas, Mandal, Bosca, Ghosh, and Hazra)

Simple addition of PFKF3 identified the enzyme central to most of my studies, which is PFKFB3. They identified PFKFB3 as an enzyme that associates with PNKP through an immunoprecipitation of PNKP. Additionally, they conducted an immunoprecipitation with PFKFB3 and LigIV to demonstrate that PFKFB3 is associated with the DNA repair complex and likely plays a role, Figure 6 (Chakraborty, Miller, Huai, Biswas, Mandal, Bosca, Ghosh, and Hazra). PFKFB3 is also a bifunctional enzyme which attaches a phosphate to F6P using its kinase activity generating fructose 2,6-bisphosphate (F2,6BP) or generating F6P from F2,6BP using the phosphatase activity. There are four enzymes in the PFKFB1-4. Of these PFKFB3 is the only member with >700-fold higher kinase activity that generate F2,6BP from F6P than the phosphatase activity. PFKFB3 also localizes to the nuclear. They found that it is not the PFKFB3 but its product F2,6BP is required to augment PNKP activity.
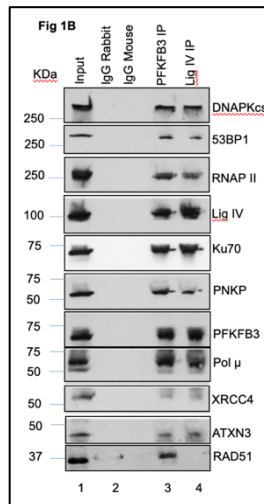
Figure 6. Immunoprecipitation of PFKFB3 and LigIV. The proteins listed on the right side of the image are what associated with PFKFB3 and LigIV

F2,6BP is a potent cofactor phosphofructo kinase 1 (PFK1), a glycolytic enzyme that converts F6P to F1,6BP(Rider, Bertrand, Vertommen, Michels, Rousseau, and Hue). This

reaction step is rate limiting in the 10-reaction glycolytic pathway. It is likely the other members of the PFKFB family are involved in glycolysis by regulating the levels of F2,6BP. These enzymes are primarily or exclusively cytoplasmic where glycolysis occurs. PFKFB3, on the other hand mostly synthesizes F2,6BP in the nucleus supplying it to PNKP. Thus, F2,6BP apparently has another important function as a regulator of DNA repair. However, There are many questions that have remained unresolved. Does PNKP bind F2,6BP directly or it acts through other proteins? Where in PNKP does F2,6BP binds? How does F2,6BP binding affect its active site? Is there any connection between IKK and PNKP since IKK is also involved in DNA reapir?

Focus of the Thesis

My main goal of this thesis is to optimize a method to generate sufficient amounts of F2,6BP for biochemical and biological studies in coordination with the Hazra group at UT Galveston.This involved optimizing the expression and purification protocol of the enzyme which makes F2,6BP, "PFKFB3." This F2,6BP I purified was then being used for subsequent assays by the Hazra group to test its effect as a cofactor of PNKP. The F2,6BP was also used to test for recovery of activity of PNKP in disease models, identifying it as a possible therapeutic. I also wanted to implement an nonradioactive PNKP phosphate activity assay to monitor F2,6BP as a cofactor of PNKP. I also wanted to investigate if PNKP and F2,6BP directly interact, through the use of BLI and nanotemper instruments. In this work I wanted to connect the DNA repair pathway of PNKP and IKK to see if there was any correlation, and if so then how does the phosphorylation of these DNA repair enzymes affect the activity and outcome of the repair.

II.Materials and Methods

A. Protein Purification Protocols

1. His-PNKP and Mutants

A plasmid containing the PNKP coding sequence, was transformed into comptetent BL21 (DE3) cells. Kanomycin was used as the antibiotic as the vector pet28a+ has the kanamycin resistant gene. The cells were left to grow on a Luria-Bertani broth/Agar plate for 16 hours. The next day the plate containing the colonies used to inoculte a 1L culture of Luria-Bertani broth supplemented with 50 µg/mL of kanamycin. The culture was shaked for 16 hours at 37°C. The next day, 1L of fresh Luria-Bertani broth supplemented with 50 µg/mL of kanamycin and 2mM IPTG was added to the overnight culture. The culture was then left to grow at roomtemp with constant shaking for 4 hours. The culture was then collected and pelleted at 4000 RPM for 30 minitues. The pellet was then removed from the centrifuge bottles with a spatula and placed into a 50mL falcon tube that was pre-incubated on ice. The pellet was then resuspended with 50mL of ice cold lysis buffer composed of 500mM NaCl, 25mM Tris-HCl pH 7.5, 10% Glycerol, 5mM beta mercaptoethanol, 10mM Imidizole, 0.1% NP-40, and 0.1mM phenylmethylsulfonyl fluoride.The pellet was resuspended using a spatula with constant stiring. The solution was then transferred to a metal sonication beaker. Sonication was completed using these settings, 50% duty power and power setting 8. Sonication was completed in a ice water bucket to keep the solution near 4°C. The solution was kept under 16°C at all times, by pausing the sonication and letting the solution cool between runs. The sonication took roughly 8 cycles or until all solid pellet was dissolved in solution. The lysate was then put into oakridge tubes and centrifugred for 30 minitues at 25000rpm at 10°C. While the lysate was spinning a 5ml Ni-NTA pre packed column was equilibrated with 50mL of Lysis buffer. The supernatant was collected and then loaded onto a prepacked 5mL Ni-NTA column using a Bio-Rad NGC Medium-Pressure Liquid

Chromatography System at 4°C. The column was then washed with 200mL of ice cold wash buffer which composed of 500mM NaCl, 25mM Tris-HCl pH 7.5, 10% Glycerol, 5mM Beta mercaptoethanol, 10mM Imidizole. Following the washing of the column the protein was eluted in 2mL fractions using a buffer composing of 500mM NaCl, 25mM Tris-HCl pH 7.5, 10% Glycerol, 5mM Beta mercaptoethanol, 250mM Imidizole. The amount of protein in each fraction was calculated using Bradford reagent. The protein elutions and previous fractions were then ran on a 12.5% SDS page gel to identify what fractions had PNKP. While the SDS gel was running a superdex 200 column was equilibrated with 150mL of the same wash buffer previously described but with no Imidizole. Following the complete of the SDS gel the fractions containined the most protein were loaded into a 5mL loop and then onto the superdex 200 column. The column was eluted with the was buffer previously described in 5mL fractions. The eluted fractions were then loaded onto a 12.5% SDS gel to confirm which fractions had PNKP. The fractions containing the highest quantity of PNKP were pooled and then bradford was completed to determine the final concentration.

1. His-PFKFB3 and Mutants

A plasmid containing the PFKFB3 coding sequence, was transformed into comptetant BL21 (DE3) cells. Kanomycin was used as the antibiotic as the vector pet28a+ has the kanamycin resistant gene. The cells were left to grow on a Luria-Bertani broth/Agar plate for 16 hours. The next day the plate containing the colonies used to innoculte a 4L culture of Luria-Bertani broth supplemented with 50 μg/mL of kanamycin. The culture was shaked for 16 hours at 37°C. The next day, 4L of fresh Luria-Bertani broth supplemented with 50 μg/mL of kanamycin and 2mM IPTG was added to the overnight culture. The culture was then left to grow at roomtemp with

constant shaking for 4 hours. The culture was then collected and pelleted at 4000 RPM for 30 minitues. The pellet was resuspended using a spatula with constant stiring. The solution was then transferred to a metal sonication beaker. Sonication was completed using these settings, 50% duty power and power setting 8. Sonication was completed in a ice water bucket to keep the solution near 4°C. The solution was kept under 16°C at all times, by pausing the sonication and letting the solution cool between runs. The sonication took roughly 8 cycles or until all solid pellet was dissolved in solution. The lysate was then put into oakridge tubes and centrifugred for 30 minutes at 25000rpm at 10°C. While the lysate was spinning a 5ml Ni-NTA pre packed column was equilibrated with 50mL of Lysis buffer. The supernatant was collected and then loaded onto a prepacked 5mL Ni-NTA column using a Bio-Rad NGC Medium-Pressure Liquid Chromatography System at 4°C. The column was then washed with 200mL of ice cold wash buffer which composed of 300mM KCl, 25mM Tris-HCl pH 7.5, 10% Glycerol, 5mM Beta mercaptoethanol, 10mM Imidizole. Following the washing of the column the protein was eluted in 2mL fractions using a buffer composing of 300mM KCl, 25mM Tris-HCl pH 7.5, 10% Glycerol, 5mM Beta mercaptoethanol, 250mM Imidizole. The amount of protein in each fraction was calculated using bradford reagent. The protein elutions and previous fractions were then ran on a 12.5% SDS page gel to identify what fractions had PFKFB3. While the SDS gel was running a superdex 200 column was equilibrated with 150mL of the same wash buffer previously described but with no Imidizole. Following the complete of the SDS gel the fractions containined the most protein were loaded into a 5mL loop and then onto the superdex 200 column. The column was eluted with the was buffer previously described in 5mL fractions. The eluted fractions were then loaded onto a 12.5% SDS gel to confirm which fractions had

PFKFB3. The fractions containing the highest quantity of PFKFB3 were pooled and then bradford was completed to determine the final concentration.

B. Enzymatic Synthesis and Purification of Fructose 2,6-Bisphosphate

The production of Fructose 2,6-Bisphosphate starts by creating a master mix of buffer B. Buffer B consists of 60mM Tris-Hcl pH 7.5, 1.5mM DTT, 5mM KP_i pH 7.5, 20mM KCl, 40 μm EDTA, 6mM $MgCl_2$, 5mM ATP, and 1mM Fructose 6-phosphate. Next, 200uL of buffer B was aliquoted into 60 tubes. Then, 100μg of PFKFB3 was added into each tube, and the tubes were then incubated at 37°C for 2 hours. The reaction was then stopped by heating the tubes at 90°C for 5 minitues.

Following the completion of the reaction, the reactions were diluted accordingly. All 60 reactions were pooled. 60mL of 10mM TEABC was then added to the pooled reactions to dilute them. Only 20mL of the diluted reaction mixture was loaded onto the FPLC, to not overload the binding capacity of the column.

A 8mL MonoQ column was equilibrated with 50mL of 10mM TEABC, or until the m/s reached a value of 0.7 through Pump A. Pump B was then equilibrated with 800mM, without the column attatched. This equilibration took place until the m/s read a value of 28. Next, 20mL of the diluted reaction mixture was loaded onto the column through pump A. After loading finished, 30mL of 10mM TEABC was passed through pump A until the m/s read 0.7. A gradient elution method was then ran which consisted of 0-29%B over 10mL, 29-31%B over 20 mL, 31-80%B over 10mL, 100%B over 4mL.

Following elution from the NGC, the fractions inbetween the ADP/ATP peaks were pooled together and dried overnight in the speedvac. The fractions were then dissolved in 20µl of

10mM TEABC and were all combined into a pre-weighted Eppendorf tube. The sample was then dried again in the speed vac and a pellet became visable after drying. The Eppendorf tube was then weighted again to determine the weight of the pellet. This resulted in ~1mg of F26,BP per 60 reactions. The following powder was stored at -80°C until needed for future assays. When the pellet was resuspended, 10mM Tris-HCl pH7.5 was used to create a final concentration of 10mM.

C. PNKP 3' Phosphatase Assay

To analyze the phosphatase activity of PNKP, we needed to detect the change in the phosphate relase that's induced by PNKP. To determine this phosphate release by PNKP, I used an oligo with a 5' TAMRA tag and a 3' Phosphate group. This oligo was then annealed to a complementary strand, which was annealed to a strand following the 3' Phosphate group. This oligo mimicked what a single strand break would be like in vivo. The sequence of the strands are "[5'-(TAMN)-TAGCATCGATCAGTCCTC-3'-P], [5'-OH-GAGGTCTAGCATCGTTAGTCA-(6-FAM)-3'], and the complementary strand [5'-TGACTAACGATGCTAGACCTCTGAGGACTGATCGATGCTA-3']."

First a 20% Urea gel was casted. This consisted of 33.6 grams of Urea, 40mL of 40% acrylamide, 8mL of 10x TBE, 300uL of 10% APS, and 30uL of TEMED. The solution was heated until all the urea was dissolved. The solution was then poured into a sequenceing gel casting apparatus. The gel was left to polymerize for 1 hour.

Next the reaction mixture was setup. A 5x mastermix consisting of 75mM Tris-HCl, 390mM KCl, 30mM $MgCl_2$, 5mM DTT, and 5mg/mL BSA was created. The PNKP stock solution was then diluted with this mastermix to 0.5pg/μl. The substrate was then diluted to

100nM using the 5x mastermix. To each tube 9µl of 5x mastermix, 27µl of miliQ water, 8µl of subrate was added. 1µl of F2,6BP or water was then added to the side of the tube. 2uL of the diluted PNKP stock was added to the other side of the tube. The reaction was then spun down for 5 seconds, and incubated on the 37°C heatblock for 15 minitues. The reaction was stopped by heating the Eppendorf tube on the 95°C heablock for 60 seconds. 2µl of the completed reaction was added to 18uL of Deionized Formamide. From the Deionized Formamide and reaction mixture, 10uL was loaded onto the gel after being heated for 30 seconds at 95°C. The gel was pre run for 45 minitues before the loading at 50W. After loading all lanes the gel was immidiently ran for 3 hours at 50W. The TAMARA tag was then imaged using the Typhoon FLA 9000 laser scanner.

D. Ppi-PFK Purificaiton and Assay

Pyrophosphate-dependent phosphotransferase was purified from potatos following a method created by (Schaftingen, Lederer, Bartrons, and Hers).First 300g of potato was homogenized in a blender with 600mL of lysis buffer containing ice cold 20mM Hepes pH8.2, 20mM Potassium Acetate, and. 2mM DTT. The homogenate was kept under 16°C for the entire homogenization process. The homogenate was then passed through a cheesecloth, containing a beaker with a stir bar. Magnesium Chloride was added to 1M and Sodium Pryrophosphate to 2mM. The pH was then adjusted to 8.2. The homogenate was then added to a 70°C water bath and left stiring, The temperature of the lysate was kept constant at 59°C for 5 minitues. The lysate was then cooled to 4°C in an ice bucket. The pH was then adjusted to 7.1. 6g of PEG 6000 was added per 100mL of lysate. The solution as left to stir at 4°C for 15 minutes. The solution was then allowed to stand for 10 minutes at 4°C, and then centrigured for 10 minutes at 4000rpm. The supernatant was then poured off into a beaker and PEG 6000 was added at

8g/100mL of supernatant. The solution was left to stir for 15 minutes at 4°C and then left to sit for 10 minutes. The solution was then centrifugred at 4000rpm for 10 minutes. The supernatant was discareded and the pellet was resuspended in 40mL of resupension buffer. The resuspension buffer consisted of 20mM Tris pH 8.2, 20mM KCl, and 2mM DTT. The solution was then centrifuged at 20000rpm for 10 minutes. The supernatant was saved. The supernatant was then passed through Q-Sepharose beads that were previously eqlibrated with resuspension buffer. The column was then washed with 100mL of resuspension buffer. The column was then eluted with the same resuspension buffer + 150mM KCl. This elutant was aliquoted and stored at -80°C.

To test the activity of Ppi-PFK, a reaction measuring the absorbance change of NADH to NAD+ was set up. Ppi-PFKwas mixed with 25mM Tris-HCl pH8.0, 5mM Magnesium Chloride, 0.2mM NADH, 50µg/mL aldolase, 1µg.mL Triosephosphate isomerase, 10µg/mL Glycerol-3-phosphate to 450uL. The reaction is then started by the addition of 50µL of 25mM Sodium Pyrophosphate. The change in A_{340} is then measured. 1uL of 10mM F2,6BP is added to the reaction before starting. The change in A_{340} with and without F2,6BP, confirms that F2,6BP is present.

E. IKK Kinase Assay

PNKP or mutant PNKP was diluted to 1µg/µL of PNKP with kinase buffer, which consisted of 20mM Tris-HCl pH 7.5, 10mM NaCl, 10mM MgCl2, 1mM DTT, 200µM ATP. 1µl of diluted protein was added to a tube with 8µl of kinase buffer. 1µL Radioactive Phosphate labeled ATP and 1µL of 50ng/µL of IKK1/2 were added to initiate the reaction. The reaction was incubated at room-temperature for 30 minutes. The reaction was stopped by adding 4x SDS loading dye and heating at 95°C for 5 minutes. The samples were then run on a 12.5% SDS-PAGE gel at 140V until the dye front was 4cm from the bottom of the gel. The free radioactive

band was cut from the gel at the bottom, and the rest of the gel was wrapped in saran wrap. The gel was left overnight in a blanked phosphor screen in a dark cabinet. The screen was then analyzed on the Typoon FLA 9000 phsphoimager to visualize the radioactive bands.

F. Protein stability by nanotemper

PNKP was diluted with a buffer containing 25mM Tris-HCl pH7.5, 150mM NaCl, 5mM $MgCl_2$, 1% glycerol, and 1mM DTT to 0.3mg/mL. Fructose-2,6-Bisphosphate was also diluted down to final concentrations of 1μM and 10μM in the PNKP solution. The samples were then loaded into capillary tubes and then onto the NanoTemper Tycho. The instrument started to denature and read absorbance values at 35°C and continued until it reached 95°C.

H. Binding assay using Bio-Layer Inferometry (BLI)

PNKP was immobilized on a BLI-His Sensor by placing the senor in buffer containing 25 mM Tris-HCl pH 7.5, 150mM NaCl, 0.02% Tween 20,and 1 mM DTT for 5 seconds. A reference senor without PNKP was used as background subtraction. Baseline was first measured by incubation of sensors in BLI buffer for 60 seconds. Binding kinetics were then measured through an association phase of 300 seconds in which 20uM of F2,6BP was incubated with the PNKP sensor complex, followed by a dissociation phase of 180 seconds in BLI buffer without Protein.

II.   Results

Chapter 1 Synthesis and purification of Fructose-2,6-Bisphosphate

Purification of Ni-NTA PFKFB3 Using Affinity Chromatography

PFKFB3 has no know roles in DNA damage and repair. Its main function is to produce

Fructose-2,6-Bisphosphate which is a known activator and repressor of the cyclic acid cycle.

Since the levels of PFKFB3 were low in disease model we wanted to enzymatically make and

purify the product of PFKFB3 (F2,6BP) to investigate the interaction between DNA repair.

I first started purifying PKKFB3 by previously used methods in our lab. This consisted

of growing the Rosetta cells with PFKFB3 plasmid to an OD of 0.6 and then inducing with

0.2mM IPTG. The cells were then left to grow overnight at 16°C and collected the next day.

This led to an extremely small yield of PFKFB3 ~1.6mg from 8L of culture. This low yield

occurred due to PFKFB3 being insoluble and most of the protein left inside the inclusion body,
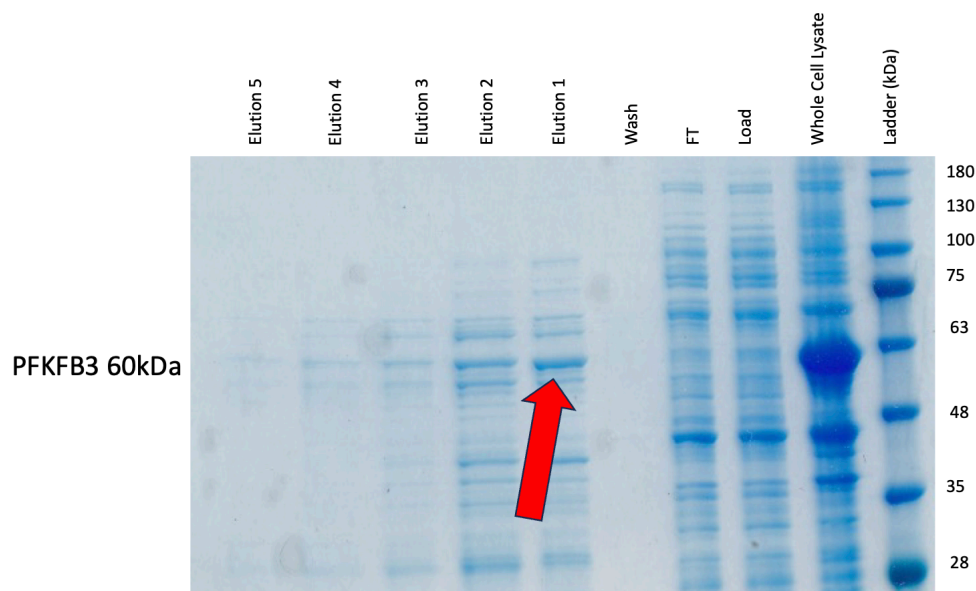
Figure 7.

Figure 7. Low-Yield Ni-NTA purification of His-PFKFB3.

To increase the yield of His-PFKFB3 I purified the protein from BL21(DE3) cells. The cells were grown for 16 hours at 37°C and then equivolume fresh LB supplemented with 2mM IPTG was added. The lysate was then clarified, bound and then eluted from Ni-NTA beads. Figure 8 shows the elutions from the Ni-NTA column ran on a 12.5% SDS page gel. The resulting elution was then loaded onto a Superdex 200 size exclusion column and was purified further. Figure 9 shows the elutions from the Superdex 200 ran on a 12.5% SDS page gel. Fractions 13-17 were collected and concentrated. The purification of PFKFB3 resulted in ~21mg of pure protein.
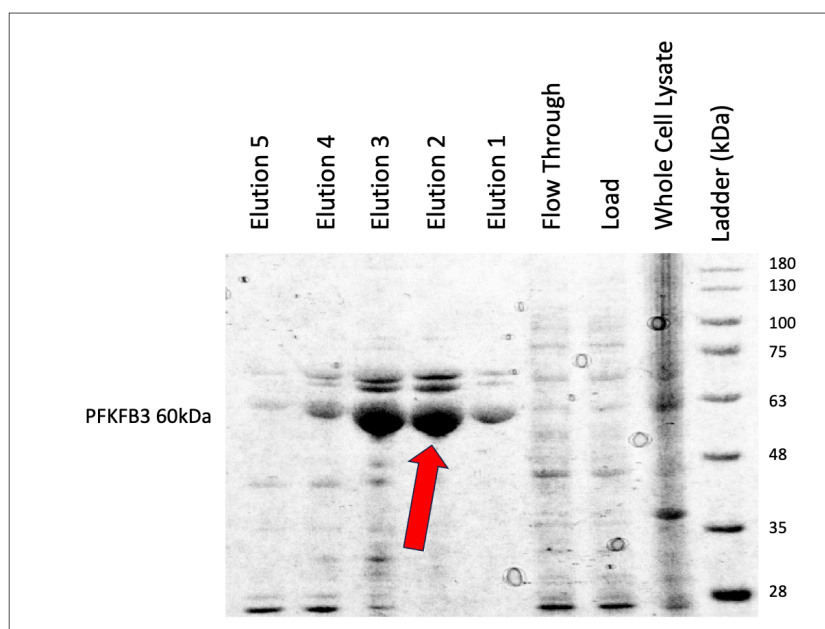

Figure 8. Ni-NTA purification of His-PFKFB3. PFKFB3 elutes mostly in fractions 2 and 3 and is seen at 60kDa
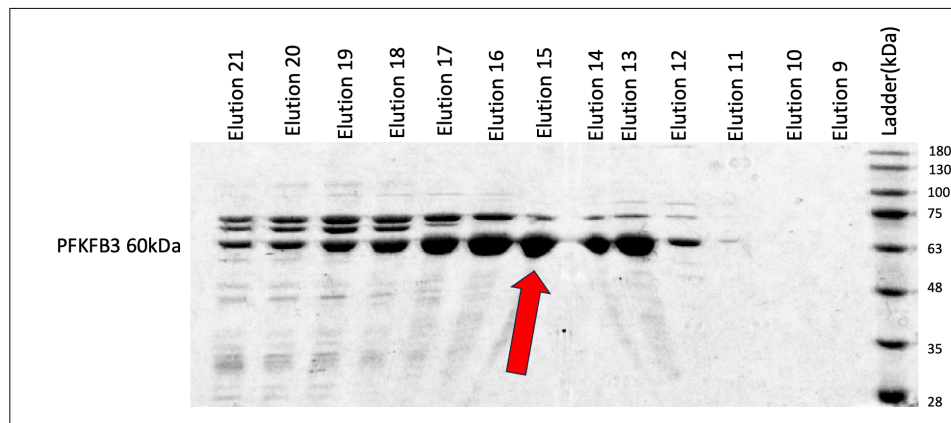
Figure 9. Gel Filtration purification of His-PFKFB3. PFKFB3 Elutes in multiple 5mL fractions, the majority of the enzyme is in fractions 13-17.

Enzymatic Synthesis and purification of Fructose-2,6-Bisphosphate

The production of Fructose-2,6-Bisphosphate took many months of optimization to get a yield acceptable for further assays. Previously work done to make Fructose-2,6-Bisphosphate was in very small concentrations and was contaminated with ADP and ATP. I developed a method to product Fructose-2,6-Bisphosphate in bulk. Figure 10, shows the 1 step enzymatic synthesis I use to create Fructose-2,6-Bisphosphate.
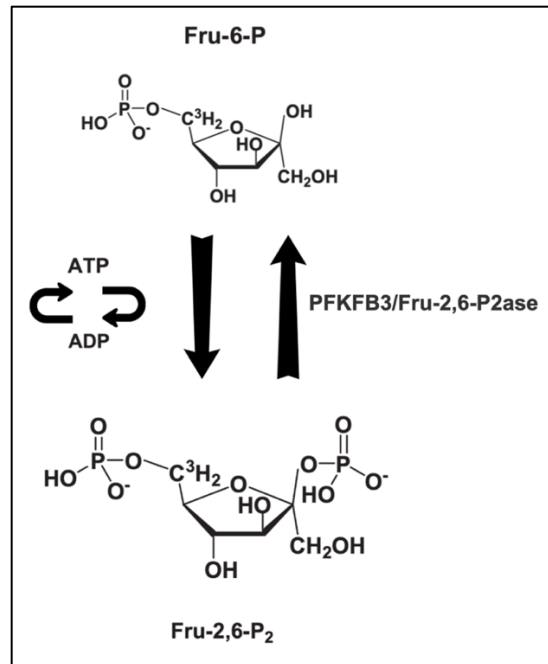
Figure. 10 Synthesis of Fructose-2,6-Bisphosphate with PFKFB3, which Fructose 6 phosphate as the substrate and ATP as phosphate donator.

I first started my optimization of making Fructose-2,6-Bisphosphate by changing the crude reaction conditions. This consisted of identifying the amount of enzyme, optimal reaction volume, concentrations of subtrate (F6P,ATP) and the temperature. To confirm the production of Fructose-2,6-Bisphosphate, I used a Ppp-PFK assay which measures the change of NADH to NAD^+. This assay developed by Schaftingen, identifies Fructose-2,6-Bisphosphate as a substrate that speeds up the conversion of NADH to NAD^+. Since NADH absorbs at 340nm wavelength, I can measure the change with and without sample to determine if Fructose-2,6-Bisphosphate is present as shown in Figure 11. I identified that 100ug of enzyme gives the best result for production of Fructose-2,6-Bisphosphate, I also identified that the reaction must take place at 37°C or no Fructose-2,6-Bisphosphate will be produced.
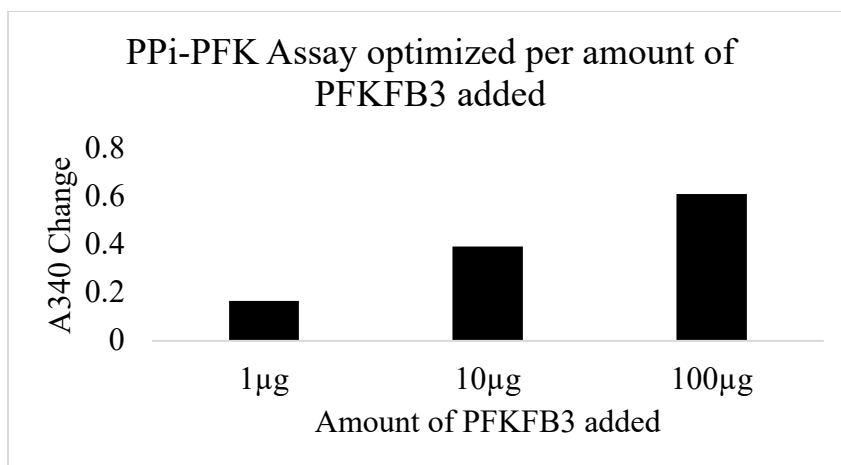
PPi-PFK Assay optimized per amount of PFKFB3 added

Figure 11 Optimization of Amount of PFKFB3 needed to make the most Fructose-2,6-Bisphosphate

Finally, 100μg of purified PFKFB3 was added to a reaction containing 5mM ATP, 1mM Fructose 6 Phosphate, 2mM DTT, 5mM kPi, 100mM Tris-HCl, 10mM MgCl2. The final volume of the reaction was 100uL. The reaction was incubated at 37°C for 2 hours. Following this the reaction mixture was heated to 95°C for 5 min to stop the reaction. This process was completed for 60 reactions at a time. Following the inactivation, 20 reactions were combined into pools and each pool was diluted with 20mL of 10mM Triethylammonium bicarbonate buffer. The diluted reactions were then loaded onto a 8mL Mono-Q column that was elaborated with 10mM Triethylammonium bicarbonate buffer. The column was then eluted with a gradient consistent of 10mM Triethylammonium bicarbonate buffer and 800mM Triethylammonium bicarbonate buffer. Triethylammonium bicarbonate buffer was selected as the salt elution gradient due to its volatility an ability to precipitate compounds in solution when evaporated. This separation resulted in 2 separate peaks, one belonging to ADP and the other ATP Figure 12. We can assume the reaction took place as the ADP peak is present, indicating the conversion of ATP to ADP in our reaction. Since Fructose-2,6-Bisphosphate does not absorb any wavelength of light we could not see a peak that corresponds to F2,6BP.
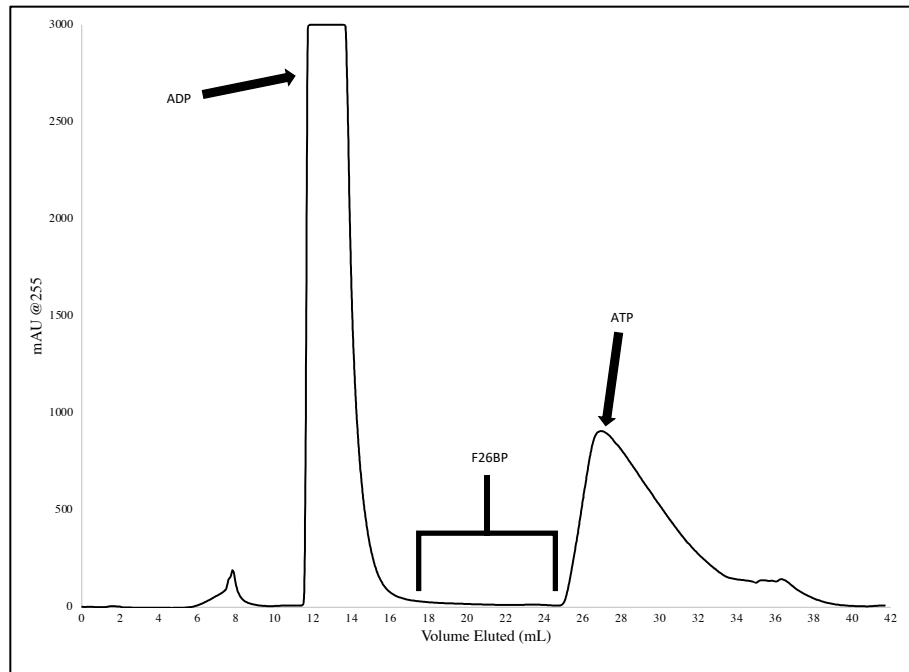
Figure 12. Separation of Fructose-2,6-Bisphosphate on 8mL MonoQ Column. 12-16mL is the ADP peak, and 26-34mL is the ATP peak. Fructose-2,6-Bisphosphate does not absorb light and there is no peak.

One of the major problems was the separation of the ADP and ATP peak when eluted from the MonoQ column. I believed that the Fructose-2,6-Bisphosphate was coming out between the peaks but did not know if there was an overlap and if the peak of the Fructose-2,6-Bisphosphate elution was containmated with ADP/ATP. To counter this I tried implementing a treatment of Pyruvate Kinase(PK), post enzymatic synthesis of Fructose-2,6-Bisphosphate. In theory the PK should turn all the ADP present in the reaction back to ATP. Thus I would be able to collect the fractions where the ADP previously was and save those as potential Fructose-2,6-Bisphosphate fractions. This partially worked out as planned, as we can see in figure 13 the ADP peak is removed. This also negatively impacted the purification as the ATP peak was greatly enhanced and spanned many fractions. This caused the loss of many fractions with Fructose-2,6-Bisphosphate and was not a preferred method.
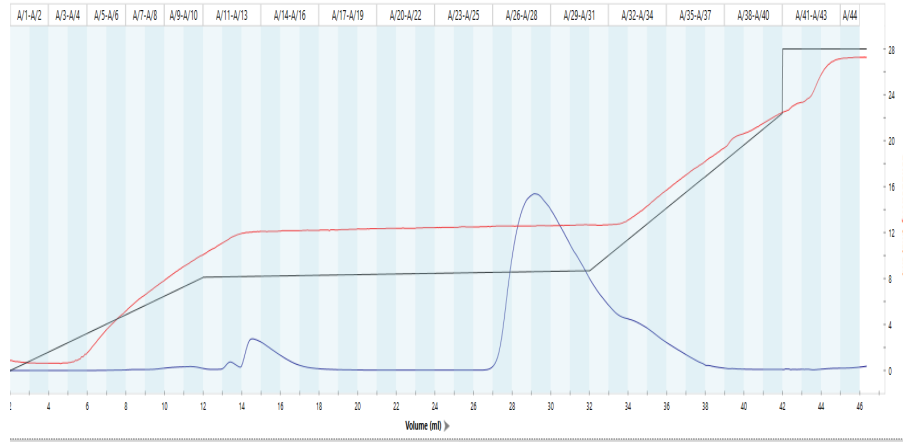
27

Figure 13 Pyruvate Kinase Treatment of the completed Fructose-2,6-Bisphosphate synthesis reaction

I then tried the same protocol but with Hexokinase instead of PK. This should have convereted the ATP back to ADP and thus have more Fructose-2,6-Bisphosphate fractions available. This method had the same problem as before, but this time the ADP peak was enlarged and spanned over too many fractions causing the Fructose-2,6-Bisphosphate elutions to be contaimined with ADP Figure 14.
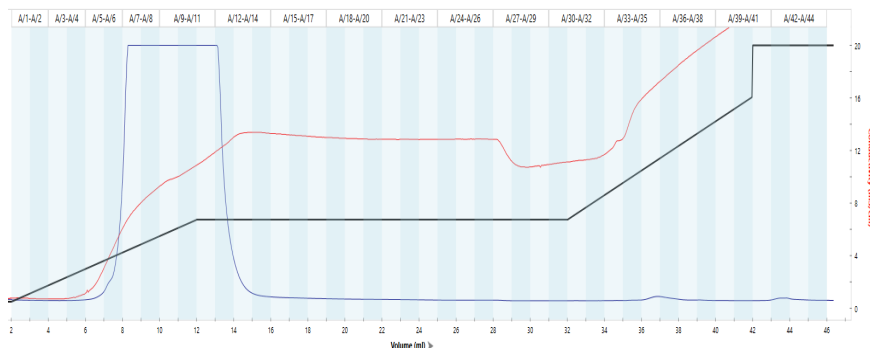
Figure 14 Hexokinase Treatment of the completed Fructose-2,6-Bisphosphate synthesis reaction

To solve this problem, I loaded less reactions on the column and changed the elution protocol to make the peaks more spread out. The fractions in-between the ADP/ATP peak were then dried and combined. From the 60 reactions completed 1mg of Fructose-2,6-Bisphosphate was obtained. The resulting product was diluted to 10mM with 10mM Tris-HCl and aliquoted

and store at -80°C. To determine if the powder we collected was actually Fructose-2,6-Bisphosphate mass spec was conducted on the sample. The mass to charge ratio of 344 indicated that Fructose-2,6-Bisphosphate was present in our sample and was successful enzymatically made and purified, Figure 15.
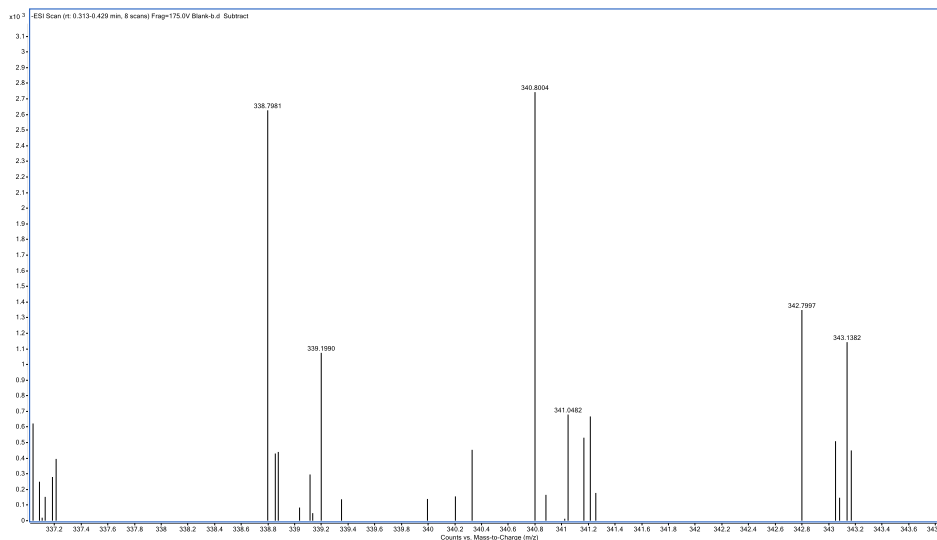
Figure 15. Mass Spec identification of Fructose-2,6-Bisphosphate. Fructose-2,6-Bisphosphate has a mass to charge ratio of 3.

In figure 16, I used a method previously described by Schaftingen. If Fructose-2,6-Bisphosphate is present in this assay then change can be observed from the conversation of NADH to NAD+. The change in UV at 340 is thus able to be quantified as NAD+ does not absorb any wavelength. This assay is extremely sensitive and does not work in the presence of Fructose 6 phosphate. From this assay and the mass spec data we can conclude that Fructose-2,6-Bisphosphate is present in the sample.

Figure 16. Fructose-6-phosphate phosphotransferase assay on MonoQ Elutions

Chapter 2: Fructose-2,6-Bisphosphate as a cofactor of PNKP

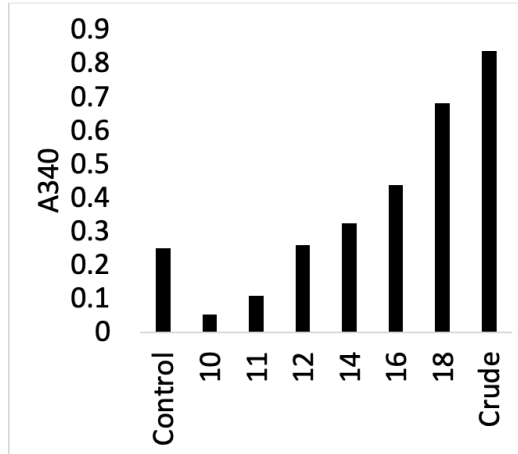To assess if Fructose-2,6-Bisphosphate had any effect on the activity of PNKP, I purified His-PNKP. His-PNKP was transformed into Rosetta cells and then induced with 1mM IPTG. The cells were spun down collection and then resuspended. The cell resuspension was then sonicated, resulting in the whole cell lysate. The whole cell lysate was then spun again to remove inclusion bodies. The supernatant was loaded onto a 5mL Ni-NTA prepacked column. The column was washed with 200mL of wash buffer and eluted in 2mL fractions. The amount of protein present in these fractions was then quantified by bradford. There was 5mg of total protein that was collected after affinity chromatography. Figure 17 shows the steps during the purification whichwere loaded onto a 12.5% SDS gel.
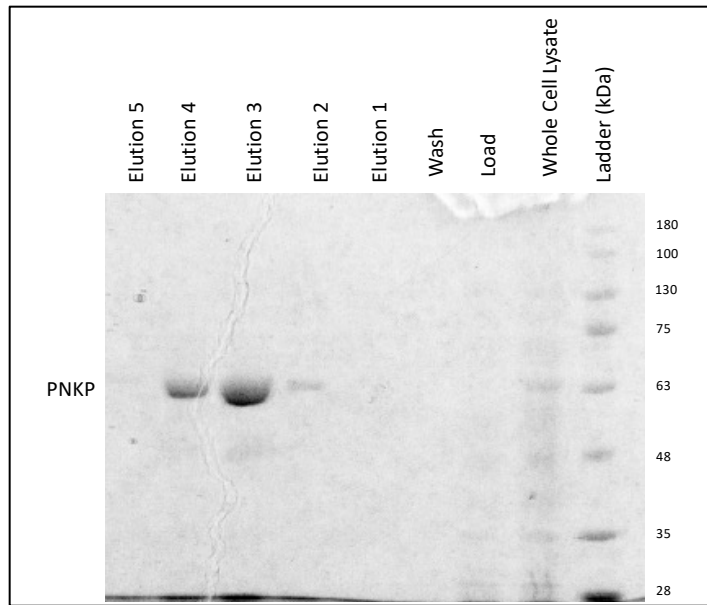
Figure 17. Ni-NTA Purification of His-PNKP

PNKP was eluted in fraction 3 and 4 and was relatively pure. To make sure the

fractions were pure PNKP, gel filtration was completed on fractions 3 and 4. Fractions 3 and 4

were spun at 13000rpm for 2 minutes at 4°C to remove any insoluble protein. The supernatant

was then injected into a 5mL loop equilibrated with elution buffer. The fractions were then

passed onto a superdex 200 size exclusion column and eluted in 5mL fractions. Figure 18 is

the resulting 12.5% SDS gel that was run on fractions containing PNKP. Fractions 15 and 16

were combined and then concentrated, which resulted in a final yield of 2mg of PNKP.

Figure 18. Gel Filtration purification of His-PFKFB3

Fructose-2,6-Bisphosphate causes increased PNKP Phosphate activity.

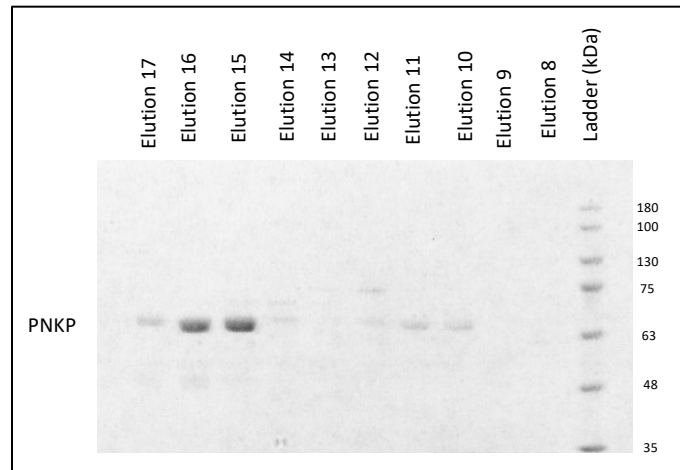To asses if Fructose-2,6-Bisphosphate increases the 3' phosphate activity of PNKP, I designed a non radioactive phosphatase assay. This assay consisited being able to detected the change in the removal of a 3' phosphate by PNKP. I achieved this by using a oligo with a 3' phosphate at one end and then a 5' TAMRA tag at the other end. The subtrate was then mixed with PNKP and ran on a sequencing gel to separate the oligo with phosphate and without phosphate. This resulted in 2 bands being present, the top band being the phosphate removed and the bottom band being with the phosphate.

In figure 19 lane 1 is with no PNKP, so only the bottom band is present which identifies that phosphate is attached to the substrate. We can assume this as the phoshphate group is negatively charged and will thus speed up the oligo's migration towards the positive electrode when a current is applied. In lane 2 we can see 3ng of PNKP causes a feint top band indicating that at this concetration of PNKP and reaction time of 13 minutes, a fraction of the phosphate is removed from the oligo. In lanes 3-5 I added in and increased the concentration

of Fructose-2,6-Bisphosphate to see the effect on the activity of PNKP. The concentration of

PNKP was kept the same through these lanes. As the concentration of Fructose-2,6-

Bisphosphate increased, so did the 3' phosphate activity of PNKP. This is indicated clearly in

lane 5 as the top band is the more prominent species, indicating that most of the phosphate was

removed.

PNKP	0ng	3ng	3ng	3ng	3ng
F26BP	0uM	0uM	1uM	100uM	1mM
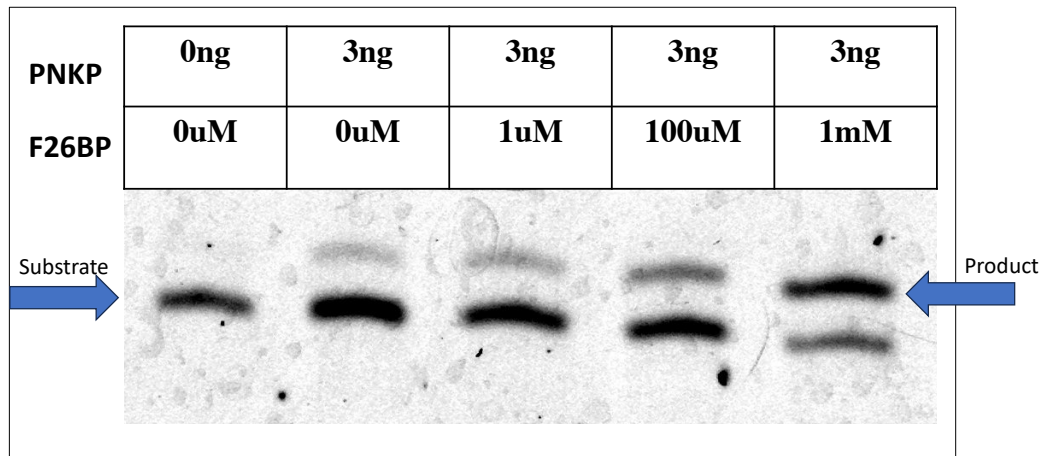
Figure 19. In Vitro PNKP assay with enzymatically synthesized Fructose-2,6-Bisphosphate

As shown in Figure 20, our collaborators used a radioactive phoshpahte assay to

analyze 3' phosphatase activity of PNKP. In their assay the 3' phosphate group was radioactive,

thus they were able to detect the free phosphate group when it was attached to the oligo, or

removed from the oligo by PNKP. Similar to my results this assay showed that with increasing

concentraions of Fructose-2,6-Bisphosphate, the 3' phosphtase activity of PNKP also increases.

Furthermore our collaberators also used, my starting material as a control Fructose 6-phosphate.

The control showed no increase in the activity.

Figure 20. Radioactive Phosphate PNKP assay with enzymatically synthesized Fructose-2,6-Bisphosphate

Another member of our lab Dr. Biswas, synthesized Fructose-2,6-Bisphosphate synthetically and I tested the activity of it on my non radioactive phosphotate assay. Similar to my results the chemically synthesized Fructose-2,6-Bisphosphate increased the 3' phosphatase activity of PNKP. In this assay I completed a timepoint experiment where I had a reaction with Fructose-2,6-Bisphosphate and without. At all timepoints there is increased 3' phosphate activity in figure 21.
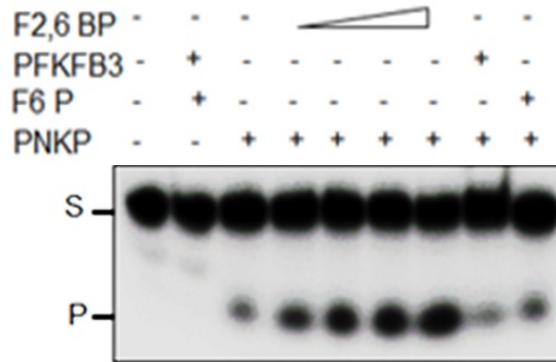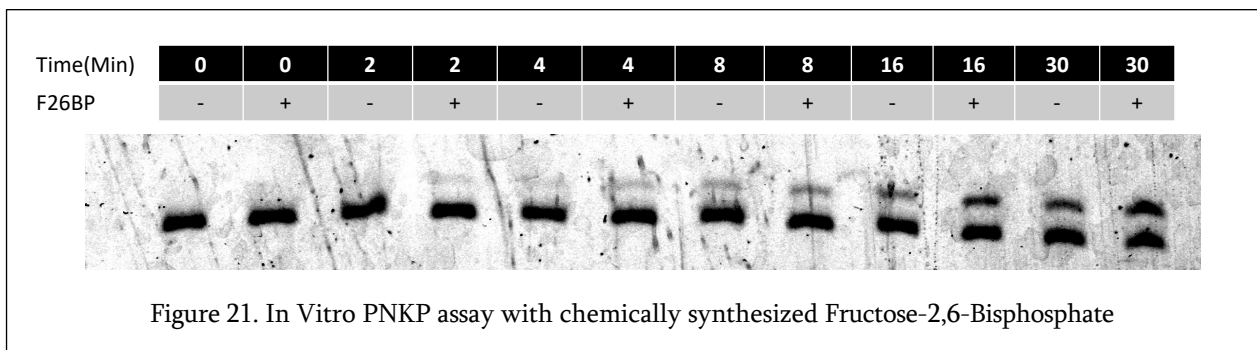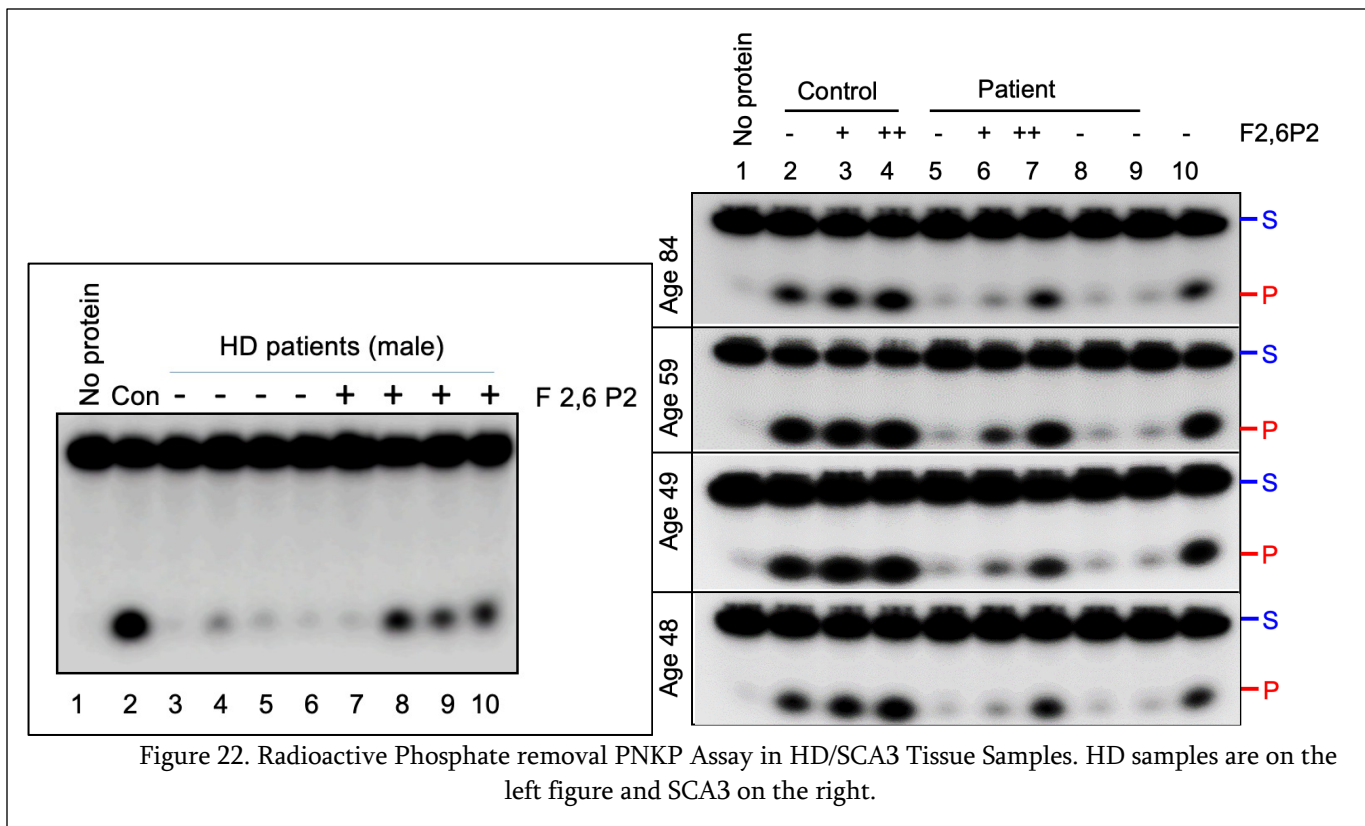

Figure 21. In Vitro PNKP assay with chemically synthesized Fructose-2,6-Bisphosphate

F2,6BP Recovers PNKP activity in Huntington Disease/Sca3 Cortex Tissue

Since our in vitro assay was working we next wanted to see if we could see any affect in human patient disease tissue. First, we looked at the levels of Fructose-2,6-Bisphosphate in

Huntington disease and SCA3 cortex patient samples. The sample was lysed and then brought down to a low pH with HCl. Since Fructose-2,6-Bisphosphate is pH sensitive this would convert it all to Fructose 6 Phosphate. The Fructose 6 Phosphate was then quantified using the "Fructose-6-Phosphate Assay Kit," from Sigma Aldrich. The reading was taken before and after the lowering of the pH and the change was observed in the amount of Fructose 6 Phosphate. From this assay the amount of Fructose-2,6-Bisphosphate in Huntington disease and SCA3 cortex patient samples was determined to be significantly less then healthy samples. Since the Fructose-2,6-Bisphosphate levels were lower in these tissues, we wanted to see if addition of Fructose-2,6-Bisphosphate would rescue the 3' Phosphate activity of PNKP. The radioactive 3' phosphate removal assay was completed in the same fashion as before with tissue extract from Huntington disease and SCA3 cortex patient samples, done by our collabertors (Hazra Group). Fructose-2,6-Bisphosphate was added into the disease models which showed a recovery in 3' Phosphate activity of PNKP. This was present throughout all age groups for the SCA3 patient samples. When no Fructose-2,6-Bisphosphate was added the activity was significantly decreased compared to the healthy samples, Figure 22.

Figure 22. Radioactive Phosphate removal PNKP Assay in HD/SCA3 Tissue Samples. HD samples are on the left figure and SCA3 on the right.

F2,6BP Enhances PNKP Stability

In the nanotemper instrument a sample of PNKP with no Fructose-2,6-Bisphosphate, and PNKP with 1uM and 10uM Fructose-2,6-Bisphosphate was added. The denaturation of the enzyme showed multiple curves with different peak heights indicating that the Fructose-2,6-Bisphosphate was interacting with PNKP and increasing the stability. When the concentration of Fructose-2,6-Bisphosphate was at its maximum the peak height was at its minimum compared to PNKP with no Fructose-2,6-Bisphosphate as seen in Figure 23. From this we can assume that F2,6BP is associating and increasing the stability of PNKP.

Figure 23. Nanotemper denaturation curve of His-PNKP with varying concentrations of F2,6BP
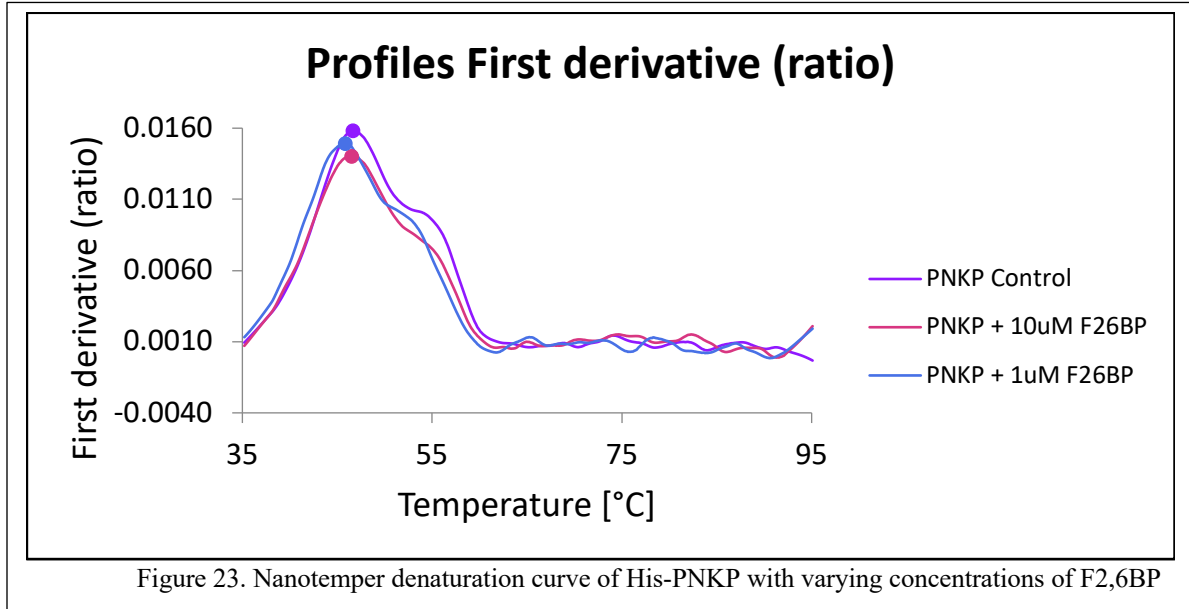
Measurement of direction interaction between PNKP and F2,6BP

A solution containing 20uM of Fructose-2,6-Bisphosphate was incubated with immobilized His-PNKP. The BLI instrument indicated a change in the light being reflected from the tip, indicating the binding of Fructose-2,6-Bisphosphate to His-PNKP, Figure 24. After the sensor was placed into buffer containing no Fructose-2,6-Bisphosphate the curve started to go down indicating that the Fructose-2,6-Bisphosphate was no longer binding to PNKP. This experiment was only completed once and needs to be repeated in order to confirm the binding.
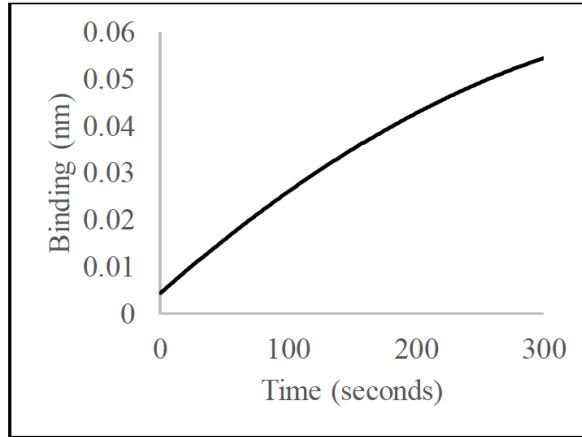
Figure 24. Bio layer interferometry binding curve of Fructose-2,6-Bisphosphate to His-PNKP

PNKP is Phosphorylated by IKK in Vitro

I next wanted to see if PNKP was being phosphorylated by IKK. Since previous studies has

shown there are 2 motifits on PNKP that could possibly interact with IKK and cause

phosphorylation of serine sites. In this assay I used WT PNKP, a double Mutant PNKP at the

hypothesized phosphorylation sites S280E and S284E, and IκBα as a control. IKK 1, 2 and IKK

heterodimer were purified and provided by a member of our lab Dr. Shahabi. This assay

confirmed our hypothesis that IKK was phosporylating PNKP, but more interestingly IKK2 was

phosphorylating PNKP much more then IKK1. The control IκBα confirmed that the

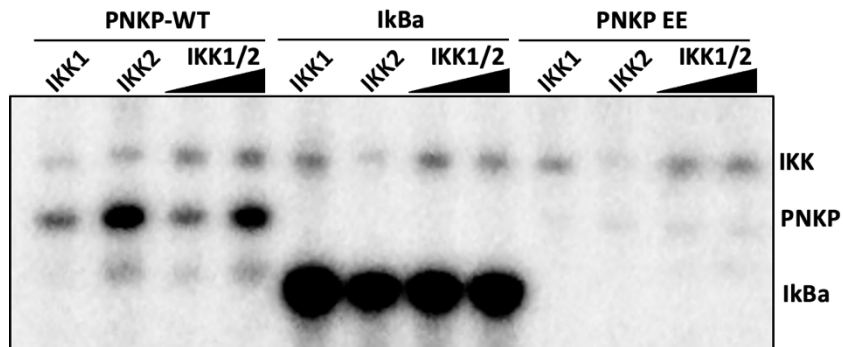phosphorylation was by IKK as it's a known subtrate of IKK. This is shown in figure 25.

Figure 25. IKK1, IKK2 and IKK1/2 Heterodimer Phosphorylation of PNKP WT and Mutant PNKP EE

IV: Discussion

The purpose of this study was to determine potential causes of increased DNA damage in Huntington's disease and SCA3. The discovery of PNKP interacting with PFKFB3 led us into a completely uncharted area of research that had never been explored before. The interaction of an enzyme involved in glycolysis had never been shown to be linked to the DNA repair pathway. Previously, PFKFB3 had only been studied as a regulator of the citric acid cycle and had no known role in the DNA repair complex. PFKFB3 is the sole known enzyme that converts the substrate Fructose 6 Phosphate to Fructose 2,6 Bisphosphate using ATP. The metabolite Fructose 2,6 Bisphosphate allosterically influences the activity of the enzymes PFK-1 and FBPase-1. In the absence of Fructose 2,6 Bisphosphate, the breakdown of glucose is inhibited. When Fructose 2,6 Bisphosphate is present, it activates PFK-1 and stimulates the breakdown of glucose. PFKFB3 has also been shown to be involved in multiple types of cancer as it is a key regulator in the survival and proliferation of cancer cells. When PFKFB3 is overexpressed, more Fructose 2,6 Bisphosphate is produced, which, results in a high rate of glycolytic flux. A high rate of glycolytic flux provides cancer cells with a quick source of energy and biosynthetic intermediates to support their rapid growth and proliferation. Higher PFKFB3 expression helps cancer cells adapt to the hypoxic conditions commonly found in solid tumors, enabling them to maintain energy production and continue proliferating even in oxygen-deprived environments. From my study I found that PFKFB3 also has another important role in the regulation of DNA repair. In the first part of my thesis, I describe how we identified PFKFB3 as a potential target for regulation of DNA repair. This identification processed first started with the discovery of reduced activity of the DNA repair enzyme PNKP in diseased Huntington disease and SCA3 tissues. From here an immunoprecipitated identified the association of PFKFB3 with the DNA repair enzyme PNKP.

The next goal of this work was to confirm the hypothesis that Fructose 2,6 Bisphosphate allosterically activates the DNA repair enzyme PNKP. The next part of my thesis describes how I purified the small molecule Fructose 2,6 Bisphosphate by using the enzyme PFKFB3. I produced Fructose 2,6 Bisphosphate by anion exchange column, separating the needed input ATP and output ADP based on charge. This separation of Fructose 2,6 Bisphosphate was done by modifying a previous protocol using a gradient of a volatile salt to elute the negatively charged molecules from the column. I established that the elution of Fructose 2,6 Bisphosphate occurs between the elution of ADP and ATP on a MonoQ column, thus resulting in a pure sample of Fructose 2,6 Bisphosphate when the volatile salt is evaporated.

In the next part of my thesis, I describe how in Vitro I test the effect of Fructose 2,6 Bisphosphate on the enzyme PNKP. I show the with increased concentrations of Fructose 2,6 Bisphosphate the 3' Phosphate activity of PNKP increases. I also show that ADP and ATP have no effect on the activity of PNKP, thus confirming the hypothesis that Fructose 2,6 Bisphosphate is increasing the activity of PNKP. I then shipped the Fructose 2,6 Bisphosphate I made to our collaborators Dr. Tapas Hazra at The University of Texas Medical Branch. Our collaborators were able to complete in vivo assays with the Fructose 2,6 Bisphosphate that I created. Our collaborators first conducted another in vitro assay which confirmed the results I showed of Fructose 2,6 Bisphosphate enhancing the activity of PNKP. Next our collaborators showed how the addition of Fructose 2,6 Bisphosphate to disease tissue increases the amount of 3' phosphate activity and recovers the DNA repair activity of PNKP. This enhancement was shown in both the disease tissue of Huntington Disease and SCA3 cortex.

In the last part of my thesis I describe how I have show the binding of Fructose 2,6 Bisphosphate to PNKP. The first experiment that shows the binding of Fructose 2,6

Bisphosphate was done on the nanotemper which shows a different denaturation curve when Fructose 2,6 Bisphosphate is bound to PNKP. To confirm this hypothesis, I showed that Fructose 2,6 Bisphosphate does bind to PNKP by utilizing immobilized His-PNKP in Bio-layer interferometry.

There are still multiple unanswered questions about how Fructose 2,6 Bisphosphate interacts with PNKP and enhances the 3' phosphate activity. Where does Fructose 2,6 Bisphosphate bind to PNKP? I could hypothesize that it could bind to the ATP binding pocket of PNKP as both small molecules possess 2 phosphate groups. This could be testing in future work by mutating the ATP binding site of PNKP and complete the in vitro assay to see if the activity of PNKP is still being enhanced by Fructose 2,6 Bisphosphate. Another experiment that is still being worked on is if the addition of Fructose 2,6 Bisphosphate to a diseased animal model will slow down and prevent the increased DNA damage that is present. Our group plans to test if adding Fructose 2,6 Bisphosphate to a Drosophila Huntington's disease models food, will slow down the disease.

References

Chakraborty, Anirban, Wyatt Miller, Weihan Huai, Tapan Biswas, Santi Mohan Mandal, Lisardo Bosca, Gourisankar Ghosh, and Tapas Hazra. *A Glycolytic Metabolite Restores DNA Repair Activity of Polynucleotide Kinase 3'-Phosphatase in Polyglutamine (PolyQ) Diseases*. preprint, Biochemistry, 26 Oct. 2023. *DOI.org (Crossref)*, https://doi.org/10.1101/2023.10.26.564220.

Chakraborty, Anirban, Nisha Tapryal, Tatiana Venkova, Nobuo Horikoshi, Raj K. Pandita, Altaf H. Sarker, Partha S. Sarkar, Tej K. Pandita, and Tapas K. Hazra. "Classical Non-Homologous End-Joining Pathway Utilizes Nascent RNA for Error-Free Double-Strand Break Repair of Transcribed Genes." *Nature Communications*, vol. 7, no. 1, Oct. 2016, p. 13049. *DOI.org (Crossref)*, https://doi.org/10.1038/ncomms13049.

Chakraborty, Anirban, Nisha Tapryal, Tatiana Venkova, Joy Mitra, Velmarini Vasquez, Altaf H. Sarker, Sara Duarte-Silva, Weihan Huai, Tetsuo Ashizawa, Gourisankar Ghosh, Patricia Maciel, Partha S. Sarkar, Muralidhar L. Hegde, Xu Chen, and Tapas K. Hazra. "Deficiency in Classical Nonhomologous End-Joining–Mediated Repair of Transcribed Genes Is Linked to SCA3 Pathogenesis." *Proceedings of the National Academy of Sciences*, vol. 117, no. 14, Apr. 2020, pp. 8154–65. *DOI.org (Crossref)*, https://doi.org/10.1073/pnas.1917280117.

Liu, Ting, Lingyun Zhang, Donghyun Joo, and Shao-Cong Sun. "NF-κB Signaling in Inflammation." *Signal Transduction and Targeted Therapy*, vol. 2, no. 1, July 2017, p. 17023. *DOI.org (Crossref)*, https://doi.org/10.1038/sigtrans.2017.23.

McLoughlin, Hayley S., Lauren R. Moore, and Henry L. Paulson. "Pathogenesis of SCA3 and Implications for Other Polyglutamine Diseases." *Neurobiology of Disease*, vol. 134, Feb. 2020, p. 104635. *DOI.org (Crossref)*, https://doi.org/10.1016/j.nbd.2019.104635.

Myers, Robert W., Walter F. Baginsky, David J. Gattermeir, Wayne M. Geissler, and Georgianna Harris. "Enzymatic Preparation of High-Specific-Activity β-d-[6,6′-3H]Fructose-2,6-Bisphosphate: Application to a Sensitive Assay for Fructose-2,6-Bisphosphatase." *Analytical Biochemistry*, vol. 406, no. 2, Nov. 2010, pp. 97–104. *DOI.org (Crossref)*, https://doi.org/10.1016/j.ab.2010.06.017.

Pradhan, Subrata, Rui Gao, Keegan Bush, Nan Zhang, Yogesh P. Wairkar, and Partha S. Sarkar. "Polyglutamine Expansion in Huntingtin and Mechanism of DNA Damage Repair Defects in Huntington's Disease." *Frontiers in Cellular Neuroscience*, vol. 16, Apr. 2022, p. 837576. *DOI.org (Crossref)*, https://doi.org/10.3389/fncel.2022.837576.

Ranganathan, Rajesh, Georgy Sapozhnikov, Wang Ni, Siwen Li, and Youqiang Song. "Recent Developments in the Role of DNA Damage Response and Understanding Its Implications for New Therapeutic Approaches in Alzheimer's Disease." *Translational Medicine of Aging*, vol. 7, 2023, pp. 52–65. *DOI.org (Crossref)*, https://doi.org/10.1016/j.tma.2023.06.004.

Rider, Mark H., Luc Bertrand, Didier Vertommen, Paul A. Michels, Guy G. Rousseau, and Louis Hue. "6-Phosphofructo-2-Kinase/Fructose-2,6-Bisphosphatase: Head-to-Head with a Bifunctional Enzyme That Controls Glycolysis." *Biochemical Journal*, vol. 381, no. 3, Aug. 2004, pp. 561–79. *DOI.org (Crossref)*, https://doi.org/10.1042/BJ20040752.

Schaftingen, Emile, Beatrice Lederer, Ramon Bartrons, and Henri-Gery Hers. "A Kinetic Study of Pyrophosphate: Fructose-6-Phosphate Phosphotransferase from Potato Tubers. Application to a Microassay of Fructose 2,6-Bisphosphate." *European Journal of Biochemistry*, vol. 129, no. 1, Dec. 1982, pp. 191–95. *DOI.org (Crossref)*, https://doi.org/10.1111/j.1432-1033.1982.tb07039.x.

Shin, Wisoo, Whitney Alpaugh, Laura J. Hallihan, Sarthak Sinha, Emilie Crowther, Gary R. Martin, Teresa Scheidl-Yee, Xiaoyan Yang, Grace Yoon, Taylor Goldsmith, Nelson D. Berger, Luiz Gn De Almeida, Antoine Dufour, Ina Dobrinski, Michael Weinfeld, Frank R. Jirik, and Jeff Biernaskie. "PNKP Is Required for Maintaining the Integrity of Progenitor Cell Populations in Adult Mice." *Life Science Alliance*, vol. 4, no. 9, Sept. 2021, p. e202000790. *DOI.org (Crossref)*, https://doi.org/10.26508/lsa.202000790.

Tsukada, Kaima, Mikio Shimada, Rikiya Imamura, Kotaro Saikawa, Masamichi Ishiai, and Yoshihisa Matsumoto. "The FHA Domain of PNKP Is Essential for Its Recruitment to DNA Damage Sites and Maintenance of Genome Stability." *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 822, Jan. 2021, p. 111727. *DOI.org (Crossref)*, https://doi.org/10.1016/j.mrfmmm.2020.111727.

Weinfeld, Michael, Rajam S. Mani, Ismail Abdou, R. Daniel Aceytuno, and J. N. Mark Glover. "Tidying up Loose Ends: The Role of Polynucleotide Kinase/Phosphatase in DNA Strand Break Repair." *Trends in Biochemical Sciences*, vol. 36, no. 5, May 2011, pp. 262–71. *DOI.org (Crossref)*, https://doi.org/10.1016/j.tibs.2011.01.006.

Yushchenko, Tetyana, Elke Deuerling, and Karin Hauser. "Insights into the Aggregation Mechanism of PolyQ Proteins with Different Glutamine Repeat Lengths." *Biophysical Journal*, vol. 114, no. 8, Apr. 2018, pp. 1847–57. *DOI.org (Crossref)*, https://doi.org/10.1016/j.bpj.2018.02.037.