PABPN1 and SKIIP: A Putative Mechanism for the Onset of Oculopharyngeal Muscular Dystrophy

Master’s Thesis

Presented to the
Biochemistry Department
Brandeis University

Gregory Petsko and Dagmar Ringe, Advisors

In Partial Fulfillment of the
Requirements for the Degree

Master of Science

By
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May 2011
**Acknowledgements:**

First and foremost, I would like to thank Professors Gregory Petsko and Dagmar Ringe for creating the Petsko-Ringe Lab and giving undergraduates like me the opportunity to conduct research. I have enjoyed my experience working in the Petsko-Ringe lab enormously and after graduation I will remember working long hours on the sixth floor of Rosenstiel with fondness.

I would also like to thank Dr. Paul Hubbard for mentoring me and teaching me how to work in a lab. My experience working with Paul cultivated my interest in research and has led me to pursue a doctorate in graduate school next year. After Paul left the lab in September, he continued helping me from across the Atlantic, telling me when I should move from something and come back to it later as well as by me to revise my thesis.

I would also like to thank the other members of the Petsko-Ringe Lab who have helped me tremendously since I first joined the lab. Everyone has been especially helpful since Paul left by guiding me and showing me how work independently. I have learned an enormous amount and I would not have made it without everyone. I especially want to thank Drs. Shulin Ju and Jacqueline Naffin and for their support, advice, and help with cloning. Jackie also helped me with editing and by urging me to stop lab work and begin writing. I would also like to thank Dr. Heather Brodkin for helping me use the FPLC and HPLC. Vincent Mecozzi helped me perform CD spec, use the FPLC, edit my thesis, as well as for his feedback discussing the project and future research.

I would also like to thank Professor Christopher Miller and Dr. Ming-Feng Tsai of the Miller Lab for allowing me to use their ITC and NanoDrop as well as for their help in designing and executing the experiment. They both also went above and beyond my expectations to suggest other assays that could be used in the future if ITC does not work. I would also like to thank Professor Douglas Theobald for giving me permission to use his refrigerated microcentrifuge while our rotor on ours is repaired.

I would also like to thank all my friends and family for their support and understanding. It is difficult to try to schedule things around lab work and everyone has been very understanding.
Abstract

Oculopharyngeal muscular dystrophy (OPMD) is an autosomal dominant disease that stems from mutations expanding the polyalanine tract at the N-terminus of PABPN1 [1]. This region is a trinucleotide repeat and OPMD has similarities to other trinucleotide repeat disorders like Huntington’s disease. In OPMD, symptoms include the development of intranuclear inclusions (INIs) in myocytes containing PABPN1, Hsp70, Hsp40, poly(A) RNA, ubiquitin, and proteasomes [2]. Some researchers hypothesize that the aggregates cause cellular toxicity that results in muscle degeneration [3].

Previous studies have found that the expanded polyalanine tract increases the propensity of PABPN1 to form aggregates [4]. In vitro fibril formation for synthetic peptides corresponding to the N-terminus of PABPN1 has been detected with a lag-phase that decreases as the length of the N-terminal polyalanine tract increases. There is speculation that this lag phase could be similar to the lag phase experienced by OPMD patients who do not develop symptoms until middle-age [1].

PABPN1 has been found to interact with ski-interacting protein (SKIIP) by a yeast-2-hybrid screen [5]. The binding was confirmed in vitro by immunoprecipitation and in vivo by a colocalization assay with GFP-tagged protein. PABPN1 and SKIIP were found to work together to activate E-box mediated transcription through MyoD [5].

Understanding the mechanism of onset for OPMD may lead to developing treatments and therapies for patients. Also, investigating this degenerative disease may help with research and ultimately treatments of other protein aggregation diseases such as Alzheimer’s disease, ALS, Parkinson’s disease, Huntington’s disease, and others.
The research presented here examines the interaction between PABPN1 and SKIIP with the goal of localizing the region of PABPN1 that interacts with SKIIP and determining how mutations may disrupt this interaction. Mutations expanding the polyalanine tract in PABPN1 may alter the interaction between PABPN1 and SKIIP, disrupting transcription and leading to symptoms of OPMD.
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<tr>
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<td>CNS</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
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<tr>
<td>HPLC</td>
<td>High-pressure liquid chromatography</td>
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<tr>
<td>INIs</td>
<td>Intranuclear inclusions</td>
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<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
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<td>ITC</td>
<td>Isothermal titration calorimetry</td>
</tr>
<tr>
<td>Kan</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>$K_d$</td>
<td>Dissociation constant</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>Ni-NTA</td>
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<td>OPMD</td>
<td>Oculopharyngeal muscular dystrophy</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
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<td>PABPN1</td>
<td>Polyadenylate binding protein nuclear 1</td>
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<tr>
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<td>PAP</td>
<td>Polyadenylate polymerase</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>Peripheral nervous system</td>
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<td>PTMs</td>
<td>Post translational modifications</td>
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<td>Size exclusion chromatography</td>
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<td>SKIIP</td>
<td>Ski-interacting protein</td>
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<tr>
<td>TB</td>
<td>Terrific broth</td>
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Introduction

Oculopharyngeal muscular dystrophy (OPMD) is a rare trinucleotide repeat genetic disease characterized by developing muscle weakness resulting in droopy eyelids (ptosis), difficulty swallowing (dysphagia), and proximal limb weakness, which onsets around age 50 or 60 [1,6]. The occurrence of OPMD is higher in some populations, with a prevalence of 1:1000 for French Canadians and 1:600 for Bukhara Jews living in Israel [6]. Currently no cure exists for OPMD and the available therapies are mainly surgeries to reduce ptosis and dysphagia [6].

The expansion of a (GCN)$_{10}$ repeat, corresponding to a polyalanine tract at the N-terminus of polyadenylate binding protein nuclear 1 (PABPN1), has been identified as the cause of OPMD [1]. PABPN1 is a 32.8 kDa protein that stimulates polyadenylate polymerase (PAP) in the nucleus, in conjunction with cleavage and polyadenylation-specific factor (CPSF) [7]. This sequence of events results in the addition of ~250 adenylate groups to the 3’ end of mRNA, which is necessary for efficient gene expression [7]. Yet it appears that PABPN1 with an expanded polyalanine tract (PABPN1$_{exp}$) has the same control on adenylation length as wild-type PABPN1 (PABPN1$_{wt}$) [2].

PABPN1$_{wt}$ has 10 alanines in the N-terminal domain. People who are homozygous for the (GCN)$_{11}$ form of the allele, with 11 alanines, exhibit autosomal recessive OPMD, whereas individuals with an allele containing (GCN)$_{12-17}$ repeats, for 12-17 alanines, exhibit autosomal dominant OPMD [1]. In the literature, the two
mechanisms proposed for the origin of these mutations are unequal crossing over between two mispaired alleles and polymerase slippage during replication [3]. The former is favored in the literature and has been suggested as the mechanism for other polyalanine expansion diseases [6]. There is evidence of a gene dosage effect because patients homozygous for a dominant OPMD mutation have more severe symptoms than heterozygotes [1]. Furthermore, individuals with longer expansions of PABPN1 tend to have more severe symptoms and an earlier onset of the disease [1]. However, compound heterozygotes for dominant and recessive alleles have more severe symptoms than heterozygotes for dominant and wild-type alleles [6].

Understanding the etiology of OPMD may lead to developing more effective therapies and treatments for patients. Furthermore, studying OPMD may help to draw parallels between other trinucleotide expansion diseases such as Huntington’s disease; as well as neurodegenerative diseases that, like OPMD, result in the accumulation of protein aggregates such as Alzheimer’s disease, ALS, Parkinson’s disease, and others.

For OPMD, these protein aggregates, known as intranuclear inclusions (INIs), occur exclusively in skeletal muscles, and were originally used as a diagnostic marker for the disease [8]. These INIs contain tubular filaments visible using electron microscopy and have been shown to contain various ubiquitinated proteins, including PABPN1, Hsp70, Hsp40, poly(A) RNA, proteasomes, and ski-interacting protein (SKIIP) [2,9].

The molecular mechanism for muscle degeneration in OPMD remains unknown. Some theories suggest that muscle degeneration may be the result of the toxicity of these proteinaceous aggregates caused by PABPN1_{exp} [3]. Other theories contend that the polyalanine tract expansion changes the properties of PABPN1 and the aggregates result
from a toxic gain of function [2]. There is some evidence for these theories as PABPN1$_{\text{wt}}$ has been found to alleviate some of the toxic effects of PABPN1$_{\text{exp}}$, perhaps by serving as an anti-apoptotic factor [10].

Another theory explaining cellular toxicity is that the cellular machinery that recognizes and ubiquitinates misfolded proteins for degradation creates localized high concentrations of misfolded proteins, increasing the likelihood of forming aggregates, thus triggering apoptosis [3]. Other theories state that the expanded polyalanine tract leads to the formation of aggregates that serve as RNA “traps,” preventing certain mRNAs from being transcribed [2]. If this later theory were true, it is possible that this would change the levels of expressed proteins in cells enough to cause cell death, but such a mechanism would be highly dependent on the relative transcription and turnover rates [2], and it fails to explain the significance of ubiquitin and proteasomes that are found within INIs.

Currently, no structure exists for full length PABPN1 in the Protein Data Bank (PDB) and no significant structural differences have been detected with circular dichroism (CD) spectroscopy for PABPN1$_{\text{wt}}$ and PABPN1$_{\text{exp}}$ [4], yet genetic analysis indicates that adding additional alanines to the N-terminus of PABPN1 causes OPMD [1]. CD spectra for N-terminal fragments of PABPN1 show an increase in $\alpha$-helical structure for PABPN1$_{+7\text{Ala}}$ compared to PABPN1$_{\text{wt}}$ and a decrease in secondary structure for PABPN1 lacking the polyalanine tract (PABPN1$_{\Delta\text{Ala}}$) compared to PABPN1$_{\text{wt}}$ [4]. This observation contradicts studies by $^1$H-NMR on N-terminal fragments of PABPN1 showing the N-terminus to be largely unstructured [11]. Computational secondary structure prediction methods agree with the NMR data and predict PABPN1 to be largely
unstructured; however, these computational prediction methods may be biased by high proline and glutamine content at the N- and C-termini [4].

The fibrils that are the hallmark of OPMD have been successfully reproduced \textit{in vitro} with PABPN1\textsuperscript{+7Ala} N-terminal peptides [4,11]. These fibrils formed slowly with a long lag phase that could be shortened by seeding fibril formation [4]. It is plausible that the delay OPMD patients experience before onset of symptoms could be similar to the lag time observed for the \textit{in vitro} formation of fibrils; however, the high concentrations of protein required to observe fibril formation were clearly non-physiological [4]. Seeding fibril formation \textit{in vitro} reduced the lag time dramatically and also lowered the concentration of peptide necessary for fibril formation [4]. This could explain the lag time before symptoms develop in OPMD patients and the rapid progression of the disease once symptoms first appear [4]. Furthermore, a truncated construct corresponding to the N-terminal 125 residues with a 17 alanine tract showed an increased tendency to form fibrils and aggregate during purification [4]. \textit{In vitro} aggregates do not appear to have amyloid-like structure by EM Congo red staining or Fourier transform spectroscopy [4].

The connection between PABPN1\textsubscript{exp} and the onset of OPMD has lead to an investigation into the polyalanine tract function and to how that function changes with length. Although inactive, PABPN1 lacking residues 1-160 (PABPN1\textsubscript{N\Delta160}) has the same affinity to poly(A) as PABPN1\textsubscript{wt} [12]. However, PABPN1 missing residues 1-113 of the N-terminus (PABPN1\textsubscript{N\Delta113}) is fully active, binding to RNA and enhancing adenylation comparable to PABPN1\textsubscript{wt} [13]; indicating that the N-terminus is not required for PABPN1’s function in adenylation. This result also means that the region D114-M160 is required for PABPN1 activity since deleting that region inactivates PABPN1. Just C-
terminal to this is the RNA recognition motif (RRM) that by sequence analysis is composed of residues I173-I244.

Researchers have tried with varying success to create in vivo models for OPMD in both mice and Drosophila. In one experiment, mice expressing human PABPN1+7Ala developed progressive muscle weakness as seen in OPMD patients; however, the INIs that are characteristic of OPMD were missing from muscle cells of the mice [14]. These mice failed to develop the phenotypes of muscle weakness present in people with OPMD; rather, the mice developed muscle weakness due to the presence of neuronal INIs, which have not been found in OPMD patients [14]. The transgenic mice in this study did not show evidence of muscle atrophy, but rather, the observed muscle weakness was due to the presence of INIs in the central nervous system (CNS), which has not been detected in OPMD patients. There have been a few cases where OPMD patients have developed a neurologic component to the disease [15] and other instances suggesting that there may be a peripheral nervous system (PNS) component [16]. Yet, the neurocellular INIs observed in this murine models contained ubiquitinated PABPN1+7Ala [14], suggesting that there may be an unknown neurological component to OPMD that has only been documented in a few patients [15,16]

Other researchers have had more success creating an OPMD mouse model. One group mated over mice over expressing B-cell CLL/lymphoma (BCL2) with mice expressing human PABPN1+7Ala under the myogen regulatory 4 promoter, the native gene promoter [17]. This group found that up-regulating BLC2 reduced apoptosis; however, it did not alleviate the symptoms of OPMD entirely. BLC2 is an anti-apoptotic factor and this research makes a connection between apoptosis and muscle weakness in OPMD. In
muscular dystrophy, the relationship between apoptosis and muscle weakness is unclear. In some forms of muscular dystrophy, apoptosis is thought to cause myopathy, but in OPMD the connection between apoptosis and myopathy is unclear [17].

Transgenic *Drosophila* expressing PABPN1<sup>+</sup>Ala/<sup>Δ</sup>K165-Y272 that lack the RRM do not develop muscle weakness [18]; however, PABPN1<sup>Δ</sup>K165-Y272 is inactive as M161-T257 corresponds to the RRM [12]. Thus it is unsurprising that *Drosophila* expressing PABPN1<sup>+</sup>Ala that lacks the RRM do not develop symptoms of OPMD, as PABPN1 was inactive [18]. It appears that the RRM is required for developing OPMD symptoms even though mutations in the N-terminus are responsible for its onset. This indicates that the polyalanine expansion alone is not enough to cause OPMD and suggests that the alanine expansion may disrupt something in a cellular process that does not occur when PABPN1 lacks the RRM. As PABPN1 is involved in the adenylation of mRNA, it is possible that PABPN1<sub>exp</sub> could disrupt something when bound to RNA so that PABPN1<sub>exp</sub><sup>Δ</sup>K161-T257 does not cause symptoms of OPMD because it is not localized to the correct place in the cell.

In 2001, Kim et al. performed a yeast-2-hybrid screen with PABPN1 to determine possible interaction partners that may help clarify the physiological basis of OPMD [5]. They found that an N-terminal fragment of PABPN1, corresponding to amino acids 1-145, bound to a 62kDa protein called ski-interacting protein (SKIIP) [5]. A GST-pull-down assay confirmed that SKIIP bound specifically to full length PABPN1 and to the N-terminal domain, not to the C-terminal domain containing the RRM was responsible for this interaction [5]. They also found that fluorescently labeled PABPN1 and SKIIP were colocalized in the nucleus of HeLa cells [5]. This interaction is intriguing as SKIIP is a
transcription factor and is involved in the Notch1 pathway [19]. Kim et al. found that PABPN1 accumulated in nuclei during myogenic differentiation, suggesting that myocyte gene expression requires the upregulation of PABPN1 during myogenesis [5]. Recently, knockdown of PABPN1 has been found to decrease the rate of myoblast differentiation and cell proliferation during myogenesis \textit{in vitro} [20]. Furthermore, PABPN1 and SKIIP were found to work together to activate E-box mediated transcription through myogenic differentiation 1 (MyoD) [5]. It is possible that the interaction between soluble PABPN1\textsubscript{wt} and PABPN1\textsubscript{exp} may change the expression levels of PABPN1\textsubscript{wt} and its function in the cell [21].

We hypothesize that the onset of OPMD comes from PABPN1\textsubscript{exp} disrupting the interaction between PABPN1\textsubscript{wt} and SKIIP and causing aggregates to form. If this is true, then there would be a different binding affinity for PABPN1\textsubscript{exp} and SKIIP than PABPN1\textsubscript{wt} and SKIIP. To test this hypothesis, genes coding for human PABPN1 and human SKIIP were cloned into expression vectors, which were used for protein growth in \textit{E. coli}. These proteins were used in conjugation with a synthetic peptide corresponding to the N-terminus of PABPN1 to look at binding affinities. I performed denaturation based fluorescence assays using ThermoFluor technique to measure the change in stability for the PABPN1-SKIIP complex. The assay uses a dye that fluoresces as it binds hydrophobic regions of proteins that are exposed to solvent during thermally induced denaturation [22]. This allows one to plot the normalized relative fluorescence and to calculate a Tm for proteins with and without putative substances that stabilize the protein fold; and hence alter the Tm of the protein. The research presented here shows how the ThermoFluor technique was used to look at binding between PABPN1 and
SKIIP. If PABPN1 binds SKIIP one would expect an increase in Tm due to the energy associated with their interaction. Synthetic peptides corresponding to the N-terminus of PABPN1 were used in an attempt to localize the region of PABPN1 that binds SKIIP. The interactions between PABPN1+1Ala and SKIIP and PABPN1wt and SKIIP differ, suggesting that the N-terminus of PABPN1 is involved in mitigating their interaction.
Materials and Methods

Table 1

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<td><strong>E. coli strains</strong></td>
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<td>BL21(DE3)</td>
<td><em>E. coli</em> B F&lt;sup&gt;+&lt;/sup&gt; dcm ompT hsdS(rB–mB–) gal λ(DE3)</td>
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<tr>
<td>Rosetta</td>
<td>F&lt;sup&gt;–&lt;/sup&gt; ompT hsdSB(rB–mB–) gal dcm pRARE2 (CamR)</td>
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<tr>
<td>Top10</td>
<td>F&lt;sup&gt;–&lt;/sup&gt; mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(araleu) 7697 galU galK rpsL (StrR) endA1 nupG</td>
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<td>XL-10Gold</td>
<td>Tetra (mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F&lt;sup&gt;+&lt;/sup&gt; proAB lacIqZΔM15 Tn10 (Tetr) Amy Camr]</td>
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<td>pET-SUMO</td>
<td>A protein expression vector using the lac operator that expresses protein with an N-terminal ubiquitin like cleavable tag containing a 6xHis tag containing Kan resistance</td>
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<tr>
<th>Peptide name</th>
<th>Sequence (N-terminal to C-terminal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide1</td>
<td>MAAAAAAAAAAAGAAGGR</td>
</tr>
</tbody>
</table>
All bacterial strains (Table 1) were grown in Terrific Broth (TB; recipe in Appendix I) with appropriate antibiotics. Antibiotics were used at working concentrations of 50µg/mL kanamycin (Kan) and 100µg/mL chloramphenicol (Cam). Antibiotics were taken from stocks of 100mg/mL Cam in 95% ethanol and 50mg/mL Kan that were stored at -20°C. All primers were synthesized by Eurofins Operon (Table 3). All restriction enzymes, buffers for digestions, and BSA used were purchased from NEB unless otherwise noted. Extinction coefficients were calculated by ProtParam.
Cloning

PABPN1

A gene of PABPN1 that was codon optimized for protein expression in *E. coli* was obtained from Dr. Paul Hubbard. This gene was ligated into pET-SUMO vector (Invitrogen) using the protocol from the manufacturer; pET-SUMO was chosen as it introduces an N-terminal 6xHis affinity tag that can be completely removed using SUMO-protease to produce a protein that has no extraneous amino acids adjacent to the polyalanine tract. This form of the protein closely resembles the mature form of the endogenously expressed protein, lacking acetylated and phosphorylated post-translational modifications (PTMs). The ligation product was then transformed into Mach1 T1 competent cells (Invitrogen) and plated onto LB/Kan selective media. DNA was purified from overnight cultures and sequenced to confirm the presence and frame of the PABPN1 gene. An additional stop codon was inserted using Quik Change XL II kit (Agilent) with primers PABPN1doubleXF and PABPN1doubleXR. An N-terminal Met was removed from the construct using Quik Change XL II kit with Met-SUMOPABPN1F and Met-SUMOPABPN1R.

PABPN1 mutants

The PABPN1 construct was mutated using Quik Change XL II kit (Agilent) with primers PABPN1-Ins1xGCA-F and PABPN1-Ins1xGCA-R to expand the polyalanine tract at the N-terminus from 10 to 11 alanines. The PCR mixture was prepared and performed following the manufacturers protocol. The PCR products were digested with *DpnI* for 1hr at 37°C and transformed into Top10 competent cells (Invitrogen). This
procedure also produced a mutant that sequencing identified as having 12 at the N-terminus instead of 11 as was expected from the primers.

The PABPN1 construct was also mutated to insert 4 alanines into the polyalanine tract at the N-terminus of the protein. This was done by performing PCR with the standard T7 promoter primer and PABPN1-Ins4xGCA-R and another PCR with the standard T7 terminator primer and PABPN1-Ins4xGCA-F. The PCR was conducted with 10x reaction buffer (5.0µL), template (0.5µL), each primer at 100µM (0.2µL each), 10mM dNTPs (1µL), DMSO (3µL), Pfu Turbo (1µL) and ddH₂O to a final reaction volume of 50.0µL. The reaction was performed with an initial denaturing step at 95°C for 2 minutes and then cycled with a 30 sec denaturing step at 95°C, 30 sec annealing step at 55°C, and a 2 minute polishing step at 72°C. After 30 cycles, a final 7 minute extension step at 72°C was performed and the reaction mixture stored at 4°C until further use. The PCR products (1µL of each) were then used in another round of PCR to amplify the 14xAla PABPN1 mutant using the standard T7 Promoter and standard T7 Terminator primers with the same cycling conditions as above. The PCR product was confirmed to be the correct size by electrophoresis on a 1% agarose gel. DNA was extracted from the gel using a DNA Gel Extraction Kit (Zymo Research). For ligation into amplifying vector pCR2.1 (Invitrogen), 7.9µL of the gel extracted DNA was incubated with 1.0µL Taq in 0.1µL 100mM dATP with 0.1µL of reaction buffer at 72°C for 20 minutes. The ligation step was performed following the protocol from the manufacturer. The pCR2.1 ligation product was transformed into Mach 1 T1 competent cells (Invitrogen) and grown overnight at 37°C on an LB/Kan selective media. A colony was isolated and grown in 2mL TB with 2µL Kan overnight at 37°C. The following day plasmid was extracted
from the overnight culture using a QIAprep Spin Miniprep Kit (Qiagen). Mutations were confirmed by sequencing using the standard SUMOF primer. A double digest was performed on the PABPN1-4xGCA mutant in pCR2.1 using *HindIII* and *XbaI*. The miniprep plasmid (33.6µL), 10x buffer #2 (4.0µL), BSA (0.4µL), 1.0µL *HindIII*, and 1.0µL of *XbaI* were combined and incubated for 2.5hr at 37°C. Digestion was confirmed by electrophoresis on a 1% agarose gel and the band corresponding to the mutant PABPN1 gene was extracted using a DNA Gel Extraction Kit (Zymo Research).

The mutant open reading frame (ORF) from pCR2.1 was then ligated into an expression vector, pET-SUMO, prepared by digesting a previous construct plasmid, pET-SUMO-PABPN1, with *HindIII* and *XbaI* as described above. The ligation reaction was carried out using T4 DNA ligase; ligase buffer (2µL), the 14xAla-PABPN1 ORF (4µL), cut pET-SUMO plasmid (1µL), T4 DNA ligase (1µL), and ddH2O (12µL) were combined to a total volume of 20µL. The reaction was incubated overnight at 16°C and transformed into Mach1 T1 competent cells (Invitrogen) and plated onto LB/Kan selective media. The sequence and frame of the mutant gene in the pET-SUMO vector was confirmed by sequencing. Constructs for pET-SUMO-PABPN1+7Ala and pET-SUMO-PABPN1ΔNterm in pCR2.1 were made following the same procedure as above using the appropriate primers.

**SKIIP**

Genomic DNA for human SKIIP was obtained from Dr. Paul Hubbard. The DNA was PCR amplified using SKIIP-R and SKIIP-R primers. The PCR was performed using 10x reaction buffer (5.0µL), template (0.5µL), 100µM primers (0.2µL each), 10mM dNTPs (1µL), and ddH2O (42.1µL). *Pfu*-HotStart polymerase (Agilent) (1µL) was added
to the mixture. PCR was performed with an initial denaturation step of 95°C for 2 minutes and then cycled with a 30 sec denaturing step at 95°C, 30 sec annealing at 55°C, and a 2 minute polishing step at 72°C. After 30 cycles, a final 7 minute polishing step at 72°C was performed and the reaction mixture was stored at 4°C. The PCR product was analyzed by electrophoresis on a 1% agarose gel and the band corresponding to the SKIIP gene was extracted using a DNA Gel Extraction Kit (Zymo Research).

Terminal adenylate groups were added to the 3’ ends of the PCR product by incubating the gel extracted ORF fragment with Taq (NEB) as described above. The product was ligated into the pET-SUMO vector (Invitrogen) following the directions from the manufacturer. The ligation product was transformed into Mach 1 T1 competent cells (Invitrogen) and plated onto LB/Kan selective media. An overnight culture (2mL) with Kan was inoculated with a single colony and grown at 37°C. DNA was extracted using the QIAprep Spin Miniprep Kit (Qiagen). The correct frame and sequence of SKIIP in the pET-SUMO vector was confirmed by sequencing.

The clone for pET24a-SKIIP that introduces a C-terminal 6xHis tag was obtained from Dr. Paul Hubbard. Sequencing was used to confirm the correct sequence and reading frame for the protein.

**Protein expression and purification**

**PABPN1**

The pET-SUMO-PABPN1 construct was transformed into BL21(DE3) competent cells (Invitrogen) and expression tests were conducted to confirm that the protein expressed. Expression was confirmed by western blot (data not shown).
For large scale expression, a colony was picked from a freshly transformed BL21(DE3) plate and used in a 6mL starter culture of TB with 6μL Kan grown overnight at 37°C. Then 5mL of the starter culture was inoculated into 1L of TB and grown at 37°C for 3.5 hours. The shaker was then cooled to 15°C for 30 minutes prior to induction with IPTG at a final concentration of 10μM, a concentration necessary to obviate the toxic effect of this protein in *E. coli* and consistent with the literature for expressing toxic proteins [23]. Cultures were grown for 16 hours at 15°C to enhance solubility of the protein. Cells were pelleted by centrifugation at 6000 rpm (Sorvall; F10 rotor) and frozen at -20°C for 1 hour. Cells were then stored at -80°C until further use.

Cell pellets were resuspended in 150mL of Ni-NTA lysis buffer at 4°C (see QIAexpressionist for details), supplemented with 600mM NaCl and 20mM imidazole. Resuspended cells were then sonicated on ice at 60% power with a pulse of 2 seconds on 1 second off for 4 minutes (Fisher Scientific; Sonic Dismembrator 500). This was followed by allowing the cells to chill on ice for 4 minutes. After this 8 minute cycle was repeated 3 times, the lysate was centrifuged at 10,000rpm for 1 hour (Sorvall; SS-34 rotor). The supernatant was poured over 5mL Ni-NTA resin (Qiagen) pre-equilibrated in a gravity flow column. The column was washed with 50mL of Ni-NTA wash buffer (see QIAexpressionist for details), supplemented with 600mM NaCl and 50mM imidazole. Partially purified protein was eluted from the column using a 10mL of Ni-NTA elution buffer (see QIAexpressionist for details), supplemented 600mM NaCl. Dry ammonium sulfate crystals (Fisher) were added slowly to the elution to make a 35% saturated solution, which was rocked at 4°C for 1 hour. The solution was then centrifuged (Beckman; GH-3.8 rotor) for 20 minutes at 3500 rpm using a tabletop centrifuge at 4°C.
The supernatant was decanted and the pellet centrifuged (Beckman; GH-3.8 rotor) for an additional 10 minutes at 3500rpm. The supernatant was again decanted and the pellet resuspended in 0.5mL of 100mM Tris pH 8.0 by rocking at 4°C for 1 hour. The protein was then dialyzed overnight in a 0.5mL cassette (Thermo Scientific) into a solution of 500mL of 100mM Tris pH 8.0. The buffer was exchanged to ensure complete removal of ammonium sulfate. The protein solution was removed from dialysis and Beer’s Law was used to calculate the concentration of protein by measuring $A_{280}$ with an extinction coefficient of $\varepsilon = 22000\,\text{M}^{-1}\text{cm}^{-1}$. Then 10% glycerol was then added and the protein was frozen in liquid nitrogen for long-term storage at -80°C.

The SUMO-tag was cleaved by adding 0.5µL SUMO protease (Invitrogen) to the reaction and rocking overnight at 4°C. This step was incorporated to the overnight dialysis step by adding 0.5µL of the protease to the protein mixture. The protease and SUMO-tag were removed by FPLC with a by Sephacryl-200 HiRes column (GE Healthcare). The fractions (1mL) were collected with a flow rate of 0.2mL/min in 100mM Tris pH 8.0 and 150mM NaCl. The fractions corresponding to pure untagged PABPN1 were determined by SDS-PAGE analysis, combined, and then concentrated. The final protein product was concentrated using an Amicon Ultra-4 Centrifugal Filter Unit and an Amicon Ultra-0.5 Centrifugal Filter Unit with Ultracel-10 membrane. The protein concentration was calculated using Beer’s Law as before with an extinction coefficient of $\varepsilon = 20500\,\text{M}^{-1}\text{cm}^{-1}$.

**SKIIP**

As the pET24a-SKIIP construct was not codon optimized for expression in *E. coli*, it was transformed into Rosetta competent cells (Invitrogen), plated onto
LB/KanCam selective media, and incubated overnight at 37°C. A 6mL starter culture of TB with 6µL Kan and 6µL Cam was inoculated with a single colony from the plate and grown overnight at 37°C. The following day 5mL of the starter culture was used to inoculate a 1L culture of TB with 1mL Kan and 1mL Cam. Cultures were grown for 3.5 hours at 37°C and then the temperature of the shaker was adjusted to 15°C to reduce aggregation of the expressed protein. Expression was induced by adding IPTG to a final concentration of 1mM 4 hours after inoculation. The cultures were grown overnight for 16 hours before the cells were pelleted by centrifugation at 6000 rpm (Sorvall; F10 rotor) at 4°C. Cell pellets were frozen at -20°C for one hour and stored at -80°C until they were used.

pET24a-SKIIP was purified by resuspending the cell pellet in 150mL of Ni-NTA lysis buffer (see QIAexpressionist for details). The cells were sonicated (Fisher Scientific; Sonic Dismembrator 500) on ice at 60% power with a pulse of 2 seconds on and 1 second off for 4 minutes. The cells were allowed to rest on ice for 4 minutes. This 8 minute sequence was repeated 3 times. The lysate was then centrifuged at 10,000 rpm (Sorvall; SS-34 rotor) for 1 hour. The supernatant was poured over 5mL Ni-NTA resin (Qiagen) pre-equilibrated in a gravity flow column. The column was washed with 50mL Ni-NTA wash buffer (see QIAexpressionist for details), supplemented with 35mM imidazole. The partially purified protein was eluted from the column with 10mL of standard Ni-NTA elution buffer (see QIAexpressionist for details). Dry ammonium sulfate crystals (Fisher) were added slowly to make a 30% saturated solution. The solution was rocked at 4°C for 1 hour and centrifuged at 3500rpm (Beckman; GH-3.8 rotor) for 20 minutes at 4°C. The supernatant was decanted and the pellet centrifuged.
(Beckman; GH-3.8 rotor) for an additional 10 minutes at 3500rpm. The supernatant was again decanted and the pellet was resuspended in 350µL of 100mM HEPES pH 7.5 and rocked at 4°C for 1 hour. The sample was loaded into a 0.5mL cassette (Thermo Scientific) and dialyzed overnight at 4°C into 500mL of 100mM HEPES pH 7.5 with 150mM NaCl. The buffer was exchanged to ensure complete removal of ammonium sulfate. The protein concentration was calculated by measuring the $A_{280}$ and using Beer’s Law with an extinction coefficient $\varepsilon = 45840M^{-1}cm^{-1}$. Then 10% glycerol was added and the protein was snap frozen in liquid nitrogen for long-term storage at -80°C. SKIIP was thawed on ice and concentrated using Amicon Ultra-0.5 Centrifugal Filter Unit with Ultracel-10 membrane.

SUMO-SKIIP was purified following the same protocol as pET24a-SKIIP except that the Ni-NTA wash buffer for SUMO-SKIIP was supplemented with 20mM imidazole instead of 35mM imidazole as for pET24a-SKIIP. Also, the extinction coefficient that used for SUMO-SKIIP was $\varepsilon = 45840M^{-1}cm^{-1}$.

**Synthetic peptide**

A synthetic peptide corresponding to the N-terminal amino acids of PABPN1 (Table 2) was purchased from GenScript with a purity of 78.1%.

The peptide was dissolved in HPLC grade acetonitrile with 0.1% TFA and diluted with water and DMSO until soluble. The peptide was loaded onto the HPLC (Agilent) and run with a similar protocol to the one conducted by GenScript to confirm the peptide purity. The peptide was injected and loaded onto a reverse phase C-18 column 4.6mm x 150mm (Zorbax) to try to increase the purity.
**Secondary structure characterization**

CD spectra were collected using a Jasco J-810-150S instrument. PABPN1 (1mg/mL) was used in 1x PBS pH 7.4 (Fisher). A scan of the buffer without protein was subtracted from the protein signal. The mean ellipticity per residue ($\theta$) was calculated using the formula

$$\theta = \frac{100 \text{(deg)}}{c \cdot \ell \cdot n}$$

where (deg) is the ellipticity measured in degrees, $c$ is the molar concentration of the sample, $\ell$ is the path length measured in cm, and $n$ is the number of amino acids in the sample sequence. The CD spectrum of the synthetic peptide was measured at 0.5mg/mL in 90mM HEPES pH 7.5 with 10% glycerol.

**Binding Assays**

Binding assays were conducted using the ThermoFluor technique, a denaturation fluorescence assay with SYPRO Orange Protein Stain (Invitrogen). The optimal dye concentration for signal was determined by an empirical approach testing various concentrations. Dye was diluted from a 5,000x stock using the same buffer of the well solution. ThermoFluor assays were conducted in HEPES (Fisher) pH 7.5 with and without NaCl (Fisher). Protein samples were buffer exchanged and reactions were prepared to a final volume of 20µL in MicroAmp Fast Optical 96-Well Reaction Plates (Applied Biosystems). The reaction wells were mixed and sealed with MicroAmp Optical Adhesion Film (Applied Biosystems) and conducted using a StepOnePlus RT-PCR machine (Applied Biosystems) with a temperature gradient from 25°C to 95°C that increased by increments of 0.3°C per minute.
Multiple concentrations of PABPN1 were used in each assay to show a shift in Tm as the concentration of PABPN1 increased so as to suggest binding to SKIIP. PABPN1 was used at a concentration of 1.5µM, 15.3µM, and 26.8µM (Figure 9). SKIIP was used at a concentration of 1.17µM. The assay was performed in 90mM HEPES pH 7.5 with 135mM NaCl and 10% glycerol. All experiments were conducted in either duplicate or triplicate and data were averaged and analyzed using StepOne Software v2.0 (Applied Biosystems) in conjunction with a Pearl script written by Dr. Melissa Landon and a macro for Microsoft Excel written by Dr. Paul Hubbard.

The binding between PABPN1+1Ala and SKIIP was assayed in 100mM HEPES pH 7.5 with 150mM NaCl (Figure 10). The assay was conducted as above except that PABPN1+1Ala was added at a final concentration of 1µM, 10µM, and 18.2µM. SKIIP was used at a final concentration of 0.78µM. The settings used for the RT-PCR machine as well as the way the data were analyzed were the same as discussed previously.

The binding of the synthetic peptide to control proteins was measured with lysozyme, glucose oxidase, catalase, alcohol dehydrogenase, trypsin, cytochrome c, myoglobin, hexokinase, and insulin were conducted with 1µL of protein at 10mg/mL in 90mM HEPES pH 7.5 with 10% glycerol to a well solution with a final volume of 20µL. The synthetic peptide (10µL) was added from a stock to a concentration of 1mg/mL to a concentration in the well of 373µM. The RT-PCR settings were the same as above. The assays were conducted in at least duplicate and the fluorescence was averaged before scaling. The control proteins were purchased from Sigma and the source organism for each protein is given in Appendix III.
ITC was conducted using a Nano Isothermal Titration Calorimeter (TA Instruments) to examine binding between PABPN1 and pET24a-SKIIP. The experiment was designed with pET24a-SKIIP in the cell at a concentration of 9.16µM and 1.0µL injections of PABPN1 at 133µM at 25°C. A mixing time of 3 minutes was allowed between injections. Prior to conducting ITC, PABPN1 and SKIIP were dialyzed overnight at 4°C in the same beaker of 100mM HEPES (Fisher) pH 7.5 with 150mM NaCl (Fisher). The proteins were dialyzed using 10,000 MW dialysis cassettes for 0.5mL samples (Thermo Scientific).
Results

Protein overexpression and purification

PABPN1

The purity of SUMO-tagged PABPN1 purified using gravity flow affinity chromatography with Ni-NTA resin and ammonium sulfate precipitation was analyzed by SDS-PAGE (Figure 1). Lane 1 is the protein after eluting from the Ni-NTA column. Lane 2 and 3 are after the ammonium sulfate precipitation step. Although SUMO-PABPN1 is 45kDa, PABPN1 runs anomalously high on SDS-PAGE [24]. The SUMO-tag was then cleaved during dialysis with protease at 4°C. Cleavage was confirmed by SDS-PAGE analysis comparing uncleaved SUMO-PABPN1 and cleaved PABPN1 (Figure 2). Cleaved PABPN1 was then separated from the SUMO-tag by FPLC. The purity of protein in peaks on the UV spectra (Figure 3) was analyzed by SDS-PAGE (Figure 4). The fractions that were determined to be pure were combined and concentrated.

SKIIP

The purity of SUMO-SKIIP was determined by SDS-PAGE following purification (Figure 5A). Cleaving the SUMO-tag from SUMO-SKIIP was conducted at many conditions without success (Figure 5A; other data not shown). A tandem cleavage with SUMO-SKIIP and SUMO-PABPN1 was performed as a control (Figure 5B), which shows the SUMO-protease is unable to cleave the SUMO-tag from SKIIP, but it
successfully cleaves the tag from SUMO-PABPN1. The pET24a-SKIIP construct was then used to express SKIIP because the C-terminal 6xHis-tag is more similar to native protein than an uncleavable 13kDa SUMO-tag. The purity of this new construct expressing SKIIP was determined by SDS-PAGE (Figure 6).

**Synthetic peptide**

Unfortunately, the many consecutive alanines in PABPN1 sequence made synthesis of an N-terminal peptide especially difficult and GenScript could only make a peptide of 78.1% purity, as determined by HPLC. This is the peptide that was used for all of the assays. An attempt to further purify the peptide was made so as to rule out the possibility that contaminants in the peptide were having a significant effect on the experiments; however, it was unsuccessful. The HPLC scan was unlike the scan obtained with GenScript. GenScript technical support attributed this disparity to the peptide being dissolved in the wrong solvent. Another peptide was purchased, but it could not be purified in time.

**Secondary structure characterization**

CD spectroscopy was used to show that PABPN1 is folded (Figure 7). The sample was found to be partly unstructured, agreeing with reference 10, but the spectrum looks as if the protein adopts some sort of secondary structure. CD spectroscopy was also conducted on the synthetic PABPN1 peptide that suggests that it also may have a secondary structure (Figure 8). The molar-ellipticity per residue is negative for α-helices at 222nm and 208nm, whereas it is positive at 190nm. Peptides and proteins that are mostly composed of β-sheets have negative peaks for the molar-ellipticity per residue at 218nm and positive value at 196nm. PABPN1 has a negative peak at 222nm, but the
spectrum was unreadable under 205nm (Figure 7). The synthetic peptide had a defined peak at 218nm suggesting that it has a secondary structure (Figure 8).

**Binding assays**

PABPN1 was titrated into 3µM SKIIP to final concentrations listed in Figure 9, and the fluorescence was measured by ThermoFluor while a temperature gradient denatured the proteins (Figure 9). The thermal denaturing signal for 1.5µM PABPN1 is typical for protein aggregates, but is an artifact of the low signal and a result of the averaging and normalization process. This is shown because the signal for 1.5µM PABPN1 and 1.17µM SKIIP is similar to the signal for 1.17µM SKIIP without PABPN1 indicating that SKIIP dominates the signal. There is an observable increase in the Tm for 1.17µM SKIIP with 26.8µM PABPN1 compared to the Tm for each 1.17µM SKIIP and 26.8µM PABPN1 separately.

PABPN1_{+1Ala} was also assayed with SKIIP at 0.78µM (Figure 10). This graph shows a slight decrease in the Tm for SKIIP with PABPN1_{+1Ala} compared to the Tm of SKIIP alone. The graph shows that PABPN1_{+1Ala} was more prone to aggregate compared to PABPN1_{wt} which is consistent with the literature. At higher concentrations of PABPN1_{+1Ala} the Tm appears to increase. This may be an artifact of the system as the PABPN1_{+1Ala} concentration is significantly higher than the concentration of SKIIP.

ThermoFluor was used to study the binding between the synthetic peptide corresponding to the first 16 amino acids of PABPN1 and SUMO-SKIIP. The peptide was used at a concentration of 676µM and SUMO-SKIIP was at a concentration of 2.67µM (Figure 11). The signal for the peptide and peptide with SUMO-SKIIP show
signs of aggregation, but the Tm increases indicating a stabilization effect for SUMO-SKIIP in the presence of the PABPN1 peptide.

Titrating the synthetic peptide into SUMO-SKIIP at 2.67µM shows a small increase in Tm for the complex over SUMO-SKIIP without the peptide (Figure 12). As the concentration of the peptide increases, it starts to dominate the signal which gives the curves for high concentrations of peptide with SUMO-SKIIP the appearance of the peptide only curve (Figure 11). This is from the peptide aggregating and changing the fluorescence.

As a control for SUMO-SKIIP, the same assay was conducted titrating the synthetic peptide into lysozyme at 14µM. The ThermoFluor assay shows that the synthetic peptide destabilizes lysozyme by lowering the Tm (Figure 13 and 14).

As additional controls, ThermoFluor assays were conducted with the synthetic peptide and various standard proteins. The synthetic peptide was shown to destabilize some proteins such as alcohol dehydrogenase, trypsin, catalase, hexokinase, insulin, cytochrome C, and myoglobin (Figures 15-18, 20-22). The assays also showed that the peptide appeared to have a destabilization affect on all proteins it was assayed with except for glucose oxidase (Figure 19) and SUMO-SKIIP (Figure 12) which appeared to have a stabilization effect.

**Calorimetry**

A preliminary ITC experiment did not give meaningful results (Figure 23). The titration curve does not show evidence of binding because the signal to noise ratio was too low. It is possible that the peaks are from heats of dilution rather than the heat of
binding between SKIIP and PABPN1. Future ITC experiments should use injections of higher concentrations of protein as well as a higher concentration of protein in the cell.
Figure 1: SDS-PAGE analysis of PABPN1 purification. Lane 1 is PABPN1 after eluting from the Ni-affinity column. Lane 2 and 3 are different amounts of protein after resuspending the pellet from the ammonium sulfate precipitation step.

Figure 2: SDS-PAGE analysis of SUMO-PABPN1 and cleaved PABPN1. Lane 1 and 2 correspond to PABPN1 before and after incubating with SUMO protease. The change in molecular weight is visible for SUMO-PABPN1 to PABPN1 and the SUMO-tag.
Figure 3

Figure 3: The trace from the FPLC of PABPN1$_{+1\text{Ala}}$ being separated from the SUMO-tag. This was done in 100mM Tris pH 8.0 with 150mM NaCl with a flow rate of 0.2mL/min.

Figure 4

Figure 4: (A): SDS-PAGE showing Lane 1 showing PABPN1$_{+1\text{Ala}}$ and the SUMO-tag before running the sample over the S200. (B): SDS-PAGE visualizing the separation on the FPLC S200 column of PABPN1$_{+1\text{Ala}}$ from the SUMO-tag shown in (A). Lane 1 is from C12, lane 2 is from D2, lane 3 is from D6, lane 4 is from D12, and lane 6 is from E5. The fractions E2 to E9 were pooled and concentrated. The separation by the column was poor but D12 and E5 were pure.
Figure 5: (A) Lane 1 visualizes the purity of SUMO-SKIIP after purification and lane 2 after incubation with the SUMO-protease showing no cleavage. (B) Lane 1 and 2 are a control showing SUMO-PABPN1 pre and post incubation with the SUMO-protease respectively. Lane 3 and 4 show pre and post incubation with the SUMO-protease for a mixture of SUMO-PABPN1 and SUMO-SKIIP respectively, illustrating that SUMO-tag is cleaved from PABPN1 and left on SUMO-SKIIP.

Figure 6: SDS-PAGE analysis of the purity of pET24a-SKIIP after purification. Lane 1 and Lane 2 show different concentrations pET24a-SKIIP on the gel.
Figure 7

Figure 7: This spectrum shows the mean molar ellipticity of PABPN1 at 1mg/mL. This spectrum suggests that PABPN1 is of predominately helical and unstructured tertiary structure.

Figure 8

Figure 8: The mean molar ellipticity of the synthetic peptide in 90mM HEPES pH 7.5 with 10% glycerol. This spectrum shows that the peptide has a tertiary structure, but the ellipticity could not be collected at short enough wavelengths to give insight into whether it is made of predominately beta-sheets or alpha-helices.
Figure 9: The fluorescence was measured, averaged, and then scaled to get the relative fluorescence. As PABPN1 is titrated into pET24a-SKIIP the fluorescence increases showing a shift in Tm for PABPN1 and pET24a-SKIIP when together over the individual curve for each. The curve for 1.5µM PABPN1 looks like a curve for an aggregated protein because of low signal.

Figure 10: Titrating PABPN1_+1Ala into pET24a-SKIIP showed that PABPN1_+1Ala was more prone to aggregation than PABPN1_wt. There is a slight decrease in the Tm of SKIIP with 1µM PABPN1_+1Ala, but this affect disappears as the concentration of PABPN1_+1Ala increases.
Figure 11: Adding peptide to SUMO-SKIIP shows an increase in Tm for the complex compared to the individual compounds.

Figure 12: Titrating the synthetic peptide into 2.67µM SUMO-SKIIP shows a small increase in Tm at 37µM peptide. At higher concentrations the peptide shows signs of aggregating.
Figure 13: A small amount of peptide was found to stabilize lysozyme by an observable shift in the Tm. However, as more of the peptide was added it appeared to destabilize lysozyme.

Figure 14: Adding the peptide to lysozyme lowers the Tm compared to lysozyme alone. This suggests that the peptide destabilizes lysozyme.
Figure 15: Comparing the affect of the synthetic peptide on the Tm of alcohol dehydrogenase

Figure 16: Comparing the affect of the synthetic peptide on the Tm of trypsin
Figure 17: Comparing the affect of the synthetic peptide on the Tm of catalase.

Figure 18: Comparing the affect of the synthetic peptide on the Tm of catalase.
Figure 19

Figure 19: Comparing the affect of the synthetic peptide on the Tm of glucose oxidase.

Figure 20

Figure 20: Comparing the affect of the synthetic peptide on the Tm of hexokinase.
Figure 21: Comparing the affect of the synthetic peptide on the Tm of insulin.

Figure 22: Comparing the affect of the synthetic peptide on the Tm of myoglobin.
Figure 23: A preliminary ITC experiment with 9.1µM pET24a-SKIIP in the cell with 1.0µL injections of 133µM PABPN1 was inconclusive. The signal to noise ratio was too small to determine whether the signal was from PABPN1 binding to SKIIP or heat of dilution.
**Discussion**

The N-terminal domain of PABPN1 (1-145) has been found to interact with SKIIP [5]. This interaction serves as the foundation for the hypothesis that the expanded polyalanine tract disrupts the interaction between PABPN1\textsubscript{exp} and SKIIP and causes onset of OPMD. Since PABPN1 and SKIIP have been found to interact, it is not surprising that the ThermoFluor show an increase in Tm for the PABPN1-SKIIP complex compared to each separately (Figure 4).

**Secondary structure analysis**

CD spectroscopy was used to show that PABPN1 was indeed folded (Figure 7) in lieu of performing the adenylation assay with RNA and PAP (protocol in Appendix II). Furthermore CD spectrum shows that the synthetic peptide adopts some secondary structure although it was unclear whether it was made of predominately \(\alpha\)-helices or \(\beta\)-sheets (Figure 8). Unfortunately, the CD spectrum was obtained using 90mM HEPES as a buffer, which had too much background so that the scan could not reach a wavelength that was low enough to determine the secondary structure of the peptide. Future CD spectra will utilize 1xPBS instead which has a lower background than 90mM HEPES.

**Binding Assays**

The ThermoFluor assays show that as PABPN1 is titrated into SKIIP there is an observable shift in the Tm (Figure 9). The green dotted line and the blue solid line are for SKIIP alone and SKIIP with a small amount of PABPN1 respectively. The Tm then
increases by 2-3°C as the ratio of PABPN1 to SKIIP increases from 1.3:1 to 22:1. This increase of 2-3°C is a significant change with ThermoFluor which is very sensitive and highly repeatable. There is a dip in the signal from 1.17µM SKIIP with 26.8µM PABPN1 because the fluorescence is the sum of two curves and the signal at that point is dominated by PABPN1.

The synthetic peptide was used to try to determine whether the N-terminus of PABPN1 interacted with SKIIP. The domain of amino acids 1-145 was known to interact with SKIIP [5], but the precise localization of the interaction is unknown. ThermoFluor assays with SKIIP and the synthetic peptide indicate that the peptide is likely binding to SKIIP (Figures 11 and 12). Figure 11 shows that the peptide is aggregated. When it is added to SUMO-SKIIP the aggregation is reduced and the spectra suggest that there is a stabilizing effect of the peptide. The stabilizing effect is more clearly observable in Figure 6, where there is a slight increase in the Tm for SUMO-SKIIP with the peptide from SUMO-SKIIP alone. The peptide that was used in this assay had an N-terminal methionine that the native protein does not have. The peptide was also not acetylated, unlike native protein that has an N-alpha acetylation [25]. Furthermore, the peptide was only 78.1% pure, which leaves the possibility that the shift in Tm is due to contaminants in the peptide rather than to the peptide itself.

As a control, ThermoFluor assays were conducted by adding 0.41µM peptide to lysozyme. It was expected that there would be either a small stabilizing effect or no effect on the Tm of lysozyme. Surprisingly, it was found that adding peptide to 0.41µM had a small stabilizing effect, but that further increasing the concentration of peptide to 4.1µM and 0.73mM both substantially destabilized lysozyme (Figure 13). This assay
was then repeated with other standard proteins and the peptide appeared to have a destabilizing effect on all proteins it was assayed with (Figures 13-18, 20-22), except for SUMO-SKIIP (Figures 11 and 12) and glucose oxidase (Figure 19). These data suggest that the N-terminus of PABPN1 may be responsible for some interaction with SKIIP but whether additional alanines disrupt this interaction is unclear.

ThermoFluor conducted with PABPN1_{+1Ala} and SKIIP suggests that PABPN1_{+1Ala} may still be able to interact with SKIIP even though there is an additional alanine at the N-terminus (Figure 10). PABPN1_{+1Ala} appears to have aggregated similar to PABPN1_{wt} (Figure 9), except that the aggregation like signal does not go away at higher concentrations of protein as it does for PABPN1_{wt}. This analysis agrees with previous research suggesting that PABPN1_{exp} has an increased tendency to aggregate [4]. Yet, adding a small amount of PABPN1_{+1Ala} to SKIIP decreased the Tm of SKIIP slightly (Figure 10), even though this effect was diminished as more PABPN1_{+1Ala} was added. This effect is small but it is significant due to the sensitive nature of the ThermoFluor technique, especially compared with adding PABPN1_{wt} to SKIIP (Figure 9). More research needs to be conducted to examine whether this change in Tm is an artifact of the system or due to a change in the PABPN1_{+1Ala}-SKIIP interaction. However, due to the sensitive nature of ThermoFluor assays, it is likely that this is not an artifact and is the result of a decreased binding interaction between PABPN1 and SKIIP.

**Calorimetry**

ITC was performed to determine the $K_D$ of binding for PABPN1 and SKIIP; unfortunately the signal to noise ratio was not high enough and the titration curve (Figure 23) is not interpretable. Further work needs to be done to determine the $K_D$ of binding
between the two proteins. It is possible that the heat of binding between PABPN1 and SKIIP was too small to be detected so higher concentrations of proteins need to be used. It is also possible that the apparent signal is from dilution, which would be clear if higher concentrations were used. Future research will use higher concentrations of protein in ITC experiments to try to expand the signal. If future research finds that PABPN1 and SKIIP do not interact by ITC it is possible that post translational modifications are necessary for their interaction.

The first task for future research continuing this project is to optimize the separation of the SUMO-tag from PABPN1. While the FPLC separates the tag and the protein using the S200 column, the separation is not good and the yield is low. Optimizing the protocol for separating the SUMO-tag from PABPN1 is crucial to being able to continue to conduct these experiments.

Previous research showing the interaction between PABPN1 and SKIIP relies on SKIIP expressed in yeast or mammalian cells which would have the correct PTMs, which were not present on the proteins used in these assays. Kim et al. [5] used PABPN1 purified from BL21(DE3) E. coli cells and SKIIP purified from C2 cells in their immunoprecipitation assays. In the yeast-2-hybrid screen, SKIIP would have been modified because yeast has the enzymes that make proteins phosphorylated and acetylated proteins. If future ITC fails to show that PABPN1 and SKIIP bind then it is possible that the PTMs of SKIIP are required for their interaction. In that case a construct expressing SKIIP in yeast will have to be used.

1: In human SKIIP, A2 is acetylated, S224, S232, and S234 are phosphorylated, N115 is N6-acetyllysine, and the initiator methionine is removed.
Additionally, it is possible that increasing the length of the polyalanine tract leads to the formation of aggregates that could sequester SKIIP, similar to the theory for the aggregates sequestering mRNA [2] and supported by SKIIP being found in the aggregates [9]. It is also possible that the aggregates decrease the amount of available PABPN1 in the cell, disrupting the interaction between PABPN1 and SKIIP and resulting in altered levels of transcription leading to myopathy.

PABPN1 was shown to increase the Tm of SKIIP, confirming previous research [5]. The preliminary results with PABPN1_{+1Ala} and SKIIP suggest that something may be different for PABPN1_{+1Ala} and SKIIP compared to PABPN1_{wt} and SKIIP. In order to examine this interaction further, these experiments will be conducted using additional PABPN1 mutants and additional synthetic peptides.

A $K_D$ between SKIIP and PABPN1 was unable to be determined from the ITC data. Future studies will utilize higher concentrations of protein in the cell and injector to increase the signal to noise ratio. If higher concentrations of PABPN1 and SKIIP are not stable in solution, then other ways of finding the $K_D$ should be examined, such as equilibrium sedimentation centrifugation with PABP1 and SKIIP. Additionally, detergents and other additives could be screened to see whether they might be used to reduce aggregation.

Unfortunately, in trying to purify the synthetic peptide to a purity greater than 78.1%, the peptide was dissolved in a solvent that made separation by HPLC impossible. A new peptide with the proper PTMs was ordered but it could not be purified and used in assays by time of publication. Future research will use a synthetic peptide of high purity to localize the binding between PABPN1 and SKIIP using ThermoFluor and ITC.
Furthermore, crystallization conditions for SUMO-PABPN1, pET24a-SKIIP, and co-crystals of a complex can be screened. Additionally, NMR can be performed with $^{15}\text{N}$- and $^{13}\text{C}$- labeled protein to examine how PABPN1$_{exp}$ changes the structure of the N-terminus which is considered to be largely unstructured. Although the activity of PABPN1 was not confirmed using an adenylation activity assay[24], future research will confirm activity with the protocol in Appendix 2 with PAP that was a generous gift from Professor James Marley of Columbia University.

While there is a lot of work left to do, I confirmed the interaction between SKIIP and PABPN1 [5] as well as taking the first steps to study how PABPN1$_{exp}$ might change its interaction with SKIIP as suggested by preliminary data. Furthermore, I have conducted preliminary research to assess the thermodynamics of the interaction between these proteins using ITC. Although there are more experiments to do, I hope to have laid a foundation for future work in this field.
Bibliography


Appendix I: Recipe for Terrific Broth (TB)

1. Add the following to 900ml distilled H₂O in a 2.8L baffled flask
   - 12g Bacto Tryptone
   - 24g Bacto Yeast extract
   - 4ml Glycerol
2. Autoclave for 20min
3. Allow to cool and add 100 mL of sterile 0.17M KH₂PO₄ and 0.72M K₂HPO₄ that was prepared separately

Note: Glycerol, KH₂PO₄, and K₂HPO₄, were purchased from Fisher. Tryptone and yeast extract were purchased from Becton, Dickinson and Company and Fisher.
Appendix II: Activity of PABPN1

The following protocol will be used to confirm activity of PABPN1. This should be done with all PABPN1 mutants as well as with PABPN1 wt.

1. Incubate an (A)20 oligo of RNA with T4 polynucleotide ligase (NEB) with [γ-32P]ATP following the directions from the manufacturer.

2. Extract the labeled 5’ labeled RNA with phenol and chloroform.

3. Setup 6 reactions with a reaction volume of 25µL in 25mM Tris-HCl pH 7.9, 50mM KCl, 2mM MgCl2, 0.05mM EDTA, 0.01% Nonidet P-40, 0.4mg/mL methylated BSA, 0.5mM ATP, 1mM dithiothreitol, 4 units of RNAguard, 80fmol of radiolabeled oligo (A)20. Add 9fmol PAP and 200fmol of PABPN1. Incubate at 37°C.

4. Setup 2 additional control reactions the same as above but one should not include PABPN1 and the other should have 90fmol of PAP.

5. Stop the each of the 6 reactions after 1 minute, 2 minutes, 5 minutes, 10 minutes, 20 minutes, and 30 minutes by adding 2x concentrated proteinase K digestion buffer and water to a final volume of 100µL. Stop the control reactions after 30 minutes in the same way. Next add 10µg of proteinase K and 2.5µg of tRNA and incubate for 30min at 37°C.

6. Precipitate RNA by adding 2.5 volumes of ethanol washed with 70% ethanol, dried, dissolved in formamide loading buffer and analyze the samples by 10-15% PAGE with a scintillation counter.
Appendix III: Control proteins

Table 4

<table>
<thead>
<tr>
<th>Protein</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol dehydrogenase</td>
<td>Bakers’ yeast</td>
</tr>
<tr>
<td>Catalase</td>
<td>Bovine liver</td>
</tr>
<tr>
<td>Cytochrome C</td>
<td>Horse heart</td>
</tr>
<tr>
<td>Glucose oxidase</td>
<td>Aspergillus niger</td>
</tr>
<tr>
<td>Hexokinase</td>
<td>Bakers yeast</td>
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<td>Insulin</td>
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<td>Myoglobin</td>
<td>Equine skeletal muscle</td>
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<tr>
<td>Trypsin</td>
<td>Bovine pancreas</td>
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Table 4: Source organisms for control proteins