THE MEIOSIS SPECIFIC AAA+ ATPASE PCH2 INTERACTS WITH THE SYNAPTONEMAL COMPLEX PROTEIN HOP1 THROUGH THE HOP1 HORMA DOMAIN

A Thesis

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Master of Science

by

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Abstract

In most sexually reproducing organisms physical linkages are required between homologous chromosome pairs in order to properly segregate them in the first meiotic division. Crossovers (CO) provide this physical linkage and help to position chromosomes at the metaphase plate for association with the meiotic spindle, which segregates the homologs. A multi-protein complex known as the synaptonemal complex (SC) facilitates homology pairing and CO formation. Defects in SC assembly or CO formation result in non-disjunction of homologs leading to aneuploid gametes, and for the budding yeast *S. cerevisiae*, decreased spore viability. The meiosis specific AAA+ (ATPases Associated with diverse cellular Activities) ATPase Pch2 (Pachytene checkpoint) facilitates proper SC assembly by regulating the localization of the axial SC element Hop1. This helps ensures that each homolog pair produces at least one CO and is subsequently properly distributed to each daughter cell. Little is known about the mechanism by which Pch2 performs this function. The research presented here establishes reliable methods for purification of Hop1 and Pch2 for *in vitro* analysis. I also show that Pch2 is able to dissociate Hop1 from DNA *in vitro* when in the presence of ATP. By constructing and purifying a Hop1 mutant allele (Hop1-HORMAΔ) I found that the N-Terminal HORMA protein-protein interaction domain of Hop1 is necessary for Pch2 interaction *in vitro.*

Biographical sketch

During my undergraduate career I worked as a student researcher in a microbiology lab studying *Helicobacter pylori* (*H.p.*), a bacterium which causes gastric ulcers. My studies with *H.p.* lasted for one and a half years before beginning a project characterizing a recently discovered species of *Bacillus*. The organism had potential applications in the agriculture and biofuels industries. I later presented my work on this organism at the national Annual Biomedical Research Conference for Minority Students (ABRCMS) in 2010, and was invited to present additional findings at two more symposiums at my university. After graduation I was accepted to the PREP program at the University of Texas Medical Branch, which is a one year post-baccalaureate program, and took a position as research tech in a virology lab. My research focused on investigating the properties of Hazara Virus as a possible low containment model for the very closely related bunyavirus, Crimean Congo Hemorrhagic Fever Virus (CCHFV), which requires BSL 4 facilities. My duties consisted of deep sequencing and development of protocols and standard operating procedures for working with Hazara virus and CCHFV.

After four years in microbiology I decided to make a change to basic science. I am currently a doctoral student at Cornell University working in Dr. Eric Alani research group. Our focus is on DNA damage repair signaling and mismatch repair mechanisms in *Saccharomyces cerevisiae* (budding yeast). The topic is exciting and important in understanding how organisms maintain genome stability and repair DNA damage. I find this type of basic science is a welcome relief

from my training in microbiology where many ideas and theories are taken for granted without the need to understand many of the underlying mechanisms.

I intend to remain in an academic setting where research interests and publication of findings is not restricted and opportunities to interact with younger scientists and the community are not only more accessible but encouraged. My objective is to be in a primary investigator position that will allow me to reach out to young disadvantaged students through programs such as the Bridges to Baccalaureate Program, which I was personally supported by, and is designed to expose junior college students from disadvantaged backgrounds to research at the university level. This program is a large part of why I became a scientist and the close personal and professional relationships I formed with the faculty, in particular my mentor, have lasted throughout my career and provided invaluable motivation and encouragement.

Dedication

This is dedicated to my parents for their love, support, encouragement and understanding. My sister and her family who were a source of comfort and peace and a home whenever one was needed. My grandparents who never doubted me, and The Old Main Gang (OMG) rumored to claim territory in and around the 4th floor biology lab of the Old Main Building at Texas Woman's University, without whom I would not be here.

Acknowledgments

I would like to acknowledge my advisor, Dr. Eric Alani, whose patience, breadth of knowledge and enthusiasm for science and life created a laboratory environment that has become the envy of my graduate colleagues. His mentorship and guidance on this work and during my time here have been invaluable and could never be adequately described writing. Dr. Joe Peters and Dan Barbash who sat on my committee and were helpful in guiding me towards an understanding of the experiments and techniques needed to perform my research. Dr. Cheng Chen who bequeathed me this project provided invaluable resources such as reagents and experimental protocols and is heavily cited throughout this thesis.

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Introduction

During meiosis in the budding yeast *Saccharomyces cerevisiae* cells undergo one round of DNA synthesis followed by two chromosomal divisions (MI, MII) to produce haploid spores. During Meiosis I in most organisms physical linkages are required between homologous chromosome pairs to bind them together in the proper orientation prior to segregation (Hunter, 2007). Crossovers (COs) provide this physical linkage and help to position chromosomes at the metaphase plate for bipolar association with the meiotic spindle which segregates the homologs (Borner et al., 2004; Hunter, 2007; Hollingsworth, 2010). A multi-protein complex known as the synaptonemal complex (SC) pairs the homologous chromosomes and facilitates CO formation. Defects in SC assembly or CO formation result in nondisjunction of homologous pairs, leading to aneuploid gametes and decreased spore viability. In humans these abnormalities result in genetic disease, miscarriage, and birth defects such as Down and Turner's syndrome. To ensure proper segregation of genetic material cells have evolved a robust system to accurately replicate and distribute DNA between daughter cells.

The SC in yeast is composed of a tripartite protein complex that begins to assemble in early prophase I (Figure 1; Hunter, 2007). The axial element Hop1 is discontinuously loaded onto chromosomes beginning in early leptotene followed by Zip1 loading during zygotene (Borner et al., 2008). This process occurs at roughly the same time that double-strand breaks occur throughout the genome that ultimately result in both COs and non COs. Zip1 localizes to chromosomal regions of sparse Hop1 abundance, this results in the appearance of domain like regions of alternating Hop1/Zip1 localization that is fully apparent in pachytene when SC assembly is complete. The third SC component, Red1, is an axial element and extends the

length of the chromosome with no apparent specificity. The regions of chromosomes where Hop1 and Zip1 form boundaries delineate sites of future CO formation (Borner et al, 2008; Joshi et al., 2009; San-Segundo and Roeder, 1999).

The meiosis specific AAA+ ATPase Pch2 is an important factor in positioning Hop1 during SC formation. Although Pch2 has been implicated in multiple meiotic processes and is known to have several direct interactions with other meiotic proteins, its role in SC assembly and subsequently CO formation may explain many of them (Chen, 2014). As I will show in the Results section, I and others (Chen et al. 2014) have shown *in vitro* that Pch2 can displace a subset of Hop1 from duplex DNA substrates but strengthens the interaction of the remaining Hop1 with DNA. This may be to ensure that promiscuously bound Hop1 is dissociated while properly localized Hop1 is reinforced (Chen et al., 2014). How Pch2 determines which regions Hop1 should and should not localize is unknown, although there is evidence indicating some sequence specificity by Hop1 (Muniyappa et al., 2000).

Synaptonemal Complex Dynamics

CO and SC formation are highly interdependent and defects in either pathway can cause defects in the other. Pch2 has been shown to be a common link between the two pathways and in *pch2Δ* cells this link can be visualized as aberrant localization of Hop1 on meiotic chromatin (Figure 2).

Hop1 forms domain like regions alternating with Zip1 and CO's occur at the boundaries of these regions. In *pch2Δ* mutants Hop1 co-localizes extensively with Zip1 and defects in both CO and SC assembly result (Borner et al. 2008). It has been proposed that Pch2 facilitates proper SC assembly by regulating the localization of Hop1 (Borner et al. 2008; Chen et al.

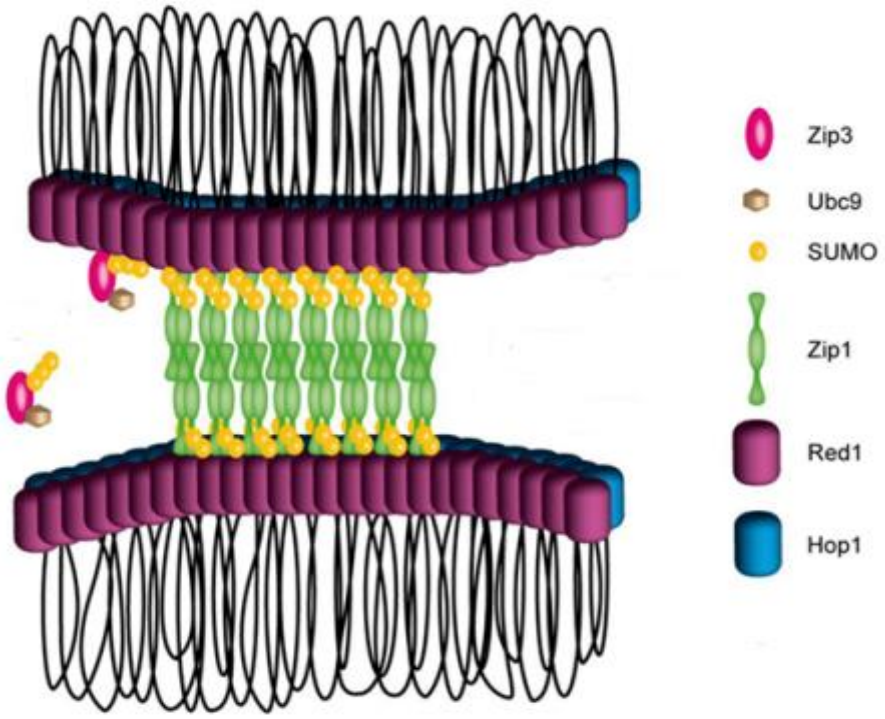


Figure 1. The synaptonemal complex (SC) is a tripartite structure. The axial/lateral elements Hop1 and Red1 bind along the chromosomal axes. Dimers of the central element Zip1 span the central region binding the homolog pairs. Zip3 foci form at boundaries of Hop1/Zip1 domains and mark sites of future crossovers (Adapted from Borner et al. 2008).

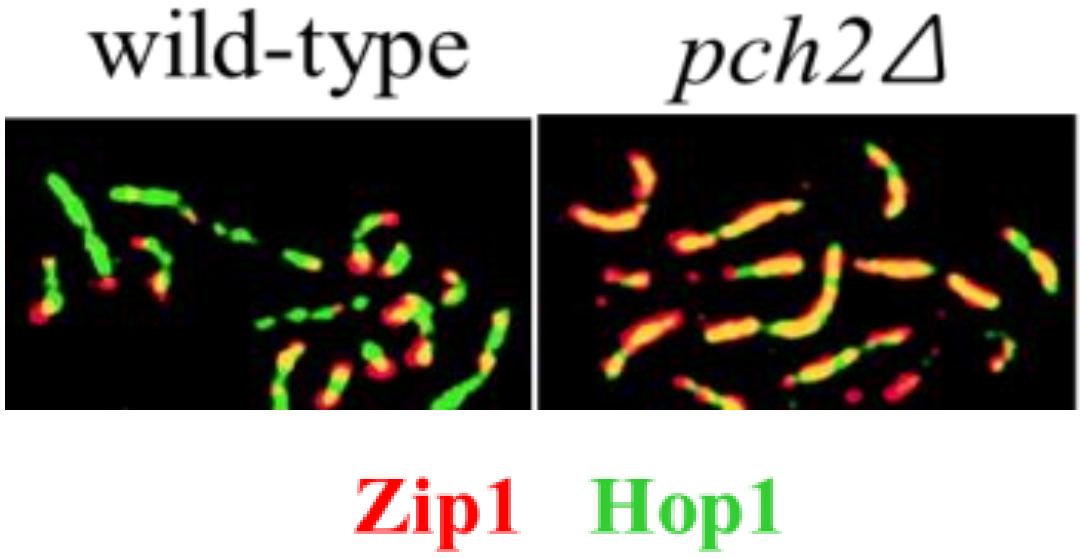


Figure 2. In *pch2Δ* mutants the domain-like organization of Hop1 and Zip1 is lost. These mutants display defects in chromatin structure and crossover regulation (Adapted from San-Segundo et al. 1999).

2014). In support of this hypothesis I show that Pch2 interacts directly with the HORMA (Hop1, Rev7, MAD2) protein-protein interaction domain of Hop1 (Aravind and Koonin 1998), and is able to displace Hop1 from linear duplex DNA *in vitro* confirming results seen by Chen et al. (2014). Interestingly, in addition to regulation of Hop1 localization, Hop1 total protein levels were shown to increase in *pch2Δ* by Western blot analysis, indicating that Pch2 plays a role in regulation of Hop1 protein abundance as well as localization (Ho and Burgess, 2011). This has led to the idea that Pch2 may function by denaturing or degrading Hop1. AAA+ ATPases such as ClpX function by extruding folded proteins through a central pore and feeding unfolded peptide chains into an associated AAA+ protease complex (Chen et al. 2014; Schmitz and Sauer, 2014). While no evidence exists for Pch2 protease function exists, the model proposed by Chen et al. (2014; reprinted in Figure 3A and 3B here) proposes that Pch2 displaces Hop1 from DNA by unfolding the protein in a manner similar to ClpX. The denatured Hop1 is then delivered to an as yet unidentified protease. Evidence for this model is based on the preliminary observation that Pch2 may undergo a large conformational change, specifically within the central pore, as observed in cryo-EM micrographs, when interacting with Hop1 (Chen et al., 2014). Similar conformational changes have been observed in AAA+ ATPases that are known to denature their substrates (Schmitz and Sauer, 2014).

The steady state kinetics performed by Chen et al. (2014) indicates that the ATP hydrolysis rate of Pch2 is not altered by interaction with DNA bound Hop1. However ATP consumption by similar AAA+ unfoldases increases dramatically when remodeling substrates (Schmitz and Sauer, 2014).

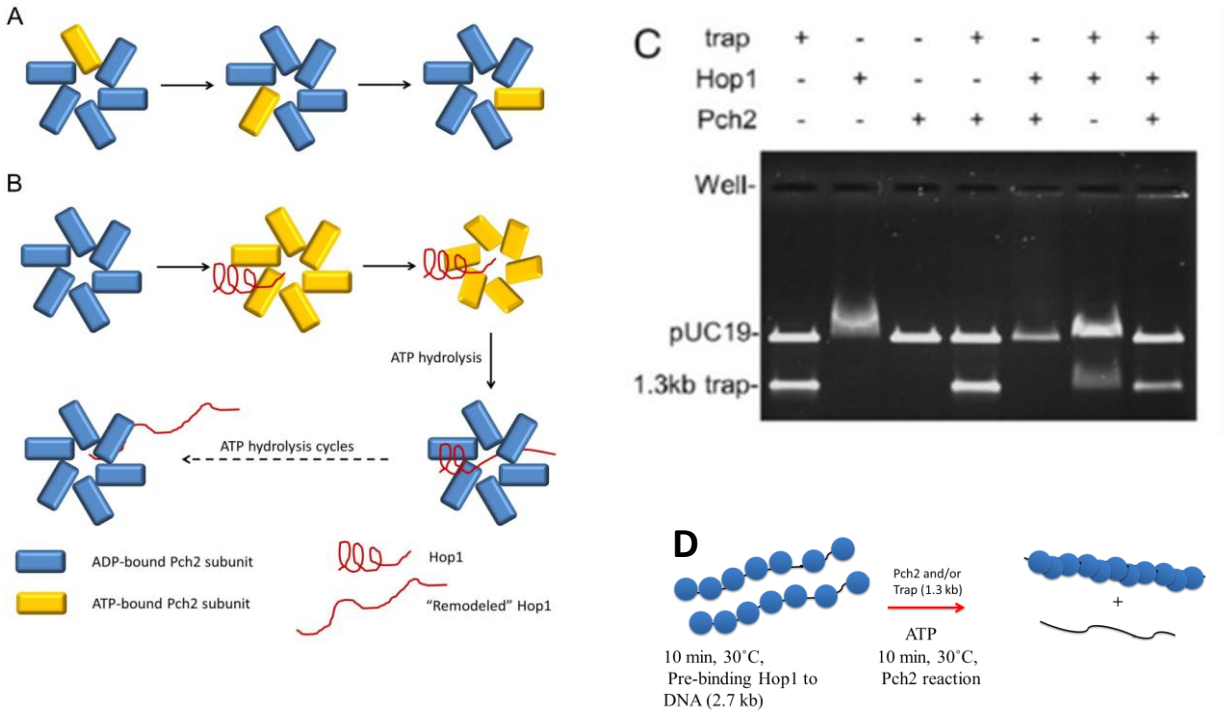
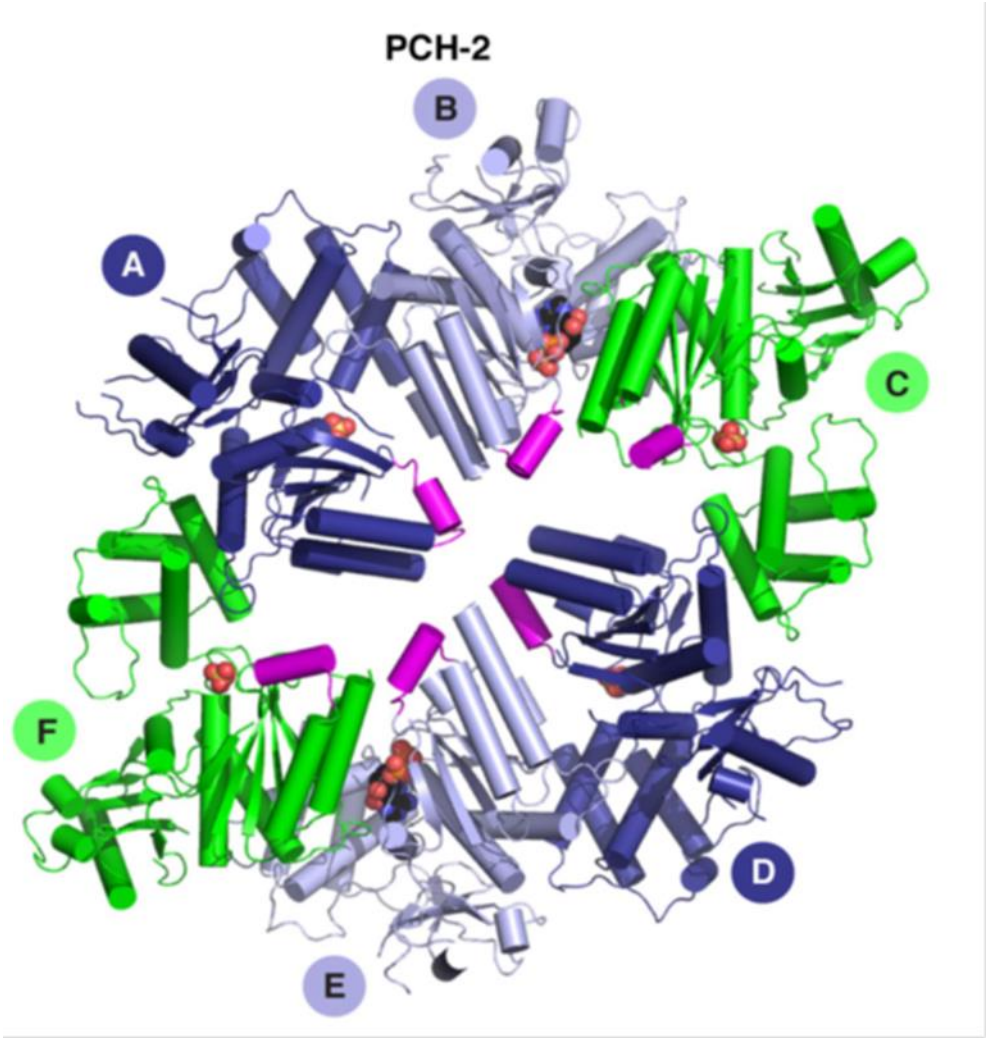


Figure 3. Molecular model of Pch2 function as proposed by Cheng (2014). (A) Pch2 subunits hydrolyze ATP relatively independently in the absence of Hop1. (B) Upon binding to Hop1, Pch2 subunits coordinate their ATP hydrolysis and bend inward to "squeeze" Hop1 through its central channel. Complete remodeling of Hop1 may take one or more ATP hydrolysis cycles. (C) 25 µl reactions in Buffer A with 60 ng *BamHI* digested pUC19 (2.7 kb), and 200 nM Hop1 were incubated at 30 °C for 10 minutes, after which 200 nM GST-Pch2 and 300 µM ATP were added as indicated in the presence of 40 ng 1.3 kb trap DNA. Reactions were then continued for 10 min at 30˚C, after which they were loaded onto a 0.7% agarose gel and analyzed as before. (Images and text are direct from the dissertation of Cheng Chen, 2014). (D) Diagram of reactions in panel C. Blue spheres represent Hop1 monomers bound to DNA. After 10 minutes of incubation with Hop1 and DNA at 30˚C ATP and Pch2 are added with or without trap DNA (1.3 kb) and incubated for an additional 10 minutes before resolving on 0.7% agarose.

Lack of increased ATP consumption by Pch2 more likely indicates a Hop1 displacement mechanism that is not dependent on denaturation. More detailed kinetic analysis will reveal if Pch2 functions as proposed by Chen et al. (2014). Additionally, the proposed protease associated with Pch2 mediated Hop1 degradation may require additional intermediate interactions (Chen et al., 2014). Interestingly, the DNA helicase/E3 ubiquitin ligase Rad5 was identified by high throughput methods to be a physical interactor of Pch2 (Yu et al. 2008). Pch2 dependent Hop1 ubiquitination by Rad5 may provide a mechanism by which Pch2 regulates Hop1 protein levels and requires further investigation.

Interestingly, Pch2 contains pore loop residues similar to that of ClpX, specifically a motif containing several key aromatic residues involved in direct substrate interaction and shown to be necessary for unfolding its substrate and threading denatured protein through the central pore (Schmitz and Sauer, 2014). This motif is also seen in the *Caenorhabditis elegans* Pch2 homolog (PCH2) as shown in Figure 4. In this thesis I speculate that these residues would likely be involved in disrupting the Hop1-DNA interactions and not large scale remodeling of Hop1 (Chen et al. 2014).

In order to explore the aspects of Hop1 / Pch2 dynamics outlined above it was necessary to develop robust methods of purifying Hop1 and Pch2 as well as methods for assaying these proteins *in vitro*. I will present empirically constructed protocols for expressing and purifying both proteins. These methods greatly improved purity, yield, activity and reduced the time required to prepare these proteins compared to previous methods. A mutant of Hop1, Hop1-HORMAΔ pEAE380, was constructed to examine the role of the HORMA domain in Pch2

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S. cer 314   GPPGTGKTTLCKALCQKLSVRREFSDGSDTIDTNYKGIIIELSCARIFSKWFGESSKNIS  373
             GPPGTGKT+LCK L Q LS+R          D    K +++E++    +FSKWF ES K +
C. ele 179   GPPGTGKTSLCKGLAQHLSIRMN--------DKYSKSVMLEINSHSLFSKWFSESGKLVQ  230
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Figure 4. *C. elegans* PCH2 structure and alignment of pore loop region of *S. cerevisiae* and *C. elegans*. The six subunits of the PCH2 hexamer form a dimer of trimers organization and have an elongated axis. Each subunit is differentially colored and labeled A-F residues are conserved exposed residues. Residues colored magenta are exposed pore loop residues located at the central pore. These were shown by Ye et al. (2015) to couple substrate interaction/work to ATP hydrolysis. (Image adapted from Ye et al. 2015).

interaction. The results of the experiments described below show that Pch2 interacts directly with Hop1 *in vitro* and that interaction requires the HORMA domain of Hop1.

Materials and Methods

Construction of Hop1-HORMAΔ Mutant

The wild type *HOP1* gene sequence from *Saccharomyces cerevisiae* was previously cloned and is maintained in the Alani lab plasmid collection as plasmid pEAE378, a pET15b insert of N-terminal, His_6 affinity tagged, wild type Hop1 (mRNA sequence). The plasmid was transformed into *E. coli.* Transformants were plated on selective LB containing 100 µg /ml Ampicillin (LB^{Amp}) and allowed to grow overnight at 37˚C. 5 ml of LB^{Amp} broth was inoculated using a single isolated colony and grown overnight at 37˚C with shaking. Plasmid was isolated from the liquid culture using the Qiagen™ Mini-Prep spin kit and sent to the Biotechnology Resource Center at Cornell for sequencing using primers AO713, AO3133, AO3135, and AO3136 found in Table 1.

The HORMA domain of Hop1 was removed from the wild type sequence using an overlap PCR method which employed three separate PCR reactions. All PCR reactions were carried out in 50 µl volumes under the following conditions: 150 µM dNTPs, 0.4 µM forward and reverse primers, 1 µg DNA template, 0.5 mM $MgCl_2$, 0.2 µl Phusion® High-Fidelity DNA Polymerase from New England Biolabs, and 10 µl of Phusion® High-Fidelity 5X Reaction Buffer. Thermocycler conditions were set to the Phusion® High-Fidelity recommended settings except for annealing temperature which was determined empirically to be 55 ˚C for all three reactions. Primers for each reaction were designed as follows: AO713 and AO3210 for reaction 1; AO714

Table 1: Primers (shown 5' to 3') used in this study

AO713	TAA TAC GAC TCA CTA TAG GG
AO714	GCT AGT TAT TGC TCA GCG G
AO3133	CTA GAG ATC ATA TGT CTA ATA AAC AAC TAG TAA AGC
AO3135	TCG AGA AGG ATG GTT CAA CAG
AO3136	ACC TTC CTT GGA TAC TAA ATG GTC
AO3209	AGA CAG AAA TAA CTA CTG AAG TTT TGT CGG CGG CAA CAT
AO3210	CGC CGA CAA AAC TTC AGT AGT TAT TTC TGT CTT TGT CT

and AO3209 for reaction 2; AO713 and AO714 for reaction 3. Each PCR reaction was analyzed by agarose gel electrophoresis and bands corresponding to the expected size were excised and DNA purified using the Qiagen™ Gel Purification Kit. The template for reactions 1 and 2 was the pET15B plasmid with wild type Hop1 insert (pEAE378). Reaction 3 template DNA was taken from gel purified PCR product of reactions 1 and 2. PCR product from reaction 3 was restriction digested using *Xho*I and *Nde*I ligated into the pET15b backbone, this ligation was used to transform *E. coli* and resulting transformants were verified by DNA sequencing as above.

Hop1 Expression

Protein expression of Hop1 was carried out in Rosetta™ (DE3) Competent *E. coli* transformed using pET15B expression vector containing wild type or mutant Hop1 inserts. 5 ml of overnight culture grown in $LB^{Amp\text{-}Cam}$ (LB^{Amp} plus 20 µg/ml chloramphenicol) was used to inoculate 500 ml $LB^{Amp\text{-}Cam}$ and placed in a shaker at 250 rpm, 37˚C and allowed to reach 0.6 OD_{600} then transferred to an 18˚C shaker and expression was induced using 500 µM IPTG. Expression was carried out for 4 hours. Cells were harvested by centrifugation at 4 krpm (Beckman™ JMI4 Rotor) for 20 minutes at 4˚C and washed twice using 25 ml wash buffer (25 mM Tris pH 7.5, 350 mM NaCl, 10% glycerol, 15 mM imidazole). Cell pellets were transferred to 15 ml conical tubes, flash frozen by submersion in liquid nitrogen and stored at -80˚C.

Hop1 Purification

This protocol is sufficient for purifying 500 ml induction equivalents of Hop1 mutant or wild type protein. All steps were performed on ice or at 4˚C. Induced cell pellets were

resuspended in 6 ml Lysis buffer (25 mM Tris pH 7.5, 350 mM NaCl, 10% Glycerol, 10 mM β-mercaptoethanol (BME), 1 mM phenylmethylsulfonyl fluoride (PMSF), 15 mM Imidazole) and sonicate at 30% duty cycle, micro-tip power 5, 1 min pulse, 2 min rest on ice, and an additional 1 min pulse. Lysate was centrifuged at 35,000 X g for 30 minutes at 4˚C. Supernatant was transferred to 1 ml Qiagen™ Ni-Agarose beads, prewashed three times in 4 ml Lysis buffer, and incubated on a rotator at 4˚C for 2 hours. Beads were pelleted by centrifugation at 5 krpm for 5 minutes at 4˚C, resuspended in 5 ml of wash buffer 1 (25 mM Tris pH 7.5, 350 mM NaCl, 10% Glycerol, 10 mM BME, 1 mM PMSF, 50 mM Imidazole) and transferred to a 10 ml gravity filter column. Beads were washed in a total of 20 ml wash buffer 1 and 3 ml wash buffer 2 (25 mM Tris pH 7.5, 350 mM NaCl, 10% Glycerol, 10 mM BME, 1 mM PMSF, 80 mM Imidazole). Protein was eluted in 2.5 ml elution buffer (25 mM Tris pH 7.5, 350 mM NaCl, 10% Glycerol, 10 mM BME, 1 mM PMSF, 160 mM Imidazole) and transferred to 50 KD concentration centrifugation columns. Concentrate was divided into 20 µl aliquots, flash frozen in liquid nitrogen and stored at -80˚C.

Pch2 Expression

GST-Pch2 was expressed by transformation of plasmid pEAE307 (2µ *amp^r URA3 leu2-d GAL1-10-GST-PCH2*) into *S. cerevisiae* strain EAY33 (*ura3-52, trp1, leu2Δ1, his3Δ200, pep4::HIS3, prb1Δ1.6R, can1, GAL*) and plated on selective media. A single colony transformant was selected and used to inoculate 100 ml of leucine dropout (LEU-DO) media and grown to saturation at 30˚C with shaking. Saturated culture was used to inoculate 6 liters of SCGL-LEU DO (3% glycerol, 2% lactate) so that the initial OD is 0.05 at OD_{600}, placed at 30˚C with shaking

and grown to 0.6 OD_{600}. GST-Pch2 expression was induced by addition of 50 ml 40% Galactose per 1 L of culture for a final concertation of 2%. Expression was carried out for 16 hours then cells were pelleted by centrifugation at 4 krmp (Beckman JMI4 rotor) at 4˚C and washed twice by resuspending in 20 ml wash buffer (100 mM Tris pH 8.0, 400 mM NaCl, 10% glycerol, 1 mM EDTA, 10 mM β-mercaptoethanol (BME), 1 mM phenylmethylsulfonyl fluoride (PMSF)). Cell pellets were weighed, resuspended in 1 ml wash buffer per 1 g cell pellet, flash frozen by dropping suspension directly into liquid nitrogen and stored at -80˚C.

Pch2 Purification

20 g of frozen yeast pellet was pulverized by grinding in a Braun™ coffee grinder for 2 minutes along with roughly equal volume of crushed dry ice. Dry ice was allowed to sublime overnight at -20˚C. All other steps were performed at 4˚C or on ice. Lysate was thawed at 4˚C on ice and resuspended in 25 ml lysis buffer (100 mM Tris pH 8.0, 500 mM NaCl, 10% glycerol, 1mM EDTA, 10 mM BME, 1 mM PMSF), centrifuged at 35,000 X g for 30 minutes. Cleared lysate was added to 2 ml of glutathione resin (Thermo Scientific, Catalog #16100) pre-equilibrated with lysis buffer and transferred to a 10 ml glass column. Flow through was saved and passed over the column 3 times, the resin was then washed with 45 ml wash buffer (100 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol, 1mM EDTA, 10 mM BME, 1 mM PMSF). GST-Pch2 was eluted from the resin in 4 ml elution buffer (100 mM Tris pH 8.0, 150 mM NaCl, 40 mM GST, 10% glycerol, 0.2% Tween-20, 1mM EDTA, 10 mM BME, 1 mM PMSF) then concentrated using 50 kD concentrating centrifugation columns. Concentrate was divided into 20 µl aliquots, flash frozen in liquid nitrogen and stored at -80˚C.

Agarose Electromobility Shift Assays (EMSA)

EMSAs were performed in 25 µl volumes using indicated concentrations of wild type or mutant Hop1 protein with constant DNA substrate concentration of 60 ng 2.7 kb pUC19 (1.4 nM) digested with *BamHI.* Final reaction conditions were as follows: 20 mM Tris pH 7.5, 0.01 mM EDTA, 2 mM MgCl2, 40 µg/ml BSA, 0.1 mM DTT, 75 mM NaCl, 10% glycerol. Reactions were incubated for 10 minutes at 30˚C then loaded on 0.7% agarose gel in 1X TAE and electrophoresed at 40 volts for 2 hours at 4˚C. Agarose gels were visualized by staining in 0.5 µg/ml ethidium bromide. EMSAs including GST-Pch2 were performed in 40 µl volumes containing indicated concentrations of protein. GST-Pch2 was prepared separately in 15 µl reaction volumes (as above: 20 mM Tris pH 7.5, 0.01 mM EDTA, 2 mM MgCl2, 40 µg/ml BSA, 0.1 mM DTT, 75 mM NaCl, 10% glycerol) and added to Hop1 reactions following the initial 10 minute incubation. GST-Pch2 reactions were incubated for an additional 20 minutes at 30˚C. When included, nucleotide concentrations were 200 µM.

***In vitro* Pull-Down Assays**

As described by Chen et al. (2014): 1.5 µg His_6-Hop1 was mixed with 2.5 µg GST-Pch2 and incubated in binding buffer (50 mM Tris pH 7.5, 50 mM NaCl, 10 mM imidazole, 10 mM BME, 0.1% Tween 20) with indicated nucleotides for 30 minutes at 4˚C. For GST-Pch2-E399Q, the conditions were the same except that 1.5 µg of mutant protein were present and 20 mM imidazole was included to reduce non-specific binding. 3 µl Magne-His Ni-Particles (Promega, equilibrated to the binding buffer) were added to the mixture and incubated for another 30 minutes. Ni-Particles were then washed 3 times with wash buffer 3 (50 mM Tris pH 7.5, 50 mM

NaCl, 10 mM BME, 0.1% Tween 20), heated at 99˚C for 5 minutes in SDS sample buffer, and visualized on a 9% SDS-PAGE gel.

Results

Construction of Hop1 Mutants

This project was developed to characterize the function of Pch2 *in vitro* with the goal of providing a mechanistic understanding of how Pch2 interacts with Hop1 during meiosis. This question was approached by identifying regions of Hop1 required for its interaction with Pch2. Figure 5A diagrams the organizations of Hop1 which consists of a C-terminal zinc finger DNA binding motif and a HORMA domain. HORMA domain containing proteins are not highly conserved at the amino acid level but display a conserved tertiary structure (Aravind and Koonin 1998; Hunter 2007; Muniyappa et al. 2000). I reasoned that due to the lack of strong conservation of amino acid sequence, mutations that disrupt specific residues may not provide meaningful information if the overall tertiary structure is not disrupted. For this reason I decided to first make a HORMA domain deletion, removing amino acids 16 to 256 in Hop1 ($Hop1^{H\Delta}$; Figure 5). In a second step I made alanine substitution mutations in two highly conserved HORMA domain residues in Hop1, R41 and E124 (shown in Figure 5 by blue (R41) and green (E124) asterisks). Hop1 proteins containing these mutations were found to be insoluble during purification and were subsequently not characterized (data not shown).

Mutations were constructed as described in Materials and Methods using overlapping heterologous primers and PCR. This method was preferred due to the ability to introduce large internal deletions within a gene. Wild type Hop1 was graciously provided by Cheng Chen of Cornell University in the form of a pET15b plasmid with wild type Hop1 ORF insert fused to an N-terminal His_6 affinity tag. Additionally, Dr. Chen provided a 2μ construct of wild type Pch2 with an N-terminal GST affinity tag.

Purification of Hop1

Wild type Hop1^{wt} and mutant Hop1$^{H\Delta}$ (Hop1-HORMAΔ) were both expressed using an *E. coli* expression system and purified by Ni-agarose affinity chromatography. Initially, Hop1 was subjected to a two-step purification procedure that employed an ssDNA-cellulose column, with the goal of maximizing purity and enriching for active Hop1 (Muniyappa et al. 2000). I found that this step greatly reduced the yield of Hop1 without significantly improving purity or quality. In addition, the original procedure included 24 hours of dialysis to remove high concentrations of Imidazole (160 mM) which I found prevented accurate determinations of protein yields using the Bradford assay (Bradford 1976). I decided to eliminate the ssDNA-cellulose procedure as well as dialysis of the final product; this resulted in significant improvement to yield and stability of both wild type and mutant Hop1 alleles. In my procedure I performed a single Ni-agarose column purification using half of the initial cellular materials, with a single dialysis step. Figure 6 shows a typical example of Hop1 purity and protein concentration using this method. The final concentrated protein ("CON" in Figure 6) has minimal contamination by other cellular proteins and little if any degradation of Hop1 based on the absence of bands on SDS-PAGE that were associated with auto-catalytic cleavage of Hop1 (Khan et al., 2013). Concentrations were determined by semi-quantitative comparison to a standard using SDS-PAGE analysis (Figure 6C). Improvements in the protocol become more apparent when yields are compared to previous attempts using a standard protocol (Table 2). The modified method increased yields by nearly 100%, and quality of the final concentrated protein was significantly improved over previous methods requiring significantly less protein to perform subsequent assays (Figure 8, upper and middle panels).

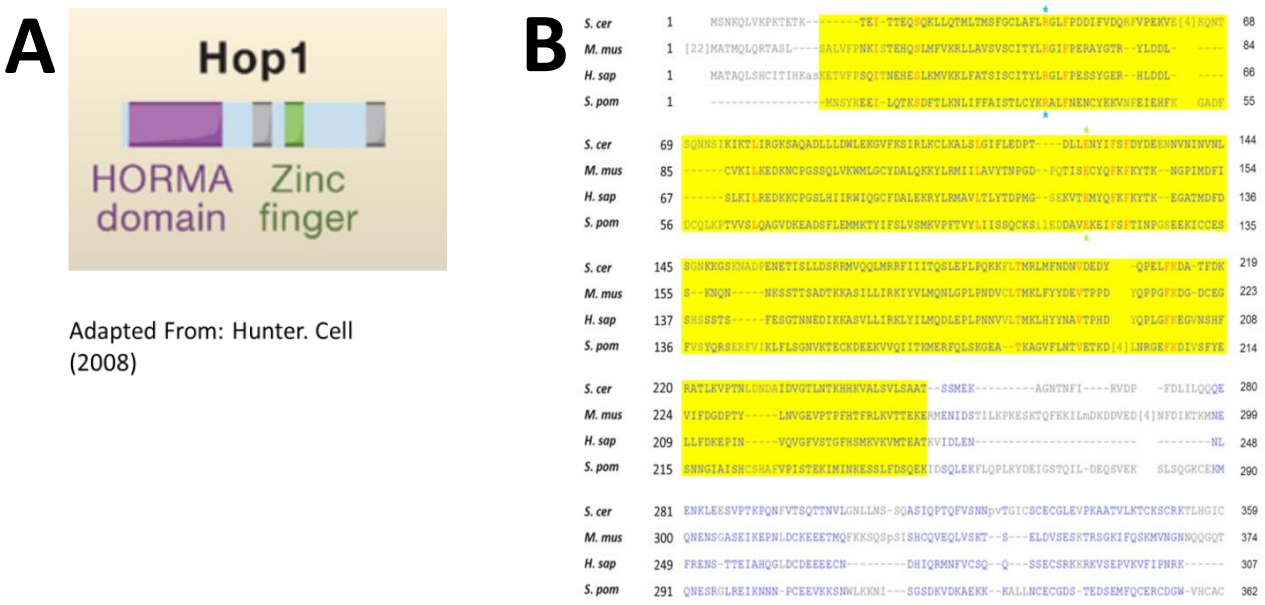

Figure 5. (A) Schematic of wild type Hop1 domain organization (Hunter, 2007). (B) Results of a multiple alignment of Hop1 homologs from the indicated species. Yellow highlighted sequence represents the HORMA domain, which was deleted to construct the Hop1-HORMAΔ mutant. Conserved amino acids shown in red. Specific residues targeted for mutation are marked with asterisks above and below the residue. *S. cerevisiae* Hop1 residues 359-608 not shown. Alignment was prepared using the NCBI web based multi-alignment tool (Altschul *et al.,* 1990; http://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi?LINK_LOC=BlastHomeLink).

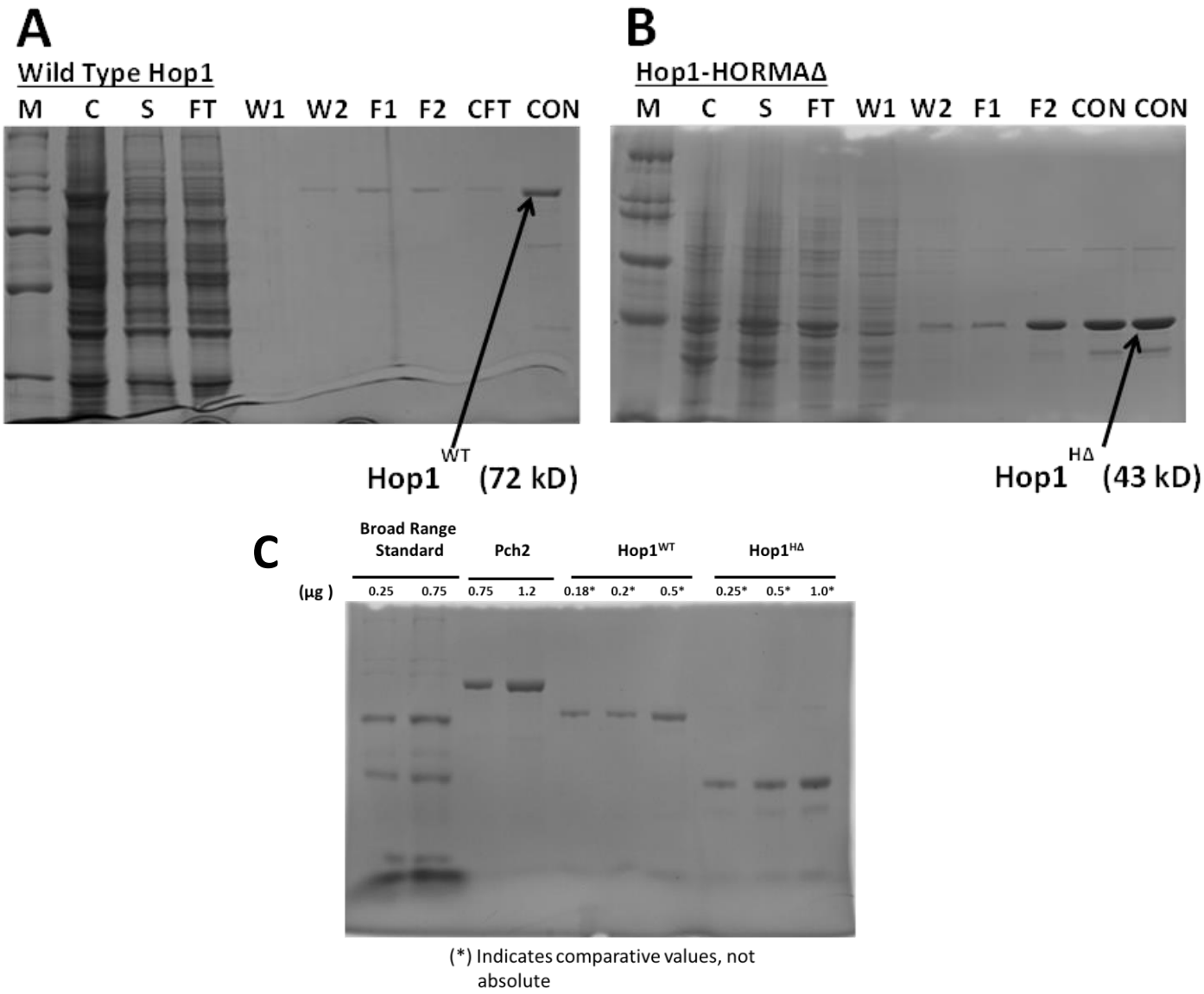

Figure 6. Representative 10% SDS-PAGE gels of wild type Hop1 (A) and Hop1-HORMAΔ (B) purification. Images represent the final protocol determined to produce the greatest yield of both proteins. Each well is loaded with 1.2 µl of the total volume the sample. The contents of each well from left to right are as follows: C, crude pre-cleared lysate; S, supernatant from first centrifugation; FT, flow-through from Ni-agarose column, W1, first wash; W2, second wash; F1 and F2, 2 ml fractions of eluate from Ni-agarose column; CFT, flow-through from 50 kD centrifugation concentrating column (omitted from right panel); CON, concentrated protein (Hop1-HORMAΔ CFT not shown). (C) Example of semi-quantitative method used for Hop1 concentration estimation. Wild type and mutant Hop1 was titrated and compared to known concentrations of BioRad™ Broadrange Standard and GST-Pch2.

Purification of Pch2

Purification of Pch2 was hampered by the inability to express it in a bacterial system such as *E. coli* due to improper folding (Cheng Chen, unpublished data). Pch2 was instead expressed using the *GAL10* promoter in *S. cerevisiae* (Chen et al. 2014). Pch2 was not expressed at high levels in this system and bands corresponding to the molecular weight of GST-Pch2 could not be identified in crude lysates of induced cells when compared to un-induced cells on an SDS-PAGE. This resulted in difficulty determining the cause for such weak expression of a GAL inducible construct because neither increases nor decreases in expression level were evident until after several steps of purification. This was overcome by preparation of large culture volumes of 6 liters, yielding approximately 40 g of yeast pellet and purifying the harvested cells in two batches of 20 g. Despite low yields the purification procedure was reproducible and sufficient to prepare Pch2 for experimentation *in vitro*. In order to reduce the high investment in time the protocol demanded I developed a slightly altered method which eliminated the need for a second column purification of PBE94 that aided in concentrating the eluate from the GSH column and removing some cellular contamination. I was able to do this by adjusting the volume and NaCl concentration of the GSH-Agarose column washes as well as that of the lysis and elution buffers. By increasing the volume of lysis and wash buffers I was able to eliminate much of the contaminating protein and could use a much stronger elution buffer with twice as much free GST, 40 mM instead of 20 mM. Elution volume from GSH-agarose was reduced 60% (4 ml instead of 10 ml) and yields were improved. Figure 7 is representative of the yields obtained from both the initial and the modified Pch2 purification procedures (Table 3). This

Protein	Crude Lysate (g)	Date	Concentration	Yield
Hop1^{WT}	25.28	06/17/2014	1 mg/ml	0.3 mg
Hop1^{WT}	23.92	10/15/2014	0.9 mg/ml	0.28 mg
Hop1^{WT}	33.32	11/15/2014	0.67 mg/ml	0.49 mg
Hop1^{WT}	38.94	11/18/2014	3.2 mg/ml	0.64 mg
Hop1^{WT}	15.17	02/03/2015	2.54 mg/ml	0.25 mg
Hop1$^{H\Delta}$	23.16	6/14/14	1.1 mg/ml	0.32 mg
Hop1$^{H\Delta}$	24.32	10/15/14	1 mg/ml	0.27 mg
Hop1$^{H\Delta}$	26.45	11/12/14	1.28 mg/ml	1.41 mg
Hop1$^{H\Delta}$	5.04	1/20/15	1.53 mg/ml	0.15 mg

Table 2. Results of purification of Hop1 and the mutant Hop1-HORMAΔ. Rows with red lettering indicate purifications that were performed after extensive modification of the purification protocol. In some instances yields appear to be similar between the two methods, but it should be noted that concentration and purity are much greater.

Protein	Starting Material	Date	Concentration	Yield
GST-Pch2	10 g	03/26/2014	0.67 mg/ml	0.49 mg
GST-Pch2	10 g	05/29/2014	0.162 mg/ml	0.21 mg
GST-Pch2	20 g	07/31/14	0.9 mg/ml	0.085 mg
GST-Pch2	**10g**	**04/22/2015**	**0.28 mg/ml**	**0.2 mg**
GST-Pch2	**20g**	**06/23/2015**	**0.93 mg/ml**	**0.28 mg**

Table 3. Representative examples of GST-Pch2 purifications with and without the PBE94 purification step. There was little difference in yield and concentration for all purifications. Rows with red lettering indicate purifications that were performed after moderate modification of the purification protocol and omit PBE94 purification. Starting material refers to frozen yeast pellet that was harvested from galactose induced cells. In some cases starting material was doubled, this was due to indications (such as extended growth time requirements, or other growth issues) that led me to believe expression would be poor.

figure shows that the PBE94 column purification did not significantly increase the concentration or purity of the protein.

Characterizing Hop1-HORMAΔ

Before Pch2 could be studied it was necessary to ensure that Hop1-HORMAΔ retained DNA binding activity. A series of experiments were performed to qualitatively assess the DNA binding capacity of purified Hop1-HORMAΔ. Agarose gel electro-mobility shift assays were performed by titration of Hop1 with linear duplex DNA (Chen et al. 2014; Materials and Methods). Purified wild type Hop1 or Hop1- HORMAΔ were added to reactions of constant DNA concentration. Hop1 was allowed to incubate with DNA substrate for 10 minutes, after which it was loaded onto an agarose gel. Hop1 bound DNA migrates more slowly than free DNA and can be seen as a shift in the position and shape of the migrating DNA band. The upper panel in Figure 8 is a titration of wild type Hop1 and Hop1-HORMAΔ. As mentioned previously, Hop1 purified using the modified protocol has significantly greater activity in assays than did that of earlier preps. This is evident in the middle panel of Figure 8 which was performed under the same conditions but with protein from the modified protocol. Nearly all DNA is shifted at 150 nM of Hop1 and is aggregated in the well. It was initially assumed based on the early evidence that Hop1HΔ had greater affinity for DNA than wild type Hop1. This assumption was later shown to not be correct; it now appears that wild type may have slightly greater affinity for DNA than Hop1-HORMAΔ (preliminary findings, data not shown). One explanation for this observation is that Hop1 lacking the HORMA domain binds less cooperatively to DNA (Byers et al*,* 1998).

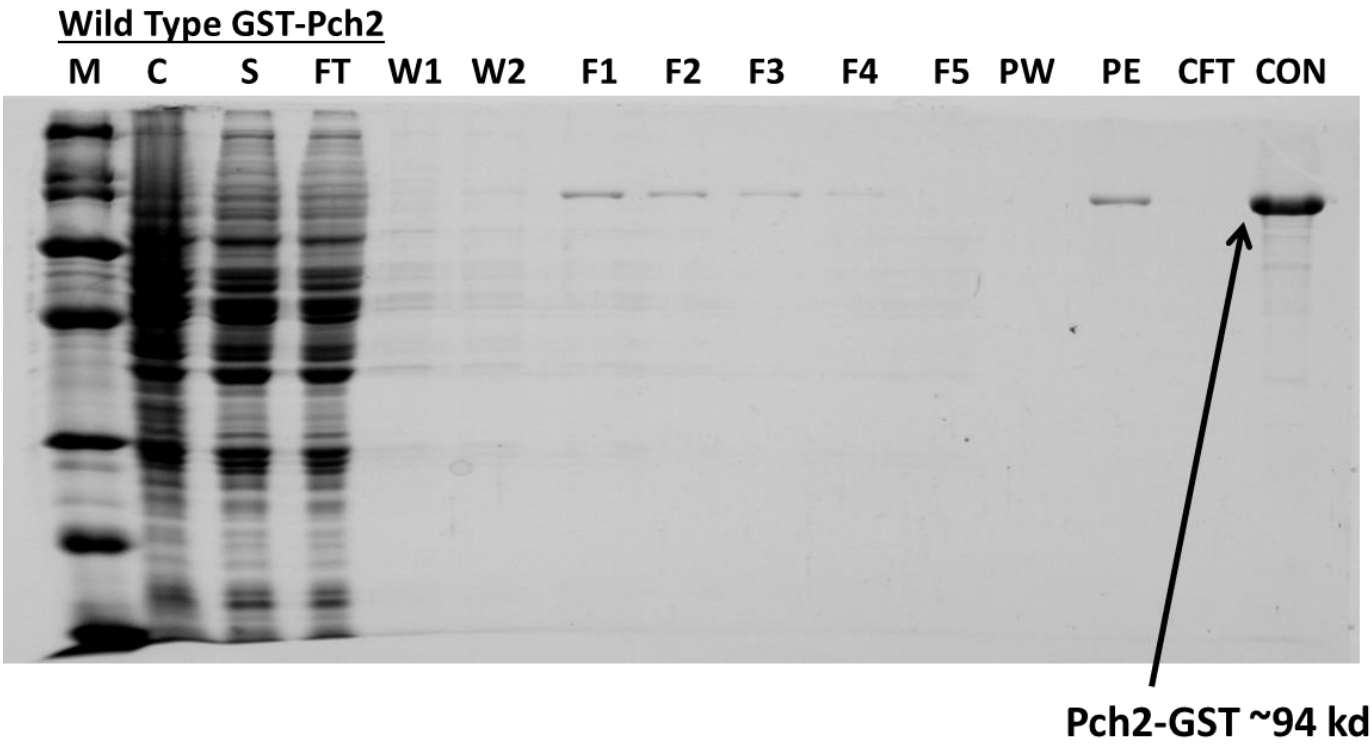

Figure 7. Representative 10% SDS-PAGE of GST-Pch2 purification. Each well is loaded with 1.2 µl of the total volume of the sample. The contents of each well from left to right are as follows: M, broad range molecular mass marker; C, crude pre-cleared lysate; S, supernatant from first centrifugation; FT, flow-through from GSH-agarose column, W1, first wash; W2, second wash; F1-F5, 1 ml eluate fractions from the GST-agarose column; PW, PBE94 (anionic exchange resin) wash; PE, PBE94 elution; CFT, flow-through from 50 kD centrifugation concentrating column; CON, concentrated protein. (Refer to Appendix for additional information and protocol for this method using PBE94.)

The bottom panel of Figure 8 shows that at lower concentrations (~50 nM) there appears to be little difference in binding affinity between Hop1 and Hop1$^{H\Delta}$. Each begins to produce noticeable shifts in band migration at roughly 75 nM. However, it is interesting to note that wild type Hop1 will produce strong mobility shifts and form protein-DNA aggregates that prevent migration into the gel at around 125 nm. However, Hop1-HORMAΔ will continue to migrate into the well even at very high concentrations and begin to plateau at the saturation point. I attribute this to the inability of Hop1-HORMAΔ to form strong protein-protein interactions with other Hop1 monomers. Based on these data and it was determined that 100 nM of Hop1 and Hop1$^{H\Delta}$ would produce comparable mobility shifts on agarose EMSA and that this concentration was would be suitable for measuring interactions with Pch2.

Pch2 Modifies Hop1-DNA Interaction

Chen et al. (2014) showed that Pch2 can remove Hop1 from duplex DNA *in vitro* (Figure 3) and that this is an ATP hydrolysis dependent reaction. This was observed as a return of DNA from a shifted-Hop1-bound complex to a condensed band with the expected mobility of 2.7 Kb dsDNA. Reduced intensity of DNA staining for these bands indicates that a portion of the DNA that went into the reaction is not observed on the gel. One hypothesis was that Pch2 is actively partitioning Hop1 into two groups and that this is leading to aggregation of a portion of the partitioned Hop1-DNA. It was also thought that Pch2 may be remodeling Hop1 in such a way as to prevent it from binding to DNA. I believe that this is not the case based on the results of Figure 3C in which it was demonstrated that Hop1 is able to bind to trap DNA following displacement by Pch2. The trap DNA, added concurrently with Pch2 and ATP to reactions containing pre-bound Hop1-DNA resulted in loss of trap DNA staining intensity with a portion of

the DNA presumably stuck in the well as was assumed for the previous experiment. It can only be concluded that since Hop1 would have been pre-bound to DNA before addition of Pch2 then any loss of trap DNA, or reduction of trap DNA mobility would be due to Hop1 that had been displaced by Pch2. To determine if the HORMA domain of Hop1 is necessary for Pch2 to displace Hop1 from DNA I used the method employed by Chen et al. (2014). As described in Materials and Methods, wild type or mutant Hop1 was incubated with 1.4 nM linear pUC19 for 10 minutes at 30˚C. After 10 minutes Hop1 reactions were treated with 200 nM, 250 nM, and 300 nM GST-Pch2 with 200 µM ATP and allowed to incubate for 10 minutes at 30˚C. Reactions were loaded on 0.7% agarose gels and visualized by staining in an ethidium bromide, 1X TAE bath. As seen by Chen et al. (2014), Pch2 appears to displace Hop1 from DNA and return the DNA substrate to normal mobility (Figure 9a). Although I was unable to completely restore DNA to its unbound mobility, these assays present sufficient evidence to conclude that a large portion of Hop1 has been displaced form the DNA substrate and some mobility has been restored. While Figure 9b shows no change in mobility under any condition. This provides compelling evidence that the HORMA domain of Hop1 plays a critical role in Pch2-Hop1 interactions that mediate Hop1 localization *in vivo*. Pch2 was unable to displace Hop1 from DNA in a reaction lacking ATP. The experiments presented here do not conclusively demonstrate a requirement for ATP since no other nucleotide was included except for ATPγS that was used to interrupt the nucleotide hydrolysis cycle of Pch2. The results show a requirement nonetheless, for a hydrolysable nucleotide such as ATP for displacement of Hop1 from DNA.

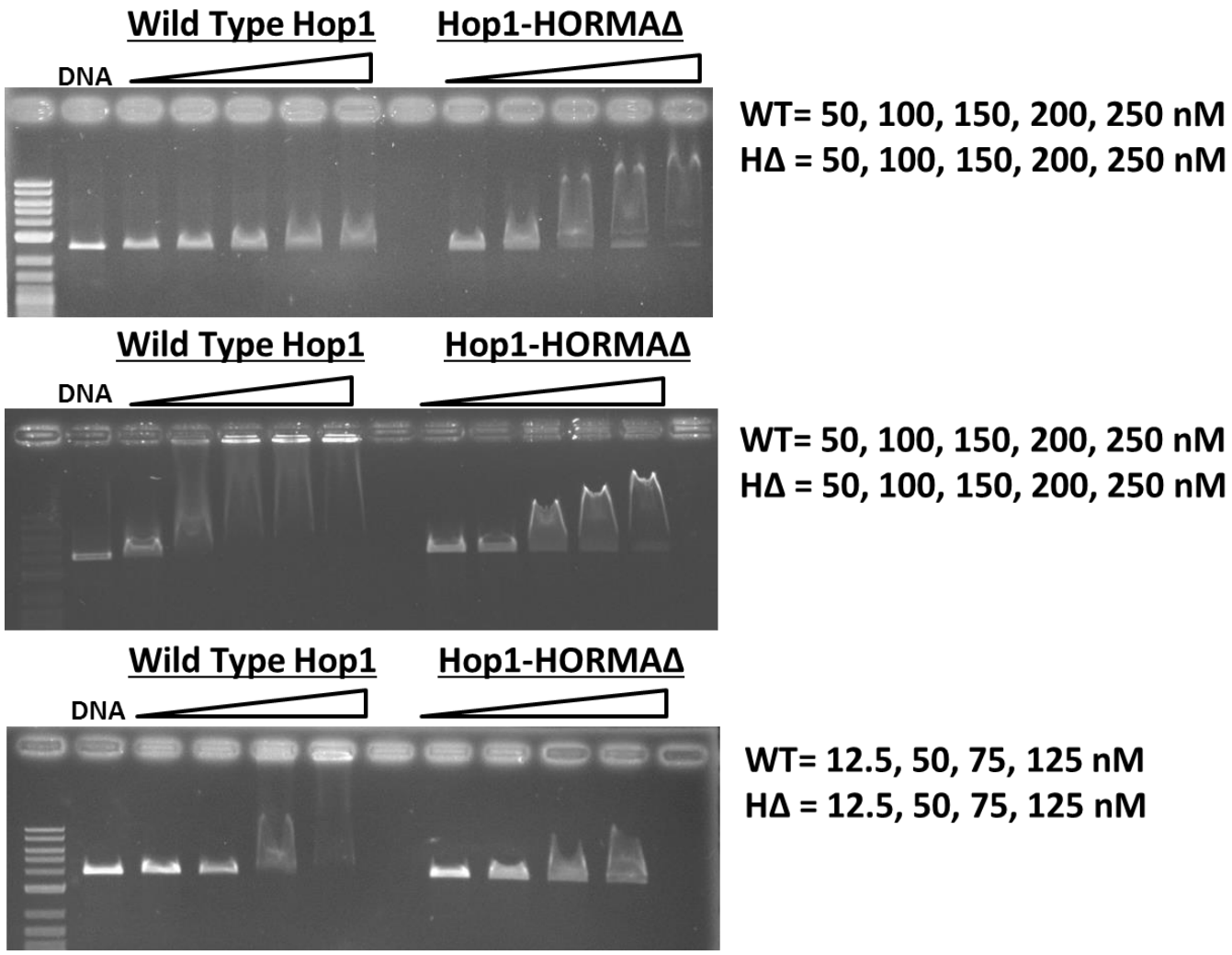

Figure 8. Agarose Electromobility Shift Assays (EMSA) of Hop1 titrations containing 1.4 nM linear duplex pUC19 (2.7 Kb) DNA with indicated concentrations of purified wild type Hop1 (left side) or Hop1-HORMAΔ (right side). Reactions were performed in 25 µl and incubated for 10 minutes at 30˚C. Results were visualized by gel electrophoresis using 0.7% agarose. Upper panel is representative of early titrations using to an un-modified protocol for purifying His_6-Hop1. Middle and bottom panels are a later batch of purified Hop1 using the modified protocol. These assays demonstrated wild type Hop1 and Hop1-HORMAΔ retain the ability to bind DNA with similar affinity. The bottom panel aided in determining the appropriate concentration of Hop1 protein for assays that included GST-Pch2.

Pch2-Hop1 *In Vitro* Interactions Require the HORMA Domain of Hop1

To determine if the HORMA domain of Hop1 interacts directly with Pch2, I performed *in vitro* Ni-pulldown assays with wild type or mutant Hop1 and GST-Pch2. By allowing Pch2 and Hop1 to interact in the presence of the non-hydrolyzable ATP analog, ATPγS, the interaction of Pch2 with Hop1 can be captured in an incomplete hydrolysis cycle that prevents Pch2 from releasing Hop1 (Chen et al. 2014). The tightly bound proteins are then pulled down from the reaction buffer with magnetic Ni resin which is bound by the N-terminal His_6 affinity tag of Hop1. Pulldowns are then visualized on 10% SDS-PAGE. Figure 10a reveals that Pch2 does not strongly associate with Hop1 in the presence of ATP and the interactions are transient. When incubated with ATPγS the transient interactions are stabilized and the proteins become locked in complex with one another. Figure 10b confirms the results seen in agarose EMSA experiments; that Pch2 does not interact with the Hop1-HORMAΔ mutant. This provides further evidence that the HORMA domain is playing key role in Pch2 mediated Hop1 localization during meiosis. It is known that Hop1 binds in a cooperative manner to dsDNA and that Hop1 monomers oligomerize along the axes of chromatin. Oligomerization is dependent on HORMA domain protein-protein interactions that bind the monomers together. A HORMA domain motif termed the “seatbelt” loop is likely the means by which Hop1 oligomerizes. The seatbelt has been shown in other HORMA proteins to bind with high affinity to their target and can be switched from an open to closed conformation by accessory proteins (Hara et al. 2010). Seatbelt motif interaction of other HORMA domain proteins are essential to many of their functions and could be an interaction that must be disrupted during Pch2 mediated Hop1-DNA displacement.

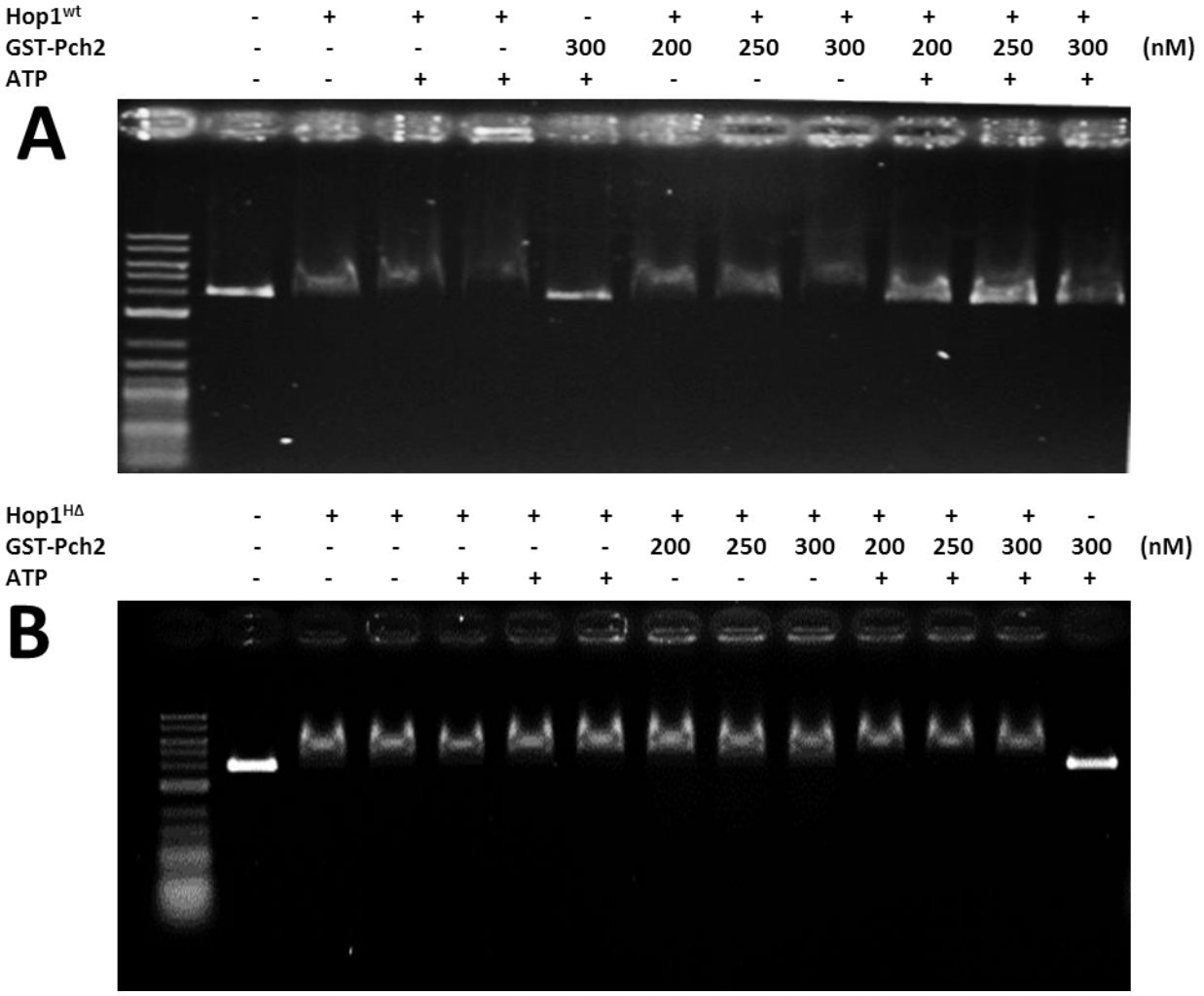

Figure 9. Agarose Electromobility Shift Assays (EMSA) of Hop1 containing 60 ng of (1.4 nM) linear duplex pUC19 (2.7 Kb) DNA with 100 nM concentrations of purified Hop1 with indicated concentrations of GST-Pch2 with or without 200 µM ATP. 100 nM of Wild type Hop1 (A) or Hop1-HORMAΔ (B) and indicated concentrations of GST-Pch2 were incubated with ATP as described in the Materials and Methods. Results were visualized as in Figure 8.

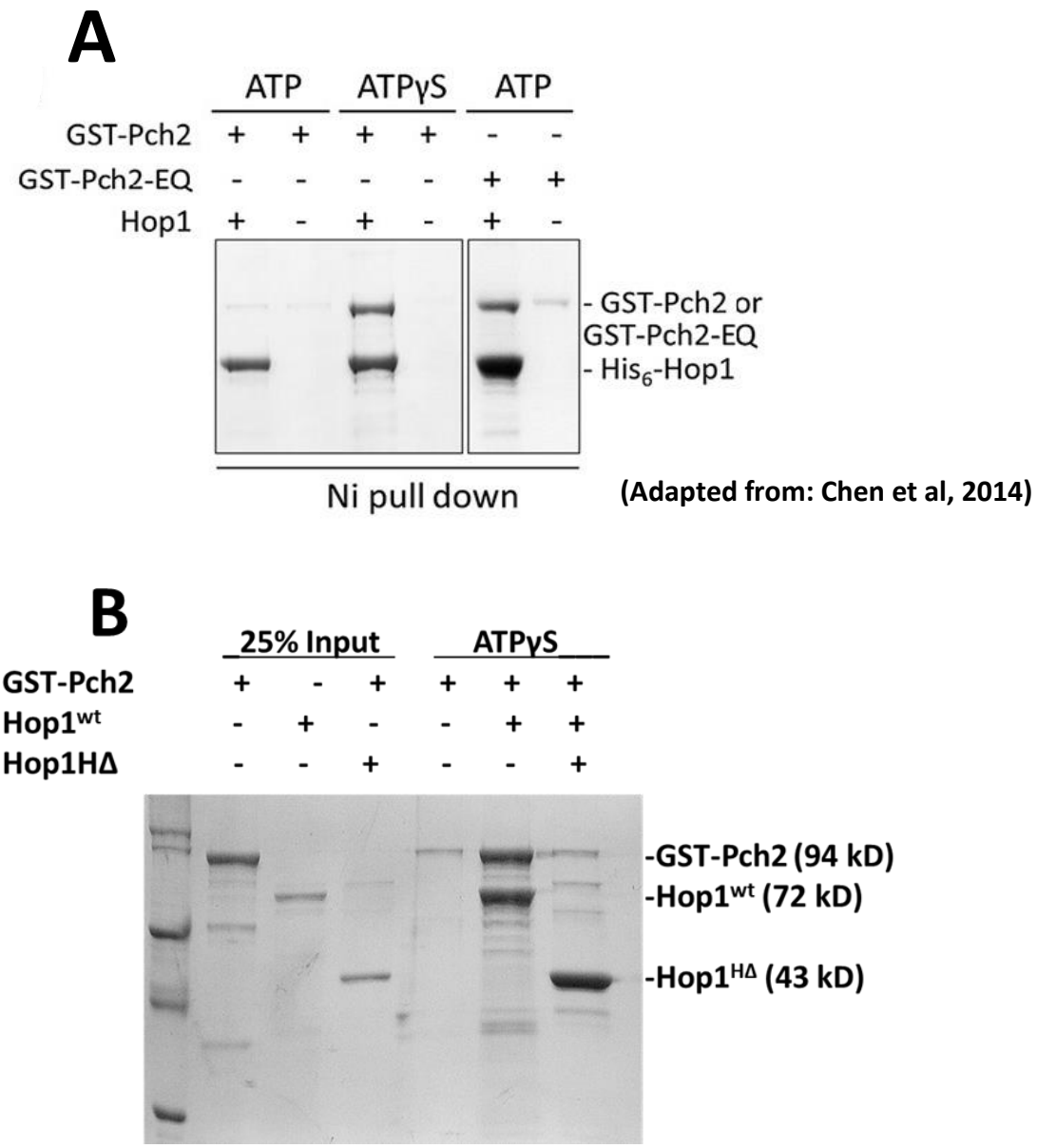

Figure 10. *In vitro* Ni-pull-down assays of (A) wild type Hop1 or (B) Hop1-HORMAΔ with GST-Pch2. 1.5 µg of purified wild type Hop1 or Hop1-HORMAΔ were incubated with 2.5 µg of purified GST-Pch2. Reactions in A include as indicated ATP and ATPγS at 200 µM, while panel B includes only ATP. GST-Pch2 binds 6His-Hop1 in the presence of ATPγS or when Pch2-EQ is substituted for wild type Pch2 which has a Walker B ATPase motif mutation which cannot hydrolyze ATP. Pch2 is then pulled down from solution using the 6His tag of Hop1. A strong interaction between Pch2 and wild type Hop1 is evident from panel A as well as panel B when ATPγS or Pch2-EQ are present, however Hop1-HORMAΔ does not appear to interact Pch2 as seen in the final lane of panel B. (Panel A adapted from Chen et al, 2014). See Materials and Methods for details.

Discussion

This project was successful in producing robust, reproducible expression and purification protocols for biologically active Hop1, Hop1-HORMAΔ, and GST-Pch2 proteins. Additionally my analysis of the Hop1-HORMAΔ mutant protein showed that the HORMA domain of Hop1 is necessary *in vitro* for interaction with Pch2. Attempts to purify point mutants of Hop1 revealed that the HORMA domain of Hop1 is likely very sensitive to mutations that may disrupt its structure. Results showed that very little Hop1- E124A and R41A remained soluble, since these residues are highly conserved and located on the surface of the HORMA domain, this could be explained as aberrant oligomerization of Hop1. Hop1 is known to form oligomers when bound to DNA and at very high concentrations can fold DNA into nucleosome-like structures *in vitro* (Khan et al. 2012, 2013). These highly conserved residues may be involved in preventing oligomerization when not in complex with DNA. Two additional mutants not mentioned in the results were also briefly investigated. The first removed the Zinc Finger domain of Hop1, Hop1-ZFΔ, and the other removed a predicted seatbelt region of the HORMA domain, Hop1-SBΔ, which has been found in other HORMA domain proteins to convert between open and closed conformations triggering binding or release of a substrate (Hara et al. 2010). These mutants were also insoluble and could not be purified lending strength to the conclusion that Hop1 may have little tolerance for HORMA domain mutations.

Agarose EMSA experiments revealed that Hop1 appeared to bind to DNA cooperatively. Although these experiments are not conclusive, a clearly non-linear decrease in mobility can be seen in wild type Hop1 titrations. This is in agreement with experiments performed by Kahn et

al. (2013) which previously demonstrated cooperative binding for Hop1. Khan et al. (2013) used filter binding assays combined with thorough kinetic analysis to determine that Hop1 binds cooperatively to dsDNA, as well as other DNA substrates. My 2.7 kb linear dsDNA substrate allows for sufficient cooperativity among Hop1 monomers and this might be critical to understand Pch2 function *in vivo*. Results obtained by Chen et al. (2014) show that upon displacement of Hop1 from DNA there appears to be no change in the DNA binding capability of the protein, however, much of the DNA substrate appears to become aggregated which prevents migration through the agarose gel in my experiments (Chen et al. 2014). This is seen as a bright fluorescent signal in wells of agarose EMSAs and appears to be partially embedded in the lower wall of the agarose wells (Figure 8). This was also seen in Chen et al. (2014) in experiments in which Pch2 displacement of Hop1 was observed in the presence of a 1.3 kb trap. This could be interpreted as re-organization of Hop1-DNA complexes by Pch2, resulting in tight, cooperatively bound Hop1-DNA complexes and the resulting excess Hop1 was not sufficient to retard the mobility of the free DNA.

When additional DNA is added however, the result was a complete reversal of this binding and Pch2 again reorganized Hop1 onto the smaller substrate, perhaps due to the necessity for monomers of Hop1 to bind more closely on the shorter substrate and increasing the likelihood of strong Hop1 cooperativity. This cooperativity may signal Pch2 to perform this organizing function, however a second possibility exists that Pch2 simply removes weakly bound Hop1 and that without any external influence Hop1 will freely associate with any DNA substrate and lack of Hop1 binding to the original 2.7 kb substrate is due to Pch2 being pre-associated with a large portion of this DNA species in solution. Pch2 remains loosely associated

throughout the reaction actively dislodging Hop1 monomers. When fresh DNA substrate is added to the reaction all available Pch2 quickly associates with the new DNA species preventing Hop1 from binding or actively removing bound Hop1. The availability of DNA binding sites is in excess of the available Hop1 and multiple Pch2 hexamers may be interacting Hop1 along a single piece of DNA substrate, this leaves a small portion of DNA with which Hop1 may interact. The aggregate DNA seen to be trapped in the wells would then be comparable to that seen in the presence excessively high Hop1 concentrations in vitro. Folding of DNA into nucleosome like substrates is a known characteristic of Hop1 (Khan et al. 2012).

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Appendix

Table of Contents

Hop1 Expression, General Protocol for 500 ml Culture Volume

Day 1

- Prepare 1 Liter of $LB^{AMP\text{-}Cam}$ (can be scaled up or down)
- Transform pET15b + Insert into competent Rossetta 2DE3 *E. coli* cells
- Plate on LB $^{Amp\text{-}Cam}$ agar and incubate O/N at $37^{O}C$

Day 2

- In the evening (~5 – 6 pm) inoculate 50 ml of $LB^{AMP\text{-}Cam}$ with a single large colony of 2DE3 *E. coli* transformant in a 100 ml Erlenmeyer flask and incubate O/N at $37^{O}C$ with shaking (~250 rpm)

Day 3

- In the morning (~8 – 9 am) inoculate 500 ml of LB $^{Amp\text{-}Cam}$ with 40 ml of the overnight culture and place back in the incubator at $37^{O}C$ with shaking (~250 rpm)
- Closely monitor the OD_{600}, when it reaches ~0.5 remove from the $37^{O}C$ incubator collect 500 µl for analysis
- Add 1 M IPTG to a concentration of 500 µm (250 µl) to induce expression
    - Spin the 500 µl sample at 3 krpm for 5 minutes and remove the supernatant
    - Freeze the sample at $-20^{O}C$
- Place the culture at $18^{O}C$ with shaking (~250 rpm) for 3-4 hours
    - Take another 500 µl sample after induction for analysis
- Remove the culture form the incubator and transfer to 2 x 250 ml centrifuge bottles and spin at 4 krpm, 20 minutes at $4^{O}C$
- Remove the supernatant and resuspend both pellets in 25 ml Lysis Buffer
    - 25 mM Tris pH 7.5, 350 mM NaCl, 10% glycerol, 15 mM imidazole
- Transfer the suspension to a 50 ml centrifuge bottle and spin as before
- Remove supernatant and resuspend the pellet in 8 – 10 ml Lysis Buffer
- Flash freeze the suspension by dropping into liquid nitrogen then transfer to $-80^{O}C$ until needed for purification
- Assay the samples taken pre and post induction for expression of the protein
- Add 20 µl SDS Loading Dye to each pellet and heat to 95 – $100^{O}C$ for ~5 minutes
- Load ~4 µl onto 10% PAGE and electrophorese at 90 – 120 volts for ~2 hours
- Stain.

Buffers , Media , ect..

- Lysis Buffer
    - Prepare 50 ml of two separate buffers, A and B
    - Buffer A : 25 mM Tris pH 7.5, 350 mM NaCl, 10% glycerol
    - Buffer B : 25 mM Tris pH 7.5, 350 mM NaCl, 10% glycerol, 15 mM imidazole
        - Mix varying volumes to achieve appropriate imidazole concentration, will be useful particularly during purification procedure

- $LB^{AMP\text{-}Cam}$
    - LB broth with 100 µg/ml Amp and 20 µg/ml Cam
    - 1 liter of LB is prepared by mixing 10 g Tryptone, 5 g NaCl, 5 g Yeast Extract in 1 Liter of ddH_2O
    - Autoclave and allow to cool then add Amp and Cam to the appropriate concentration
        - Typically Amp is 100 mg/ml stock (add 1 ml/L)
        - Cam is typically 20 mg/ml stock (add 1 ml/L)
- Cam
    - 20 mg/ml Chloramphenicol Stock is prepared in 100% EtOH and can be stored at $4^{O}C$, better if stored at $-20^{O}C$

Pch2 Induction (Galactose)

NOTE! *Plasmid pEAE307 in strain EAY33 will be used for Pch2-GST expression! All solutions need to be of biochem quality! (ie: Prepared in acid washed glass from high quality/purity reagents.)*

- This protocol should take approximately 9-10 days and require careful attention to details
- pEAE307 has two selection markers, URA3 and LEU2, LEU2 is a weakly selectable hypomorphic marker used for obtaining high copy number transformants to ensure the greatest amount of Pch2 protein is obtained from the procedure
- Remember to use dry ice when working with harvested cells and during purification to reduce protein degradation

Materials, media, and solutions

Note! *Refer to Preparation instructions for solution and media preparation and components!*

- 1 LEU DO plate
- 1 URA DO plate
- 100 ml LEU DO liquid medium
- 4 L SCGL-LEU DO liquid medium
- 200 ml 1 M Tris buffer pH 8.0
- 100 ml Lysis Buffer (100mM Tris pH 8.0, 400mM NaCl, 10% glycerol, 1mM EDTA)
- 80 ml 40% Lactic Acid (20 ml/L for SCGL medium only)
- 200ml 40% Galactose
    - ***Note!*** *DO NOT autoclave galactose, it breaks down at high temps! Filter in a 150ml bottle top filter!*
- 300 ml 40% Glucose (50 ml/L for glucose media)
- 240 ml 50% Glycerol (60 ml/L SCGL)
- 1.0 ml PEG/LiAc/TE (40% PEG 4000, 0.1M LiAc , 0.01M Tris pH 7.5, 0.01M EDTA)
- 4.5 g Amino Acid Mix (LEU DO)

Transformation of EAY33 with pEAE307

- Transform pEAE307 into EAY33 using sleazy plate transformation
    - Grow EAY33 on YPD until a single large colony can be harvested ~2 days if starting from frozen stock
    - Scoop up colony of yeast off the plate and spin down in 500ul ddH_2O and remove supernatant
    - Resuspend in 500ul LiAc/TE
    - Spin down and resuspend in 200ul LiAc/TE
        - Should be enough for 4 transformations
    - Transfer 50ul of cell suspension to tubes containing ~36ug of carrier ssDNA (10ul salmon sperm DNA) and 0.1-1.0ug transforming DNA (plasmid)
    - Mix by hand for ~5 seconds then add 300ul PEG/LiAc/TE
    - Incubate for 30 minutes at 30^{O}C with shaking
    - Incubate for 15 minutes in 42^{O}C water bath
    - Spin down and resuspend in 500ul of ddH_2O
    - Spin down and resuspend in 100ul of ddH_2O

- Plate cells on URA DO medium, 2-3 days
- Select ~4 single large colonies and streak to singles on URA DO medium, ~2 days
- Replica plate onto LEU DO medium, 2-3 days
  - Select a colony that grow on both URA DO and LEU DO media and make ~4 patches on URA DO for frozen stocks, ~2 days

Induction of GST-Pch2

- Inoculate a large LEU^+ colony in 5 ml starter culture of LEU DO liquid glucose medium and incubate O/N at 30C
- Use the 5 ml culture to inoculate a 100 ml culture of LEU DO glucose medium and grow overnight at 30C until saturation, sometime the following evening (~1 x 10^8 cells/ml)
- Take OD_{600} in a 1:50 dilution and use this to inoculate 4 x 1 L cultures of SCGL-LEU DO (3% glycerol, 2% Lactate) so that the initial OD is 0.05 at OD_{600}
- Incubate at 30C O/N when the OD_{600} reaches 0.6 - 0.75 (likely around early evening) take an 8ml sample of the uninduced culture and begin induction of the rest by adding 50 ml 40% Galactose per 1 L of culture medium
- After 16 – 18 hours of induction take a 5 ml post induction sample and harvest the rest of the cells
  - Spin down at 4krpm @ 4C in pre-weighed 1 L centrifuge bottles
  - Remove supernatant and weigh to determine grams of cells in pellet
  - Wash in 20ml lysis buffer and pellet again
  - Remove supernatant and resuspend in lysis buffer volume equal to grams in ml (ie: 20g cell pellet = 20ml lysis buffer)
  - Flash freeze suspension by dripping in liquid N_2 and store at -80C

Solutions and Reagents Preparation

- **40% Lactic Acid (w/v) 1.16 L**
    - Mix 225 grams of NaOH pellets in approximately 400 ml dH_2O let cool to 4^oC
    - Add NaOH to a two liter beaker containing 650 g 85% Lactate solution
        - **Note!** Extremely exothermic! Add NaOH very slowly-Lactate solution should be on a stirrer and bathed in ice!
    - Check pH it should be approximately 5.5 and adjust if necessary
    - Then bring volume to 1160 ml with dH_20 and autoclave
- **40% Galactose (w/v) 220 ml**
    - Mix 88 g of galactose (US Biological Cat# G-1030) into 100 ml ddH20 while gently heating with stirring
    - Remove from heat once galactose is in solution and adjust volume to 220 ml
    - Filter through a 150 ml bottle top filter
- **50% glycerol (v/v) 500 ml**
    - Add 250 ml glycerol stock (99.99%) to 250 ml ddH_2O and autoclave
- **Amino Acid Drop Out Mix**
    - Mix thoroughly and add at **0.87 g/liter**

Adenine	**800 mg**
Uracil	**800 mg**
Tryptophan	**800 mg**
Histidine	**800 mg**
Arginine	**800 mg**
Methionine	**800 mg**
Tyrosine	**1.2 g**
Lysine	**1.2 g**
Phenylalanine	**2.0 g**
Threonine	**8.0 g**

- **SCGL-LEU DO Media 1 Liter**
    - Add 7 grams of Yeast Nitrogen Base (US Biological Cat # Y2025, contains ammonium sulfate but lacks amino acids) to a 2 L E. flask
    - Add 0.87 g amino acid drop out mix (all amino acids except those that are required for plasmid selection)
    - Autoclave in 900 ml ddH2O
    - Add 60 ml of sterile 50% (v/v) glycerol and 50 ml of sterile 40% (w/v) lactate pH 5.5
        - **Note!** Glycerol and lactate were purchased from Sigma! Both stocks were autoclaved to sterilize!
- **Minimal Glucose Media 1 L**
    - Add 7 grams of Yeast Nitrogen Base (US Biological Cat # Y2025, contains ammonium sulfate but lacks amino acids) to a 2 L E. flask
    - Add 0.87 g amino acid drop out mix (all amino acids except those that are required for plasmid selection)
    - Autoclave in 950 ml ddH2O
    - Add 50 ml of sterile 40% glucose solution after autoclaving
- **Lysis Buffer 250 ml**
    - Final Concentration: 100 mM Tris pH 8.0, 400 mM NaCl, 10% glycerol, 1mM EDTA
        - 25 ml 1 M Tris pH 8.0
        - 20 ml 5 M NaCl
        - 50 ml 50% glycerol
        - 0.5 ml 0.5 M EDTA
        - 154.5 ml ddH_2O
- **LiAc/1X TE 1.0 ml**
    - 100ul 1M LiAc
    - 20ul 50X TE
    - 880ul ddH_2O
- **PEG 4000/LiAc/TE 1.0 ml**
    - 800ul 50% PEG 4000
    - 100ul 1M LiAc
    - 20ul 50X TE
    - 80ul ddH_2O

Purification of Hop1-His6

500 ml induction equivalent purification strategy

- All steps performed on Ice or at 4°C
- Each Buffer contains 0.7 µl/ml 10 mM BME and 5 µl/ml of 200 mM PMSF (1 mM final)
    - ***!EXCEPT!*** for lysis buffer used to equilibrate Ni-Beads.
    - **** Volume depends on quantity available***

Solutions:

Buffer A: 25mM Tris pH 7.5, 350mM NaCl, 10% Glycerol.

Buffer B: 25mM Tris pH 7.5, 350mM NaCl, 10% Glycerol, 1M Imidazole.

- **Lysis Buffer (22.5 ml, 15 mM Imidazole)**
    - 22.165 ml Buffer A
    - 0.335 ml Buffer B
    - 15.75 µl BME
    - 112.5 µl PMSF
- **Wash Buffer 1 (20 ml, 50 mM Imidazole)**
    - 19 ml Buffer A
    - 1 ml Buffer B
    - 14 µl BME
    - 100 µl PMSF
- **Elution Buffer (2.4 ml, 160 mM Imidazole)**
    - 2.02 ml Buffer A
    - 385 µl Buffer B
    - 1.68 µl BME
    - 12 µl PMSF
- **1.5 ml Ni-Agarose Beads**
    - Wash 3.0 ml 50% Bead slurry in 15 ml conical tube three times to remove storage solution (EtOH).
        1. Spin at 2 k for 1 min and settle on ice for 2 min, remove supernatant.
        2. Re-suspend in 4 ml of Lysis buffer w/o BME/PMSF/PI
        3. Repeat X 3

Protocol

1. Re-suspend cell pellet in a total volume of 6 ml Lysis Buffer and divide equally between 15 ml conical tubes.
2. Re-pellet suspension at 4 Krpm for 20 min.
3. Re-suspend again by bringing to a total volume of 6 ml Lysis buffer.
4. Clean sonication needle with water and EtOH and allow to dry.
5. Sonicate samples at 30% duty cycle, micro-tip power 5, 1 min pulse, 2 min rest on ice, and an additional 1 min pulse.
    a. **Perform this on ICE to prevent sample heating and protein degradation!**
6. Transfer to high speed centrifuge bottles and centrifuge at 15.5 Krpm for 30 min at 4°C.
7. Transfer supernatant to Ni-Agarose beads in a 15 ml conical tube and place on agitator for 2 hours at 4°C.
8. Centrifuge at 3 krpm for 2 min at 4°C and settle on ice for 5 min.
9. Remove supernatant (Flow Through) and re-suspend beads in 4 ml Wash Buffer 1 and transfer slurry to a 5 ml gravity column.
10. Continue to wash with Wash Buffer 1 until all is used.
11. Add Elution Buffer 1.
12. Bradford 2 ul to determine protein concentration of crude lysate, lysate supernatant, flow through, wash 1, wash 2, and elution.
13. Analyze all of the above on a 10% SDS-PAGE (8 µl of sample to 5 µl of SDS loading buffer, load 2 µl on gel)
14. Dialyze 2-3 hrs in 500 ml of [25 mM Tris pH 7.5, 300 mM NaCl, 10% glycerol, 10 mM BME, 1 mM PMSF].
15. Transfer to 1 L of [25 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol, 10 mM BME, 1 mM PMSF] and dialyze overnight.
16. Collect sample and concentrate in 2 ml 30 kd centrifuge concentration column at 4^{O}C.
    a. Load sample on column and spin at 7.5 Krpm for 15 minutes or until volume is reduced ~ 2.5 – 3.5 fold.
    b. Perform collection spin for 5 min at 2.5 Krpm.
    c. Determine final concentration by Bradford.
    d. Aliquot (~20 µl/ea.) and flash freeze in liquid nitrogen.
    e. Store at -80^{O}C.

Modified Purification of Hop1-His6

500 ml induction equivalent purification strategy

- All steps performed on Ice or at 4°C
- Each Buffer contains 0.7 µl/ml 10 mM BME and 5 µl/ml of 200 mM PMSF (1 mM final)
  - ***!EXCEPT!*** for lysis buffer used to equilibrate Ni-Beads.
  - **** Volume depends on quantity available***

Solutions:

Buffer A: 25mM Tris pH 7.5, 350mM NaCl, 10% Glycerol.

Buffer B: 25mM Tris pH 7.5, 350mM NaCl, 10% Glycerol, 1M Imidazole.

- **Lysis Buffer (22.5 ml, 15 mM Imidazole)**
  - 22.165 ml Buffer A
  - 0.335 ml Buffer B
  - 15.75 µl BME
  - 112.5 µl PMSF
- **Wash Buffer 1 (20 ml, 50 mM Imidazole)**
  - 19 ml Buffer A
  - 1 ml Buffer B
  - 14 µl BME
  - 100 µl PMSF
- **Wash Buffer 2 (2.4 ml, 80 mM Imidazole)**
  - 2.21 ml Buffer A
  - 190 µl Buffer B
  - 1.68 µl BME
  - 12 µl PMSF
- **Elution Buffer (2.4 ml, 160 mM Imidazole)**
  - 2.02 ml Buffer A
  - 385 µl Buffer B
  - 1.68 µl BME
  - 12 µl PMSF
- **1.5 ml Ni-Agarose Beads**
  - Wash 3.0 ml 50% Bead slurry in 15 ml conical tube three times to remove storage solution (EtOH).
    4. Spin at 2 k for 1 min and settle on ice for 2 min, remove supernatant.
    5. Re-suspend in 4 ml of Lysis buffer w/o BME/PMSF/PI
    6. Repeat X 3

Protocol

1. Re-suspend cell pellet in a total volume of 6 ml Lysis Buffer and divide equally between 15 ml conical tubes.
2. Re-pellet suspension at 4 Krpm for 20 min.
3. Re-suspend again by bringing to a total volume of 6 ml Lysis buffer.
4. Clean sonication needle with water and EtOH and allow to dry.
5. Sonicate samples at 30% duty cycle, micro-tip power 5, 1 min pulse, 2 min rest on ice, and an additional 1 min pulse.
    a. **Perform this on ICE to prevent sample heating and protein degradation!**
6. Transfer to high speed centrifuge bottles and centrifuge at 15.5 Krpm for 30 min at 4°C.
7. Transfer supernatant to Ni-Agarose beads in a 15 ml conical tube and place on agitator for 2 hours at 4°C.
8. Centrifuge at 3 krpm for 2 min at 4°C and settle on ice for 5 min.
9. Remove supernatant (Flow Through) and re-suspend beads in 4 ml Wash Buffer 1 and transfer slurry to a 5 ml gravity column.
10. Continue to wash with Wash Buffer 1 until all is used.
11. Add Wash Buffer 2.
12. Add Elution Buffer 1.
13. Analyze all of the above on a 10% SDS-PAGE (8 µl of sample to 5 µl of SDS loading buffer, load 2 µl on gel)
14. Collect sample and concentrate in 2 ml 30 kd centrifuge concentration column at 4^{O}C.
    a. Load sample on column and spin at 7.5 Krpm for 15 minutes or until volume is reduced ~ 2.5 – 3.5 fold.
    b. Perform collection spin for 5 min at 2.5 Krpm.
    c. Determine final concentration by Bradford.
    d. Aliquot (~20 µl/ea.) and flash freeze in liquid nitrogen.
    e. Store at -80^{O}C.

GST-Pch2 Two-Step Purification Protocol

Day 1

Note! Only during the ***Day 1*** – *Lysis* phase of the purification can metals or non-sterile equipment be used. Beginning ***Day 1*** - *Glass Column Prep* ***ALL*** equipment and reagents ***MUST BE*** of biochemical quality.

Materials and Reagents

- ~0.2 – 0.3 lbs dry ice, in an ice bucket, preferably dry
- 10 g frozen yeast pellets from Pch2-GST Induction
- Plastic wrap
- Large spatula/scoopula washed 3 - 4 x in ddH_2O (metal is fine for use here)
- 50 ml conical tube (sterile plastic / Biochem quality)
- Braun Coffee Grinder
- Hammer
- 10 ml Glass Filter Column
- Filter column cap with working syringe
- 20 ml Lysis Buffer (500 mM NaCl, 10 mM BME, 1 mM PMSF)

Protocol

- Pulverize the dry ice in ice bucket using the hammer until it is a fine white powder
- Precool spatula, and 50 ml tube on the dry ice
- Add dry ice to the lid and the bottom of the coffee grinder and allow to cool
- While equipment is cooling, retrieve frozen yeast pellets from the freezer and place plastic wrap over the tray of the scales
- Weigh out approximately 10 g of yeast pellets on the plastic wrap
- Add enough dry ice to the coffee grinder to cover the bottom and most of the blades
- Layer yeast on top of dry ice
- Add another layer of dry ice over the yeast
    - Remember to keep the lid iced as well during this process
- Grind for 2 minutes with "rough banging" of the grinder on the bench to dislodge contents and prevent accumulation of lysate
    - Grind for 30 seconds, stop pounding the grinder and power down for ~5 seconds then resume grinding and pounding.
        - ❖ Repeat for 2 minutes, can use a lab book or towels to absorb the shook from the pounding
- Scoop as much of the lysate as possible into the 50ml tube and store O/N at $^{-}20^{O}C$
    - The dry ice sublimes during the night
    - Do not tighten the lid on the tube completely, this will allow the CO_2 to escape
- Prepare class column for use on day 2
    - Wash 10 ml glass filter column with ddH_2O 3 – 4 times
    - Ensure while washing that the tubing does not leak, is not clogged, and that the syringe works and can prime the syphon

        - Also, make sure that no bubbles accumulate at the bottom column in the rubber hose fitting, if bubbles are present flush them out with ddH_2O before washing in Lysis Buffer
    - Complete rinsing by leaving a small amount of ddH_2O in the column and the rubber tubing and attach the column to the pump in the 4^OC room
    - Allow the pump to withdraw nearly all of the ddH_2O then stop the flow and fill the column with Lysis Buffer to the top and attach the cap
    - Place the aspirating tube into the remainder of the lysis buffer and restart the pump
    - Use the syringe to begin pulling buffer into the column
    - Once the reservoir of buffer is drained off, stop the pump and clamp the tubes to prevent draining of the column
        - Can store at 4^OC until needed

Day 2

Note! Prepare all buffers before beginning and store them at 4^OC. Add BME and PMSF just before using, ***DO NOT*** add PMSF to the PBE94 Elution Buffer, the next step is for thrombin cleavage of the GST tag. The wash for the PBE94 column is also reduced in PMSF by a factor of 10.
Pay special attention to how quickly the GSH column drains and aim for ~15 – 20 ml per hour. ***Do not let the columns dry with protein in them!***
REUSE the PBE94 Beads! They are no longer being commercially produced.

Materials

- 2 x 50 ml centrifuge bottles
- 5 ml Gravity column
- Good supply of:
    - 1.5 ml microfuge tubes
    - 0.2 ml PCR tubes
    - 15 ml Falcon tubes

Reagents

❖ **Glutathione Agarose Beads**

- 2 ml Glutathione agarose beads 50% slurry (1 ml of beads)
- 30 ml Lysis Buffer **(500 mM NaCl** 100 mM Tris pH 8.0, 10% glycerol, 1 mM EDTA, , 10 mM BME, 1 mM PMSF)
- 20 ml Wash Buffer **(160 mM NaCl**, 100 mM Tris pH 8.0, 10% glycerol, 1 mM EDTA, 10 mM BME, 1 mM PMSF)
- 10 ml Elution Buffer **(160 mM NaCl, 20 mM glutathione,** 100 mM Tris pH 8.0, 10% glycerol, 1 mM EDTA, 10 mM BME, 1 mM PMSF)

❖ **PBE94**

- 0.5 ml PBE94 Beads **(Stored @ 1 M NaCl, 20% EtOH,** 100 mM Tris pH 8.0, 10% glycerol)
- 12 ml PBE94 Wash Buffer **(1 M NaCl, 0.1 mM PMSF,** 100 mM Tris pH 8.0, 10% glycerol, 1 mM EDTA, 10 mM BME)

- 4 ml Wash Buffer **(<u>200 mM NaCl, 0.1 mM PMSF,</u>** 100 mM Tris pH 8.0, 10% glycerol, 1 mM EDTA, 10 mM BME)
- 1.2 ml Elution Buffer **(<u>500 mM NaCl, 0 mM PMSF,</u>** 100 mM Tris pH 8.0, 10% glycerol, 1 mM EDTA, 10 mM BME)
- 3 ml Dilution Buffer **(<u>0 mM NaCl, 0 mM PMSF,</u>** 100 mM Tris pH 8.0, 10% glycerol, 1 mM EDTA, 10 mM BME)
- 3 ml 20% EtOH in ddH_2O

<u>Protocol</u>

- Wash 1 ml Glutathione beads with 4-5 volumes Lysis Buffer (beads are 50% slurry, start with 2 ml)
    - Centrifuge at 4^{O}C, 1 minute, 600rpm
    - Wash 3 times
        - Can use centrifuge in the fly room
- Wash 500ul PBE94 (REUSE!) with 1.5ml PBE94 Wash Buffer (1 M NaCl) then again with 4ml Wash Buffer (160 mM NaCl)
    - Leave in enough Wash Buffer to prevent drying and place at 4^{O}C for later use
- Let lysate warm on ice for about 5 minutes and resuspend in 15 ml Lysis Buffer
    - Spin at 17krpm, 4^{O}C, 35 minutes in 50 ml centrifuge bottles
    - Remove lipids from surface of the supernatant by sticking a pipet tip in and swirling around the surface of the supernatant then discarding the tip, do not attempt to aspirate the lipids
    - Transfer supernatant to clean tube and take 10 µl sample for analysis
- Add beads to supernatant and place on rotator at 4^{O}C for 2 hours and seal the cap with parafilm
    - Collect 10 µl sample after incubation
- Transfer to glass column and drain flow through
    - 1X power on Pump set dial to 4 = 15-20 ml per hour
- Wash with 15 ml Wash Buffer
    - Collect 10 µl for analysis
- Elute with 10 ml elution buffer with 20 mM glutathione
    - collect 5 fractions of 2 ml each
- Bradford all fractions (10 -15 µl) save 10 µl for gel analysis
- Pool fractions for PBE94 2nd purification
- Load pooled fractions on gravity column with PBE94 and collect 10 µl of the flow through
- Wash with 4 ml Wash Buffer (200 mM NaCl, 10 mM BME, **0.1 mM PMSF**)
    - Collect 10 µl for analysis
- Elute with 1.2 ml Elution Buffer (500 mM NaCl, 10 mM BME, **0 mM PMSF**)
    - Collect 1 fraction
    - Save 10 µl for gel analysis
- Dilute PBE94 fraction with 1.5 volumes of no salt buffer (~ 3 ml)

- **!!!! Do this step slowly with constant stirring and firm pipet control. This can cause the protein to precipitate!!**

! This is the stopping point for Pch2-GST ! For removal of the GST tag continue with the following steps and complete on the 3rd day.

! ENSURE TOTAL PROTEIN IS >500 µg BEFORE CONTINUING!

- Add 1/20th total volume thrombin T7 and place on 4°C rotator O/N
- Prepare and wash 0.4 ml PBE94 column for use on day 3.
- Clean the PBE94 resin for reuse by vortex or pipetting up and down in 3 – 4 volumes PBE94 Wash Buffer (1 M NaCl, 10 mM BME, **0.1 mM PMSF**)
    - Remove wash by spinning down at 600 rpms
        - Repeat 3 – 4 times
        - If not performing thrombin cleavage resuspend PBE94 in 20% EtOH solution and store at 4°C
        - If continuing to proteolysis:
            - Transfer resin to clean gravity column and run 4 ml Wash Buffer (160 mM NaCl, 10 mM BME, 1 mM PMSF) through the column
            - Leave enough wash buffer to keep the resin from drying store O/N at 4OC

Day 3

- Run thrombin incubation through PBE94 column and wash with 3ml 200mM NaCl
- Elute with 0.5ml 500mM NaCl
- Incubate with 15ul glutathione resin, 1 hour 2 times.
- Run through a small gravity column, collect flow through, aliquot and freeze.
- **Run all samples on PAGE**

GST-Pch2^{Wt} Purification (10g Yeast Pellet)

Reagents

- **Glutathione Agarose Beads** (**2 ml GST beads**, 4 ml 50% slurry)
  - ~500 ml **Lysis Buffer/PBE94 Elution Buffer (500 mM NaCl**, 100 mM Tris pH 8.0, 10% glycerol, 1 mM EDTA,
    10 mM BME, 1 mM PMSF)
  - 75 ml **Wash Buffer (150 mM NaCl,** 100 mM Tris pH 8.0, 10% glycerol, 1 mM EDTA, 10 mM BME, 1 mM PMSF)
  - 10 ml **Elution Buffer (150 mM NaCl, 40 mM GST, 0.2% Tween-20,** 100 mM Tris pH 8.0, 10% glycerol, 1 mM EDTA, 10 mM BME, 1 mM PMSF)
  - ***Optional: PBE94*** *(**1 ml PBE94** final bead volume)*
  - *15 ml **Wash Buffer (1 M NaCl,** 100 mM Tris pH 8.0, 10% glycerol, 1 mM EDTA, 10 mM BME, 1 mM PMSF)*
  - *8 ml **Wash Buffer (200 mM NaCl,** 100 mM Tris pH 8.0, 10% glycerol, 1 mM EDTA, 10 mM BME, 1 mM PMSF)*
  - *~8 ml **Dilution Buffer (0 mM NaCl**, 100 mM Tris pH 8.0, 10% glycerol, 1 mM EDTA, 10 mM BME, 1 mM PMSF)*

Protocol

- **Glutathione**
  - Wash **2 ml GST Beads** with **8 ml Lysis Buffer (500 mM NaCl)**, 3 times
    - Equilibrate GST Beads with **25ml Wash Buffer (150 mM NaCl)**
      - Perform **wash and equilibration** in 10 ml glass vacuum column
  - Resuspend **Lysate** in **2:1 (vol : wt) Lysis Buffer (500 mM NaCl)**
    - ***Ex**: Yeast pellet is 50 g of a 1:1 slurry of yeast cells and buffer, add lysis buffer to a final volume of 75 ml.*
    - Spin crude lysate for 35 minutes at 17krpm and 4^{O}C, in 50 ml centrifuge bottles (remove lipids with pipet tip, do not aspirate lipids)
    - Transfer to glass column and drain the flow through
      - Run the flow through over the column 2 – 3 times
  - Wash three (*or more*) times with 15 ml **Wash Buffer (150 mM NaCl)** until OD_{280} reaches base line
  - Elute 2 times with 2x resin volumes of **Elution buffer (150 mM NaCl, 40 mM GST, 0.2% Tween-20)**
    - *4 ml for 2 ml of GST-Agarose.*
    - ***Examine all washes, flow through, lysate, and elution samples** on SDS-PAGE and Bradford to determine if PBE94 clean up will be required.*
    - ***If NOT**, then concentrate on 50 kD centrifuge colums and store as 10 µl aliquotes in the -80^{O}C*

  - ***Optional: PBE94***
    - *Wash **1 ml PBE94** with 3 ml **Wash Buffer (1 M NaCl)** then with 8 ml **Wash Buffer (150 mM NaCl)***
    - *Load on **PBE94 gravity column***
    - *Wash with 8 ml **Wash Buffer (200 mM NaCl)***
    - *Elute with 4.8 ml **Elution Buffer (500 mM NaCl)***
    - *Dilute with 1.5 volumes **Dilution Buffer (0 mM NaCl)** ~7.2 ml*
    - *Concentrate on **Ultra-15 centrifuge column (50 kD)** and aliquot*

Agarose DNA binding assay of Hop1^{Wt} and GST-Pch2^{WT}

- 25 µl per Hop1 rxn
- 40 µl per Hop1+Pch2 rxn
- 75 mM NaCl
- 2.7 kb pUC18 linear DNA substrate (60 ng / rxn).
- GST-Pch2^{WT} (90.7 ng / pmol)
- Hop1^{Wt} 71.50 ng / pmol)
- Hop1$^{H\Delta}$ 1.70 ng/µl ≈ 1 pM *(42.54 ng / pmol)*
- **10X Agarose DNA Binding Buffer**: 150 mM Tris pH 7.5, 450 mM NaCl, 10% glycerol, 0.1 mM EDTA, 400 µg/ml BSA.
    - **Final Rxn Conditions For Hop1 DNA Binding**: *20 mM Tris pH 7.5, 75 mM NaCl, 10% glycerol, 2 mM $MgCl_2$, 0.01 mM EDTA, 32 mM Imidazole, 40 µg/ml BSA, 0.1 mM DTT*. ***(+/- 200 µM Nt's)***

Hop1^{WT}: 03/31/15 prep 0.38 µg/µl in 350 mM NaCl, 160 mM Imidazole and 10% glycerol

GST-Pch2^{WT} : 6/23/15 prep 0.92 µg/µl in 150 mM NaCl, 10% glycerol, 40 mM GST

- *8 pmols / 1.5 µl = 0.79 µl protein stock / 0.71 µl Storage buffer*

Rxn Master Mix (per 1 rxn)	**Mock & 8 pmols Pch2-GST Rxn Mix (per rxn)**
- 2.5 µl 10X Agarose Rxn Buffer	- 1.5 µl 10X Pch2 Rxn Buffer
- 3.5 µl 50% Glycerol	- 1.3 µl 50% glycerol
- 2 µl 25 mM MgCl2	- 1.5 µl 25 mM MgCl2
- 0.5 µl 10 mM ATP	- 0.3 µl 10 mM ATP
- 0.25 µl 10 mM DTT	- 0.15 µl 10 mM DTT
- 0.35 µl linear pUC19 (171.2 ng/µl)	- *0.0 µl DNA*
- 10.9 µl ddH2O	- 3.25 µl ddH2O
- 5 µl of Hop1 Dilution	- 7 µl Pch2-GST dilution
- ***25 µl Total Volume***	- ***15 µl Total***

- Incubate at 30^{o}C for 10 min after Hop1^{Wt} Rxn is completed
- Place on ice while adding Pch2 Rxn Mix and return to 30^{o}C for an additional 15 min
- Remove to ice and electrophorese as follows
    - Load on **0.7%** agarose gel and migrate at 40 volts for 2 hours at 4 ^{o}C