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New Insights into the Assembly Mechanism of an RNA Polymerase III-Specific Transcription Complex on a *Drosophila* U6 snRNA Gene Promoter

A dissertation submitted in partial satisfaction of the
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by

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The Dissertation of Ann Marie Moreno is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

This dissertation is dedicated to every person who ever believed in me, especially my parents, John and Mary Moreno and my husband, Scott Hurlburt. I thank God for His many blessings, for giving me a love and appreciation for His creation, and for giving me a clear purpose to take those blessings and bless others.

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Chapter 1, in full, is a reprint of a manuscript submitted for publication. SNAPc interacts with Bdp1 on a U6, but not a U1, snRNA gene promoter to establish a stable protein-DNA complex with TBP. Verma, N., Hurlburt, A.M., Phan, P., Wolfe, A., Kim, M., Kang, Y.S., Kang, J.J., and Stumph, W.E. The dissertation author, designated above as Hurlburt, A.M., was a primary researcher and co-first author of this paper.

The material in Chapter 2, is not intended to be submitted for publication at the moment. The dissertation author was the primary researcher and author of this chapter.

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ABSTRACT OF THE DISSERTATION

New Insights into the Assembly Mechanism of an RNA Polymerase III-Specific Transcription Complex on a *Drosophila* U6 snRNA Gene Promoter

by

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In metazoans, the genes that code for the U6 small nuclear RNA (snRNA) have promoters that consist of a proximal sequence element (PSE) and a TATA box. The PSE is recognized by the multi-subunit small nuclear RNA activating protein complex (SNAPc). SNAPc forms a complex on the U6 promoter with TFIIIB, an RNA polymerase III-specific

general transcription factor that includes TBP, Brf1 (Brf2 in vertebrates), and Bdp1. Here we show that, in the fruit fly *Drosophila melanogaster*, DmSNAPc directly recruits Bdp1 to the U6 promoter. We also demonstrate that an 87-residue region of Bdp1 is sufficient for this recruitment and is, furthermore, sufficient to recruit TBP to the TATA box. The non-conserved N-terminal tail of TBP also plays a role in stabilizing TBP incorporation into the DmSNAPc-Bdp1 complex. While Bdp1 recruitment by DmSNAPc is independent of the presence of TBP, Brf1, or a TATA box, it does require that DmSNAPc be bound to a U6 gene PSE rather than a PSE derived from a U1 gene (which is transcribed by RNA polymerase II). We also confirmed that Brf1 is present at U6 snRNA genes but not at U1 genes. These findings further develop the concept that DmSNAPc adopts different conformations upon binding U6 and U1 gene PSEs, and that these different DmSNAPc conformations lead to the subsequent recruitment of distinct general transcription factors and RNA polymerases for U6 and U1 gene transcription.

GENERAL INTRODUCTION

Characteristics of snRNA promoter regions and the RNA polymerases they recruit

Small nuclear RNAs (snRNAs) are non-protein coding RNAs responsible for pre-mRNA splicing and the processing of several other types of RNA [Kass et al., 1990; Guthrie, 1991; Sharp, 1994]. The snRNA genes are of unique interest because they can be classified into two categories. The first class is transcribed by RNA polymerase II (Pol II) and include U1, U2, U4 and U5 snRNA genes while the second class is transcribed by RNA polymerase III (Pol III) and includes U6 and 7SK RNAs [Dahlberg and Lund, 1988; Parry et al., 1989; Lobo and Hernandez, 1994; Hernandez, 2001]. This difference in polymerase recruitment is intriguing because both classes have a similar promoter structure and are more similar to each other than they are to the promoters of any other genes transcribed by Pol II or Pol III [Dahlberg and Lund, 1988; Lobo and Hernandez, 1994].

Figure G.1 depicts the promoter regions of snRNAs from vertebrates, plants and *Drosophila*, which is the model system for the research in this dissertation. The snRNA promoters of various higher eukaryotes all display a promoter element that is 40-75 base pairs upstream of the transcriptional start site [Das et al., 1987; Dahlberg and Lund, 1988; Parry et al., 1989; Zamrod et al., 1993]. This element is called the proximal sequence element (PSE) and is required for transcription initiation of snRNA genes [Zamrod et al., 1993; McNamara-Schroeder et al., 2001; Lai et al., 2005]. Vertebrate snRNA genes transcribed by Pol II have a single promoter element while those transcribed by Pol III have a PSE as well as a conserved TATA box, 20-30 base pairs upstream of the transcriptional start site [Zamrod et al., 1993]. This difference in structure suggested that the TATA region may be required for polymerase specificity in vertebrates. Several groups tested this theory by either mutating the TATA region to an unrelated sequence or by placing the TATA sequence within the promoter of Pol

II transcribing genes. As a result, Pol III recruitment was either diminished or established, respectively [Mattaj et al., 1988; Lobo and Hernandez, 1989]. This suggests that the TATA region is required for the recruitment of Pol III over the recruitment of Pol II in vertebrates. In plants, it was found that the polymerase specificity is determined by the spacing of the upstream element (USE) from the TATA box, (32-36 nucleotide spacing for Pol II versus 23-26 nucleotides for Pol III) [Waibel and Filipowicz, 1990]. In both vertebrates and plants, mutating or interchanging the PSE/USE between gene classes had little to no effect on polymerase specificity [Lobo et al., 1989; Waibel and Filipowicz, 1990].

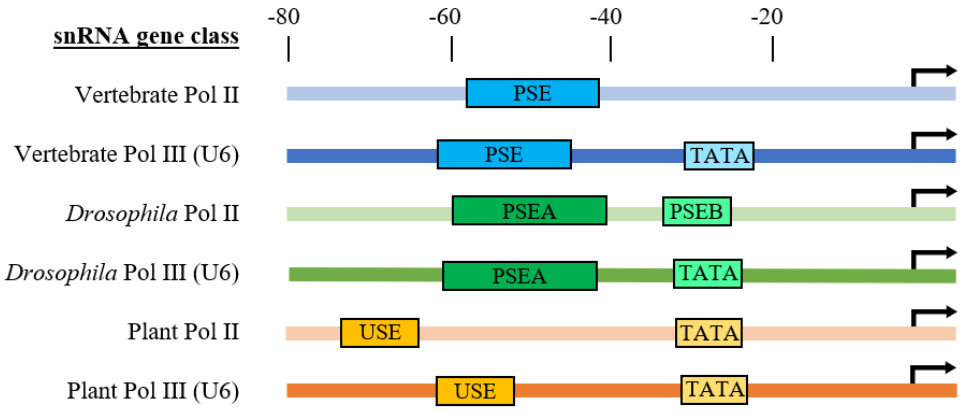

Figure G.1: Comparison of Pol II and Pol III snRNA promoter regions in various organisms. The snRNA gene promoter structure is similar throughout various organisms. Each contains either a proximal sequence element (PSE/A) or an upstream element (USE). These elements are 40-75 base pairs upstream of the transcription start site (indicated with a black arrow). In addition, most of the genes also have either a TATA box or a secondary proximal sequence element (PSEB). These elements are 20-30 base pairs upstream of the transcription start site.

However, the same does not appear to be true for *Drosophila* snRNA genes. As depicted in Figure G.2, *Drosophila* have a conserved promoter element referred to as the proximal sequence element A (PSEA) which is 21 nucleotides in length [Das et al., 1987; Zamrod et al., 1993; Hernandez et al., 2007]. Furthermore, in flies there is a second promoter element about 20-30 nucleotides upstream of the transcriptional start site which is distinct

between the two classes [Zamrod et al., 1993; Hernandez et al., 2007; Hung and Stumph, 2011]. In the snRNA genes transcribed by Pol II, this 8-nucleotide element is termed the proximal sequence element B (PSEB) and is conserved at 8 nucleotides away from the PSEA [Zamrod et al., 1993; Hernandez et al., 2007; Hung and Stumph, 2011]. In genes transcribed by RNA polymerase III, the promoters contain a canonical TATA box which is 12 nucleotides away from the PSEA [Das et al., 1987]. Research investigating these promoter elements found that the conserved *Drosophila* PSEA is the primary determinant of RNA polymerase specificity, as opposed to the presence or absence of the TATA box.

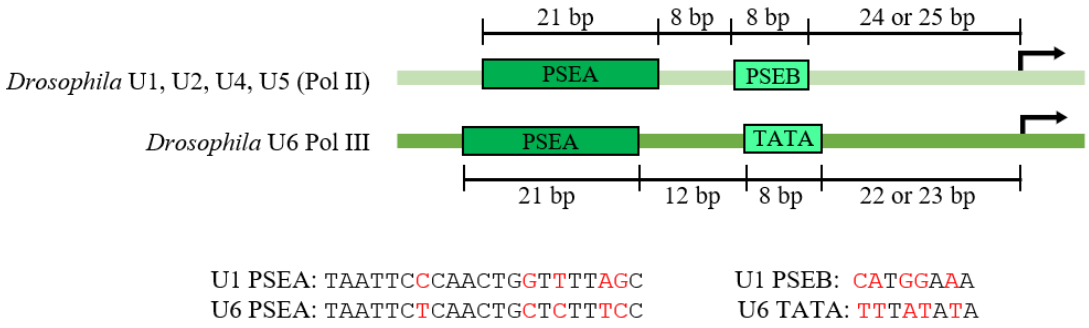

Figure G.2: Comparison of Pol II and Pol III snRNA promoter regions in *Drosophila*. Structural comparison of the promoter regions of the Pol II and Pol III gene class along with sequence comparison of the specific genes used in these studies (U1:95Ca and U6:96Ab). Nucleotide differences are indicated in red.

This research included both *in vitro* and *in vivo* studies in *Drosophila melanogaster* involving switching between the U1 PSEA and the U6 PSEA, which differ by 5 nucleotides (see sequence in Figure G.2). *In vitro*, the switch produced a result where if a U1 PSEA was present, the gene was transcribed by Pol II and if the U6 PSEA was present, the gene was transcribed by Pol III [Jensen et al., 1998, McNamara-Schroeder et al., 2001]. On the contrary, while switching the PSEB and TATA box or altering the spacing of the promoter elements reduced transcription, it did not affect polymerase specificity [Zamrod et al., 1993; Su et al., 1997; Lai et al., 2005]. *In vivo*, switching the PSEA sequences resulted in a nearly

complete loss of transcription activity [McNamara-Schroeder et al., 2001; Lai et al., 2005]. Therefore, though there is a difference of only five nucleotides, it is clear that this region is required for the recruitment of either Pol II or Pol III for the initiation of transcription on snRNA genes. This led to a further investigation of the transcription factors that are recruited to this region.

The components of the snRNA transcriptional pre-initiation complex SNAPc:

In order for transcription to occur, several transcription factors are required to assemble on the snRNA promoters which ultimately recruit the appropriate polymerase for the initiation of transcription. The PSE-binding protein or small nuclear RNA activating protein complex (SNAPc) is a transcription factor that binds to the PSEA region of the snRNA promoters and nucleates the assembly of the pre-initiation complex (PIC). SNAPc is required for the transcription of snRNA genes U1, U2, U4, and U5 by Pol II and U6 by Pol III [Sadowski et al., 1993; Goomer et al., 1994; Yoon et al., 1995]. In humans, this protein is comprised of five major subunits named for their apparent molecular weight; SNAP19, SNAP43, SNAP45, SNAP50, and SNAP190 [Bai et al., 1996; Henry et al., 1996; Sadowski et al., 1996; Yoon and Roeder, 1996; Henry et al., 1998].

In *Drosophila melanogaster* (Dm), DmSNAPc is comprised of three highly conserved subunits homologous to human SNAP43, SNAP50 and SNAP190 subunits [Wang and Stumph, 1998; Li et al., 2004]. The PSEA region has a 5’ end which is conserved in insects and a 3’ end which is less conserved [Hernandez et al., 2007; Kang et al., 2014]. Site-specific protein-DNA photo-cross-linking research produced an architectural model (Figure G.3) indicating that DmSNAP190 contacts most of the PSEA sequence while DmSNAP43 and

DmSNAP50 only interact with the 3' half of the PSEA and sequences further downstream of that [Wang and Stumph, 1998, Li et al., 2004, Lai et al., 2005]. In addition, the data determined that the PSEA sequence acts as a differential allosteric effector of the conformation of DmSNAPc (Figure G.3). For example, DmSNAP43 cross-links to DNA up to 20 nucleotides downstream of a U1 PSEA, but only 5 nucleotides downstream of a U6 PSEA [Wang and Stumph, 1998, Li et al., 2004, Lai et al., 2005].

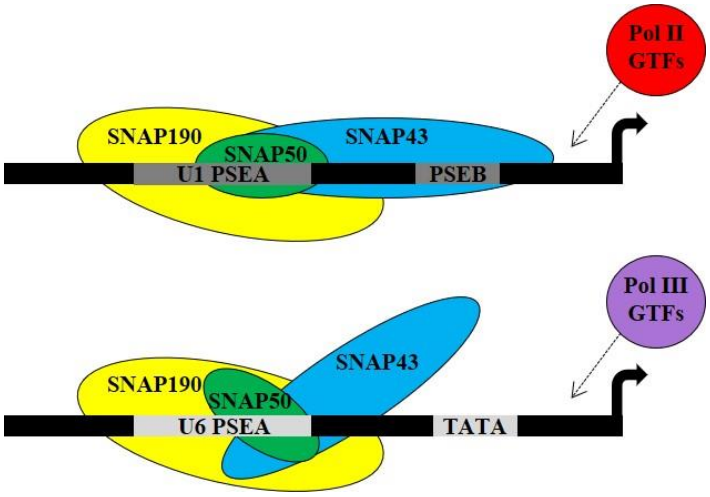

Figure G.3: Working model of DmSNAPc conformation when bound to either a U1 PSEA or a U6 PSEA. This model shows differences in the interactions between DmSNAPc and the PSEA sequences as determined by protein-DNA photo-cross-linking data [Wang and Stumph, 1998, Li et al., 2004, Lai et al., 2005]. It suggests that different conformations recruit different general transcription factors that in turn will recruit different RNA polymerases.

General Transcription Factors

Messenger RNAs transcribed by Pol II have a well-studied PIC that is known to include general transcription factors (GTFs) TFIIA, TFIIB, TBP, TFIIE, TFIIF and TFIIH [Kuhlman et al., 1999]. Each GTF plays a role in preparing the DNA for transcription and recruiting the appropriate polymerase. These factors are also involved in the Pol II transcription of human U1 snRNA genes [Kuhlman et al., 1999], but their involvement in *D.*

melanogaster U1 snRNA gene transcription has not been studied, with the exception of TBP which is required despite the lack of a TATA box [Zamrod et al., 1993; Barakat and Stumph, 2008].

GTFs involved in the transcription of genes by Pol III vary with the type of promoter structure. In higher eukaryotes, type I promoters (such as 5S rRNA genes) require TFIIIA, TFIIIB, and TFIIIC. Type II promoters (such as tRNA genes) require TFIIIB and TFIIIC. Both type I and type II promoters are internal to the gene sequence. On the other hand, type III promoters (such as U6 snRNA genes) are externally located in the 5' flanking DNA and require TFIIIB together with SNAPc to initiate transcription [Willis, 1993; Geiduschek and Kassavetis, 2001; Hernandez, 2001; Schramm and Hernandez, 2002].

Drosophila melanogaster TFIIIB (TBP or TRF1, Brf1, and Bdp1)

The U6 snRNA promoter in *D. melanogaster* requires TFIIIB which is composed of TBP, Brf1 and Bdp1. The TATA-binding factor (TBP) was thought to be the universally required transcription factor at all eukaryotic promoters until TBP-related factors were discovered [Margottin, 1991; White 1992; Willis, 1993]. One such factor, the TBP related factor 1 (TRF1) was found to have ~60% amino acid identity to the *Drosophila* TBP C-terminal core domain [Crowley et al., 1993]. Further research found that TRF1, not TBP, is required for the transcription of both type I and type II *Drosophila* Pol III promoters and it was believed to be required for type III promoters as well [Takada et al., 2000]. However, a genome wide screen for TRF1 binding sites revealed a much lower than expected signal at three U6 snRNA gene loci [Isogai et al., 2007].

Therefore, our lab performed an additional study that showed that TBP had a much higher occupancy than TRF1 on U6 snRNA gene loci [Verma et al., 2013]. Furthermore, depletion of TBP inhibited U6 snRNA transcription *in vitro* while depletion of TRF1 only inhibited tRNA transcription [Verma et al., 2013]. This study suggested that there was a differential preference of TBP factors depending on the type of Pol III promoter present.

In addition, the structure of the U6 promoter also plays a role in successfully recruiting TBP. For instance, when a U1 PSEA is put in place of the U6 PSEA on a U6 snRNA promoter, TBP can no longer be recruited [Barakat and Stumph, 2008] and transcription is reduced to an essentially non-detectable level [McNamara et al., 2001; Lai et al., 2005]. Since DmSNAPc takes on different conformations depending on the PSEA sequence present, this result suggested that the conformation of DmSNAPc on the U6 PSEA and the DNA accessible to recruited transcription factors is important for the recruitment of TBP and possibly other transcription factors.

In addition to TBP, *Drosophila* TFIIIB also contains Brf1 and Bdp1. Brf1 is a TFIIB related transcription factor [Colbert and Hahn, 1992]. Mammals have two forms, Brf1 (utilized in type I and II promoters) and Brf2 (utilized in type III promoters), while *Drosophila* have only one form, Brf1. The N-terminal region of Brf, which is homologous to TFIIB, is required for recruiting Pol III [Kassavetis et al., 1997], while the C-terminal region is required for recruiting and stabilizing the other components of TFIIIB [Cabart and Murphy, 2001].

Bdp1 is unique to the Pol III transcription machinery as no homologous factor is known to be involved in transcription by Pol I or Pol II. Bdp1 contains an evolutionarily conserved region termed the SANT domain which serves as the binding domain between

Bdp1 and Brf1 in yeast [Kassavetis et al., 2006; Saida 2008]. While Brf1 is known to recruit Pol III, there are implications that Bdp1 may play a role as well [Kassavetis et al., 1997; Abascal-Palacios, 2018]. In addition, Brf1 and Bdp1 have both been shown to have post-Pol III recruitment roles such as involvement in promoter opening [Geiduschek and Kassavetis, 2001; Kassavetis et al., 2001, Gouge et al., 2017; Vorlander et al., 2018].

In yeast, structural work from other labs has revealed more information about how these components of TFIIIB interact with each other and the DNA. TBP is the only protein that can interact with the DNA on its own, though the interaction is weak. Once TBP is bound, Brf1 is recruited to the U6 snRNA TATA box by the conserved C-terminal domain of TBP, creating a Brf1-TBP-DNA complex stabilized by the Brf1 helical pin sequence [Colbert et al., 1998; Cabart and Murphy, 2001; Abascal-Palacios et al., 2018]. The complex is further stabilized from the incorporation of Bdp1 into the complex. This recruitment occurs through Bdp1 interactions with TBP and interactions between Brf1 and the conserved SANT domain of Bdp1 [Kassavetis et al., 1992; Colbert et al., 1998; Saida, 2008]. These studies have shown that TFIIIB encompasses the promoter region in the form of a stable ring, connecting the sequences flanking the TATA box around which it is centered [Colbert et al., 1998; Abascal-Palacios et al., 2018; Vorlander et al., 2018].

In addition, studies in human have shown similar stabilizing interactions between TBP and Brf2 [Ma and Hernandez, 2002; Hinkley et al., 2003; Gouge et al., 2015; Gouge et al., 2017]. Together, TBP and Brf2 increase the affinity for Bdp1 binding [Gouge et al., 2017]. Incorporation of Bdp1 leads to structural rearrangements that loosens the interaction between Brf2-TBP and the GR element, which is upstream of the TATA box, but increases the overall stability of the TFIIIB complex through anchoring from the conserved SANT

domain of Bdp1 [Gouge et al, 2017]. Bdp1 binding was reduced with mutation of either Bdp1 invariably conserved R334 residue or a mutation in TBP E191K residue, suggesting an interaction between TBP and Bdp1 [Gouge et al., 2017]. These mutations also reduce Pol III transcription though residual activity suggested that a factor in addition to TBP, such as SNAPc, may contribute to Bdp1 recruitment [Gouge et al., 2017].

It has been shown that SNAPc can interact with and recruit both TBP and Brf2 and together they stabilize SNAPc on the promoter aiding in the assembly of the PIC [Ma and Hernandez, 2002; Hinkley et al., 2003]. Before the work of this dissertation began, no direct interaction between SNAPc and Bdp1 had been observed, though a recent study confirmed that this interaction does occur on the U6 snRNA promoter in humans [Gouge et al., 2017]. In addition, the study showed that the removal of the Bdp1 N-terminal "linker" (located just upstream of the SANT domain) reduced Bdp1 binding to the Brf2-TBP-DNA complex as well as its interaction with SNAPc and further showed a defect in promoter opening [Gouge et al., 2017]. Though these studies have built upon the understanding of PIC formation, there are still many gaps in the structural model for this transcription machinery.

Questions investigated by the work described in this dissertation

The main goal of this dissertation was to investigate the mechanisms in which transcription factors assemble to form the pre-initiation complex for transcription of *Drosophila melanogaster* snRNA genes. It is of particular interest that some snRNA genes such as U1 are transcribed by Pol II while others such as U6 are transcribed by Pol III.

The work in Chapter 1 focuses on understanding interactions between the components of the U6 snRNA PIC and specific protein regions required for their

recruitment. An interaction between DmSNAPc and Bdp1 in the absence of TBP, Brf1 or a TATA box sparked an investigation to determine which region of Bdp1 is required for the interaction with DmSNAPc and how the specific sequence of the U6 PSEA contributes to this interaction.

N- and C-terminal Bdp1 truncation mutations were created and tested for their binding ability using electrophoretic mobility shift assays (EMSAs). These truncations were also tested for their ability to recruit TBP to the DmSNAPc-Bdp1 complex. From these results, a specified region of Bdp1 was tested for sufficiency in binding DmSNAPc as well as its ability to recruit TBP to the complex as analyzed through EMSA. TBP truncations were also utilized to determine the role of the evolutionarily non-conserved TBP N-terminal tail in DmSNAPc-Bdp1-TBP complex formation.

Chapter 1, in full, is a reprint of a manuscript that has been submitted for publication. The dissertation author is a primary researcher and co-first author of this paper.

In order to further understand the interactions described in Chapter 1, Chapter 2 focuses on confirming the proteins present on the U6 promoter and which protein domains are in close proximity to each other. Preliminary experiments utilizing cross-linking mass spectrometry (CXMS) were performed on the subunits of DmSNAPc free of DNA or in the presence of wildtype or mutant U6 DNA. In the future, this information can then be compared to CXMS experiments with DmSNAPc in the presence of other general transcription factors to further refine the model of PIC formation. Chromatin immunoprecipitations assays followed by quantitative polymerase chain reaction were also used to assess the presence of TBP, Bdp1 and Brf1 on U6 snRNA promoter DNA. The results presented in Chapter 2 are not yet intended to be submitted for publication due to

their preliminary nature. The dissertation author is the primary researcher and author of this chapter.

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CHAPTER 1

SNAPc interacts with Bdp1 on a U6, but not on a U1, snRNA gene promoter to establish a stable protein-DNA complex with TBP

INTRODUCTION

RNA polymerase III (Pol III) promoters were originally identified as DNA sequences internal to the 5S rRNA and tRNA genes (that is, located downstream of the transcription start site); however, many genes transcribed by Pol III have promoters that are located in the 5'-flanking DNA [Geiduschek and Tocchini-Valentini, 1988; Schramm and Hernandez, 2002; Willis, 1993]. Pol III genes with external promoters include metazoan genes that code for U6 and U6 atac snRNAs, 7SK RNA, $tRNA^{sel}$, H1 and MRP RNAs, as well as a number of other small non-coding RNAs [Baer et al., 1990; Canella et al., 2010; Carbon and Krol, 1991; Hung and Stumph, 2011; Jawdekar and Henry, 2008; Kunkel et al., 1986; Murphy et al., 1987; Myslinski et al., 2001; Yuan and Reddy, 1991]. These gene-external Pol III promoters are bipartite, consisting of both a TATA box and a proximal sequence element (PSE) centered about 30 base pairs (bp) and 55 bp upstream of the transcription start site respectively [Hung and Stumph, 2011; Jawdekar and Henry, 2008; Schramm and Hernandez, 2002].

The TATA box in this class of Pol III genes is an interaction site for the three-subunit general transcription factor TFIIIB [Kang et al., 2016; Saxena et al., 2005] that in mammals consists of TBP, Brf2, and Bdp1. Brf2 is paralogous to the more evolutionarily ancient Brf1 protein. Whereas Brf1 is used at mammalian Pol III-transcribed genes with internal promoters (e.g., tRNA and 5S RNA genes), Brf2 is instead used specifically at genes with upstream promoters (e.g., U6 and 7SK) [Schramm et al., 2000; Teichmann et al., 2000; Willis, 2002].

In contrast, no protein orthologous to mammalian Brf2 is encoded in the *Drosophila melanogaster* genome; fruit flies therefore utilize Brf1 at both upstream and internal Pol III promoters [Isogai et al., 2007]. However, an interesting difference does exist in the basal

transcription machinery utilized at fruit fly Pol III-transcribed genes that have external versus internal promoters: those with internal promoters utilize the TBP-related factor TRF1 [Isogai et al., 2007; Takada et al., 2000], but those with external promoters such as U6 employ the canonical TBP [Verma et al., 2013]. Thus, in flies the TFIIIB complex at tRNA and 5S RNA promoters consists of TRF1, Brf1, and Bdp1, but at U6 and U6-like promoters TFIIIB consists of TBP, Brf1, and Bdp1.

The PSE is the binding site for the multi-subunit small nuclear RNA activating protein complex (SNAPc) [Henry et al., 1995; Sadowski et al., 1993], also known as PTF [Murphy et al., 1992; Yoon et al., 1995]. Three subunits of SNAPc (SNAP43, SNAP50, and SNAP190) appear to be conserved throughout metazoan evolution [Hung and Stumph, 2011]. Beyond their role in Pol III transcription, the PSE and SNAPc are also essential for the expression of snRNA genes that are transcribed by RNA polymerase II (Pol II) (e.g., U1 to U5 genes) [Egloff et al., 2008; Henry et al., 1998; Hung and Stumph, 2011; Jawdekar and Henry, 2008; Parry et al., 1989].

Studies by our lab have shown that the PSEs (more specifically referred to as PSEAs in fruit flies) of *D. melanogaster* U1 and U6 snRNA genes are not interchangeable. That is, a U1 PSEA does not support Pol III transcription of a U6 gene, and a U6 PSEA does not support Pol II transcription of a U1 gene, even though they are identical at 16 of 21 nucleotide positions and both are bound by *D. melanogaster* SNAPc (DmSNAPc) [Barakat and Stumph, 2008; Hung and Stumph, 2011; Jensen et al., 1998; Lai et al., 2005; McNamara-Schroeder et al., 2001]. Furthermore, site-specific protein-DNA photo-cross-linking studies have indicated that DmSNAPc cross-links in a distinctive manner to the PSEA of a U6 gene versus the PSEA of a U1 gene [Hung and Stumph, 2011; Kang et al., 2014; Kim et al., 2010a, b; Lai et al.,

2005; Li et al., 2004; Wang and Stumph, 1998]. The above data have led to a model in flies in which the specific PSEA sequence, U1 or U6, acts as an allosteric effector of DmSNAPc conformation that leads to recruitment of different sets of general transcription factors (GTFs) and ultimately to RNA polymerase specificity [Hung and Stumph, 2011; Kang et al., 2014; Kim et al., 2010a, b; Li et al., 2004; Wang and Stumph, 1998].

Early studies in the human system revealed that SNAPc recruited TBP to the U6 gene promoter through cooperative binding interactions [Mittal and Hernandez, 1997]. It was also observed that SNAPc interacted with and recruited Brf2 to U6 promoter DNA [Hinkley et al., 2003]. By acting together, SNAPc and Brf2 incorporated limiting levels of TBP into a more stable SNAPc-Brf2-TBP-U6 promoter DNA complex [Hinkley et al., 2003; Ma and Hernandez, 2002; Saxena et al., 2005]. No direct interactions between Bdp1 and SNAPc were observed in those studies. However, a recent report that appeared while this manuscript was being prepared did detect an interaction between SNAPc and Bdp1 [Gouge et al., 2017].

Here we describe experiments that indicate that DmSNAPc recruits Bdp1 to the U6 promoter when DmSNAPc is bound to a U6 PSEA but not when DmSNAPc is bound to a U1 PSEA in an otherwise identical U6 promoter context. This finding supports and extends our model of RNA polymerase specificity by providing a molecular rationale for DmSNAPc recruitment of Pol III GTFs to the U6 promoter but not to the similar U1 promoter. We also find that DmSNAPc and Bdp1 together can recruit TBP to form a stable DmSNAPc-Bdp1-TBP complex on the U6 promoter, and we have identified an 87-amino-acid region of Bdp1 that is sufficient for the formation of this complex.

MATERIALS AND METHODS

DmSNAPc expression constructs

The preparation of untagged constructs encoding wild type DmSNAP43, DmSNAP50, and N-terminal His_6-FLAG-tagged DmSNAP190 constructs under the control of the copper-inducible metallothionein promoter have been previously described [Hung et al., 2009]. Expression plasmids for each of the DmSNAPc subunits were used to co-transfect *D. melanogaster* S2 cells as previously described [Hung et al., 2009]. Subunit co-expression was induced with copper sulfate and confirmed by immunoblotting with anti-FLAG M2 monoclonal antibodies (Sigma #A9469) or antibodies made against synthetic peptides corresponding to amino acid sequence at or near the C-terminus of the wild type proteins [Li et al., 2004].

TFIIIB expression constructs

Constructs that encode full-length C-terminal V5-His_6-tagged TBP or Brf1 and C-terminal FLAG-Myc-His_6-tagged Bdp1 under the control of the copper-inducible metallothionein promoter have been previously described [Kang et al., 2016]. Bdp1 and TBP truncation constructs were prepared by PCR, restriction digestion, and re-cloning as previously described for the DmSNAPc subunit truncation constructs [Hung et al., 2009]. End points of the Bdp1 and TBP truncations were chosen to be within evolutionarily non-conserved regions that were flanked by evolutionarily conserved regions of the corresponding proteins. Expression plasmids were then used to generate stably transfected S2 cell lines [Hung et al., 2009]. Copper sulfate induced expression was confirmed by immunoblotting

with anti-V5 monoclonal antibodies (Life Technologies #R960-25) for TBP and Brf1 detection and anti-FLAG M2 monoclonal antibodies (Sigma #A9469) for Bdp1 detection.

Nickel-chelate chromatography

Following copper sulfate induction, cells (eight 15 cm diameter plates per cell line grown to ~100% confluency) were lysed in CelLytic M lysis buffer (Sigma #C2978) containing 1% protease inhibitor cocktail (Sigma # P8340). Lysates were then adjusted to a NaCl concentration of 0.5 M prior to incubating with ProBond resin (Life Technologies #R80101) for 2 hours for DmSNAPc, Brf1, and TBP proteins and 30 minutes for Bdp1 proteins to allow the capture of the His_6-tagged proteins. The resins were then washed three times in 50 mM sodium phosphate buffer (pH 8.0), 0.5 M NaCl, 20 mM imidazole and then once in HEMG-100 buffer (25 mM HEPES K+ (pH 7.6), 0.1 mM EDTA, 12.5 mM $MgCl_2$, 10 µM $ZnCl_2$, 10% glycerol, 100 mM KCl) containing 0.5 mM phenylmethylsulfonyl fluoride and 20 mM imidazole. The proteins/complexes were eluted from the resin with 750 mM imidazole in HEMG-100 buffer followed by addition of 3 mM dithiothreitol to each elution and then dialyzed against HEMG-100 buffer without imidazole but containing 1 to 3 mM dithiothreitol. For some experiments, untagged full-length recombinant Bdp1 was utilized that was custom-expressed in bacteria and purified by GenScript (www.genscript.com).

Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed as previously described [Hung and Stumph, 2012]. Briefly, reactions were carried out in 21 µl volumes in HEMG-100 buffer containing 5 mM dithiothreitol. Each reaction also contained 2 µg of poly (dG-dC) (Sigma #P9389). Reactions

were incubated with ^{32}P-labeled DNA probes (described in the Results and Figure legends) for 30 minutes at 25^{o}C. For antibody-induced supershifts, the antibodies were added halfway through the 30 minute incubation. Complexes were then run on 4% non-denaturing polyacrylamide gels in 25 mM Tris, 190 mM glycine, 1 mM EDTA (pH 8.3) and detected by autoradiography.

RESULTS

In recent work, our lab found that *D. melanogaster* TFIIIB, consisting of TBP, Brf1, and Bdp1, could be assembled *in vitro* on the TATA box of the U6:96Ab snRNA gene promoter independently of the presence of DmSNAPc [Kang et al., 2016]. However, mutation of the DmSNAPc binding site (the PSEA) resulted in the inactivation of U6 gene transcription in cells [Lai et al., 2005], suggesting that recruitment of TFIIIB *in vivo* is dependent upon DmSNAPc. To better understand the biochemical mechanisms behind this recruitment, we decided to investigate whether interactions between DmSNAPc and TFIIIB could be identified on the U6 promoter *in vitro*.

Bdp1 can be recruited to the U6 promoter by DmSNAPc

We first investigated whether interactions between DmSNAPc and each of the TFIIIB subunits could be detected by EMSAs. His_6-tagged proteins (TBP, Brf1, or DmSNAPc (DmSNAP43, DmSNAP50, and DmSNAP190 together)) were overexpressed in *D. melanogaster* S2 cells and partially purified by nickel-chelate chromatography as described in Materials and Methods. Bdp1 was expressed as a tagged protein in S2 cells or was obtained in

an untagged form after expression in E. coli. The EMSA probe contained the wild type DNA sequence from positions -75 to -7 relative to the U6:96Ab gene transcription start site.

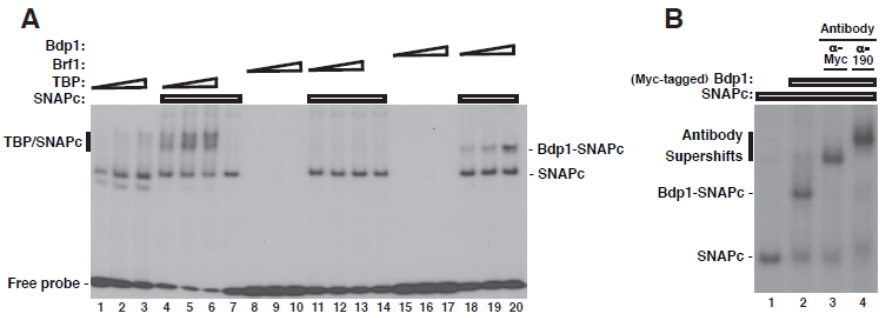

Figure 1.1: Assembly of individual subunits of TFIIIB on the U6 snRNA gene promoter with DmSNAPc. (**A**) Autoradiogram of an EMSA performed with a ^{32}P-labeled DNA probe that contained U6 promoter DNA from position -7 to -75 base pairs upstream of the transcription start site. Reactions shown in lanes 4-7, 11-14, and 18-20 each contained 4 µl of DmSNAPc. Reactions run in lanes 1-3 and 4-6 contained TBP in increasing amounts (1, 1.7, or 2.5 µl) as indicated above each three-lane set. Lanes 8-10 and 11-13 contained Brf1 in increasing amounts (1, 2, or 4 µl) as indicated above the respective lanes. (The Brf1 was active as indicated by its ability to form Brf1-TBP complexes and Brf1-Bdp1-TBP complexes [Kang et al., 2016 and data not shown]). Lanes 15-17 and 18-20 contained recombinant Bdp1 in increasing amounts (25, 50, or 100 ng). The identities of the shifted bands are indicated alongside the panels. (**B**) EMSA supershifts with antibodies specific for DmSNAPc and (Myc-tagged)-Bdp1. Reactions shown in each lane contained 1.5 µl of DmSNAPc, while those in lanes 2-4 each additionally contained 4.5 µl Bdp1. Lanes 3 and 4 contained 1.5 µl of either anti-Myc antibody or anti-DmSNAP190 antibody respectively. Lane 1 is from the same gel and length of autoradiogram exposure as lanes 2-4. Free probe was run off the gel to obtain better resolution between shifted bands.

Figure 1.1A shows that TBP (lanes 1-3) and DmSNAPc (lanes 7 and 14) each bound individually to the DNA probe. This was expected since the probe contained both a PSEA (-63 to -43) and a TATA box (-30 to -23). Despite the significant difference in molecular size, the TBP band ran just barely ahead of the DmSNAPc band (probably due to TBP bending the DNA). When DmSNAPc and TBP were both added to the reactions (Figure 1.1A, lanes 4-6), the TBP band no longer appeared. Instead, bands of slower mobility were observed, and the amount of free probe remaining was drastically reduced. This suggests that there was at least a certain degree of cooperative binding between DmSNAPc and TBP to the DNA fragment. This is similar to the findings of others who observed fairly weak cooperative binding of

human TBP and SNAPc to a human U6 gene promoter, but this effect was considerably strengthened by the addition of Brf2 [Hinkley et al., 2003; Ma and Hernandez, 2002; Saxena et al., 2005].

Brf1, on the other hand, did not bind to DNA by itself, nor was there any evidence that it could stably interact with DmSNAPc on the DNA (Figure 1.1A, lanes 8-13). As expected, Bdp1 alone did not bind to DNA (lanes 15-17), but the bacterially-expressed Bdp1 stably interacted with DmSNAPc to produce a supershifted band corresponding to a Bdp1-DmSNAPc complex (lanes 18-20). To verify that the supershifted band contained both DmSNAPc and Bdp1, we expressed Bdp1 with a C-terminal Myc tag in S2 cells. When the tagged Bdp1 was added to an EMSA reaction that contained DmSNAPc, the same Bdp1-DmSNAPc supershifted band was observed as previously seen with the bacterially-expressed Bdp1 (Figure 1.1B, lane 2, and data not shown). This band was further supershifted by antibodies directed against the Myc tag on Bdp1 or against a C-terminal epitope of DmSNAP190 (lanes 3 and 4 respectively). These results revealed that DmSNAPc and Bdp1 together formed a stable complex on the U6 promoter even though Bdp1 had no detectable DNA binding activity of its own.

Recruitment of Bdp1 to the U6 promoter by DmSNAPc involves a region of Bdp1 just C-terminal of the Bdp1 SANT domain

We next employed N-terminal and C-terminal truncations of Bdp1 to map a region of Bdp1 required for its recruitment by DmSNAPc. The most prominent feature of Bdp1 is the evolutionarily conserved SANT domain that extends between amino acids 346 and 403 in the *D. melanogaster* protein ("FL" in Figure 1.2A). In yeast, the Bdp1 SANT domain is involved

in interacting with Brf1 [Kassavetis et al., 2006; Saida, 2008]. The truncation constructs shown in Figure 1.2A were expressed in S2 cells, subjected to purification by nickel-chelate chromatography, and used in EMSAs with wild type DmSNAPc (Figure 1.2B-D).

Initially, nine Bdp1 expression constructs with deletions at either the N-terminus (Figure 1.2A, constructs A-D) or with deletions at the C-terminus (constructs E-I) were prepared. The shortest construct in each case was truncated just beyond the SANT domain. The EMSA results indicated that constructs A through D each formed a slower-migrating complex on the DNA when added together with DmSNAPc (Figure 1.2B, lanes 3-6). Thus, amino acid residues C-terminal of position 424 were sufficient for Bdp1 recruitment by DmSNAPc, and the SANT domain was not a requirement for Bdp1 recruitment.

When the C-terminal Bdp1 truncation constructs were used in the EMSAs, the constructs that contained the first 615 or 510 residues from the N-terminus of the protein (constructs E and F) formed a slower-migrating complex with DmSNAPc on the U6 promoter fragment (Figure 1.2B, lanes 9 and 10). However, Bdp1 truncations that left 464 N-terminal amino acid residues or fewer (constructs G, H, and I) were unable to form a slower-migrating complex (Figure 1.2B, lanes 11-13). These latter results implicated a region of Bdp1 lying between residues 465 and 510 as being essential for Bdp1 recruitment by DmSNAPc.

Based upon these results with the initial nine constructs, two additional N-terminal truncation constructs were prepared that deleted regions of Bdp1 just beyond the SANT domain (Figure 1.2A, constructs J and K). Neither of these constructs was able to form a slower-migrating complex with DmSNAPc (Figure 1.2C, lanes 5 and 6). Thus, a region of Bdp1 between residues 424 and 465 is important for Bdp1 interaction with DmSNAPc. The

results from the N-terminal and C-terminal truncations, when taken together, implicate a region of Bdp1 between residues 424 and 510 as required for Bdp1 interaction with DmSNAPc on the U6 promoter.

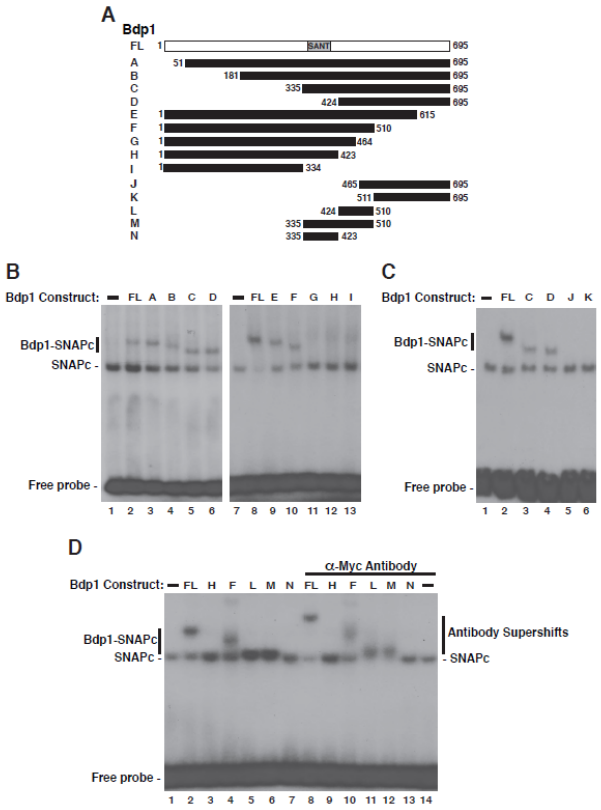

Figure 1.2: A region of Bdp1 C-terminal of the SANT domain is required for its recruitment by DmSNAPc. (**A**) Truncation constructs used to map a region of Bdp1 required for its interaction with DmSNAPc on the U6 promoter. The top rectangle represents full-length (FL) fly Bdp1 (695 amino acid residues). The location of the highly-conserved SANT domain is also indicated. The black bars beneath represent the truncation constructs utilized in EMSA reactions. All constructs were expressed in S2 cells and were tagged with Flag-Myc-His_6 epitopes at the C-terminus. The numbers at each end of the bars indicate the extent of the wild type amino acid residues present in the expressed constructs. (**B**) EMSAs showing the ability of the full-length and truncated Bdp1 constructs to assemble with DmSNAPc on U6 promoter DNA. The identities of the shifted bands and free probe are indicated to the left of the panels. A constant amount of DmSNAPc (3 µl) was used in all reactions. The particular Bdp1 truncation construct utilized in each reaction is indicated above the respective lanes. The amount of each Bdp1 construct added was adjusted to make the intensities of the Bdp1-DmSNAPc signals similar in each lane and varied between 1.5 µl and 3.0 µl in the left panel and between 3 µl and 5 µl in the right panel. Lanes 1 and 7 show reactions with DmSNAPc but no Bdp1. (**C**) EMSAs as described in (B) but with two additional Bdp1 constructs (J and K). Constructs C and D were included as positive controls. The amount of Bdp1 construct added to each reaction varied between 0.75 µl and 2.5 µl. (**D**) EMSAs as described in (B) but including more extensively-truncated Bdp1 constructs (L, M, and N) together with constructs H and F as negative and positive controls respectively. The amount of Bdp1 construct added to each reaction varied between 2.5 µl and 3.0 µl. The reactions loaded in lanes 8 to 14 furthermore contained antibody against Myc-tagged Bdp1 (4 µl anti-Myc).

To explore whether this same 87-amino-acid region of Bdp1 was sufficient for interaction with DmSNAPc in the context of the U6 promoter, three additional constructs were created (Figure 1.2A, constructs L-N). Construct L contained only the 87-amino-acid region between residues 424 and 510; construct M contained this region plus the SANT domain; and construct N contained only the SANT domain, which should be a well-structured domain [Gouge et al., 2017] but possibly not involved in the interaction with DmSNAPc.

When EMSAs were performed (Figure 1.2D), full-length Bdp1 and construct F (positive controls, lanes 2 and 4) each produced a band migrating slower than DmSNAPc alone (lane 1), whereas construct H (negative control, lane 3) and construct N (only the SANT domain, lane 7), did not. Interestingly, constructs L and M, which both contained amino acid residues 424 to 510, each produced a darker band that had a slightly retarded mobility compared to that obtained with constructs that did not interact with DmSNAPc (compare lanes 5 and 6 with lanes 3 and 7). The small magnitude of the change in mobility is likely due to the small size of constructs L and M. However, the increase in band intensity suggests that Bdp1 residues 424-510 alone are capable of stabilizing the interaction of DmSNAPc with U6 promoter DNA.

To produce a more distinct shift, antibodies against the Myc tag on the Bdp1 constructs were added to the same series of EMSA reactions (Figure 1.2D, lanes 8-14). Although the antibodies appear to have partially dissociated the complexes (compare lanes 8, 10, 11, and 12 with lanes 2, 4, 5, and 6 respectively), the bands obtained with constructs L and M (upon the addition of anti-Myc) were significantly slower in mobility compared to the bands seen with constructs H and N or DmSNAPc alone (compare lanes 11 and 12 with 9, 13, and 14). Altogether, the findings presented in Figure 1.2 provide substantial evidence that a

region of Bdp1 between residues 424 and 510 is necessary and sufficient for Bdp1 interaction with DmSNAPc on the U6 promoter. Furthermore, these experiments detected no role for the SANT domain in this interaction.

Bdp1 recruitment by DmSNAPc is dependent upon a U6 PSEA versus a U1 PSEA

We have previously shown that the PSEAs of *Drosophila* U1 and U6 genes are not functionally interchangeable for snRNA transcription even though they differ at only 5 of 21 nucleotide positions and are both recognized and bound by DmSNAPc. The U1 PSEA is only able to recruit Pol II, and the U6 PSEA is only capable of recruiting Pol III, as exchange of the PSEAs inactivated snRNA expression *in vivo* and switched the polymerase specificity *in vitro* [Barakat and Stumph, 2008; Hung and Stumph, 2011; Jensen et al., 1998; Lai et al., 2005; McNamara-Schroeder et al., 2001]. Furthermore, evidence accumulated by site-specific protein-DNA photo-cross-linking has strongly suggested that DmSNAPc binds in different conformations to the U1 and U6 PSEAs [Hung and Stumph, 2011; Kang et al., 2014; Kim et al., 2010a, b; Lai et al., 2005; Li et al., 2004; Wang and Stumph, 1998].

To investigate the molecular mechanism of the Pol III specificity of U6 snRNA gene promoters, we next performed experiments to determine whether the ability of DmSNAPc to recruit Bdp1 is dependent upon a U6 PSEA (that specifically recruits Pol III) versus a U1 PSEA (that recruits Pol II). To do this, DNA EMSA probes were utilized in which the U6 PSEA was converted to a U1 PSEA (or vice versa) by means of five base changes (Figure 1.3A). When DmSNAPc was bound to the wild type U6 promoter probe, the addition of increasing amounts of Bdp1 resulted in the formation of a DmSNAPc-Bdp1 complex on the DNA (Figure 1.3B, lanes 1-7). In contrast, when a probe was used that contained the U1

PSEA in the context of the U6 promoter, DmSNAPc itself bound very efficiently to the U1 PSEA but astonishingly was unable to recruit Bdp1 (Figure 1.3B, lanes 8-14).

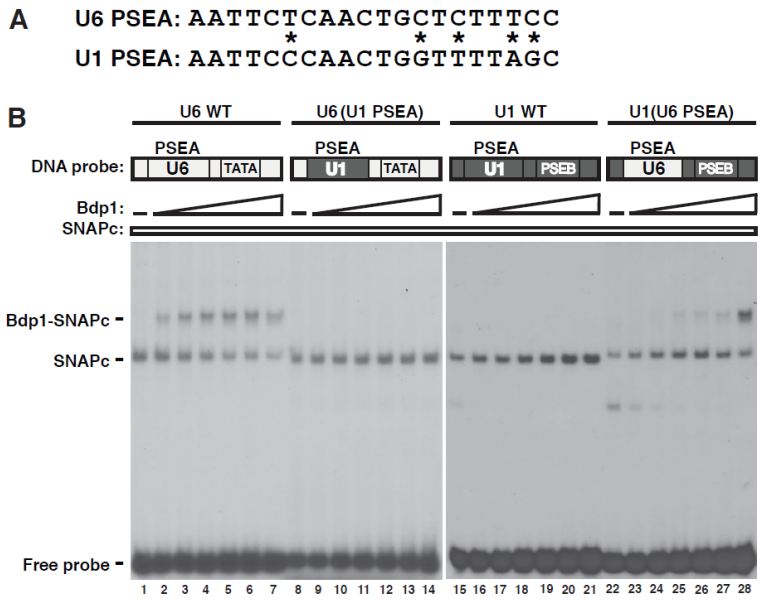

Figure 1.3: A Pol III-specific PSEA is necessary and sufficient for Bdp1 recruitment by DmSNAPc. (**A**) The five nucleotide differences between the U6 and U1 PSEA sequences used for the EMSAs in (B) are indicated by asterisks. (**B**) Increasing amounts of Bdp1 were added to the reactions containing a constant amount of DmSNAPc with either wild type U6 promoter DNA (lanes 1-7) or with a probe (lanes 8-14) that was identical except that it contained five nucleotide changes that converted the U6 PSEA to a U1 PSEA. The panel to the right shows a reciprocal experiment that employed a wild type U1 promoter DNA probe (sequences from -75 to -7 relative to the U1 transcription start site) (lanes 15-21) or a U1 probe in which the U1 PSEA was switched to a U6 PSEA (lanes 22-28). Lanes 2-7, 9-14, 16-21, and 23-28 contained bacterially-expressed Bdp1 in increasing amounts as follows: 12.5, 25, 50, 75, 100, and 200 ng.

As confirmation of these results, we performed the reciprocal experiment in which 5 base changes were made in the U1 promoter that converted the U1 PSEA to a U6 PSEA. As expected from the preceding result, when the DNA probe contained entirely wild type U1 promoter sequences, DmSNAPc bound to the DNA but was unable to recruit Bdp1 (Figure 1.3B, lanes 15-21.) However, when five base changes were made that switched the U1 PSEA to a U6 PSEA in the context of the U1 promoter, DmSNAPc successfully recruited Bdp1 (Figure 1.3B, lanes 22-28). These results support the concept that the sequence of the PSEA

plays a role in Bdp1 recruitment by differentially affecting the conformation of DmSNAPc, thereby enabling DmSNAPc to recruit Bdp1 to the U6 promoter but not to the U1 promoter.

We did notice that Bdp1 was reproducibly recruited with less efficiency when the U6 PSEA was used in the context of the U1 promoter until a critical concentration of Bdp1 was employed. This suggests that other nucleotides in the U6 promoter may contribute to the efficiency of Bdp1 recruitment.

The DmSNAPc-Bdp1 complex can recruit TBP to the U6 promoter

To follow up the observation that DmSNAPc can recruit Bdp1 to the U6 promoter, we next examined whether the DmSNAPc-Bdp1 complex might play a direct role in the recruitment of TBP to U6 promoter DNA. For the EMSA experiments shown in Figure 1.4A, sets of three lanes were assayed in parallel that contained the same gradually increasing amounts of TBP but with DmSNAPc added (lanes 3-5), both DmSNAPc and Bdp1 added (lanes 6-8), or just TBP by itself (lanes 9-11). Two lanes (12 and 13) contained still higher amounts of TBP with no additional proteins.

Reactions that contained TBP alone exhibited only very weak interactions with the DNA (Figure 1.4A, lanes 9-13). When the same amounts of TBP used in lanes 9-11 were added to a constant amount of DmSNAPc (Figure 1.4A, lanes 3-5), clear DmSNAPc bands were observed together with a very light smear of slower-migrating bands that likely corresponded to DNA fragments co-occupied by DmSNAPc and TBP (as seen more clearly in Figure 1.1A, lanes 4-6, an experiment in which higher amounts of TBP were used). Importantly, when Bdp1 was present together with DmSNAPc and TBP, even the lowest amount of TBP was sufficient to form a strong higher-order complex of slower mobility

(Figure 1.4A, lanes 6-8). Moreover, essentially all the DmSNAPc band-shift seen in lanes 3-5 was supershifted into the higher order complex in lanes 6-8. These results suggest that DmSNAPc, Bdp1, and TBP bind cooperatively to the U6 promoter.

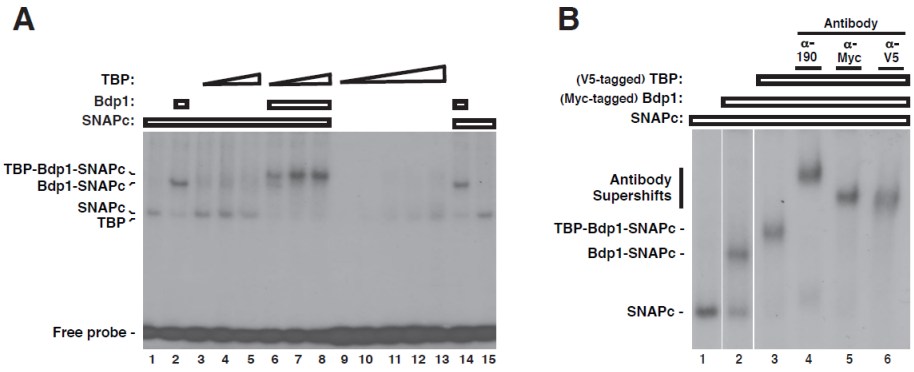

Figure 1.4: The DmSNAPc-Bdp1 complex efficiently recruits TBP to the U6 promoter. (**A**) EMSA reactions with a wild type U6 promoter DNA probe and increasing amounts of TBP (0.25, 0.5, and 0.75 µl) added either alone (lanes 9-11), or in the presence of 3 µl DmSNAPc (lanes 3-5), or in the presence of both 3 µl DmSNAPc and 2.5 µl Bdp1 (lanes 6-8). Lanes 12 and 13 show reactions with higher amounts of TBP (1 µl and 1.5 µl respectively) to reveal the binding of TBP alone to the probe. Lanes 1 and 15 show reactions with DmSNAPc alone, and lanes 2 and 14 show reactions that contained DmSNAPc and Bdp1 together but no TBP. (**B**) EMSAs with specific antibody supershifts are shown. Each reaction contained 3 µl DmSNAPc; lanes 2-6 each contained 2.5 µl of Bdp1; and lanes 3-6 each contained 0.5 µl of TBP. The TBP-Bdp1-DmSNAPc complex (lane 3) was supershifted by 1.5 µl of antibody against either DmSNAPc (anti-190, lane 4), Bdp1 (anti-Myc, lane 5), or TBP (anti-V5, lane 6). Free probe was run off the gel to improve the resolution among the shifted bands. Lanes 1 and 2, although non-adjacent, are both from the same gel as lanes 3-6.

The presence of all three proteins in the higher order complex was confirmed by antibody supershifts (Figure 1.4B). The TBP-Bdp1-DmSNAPc complex migrated more slowly than the Bdp1-DmSNAPc complex (Figure 1.4B, lane 3 vs. lane 2), and this complex was further supershifted by antibodies against either DmSNAP190, the Myc epitope on Bdp1, or the V5 epitope on TBP (Figure 1.4B, lanes, 4, 5, and 6 respectively). The above experiments revealed that DmSNAPc and Bdp1 together were capable of efficiently recruiting TBP to the wild type U6 promoter.

TBP recruitment by the DmSNAPc-Bdp1 complex requires a TATA box

We next investigated whether TBP recruitment under such circumstances was also dependent upon the presence of the TATA box in the U6 promoter. For this, a DNA EMSA probe was used that was identical to the wild type probe except the TATA sequence (TTTATATA) was mutated to GGGACCTC. As expected, TBP by itself was able to bind to the wild type probe but did not bind to the mutant TATA probe (Figure 1.5A, lanes 1 and 2). On the other hand, DmSNAPc bound equally well to both the wild type and mutant TATA probes (Figure 1.5A, lanes 3 and 4). Furthermore, the mutation of the TATA sequence had no effect on the recruitment of Bdp1 by DmSNAPc (Figure 1.5A, lanes 5 and 6). In stark contrast, the DmSNAPc-Bdp1 complex effectively recruited TBP to the wild type promoter but was unable to recruit TBP to the mutant TATA promoter (Figure 1.5A, compare lanes 7 and 8). These results indicated that the recruitment of TBP, but not Bdp1, to the U6 promoter was dependent upon the presence of a TATA sequence.

The TBP N-terminal tail contributes to the stability of the DmSNAPc-Bdp1-TBP complex on U6 promoter DNA

Earlier work by others in the human system indicated that the non-conserved N-terminal tail of TBP played a role to increase the efficiency of human U6 gene transcription [Mittal and Hernandez, 1997]. We therefore examined whether removing portions of the N-terminal tail of *D. melanogaster* TBP would affect TBP recruitment by the DmSNAPc-Bdp1 complex. The three TBP N-terminal truncation constructs shown in Figure 1.5B were expressed in S2 cells, partially purified by nickel-chelate chromatography, and tested by EMSA. Each of the three constructs retained the ability to bind to the U6 DNA probe when

assayed by EMSA in the absence of any other proteins (Figure 1.5B, lanes 9-12).

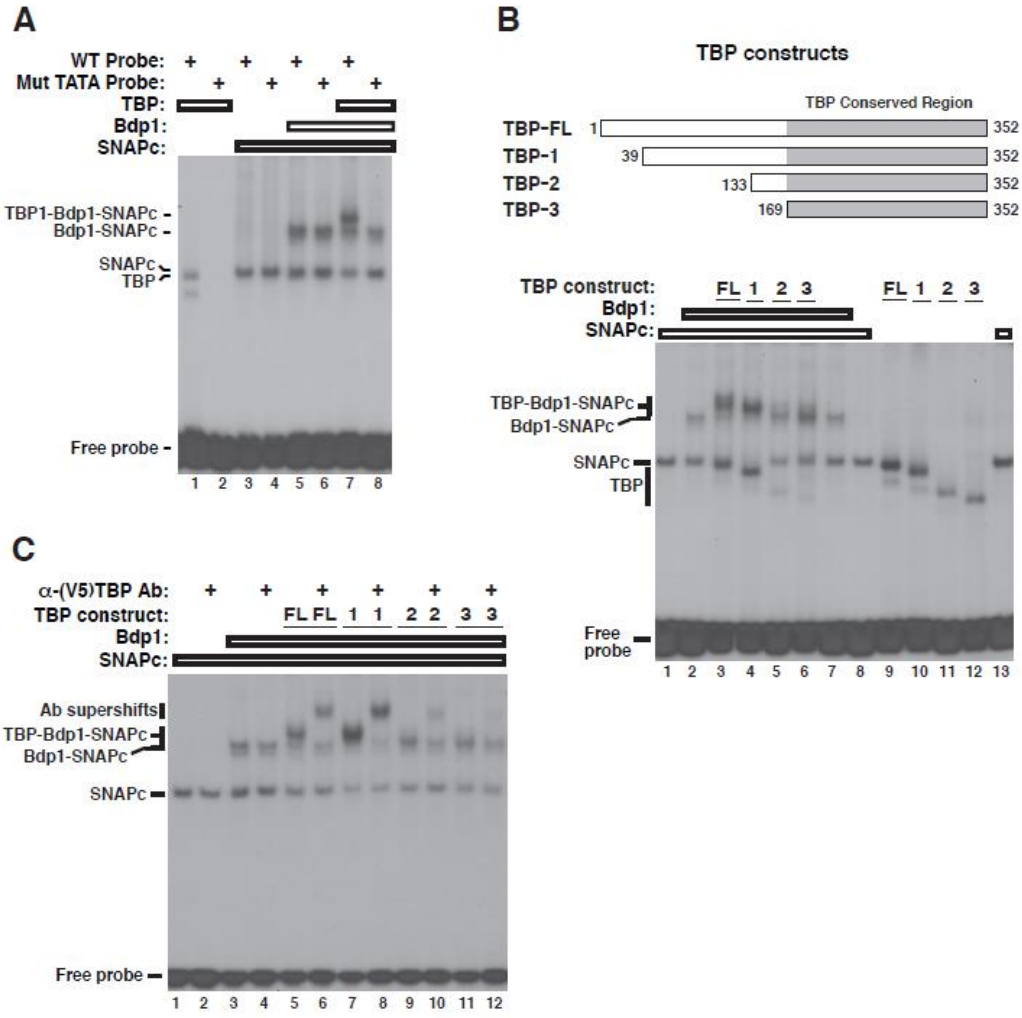

Figure 1.5: TBP recruitment to the U6 promoter by DmSNAPc-Bdp1 is stabilized by the TATA box and the TBP N-terminal tail. (**A**) An EMSA is shown that utilized either the wild type U6 promoter probe (odd numbered lanes) or a probe that was identical to the wild type sequence with the exception of a mutated TATA box (even numbered lanes). Each probe was incubated with either 3 µl of DmSNAPc alone (lanes 3 and 4), 3 µl of DmSNAPc together with 2 µl of Bdp1 (lanes 5 and 6), or 3 µl of DmSNAPc together with 2 µl of Bdp1 and 0.4 µl of TBP (lanes 7 and 8). Each probe was also incubated with 1 µl of TBP alone (lanes 1 and 2). The identity of the shifted bands and free probe are indicated to the left of the panel. (**B**) Full length TBP (TBP-FL, 352 amino acids) and three N-terminal truncation constructs (TBP-1, -2, and -3) are diagrammed at the top. The location of the evolutionarily conserved C-terminal region is indicated with shading. An EMSA to examine recruitment of the TBP constructs is shown below. Reactions included 4 µl of DmSNAPc (lanes 1-8 and 13), 3 µl of Bdp1 (lanes 2-7), and the TBP constructs as indicated above the individual lanes (0.5, 2.0, 2.5, and 7.0 µl of the respective constructs in lanes 3-6, and 0.8, 3.2, 4.0, and 7.0 µl in lanes 9-12). (**C**) EMSA as in (B), except that 1.5 µl of antibody directed against the V5 tag on the TBP constructs was added to the even-numbered lanes. Reactions also contained 4 µl DmSNAPc (lanes 1-12), 3 µl Bdp1 (lanes 3-12), and the TBP constructs as indicated above lanes 5-12 (0.4, 1.6, 3.0, and 7 µl of TBP-FL, -1, -2, and -3 respectively).

In reactions that contained DmSNAPc and Bdp1, full-length TBP and truncation construct TBP-1 clearly formed a complex of slower mobility compared to the band obtained with DmSNAPc and Bdp1 (Figure 1.5B, compare lanes 3 and 4 to lane 2). However, it was difficult to discern whether the two shorter constructs (TBP-2 and TBP-3, lanes 5 and 6) were forming a complex of slower mobility. To better resolve this issue, EMSA supershift reactions were performed that included antibody against the V5 tag present at the C-terminus of the TBP constructs (Figure 1.5C).

The anti-V5 antibody had no effect on the bands containing DmSNAPc alone or DmSNAPc plus Bdp1 (Figure 1.5C, lanes 1-4). However, the anti-V5 antibody strongly supershifted the bands containing full-length TBP or TBP-1 (Figure 1.5C, lanes 6 and 8 compared to lanes 5 and 7 respectively). A weak supershift was observed with TBP-2 (lane 10) and a barely visible supershift was obtained with TBP-3 (lane 12). These results imply that the N-terminal tail of TBP is not absolutely required for incorporation of TBP into the DmSNAPc-Bdp1-U6 DNA complex, but the tail nonetheless significantly increased the overall stability of the complex.

Identification of a region of Bdp1 sufficient for TBP recruitment in the presence of DmSNAPc

We next investigated whether a specific region of Bdp1 is involved in recruiting TBP to the U6 promoter. For this purpose, we first utilized the six N-terminal or C-terminal truncation constructs of Bdp1 that were found to assemble with DmSNAPc on the U6 promoter (constructs A through F, as determined in Figure 1.2). The upper panels of Figure 1.6 show that each of these Bdp1 truncation mutants was able to recruit TBP to form a higher

order complex on the U6 promoter (bands indicated with small circles). From those results we surmise that an 87-amino-acid region of Bdp1 (residues 424 to 510), together with sequences lying either N-terminal or C-terminal of that region, can cooperate with DmSNAPc to recruit TBP to the U6 promoter.

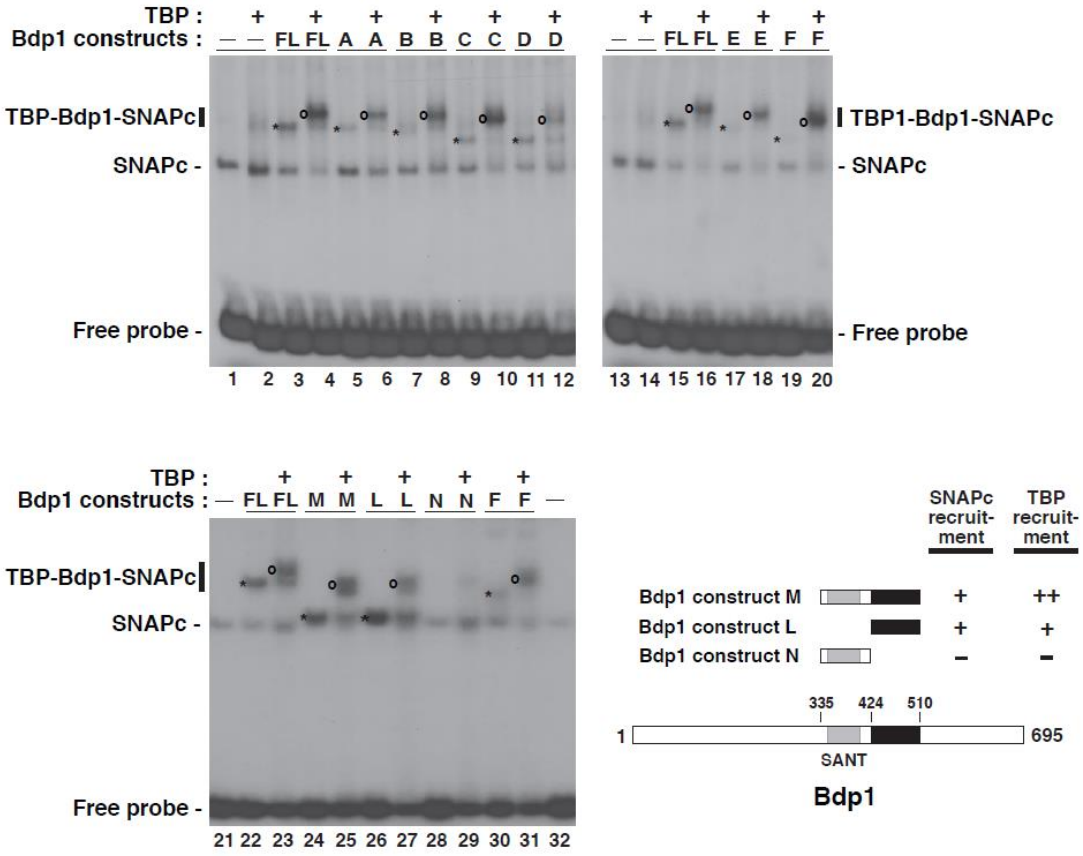

Figure 1.6: An 87-residue region of Bdp1 just C-terminal of the SANT domain is sufficient for TBP recruitment in the presence of DmSNAPc. EMSAs are shown that utilized various Bdp1 truncation constructs together with DmSNAPc and full-length TBP. The particular Bdp1 construct utilized in each lane (refer to Figure 1.2A) is indicated above the respective lanes and varied between 2 µl and 7 µl. Reactions run in all lanes contained DmSNAPc (3 µl). TBP (0.5 µl) was added to the reactions loaded in the lanes indicated by plus signs (+) above each panel. The small circles indicate the positions of the bands that correspond to the complexes formed by DmSNAPc together with TBP and the various truncation constructs of Bdp1. The asterisks, on the other hand, indicate the bands that correspond to DmSNAPc-Bdp1 complexes on the DNA that do not include TBP (as seen in Figure 1.2). The lower right diagram summarizes the findings that a region of Bdp1 lying C-terminal of the SANT domain can form a complex with DmSNAPc that is sufficient to recruit TBP.

To determine whether that 87-amino-acid region of Bdp1 (residues 424 to 510) alone was sufficient for TBP recruitment in the context of DmSNAPc, we used Bdp1 constructs L, M, and N that contained only the SANT domain and/or residues 424 to 510 (Figure 1.2 and lower right diagram of Figure 1.6). Constructs M and L efficiently formed a complex with DmSNAPc that recruited TBP to the U6 promoter (Figure 1.6, lanes 24-27). In contrast, construct N was unable to cooperate with DmSNAPc to recruit TBP (Figure 1.6, lanes 28-29). Altogether, these results indicate that residues 424 to 510 are necessary and sufficient for Bdp1 to interact with DmSNAPc and to recruit TBP.

We did notice a reproducible reduction in the intensity of the TBP-Bdp1-DmSNAPc band in the case of constructs that lacked the SANT domain when compared to similar constructs that contained the SANT domain. Compare, for example, constructs D and L (that lack the SANT domain) to constructs C and M (that contain the SANT domain), respectively (Figure 1.6, lane 12 vs. lane 10, and lane 27 vs. lane 25). Thus, the Bdp1 SANT domain likely contributes to the ability of the DmSNAPc-Bdp1 complex to recruit TBP. This is not surprising because crystal and cryo-EM structures indicate that the human and yeast Bdp1 SANT domains directly contact TBP [Abascal-Palacios et al., 2018; Gouge et al., 2017; Vorlander et al., 2018]. However, under the conditions of our assay, we find that the region of Bdp1 that lies downstream of the SANT domain is essential for TBP recruitment. These conclusions are summarized schematically on the lower right side of Figure 1.6. Based upon our results, it is possible that residues 424-510 of fly Bdp1 may interact with the N-terminal tail of TBP, as well as with DmSNAPc.

DISCUSSION

Although progress has been reported through studies in the human system toward understanding how SNAPc and TFIIIB form a stable complex on U6 snRNA gene promoters, much remains unknown. SNAPc was found to stabilize the binding of TBP to the U6 promoter TATA box by means of cooperative binding interactions [Hinkley et al., 2003; Ma and Hernandez, 2002; Mittal and Hernandez, 1997; Saxena et al., 2005]. We likewise observed weak cooperative binding of DmSNAPc and TBP to U6 promoter DNA (Figure 1.1). More interestingly, we also detected an interaction between promoter-bound DmSNAPc and Bdp1, an interaction only recently observed in the human system and published [Gouge et al., 2017] while our work was in progress [Roy-Engel, 2017]. By truncation experiments, a region of Bdp1 required for recruitment by DmSNAPc was localized to residues 424 to 510 of *D. melanogaste*r Bdp1, a region of the protein lying just C-terminal of the highly conserved SANT domain (Figure 1.2).

In the human system, Gouge et al. [Gouge et al., 2017] reported that Bdp1 constructs containing the SANT domain and sequences either N-terminal or C-terminal of the SANT domain could interact with SNAPc. Furthermore, deletion of the N-terminal sequences appeared to be more detrimental to this interaction than deletion of C-terminal sequences. In contrast, we did not detect a stable interaction between DmSNAPc and the N-terminal domain of Bdp1 on the U6 promoter, even with constructs that contained the SANT domain (for example, see Figure 1.2, constructs G and H). Thus, in work in the fly system described here, a DmSNAPc interaction was observed with Bdp1 sequences only C-terminal of the SANT domain. Presumably, these differences could be due to species-specific variations, differences

in the stringency of the assays, or other unknown factors.

In additional work, we found that the DmSNAPc-Bdp1 complex was able to recruit TBP to the TATA box with much greater efficiency than DmSNAPc alone (Figure 1.4A). Thus, Bdp1 apparently acts as a bridge between DmSNAPc and TBP to establish a quaternary complex of the three proteins tightly bound to the U6 promoter DNA (illustrated in Figure 1.7A). Residues 424 to 510 of Bdp1, a region just C-terminal of the SANT domain, was primarily required for this interaction, although inclusion of the Bdp1 SANT domain provided further stability to the complex (Figure 1.6). Not surprisingly, TBP recruitment depended upon the conserved TATA box (Figure 1.5A, illustrated in Figure 1.7A,B), but significantly, the evolutionarily non-conserved N-terminal tail of TBP considerably stabilized the DmSNAPc-Bdp1-TBP complex on the DNA (Figure 1.5C), reminiscent of findings in the human system [Mittal and Hernandez, 1997].

Perhaps most interestingly, we found that DmSNAPc must be bound to a U6 PSEA in order to recruit Bdp1 (Figure 1.3). A 5-base-pair change that converted the U6 PSEA to a U1 PSEA in the otherwise complete context of the U6 promoter abolished the ability of bound DmSNAPc to recruit Bdp1, even though DmSNAPc was still efficiently bound. This suggests that, when DmSNAPc is bound to a U1 PSEA, the Bdp1-interaction surface of DmSNAPc is occluded or wrongly disposed to interact with Bdp1 (Figure 1.7C). This finding provides new evidence to support a model of RNA polymerase specificity on snRNA genes previously proposed by our lab [Hung and Stumph, 2011; Kang et al., 2014; Kim et al., 2010a, b; Li et al., 2004; Wang and Stumph, 1998] in which the specific DNA sequence of the PSEA affects DmSNAPc conformation and determines its ability to recruit only Pol III general transcription factors to the U6 gene and Pol II general transcription factors to the U1 gene.

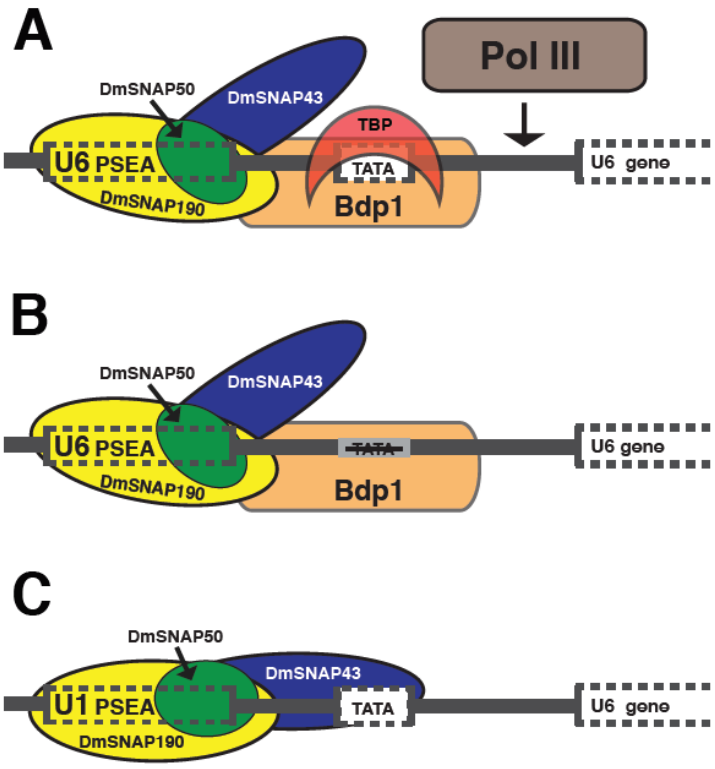

Figure 1.7: Five essentials that contribute to forming a stable snRNA-specific protein-DNA complex on the bipartite *Drosophila* U6 gene promoter: (1) an snRNA PSEA that is Pol III-specific; (2) a TATA box; (3) DmSNAPc (3 subunits); (4) TBP; and (5) Bdp1 as a factor to bridge between DmSNAPc and TBP. (**A**) The wild type scenario for a fly U6 snRNA gene is shown. DmSNAPc binds to a U6 PSEA in a conformation that allows it to recruit Bdp1 and subsequently TBP. (**B**) Mutation of the TATA box interferes with TBP recruitment but does not affect Bdp1 recruitment by DmSNAPc. (**C**) Switching the U6 PSEA to a U1 PSEA alters the conformation of DmSNAPc and interferes with its ability to recruit Bdp1. In each of the diagrams, the DmSNAPc subunits as well as Bdp1 and TBP are depicted at locations on the promoter sequences based upon site-specific protein-DNA photo-cross-linking experiments [Hung and Stumph, 2011; Kang et al., 2016; Lai et al., 2005; Wang and Stumph, 1998; Li et al., 2004; Kim et al., 2010a; Kim et al., 2010b; Kang et al., 2014].

In summary, when DmSNAPc binds to a U6 promoter, it exists in a conformation whereby it can recruit Bdp1 and subsequently TBP for Pol III transcription (Figure 1.7A). If the promoter contains a U6 PSEA but no TATA box, Bdp1 can be recruited but not TBP (Figure 1.7B). If the promoter were to contain a U1 PSEA instead of a U6 PSEA, Bdp1 could not be recruited and hence TBP also would not be effectively recruited (Figure 1.7C). These

two independent "checkpoints" effectively prevent Pol III from being recruited to U1-U5 snRNA gene promoters.

Altogether, our results indicate that a network of very specific molecular interactions occur that cooperatively establish a stable protein-DNA complex on the fly U6 promoter that is specific for Pol III transcription. This interaction network must involve at least five important conserved components: a Pol III-specific PSEA, DmSNAPc (bound to that Pol III-specific PSEA), Bdp1, TBP, and a TATA box (Figure 1.7).

ACKNOWLEDGEMENTS

Chapter 1, in full, is a reprint of a manuscript submitted for publication. SNAPc interacts with Bdp1 on a U6, but not a U1, snRNA gene promoter to establish a stable protein-DNA complex with TBP. Verma, N., Hurlburt, A.M., Phan, P., Wolfe, A., Kim, M., Kang, Y.S., Kang, J.J., and Stumph, W.E. The dissertation author, designated above as Hurlburt, A.M., was a primary researcher and co-first author of this paper.

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CHAPTER 2

Investigating pre-initiation factors and protein-protein interactions on the *Drosophila melanogaster* U6 snRNA gene promoter

INTRODUCTION

The *Drosophila melanogaster* snRNA genes consist of two classes. The U1, U2, U4, and U5 genes are transcribed by RNA Polymerase II (Pol II) while the U6 gene is transcribed by RNA Polymerase III (Pol III) [Dahlberg and Lund, 1988; Parry et al., 1989; Lobo and Hernandez, 1994; Hernandez, 2001]. Both gene classes have similar bipartite promoter structures consisting of a 21-base pair proximal sequence element A (PSEA) and a secondary element which is a proximal sequence element B (PSEB) in Pol II transcribed genes or a TATA box in Pol III transcribed genes (See Figure G.2) [Das et al., 1987; Zamrod et al., 1993; Hernandez et al., 2007].

In order for transcription to occur, both types of genes require the multi-subunit small nuclear RNA activating protein complex (DmSNAPc) [Sadowski et al., 1993; Goomer et al., 1994; Yoon et al., 1995] that consists of DmSNAP43, DmSNAP50, and DmSNAP190 and binds to the PSEA [Wang and Stumph, 1998; Li et al., 2004; Lai et al., 2005]. Though required by all snRNA genes, protein-DNA photo-cross-linking experiments have shown that DmSNAPc takes on a different conformation when bound to a U1 PSEA versus a U6 PSEA, which only differ by five nucleotides (See Figure G.3) [Wang and Stumph, 1998, Li et al., 2004, Lai et al., 2005; Kim et al., 2010a; Kim et al., 2010b; Hung et al., 2011; Doherty et al., 2012; Kang et al., 2014]. In cellular transfection assays, when the U1 PSEA was changed into a U6 PSEA, and vice versa, the promoters became transcriptionally inactive. When done *in vitro*, there was a respective switching of polymerase specificity such that the U1 PSEA recruited Pol II and U6 recruited Pol III, irrespective of the remaining promoter context [Jensen et al., 1998, McNamara-Schroeder et al., 2001; Lai et al., 2005]. This revealed that the PSEA sequence is a major determinant of the differential polymerase recruitment. Together

with the protein-DNA photo-cross-linking results, this suggests that differences in the conformation of DmSNAPc are required for the recruitment of distinct sets of Pol II or Pol III general transcription factors.

Genes transcribed by Pol III were thought to all require the TATA binding factor (TBP) [Margottin, 1991; White 1992; Willis, 1993]; however, later studies in fruit flies showed that the *Drosophila* tRNA and 5S RNA genes require the TBP related factor, TRF1, for transcription [Takada et al., 2000]. Despite this, the *Drosophila* U6 genes utilize TBP [Verma et al., 2013]. This difference in utilization of TRF1 or TBP for Pol III transcription is likely due to their difference in promoter structure since U6 genes have promoters external to the gene sequence that are recognized by DmSNAPc, while tRNA and 5S genes have promoters internal to the gene sequence that are recognized by TFIIIC (and TFIIA in the case of 5S genes) [Willis, 1993; Geiduschek and Kassavetis, 2001; Hernandez, 2001; Schramm and Hernandez, 2002]. This presents different modes of pre-initiation complex (PIC) assembly even though both genes require a form of the transcription factor TFIIIB and the same polymerase is ultimately recruited.

In humans, TFIIIB at U6 promoters consists of TBP, Brf2 (paralogous to Brf1 and specific to mammalian Pol III-transcribed genes with upstream promoters), and Bdp1 [Schramm et al., 2000; Teichmann et al., 2000; Cabart and Murphy, 2001; Willis, 2002]. Because insects contain only a single Brf-related gene (corresponding to Brf1), *Drosophila* would be expected to utilize Brf1 for U6 transcription. Indeed, a whole-genome chromatin immunoprecipitation (ChIP) analysis indicated that DmBrf1 was present at U6 and other gene-external Pol III promoters [Isogai et al., 2007]. However, recent EMSA experiments in our lab have been unable to detect the formation of a DmSNAPc-Bdp1-TBP-Brf1 complex on

the U6 promoter, even though DmSNAPc-Bdp1-TBP and Bdp1-TBP-Brf1 complexes were easily detected [unpublished results]. Moreover, no experiments have been carried out to examine the Bdp1 occupancy of these promoters in *Drosophila in vivo*. Thus, it was important to reconfirm the occupancy of the proteins and determine how they interact with each other and the DNA in order to establish a mechanistic model for U6 transcription by Pol III.

Here, ChIP assays confirm that Brf1 is present both at U6 and tRNA genes *in vivo*. U6 snRNA genes, which are transcribed by Pol III, are also occupied by Bdp1 and TBP. On the other hand, U1 snRNA genes, which are transcribed by Pol II, are occupied by TBP, but are not occupied by Brf1 or Bdp1. To further understand the details of these interactions and why they are different for each gene class, preliminary data from cross-linking mass spectrometry analysis is presented. These experiments describe the beginning stages of understanding how the conformation of DmSNAPc may lead to different interactions between different sets of general transcription factors.

MATERIALS AND METHODS

Chromatin immunoprecipitations (ChIPs)

ChIPs were performed as previously described [Barakat et al., 2008]. Polyclonal antibodies were produced in rabbits against bacterially expressed TBP as previously described [Barakat et al., 2008]. Antibodies against bacterially expressed Brf1 and Bdp1 were prepared by GenScript. DNA primers for quantitative polymerase chain reaction (qPCR) analyzed two *Drosophila melanogaster* U6 gene loci (U6:96Ab and U6:96Ac), one U1 gene loci (U1:95Ca), two tRNA gene loci (tRNA:CR30206 and tRNA:CR30509), and one non-specific gene sequence as a negative control [described in Verma et al., 2013 supplementary material].

FLAG purification

Following copper sulfate induction, cells were lysed in CelLytic M lysis buffer (Sigma #C2978) containing 1% protease inhibitor cocktail (Sigma #P8340). Lysates were adjusted to a NaCl concentration of 0.35 M prior to incubating with anti-FLAG M2 affinity gel (Sigma #A2220) overnight to allow for the capture of the FLAG-tagged proteins. The gel was then washed three times in 0.05 M Tris-HCl (pH 7.4), 0.35 M NaCl, and then three times in HEMG-100 buffer (25 mM HEPES K+ [pH 7.6], 0.1 mM EDTA, 12.5 mM $MgCl_2$, 10 µM $ZnCl_2$, 10% glycerol, 100 mM KCl). The proteins/complexes were eluted from the gel by competition with 3X FLAG peptide (Sigma #F4799) at a concentration of 200 µg/ml in HEMG-100 buffer. The FLAG peptide was then removed through dialysis in HEMG-100 buffer.

Protein-protein cross-linking

Ideally, at least 10-50 picomoles of FLAG purified DmSNAPc should be incubated at 25°C for 30 minutes with a 10-35 fold molar excess of either wildtype U6 promoter DNA (sequence from position -75 to -7) or, as a negative control, U6 promoter DNA with a fully mutated PSEA and TATA box. For the specific experiments shown in Figure 2.2 B and 2.3 B and C, 3.8 picomoles of protein and 125 picomoles of DNA were incubated together in a 510.5 µl total volume of 25 mM HEPES K+ [pH 7.6], 0.1 mM EDTA, 12.5 mM $MgCl_2$, 10 µM $ZnCl_2$, 10% glycerol, 100 mM KCl, and 8 mM NaCl. After the 30 minute incubation, 2-5 mM of bis(sulfosuccinimidyl)suberate (BS3) cross-linking reagent (Thermo Fisher #21585) was added for 30 minutes at 25°C followed by 20 mM of ammonium bicarbonate quenching reagent for an additional 5 minutes. A minimum of 9 µl of the sample was loaded onto a 10%

denaturing polyacrylamide gel and detected with antibodies against either DmSNAP43 or DmSNAP190. The remainder of the sample was sent to Dr. Jeff Ranish at the Institute for Systems Biology for mass spectrometry analysis.

Immobilized Template Assay

The assay utilized 4 µg of double stranded wildtype U6 promoter DNA (-121 to +10) or U6 promoter DNA with a fully mutated PSEA and TATA box conjugated with a N-terminal biotin label on the upper strand, in a solution of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 2 M NaCl. DNA sequences are indicated below with the PSEA and TATA regions designated in red.

Wildtype U6 promoter DNA (-121 to +10)

5′ AAAGGAAATTTTGAAAATCTTAAGCAGAGGGTTCTTAAGACCATTTGCCAATTCTTATAATTCTCAACTGCTCTTTCCTGATGTTGATCATTTATATAGGTATGTTTTCCTCAATACTTCGTTCTTGCTT 3′

3′ TTTCCTTTAAAACTTTTAGAATTCGTCTCCCAAGAATTCTGGTAAACGGTTAAGAATATTAAGAGTTGACGAGAAAGGACTACAACTAGTAAATATATCCATACAAAAGGAGTTATGAAGCAAGAACGAA 5′

Mutant U6 promoter DNA (-121 to +10)

5′ AAAGGAAATTTTGAAAATCTTAAGCAGAGGGTTCTTAAGACCATTTGCCAATTCTTACCTGATAGGTGACCAGGACTATGATGTTGATCAGGGACCTCGGTATGTTTTCCTCAATACTTCGTTCTTGCTT 3′

3′ TTTCCTTTAAAACTTTTAGAATTCGTCTCCCAAGAATTCTGGTAAACGGTTAAGAATGGACTATCCACTGGTCCTGATACTACAACTAGTCCCTGGAGCCATACAAAAGGAGTTATGAAGCAAGAACGAA 5′

The DNA was immobilized on 800 µg of streptavidin coated magnetic beads blocked with 2.5 mg of bovine serum albumin. The beads were then washed three times with HEMG-100 buffer (25 mM HEPES K+ [pH 7.6], 0.1 mM EDTA, 12.5 mM $MgCl_2$, 10 µM $ZnCl_2$, 10% glycerol, 100 mM KCl). A protein solution was added to the beads that contained 60 µl of DmSNAPc in HEMG-100 buffer, 3 mM DTT, and 14 µg of poly dG-dC. Unbound protein was washed away three times with HEMG-100 buffer. Bound protein was eluted with sodium dodecyl sulfate (SDS) and heat. 10% of the sample after each incubation and wash was loaded on a 10% denaturing polyacrylamide gel and detected using an antibody against DmSNAP43 or DmSNAP190.

RESULTS AND DISCUSSION

Occupancy of TFIIIB components on Pol II and Pol III transcribed genes

Previous work in the lab has shown that DmSNAPc can recruit Bdp1 which then recruits TBP to the U6 snRNA promoter *in vitro* (see Chapter 1). However, we have been unable to detect co-occupancy of DmSNAPc and Brf1 (the third component of TFIIIB) by EMSAs that included the other two TFIIIB components. Work by other labs has shown that TRF1 and Brf1 colocalize to *Drosophila* Pol III-transcribed genes and work as an alternative TFIIIB complex both *in vitro* and *in vivo* [Takada et al., 2000; Isogai et al., 2007]; however, work in our lab has shown that while this is true for tRNA genes, U6 snRNA genes recruit TBP rather than TRF1 [Verma et al., 2013]. The following experiments confirm the occupancy of the components of TFIIIB (TBP, Bdp1 and Brf1) on *Drosophila melanogaster* U6 snRNA promoters *in vivo*. ChIP assays on *Drosophila* S2 cells were performed that specifically immunoprecipitated DNA bound by TBP, Bdp1 or Brf1. One U1 gene locus, two U6 gene loci, and two tRNA gene loci, as well as a non-specific region of the genome, were targeted for analysis.

The amount of DNA detected by qPCR at each locus was compared against the total amount of DNA input into the experiment to produce the percentage of total DNA presented in Figure 2.1. TBP (red bars) was specifically detected on both U1 and U6 promoters but was at background levels for tRNA promoters. This is in accordance with a previous study by our lab [Verma et al., 2013]. The blue and purple bars indicate the occupancy of Brf1 and Bdp1 respectively. Each of these proteins exhibited high levels of occupancy at both the U6 and tRNA gene promoters, but not at the U1 promoter. This confirms that Brf1 and Bdp1 are utilized at U6 promoters as well as at tRNA promoters. This is expected since these

components are unique to Pol III transcription. Differences between loci of the same genes were observed (lower signals for U6:96Ab compared to U6:96Ac, and CR30206 compared to CR30509) and are most likely due to their different expression efficiencies within the S2 cells. This study reconfirms that Brf1 is utilized at U6 promoters, and for the first time establishes that Bdp1 is utilized at U6 promoters *in vivo*.

Because our lab has not been able to show that DmSNAPc and Brf1 are capable of binding simultaneously to the U6 promoter (together with Bdp1 and TBP), this may indicate that regulatory events determine which proteins bind and when. This regulation may restrict the ability for DmSNAPc and Brf1 to bind at the same time. DmSNAPc may act as the nucleating factor which begins PIC formation but may not be present after Brf1 is recruited to the complex for the recruitment of Pol III. Further work needs to be done to assess these possibilities.

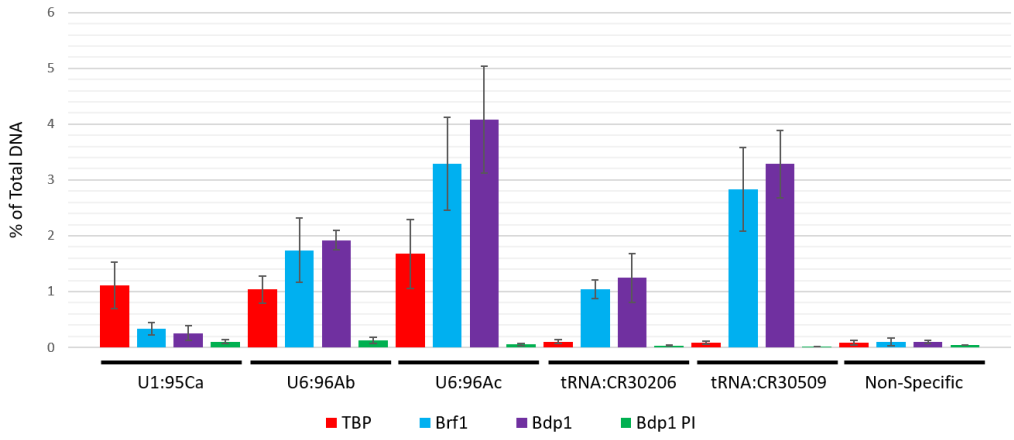

Figure 2.1: Bdp1 and Brf1 specifically occupy Pol III transcribed genes while TBP occupies both Pol II and Pol III transcribed snRNA, but not tRNA, genes. Bar graph representation of the average of three qPCR reactions following ChIP assays. Antibodies against TBP, Brf1, Bdp1 were used to immunoprecipitate chromatin bound to the respective proteins. Pre-immune (PI) sera was used as a negative control. The chromatin was then identified using primers targeting a single U1 gene, two U6 genes, and two tRNA genes indicated below each data set. A non-specific DNA sequence was also targeted as a negative control. Signals were normalized to the percent of total DNA input into the experiment. Each protein analyzed is assigned to a specific color as indicated in the key.

Cross-linking mass spectrometry (CXMS) of DmSNAPc

Collectively, data from our lab and others have shown that U1 and U6 promoters recruit a specific and distinct set of transcription factors that are required for Pol II or Pol III recruitment. Therefore, understanding how these proteins interact with each other and the DNA is pivotal in understanding this mechanism. Efforts towards gaining detailed information on protein-protein interactions within the PIC are moving forward but these interactions are still not well understood. This information is typically best gained through crystallography; however, previous work in the lab to obtain large amounts of the *Drosophila* SNAPc subunits has been unsuccessful. Therefore, an alternative technique termed cross-linking mass spectrometry (CXMS) was utilized which has been used by other labs to examine the interactions between subunits of large protein complexes such as TFIIH and Mediator [Luo et al., 2015; Robinson et al., 2016].

This technique cross-links protein complexes with a BS3 amine reactive bifunctional cross-linker (11.4 angstroms) that creates cross-links between the amino groups of lysines within a 5-30 angstrom distance. Proteolysis using trypsin, which cuts after lysines and arginines, gives peptides of particular masses. The cross-linked peptides are analyzed using mass spectrometry. The masses identified are then checked against a database containing the predicted mass of every possible cross-link in the *Drosophila* proteome. This identifies the particular lysines that have been cross-linked and can be used to create a cross-link map. The map can be used to consider restraints for structural model selection. Our lab has begun utilizing this technique to detect which domains of DmSNAPc are in close proximity to each other under different conditions.

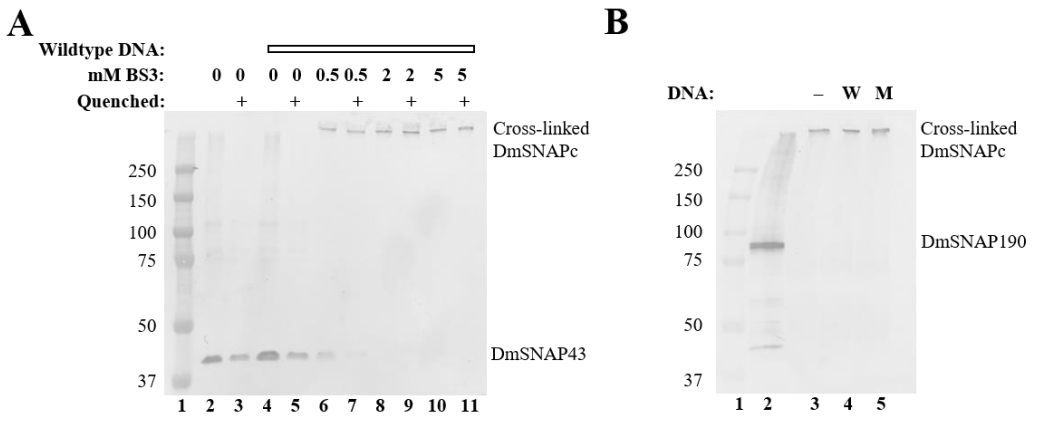

Figure 2.2: Western blots testing the efficiency of the cross-linking reagent, BS3 under various conditions. (**A**) 18 µl of DmSNAPc was incubated in the absence (lanes 2-3) or presence of a 10 fold molar excess of wildtype U6 promoter DNA (0.135 µg) (lanes 4-11). Reactions were then incubated for 30 minutes with either 0, 0.5, 2, or 5 mM of cross-linking reagent, BS3 (as indicated above each lane). 9 µl of the 27 µl reaction was loaded onto the gel in the absence or presence of the quenching reagent, ammonium bicarbonate as indicated above the lanes. Samples were run on a 10% denaturing gel and detected using an antibody against the DmSNAP43 subunit. (**B**) 500 µl of DmSNAPc was incubated in the absence (lane 3) or presence of a 25 fold molar excess (5.25 µg) of either wildtype (W) or mutant (M) U6 promoter DNA (lanes 4 and 5 respectively). Reactions were then incubated with 2 mM BS3 and quenched. 9 µl of the 510.5 µl reaction were loaded onto the gel (lanes 3-5). Lane 2 contains DmSNAPc without BS3 as a control. Samples were run on a 10% denaturing polyacrylamide gel and detected using an antibody against the DmSNAP190 subunit.

The three subunits of DmSNAPc (DmSNAP43, DmSNAP50 and DmSNAP190) were co-expressed in *D. melanogaster* S2 cells and co-purified using FLAG affinity purification. The proteins were then utilized in cross-linking reactions containing either no DNA, wildtype U6 promoter DNA (sequence from position -75 to -7), or U6 DNA with fully mutated PSEA and TATA sequences. First, cross-linking efficiency was confirmed by running the samples on a western blot with detection by an antibody against the DmSNAP43 subunit (Figure 2.2A). Reactions including DmSNAPc with and without wildtype U6 DNA and with and without the cross-linking quenching reagent, ammonium bicarbonate, were used as controls to ensure that the addition of DNA and/or the quenching reagent did not affect the migration of the protein itself through the gel (Figure 2.2A, lanes 2-5). DmSNAPc with wildtype U6 DNA was then incubated with 0.5 mM cross-linking reagent, BS3, (lanes 6-7), 2 mM BS3 (lanes 8-

9), and 5 mM BS3 (lanes 10-11) and tested with and without quenching. Addition of the cross-linking reagent shifted the DmSNAP43 protein band to the top of the gel, indicating that the complex was successfully cross-linked (compare lanes 6-11 with 2-5). It was also observed that some uncross-linked protein was still visible in lanes 6 and 7 which used only 0.5 mM BS3. Therefore, 2 mM BS3 was selected as the ideal concentration for future experiments.

In a follow-up full-scale experiment (Figure 2.2B), 500 µl DmSNAPc (approximately 3.8 picomoles) was incubated with either 5.25 µg wildtype U6 promoter DNA, 5.25 µg mutant promoter DNA, or with no DNA in a total volume of 510.5 µl per reaction. This was followed by the addition of 100 mM BS3 to a final concentration of 2 mM. A 9 µl aliquot of the 510.5 µl sample was run on a western blot with detection using an antibody against DmSNAP190 (Figure 2.2B). The blot confirmed that cross-linking of DmSNAPc was achieved in all conditions (lanes 3-5). The remainder of each (~500 µl) sample was sent to Dr. Jeff Ranish's lab at the Institute for Systems Biology (ISB) for CXMS.

Data from CXMS analysis is presented in Figure 2.3. Parts A, B, and C of the figure correspond to cross-linking without DNA, with wildtype U6 DNA, and with mutant U6 DNA, respectively. Due to sample mishandling, the map presented in Figure 2.3A is from an experiment done previous to that described in Figure 2.3B and C, using a DmSNAPc sample that is believed to have been purer and at a higher concentration. These cross-linking maps display the cross-links (red lines) between the lysines (green dots) of different subunits of DmSNAPc.

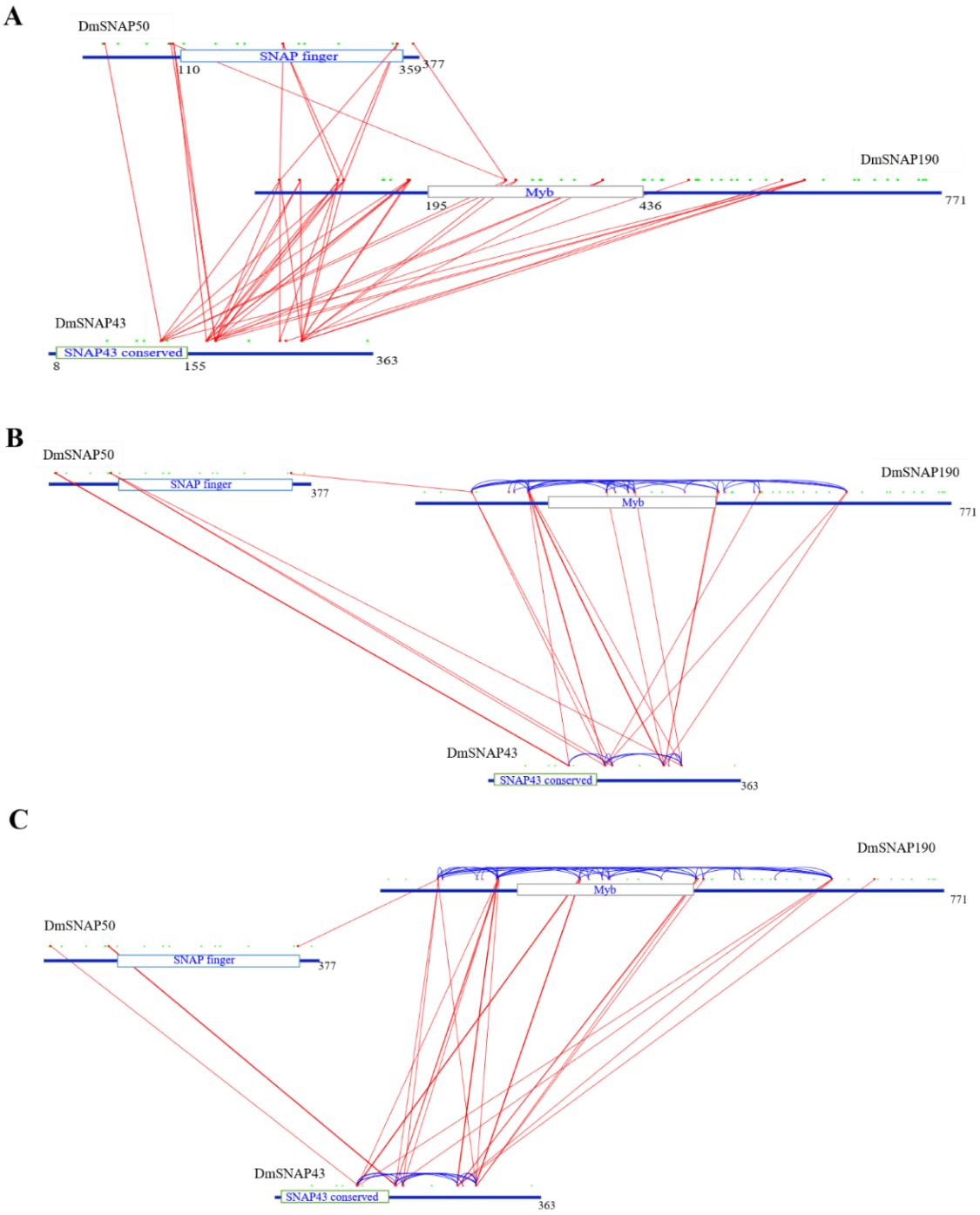

Figure 2.3: Cross-linking maps of DmSNAPc in three different conditions. Maps produced by cross-linking mass spectrometry data. Red lines represent cross-links between the lysines (indicated by green dots) of either the DmSNAP43, DmSNAP50 or DmSNAP190 subunits of DmSNAPc. Blue lines represent intra-cross-links within a single subunit but are not shown in (A). Each subunit is represented by a blue bar and conserved regions are indicated. (**A**) Map of DmSNAPc free of DNA. (**B**) Map of DmSNAPc with wildtype U6 promoter DNA. (**C**) Map of DmSNAPc with U6 promoter DNA containing full mutations of the PSEA and TATA box.

When no DNA was present, 47 cross-links between lysines were detected with less than a 1% false discovery rate. This first set of data acts as a control to reveal the cross-linking pattern of DmSNAPc in the absence of any DNA. When the experiment was performed under conditions in which greater than ~95% of the DmSNAPc was expected to bind to wildtype U6 promoter DNA (assuming a minimal K_a of 10^8, which is a low affinity for a sequence-specific DNA-binding protein), only 19 DmSNAPc inter-subunit cross-links were reliably detected. When U6 DNA with a mutant PSEA and TATA box was used under otherwise identical conditions, 22 cross-links were detected. The blue lines (only present in Figure 2.3B and C) represent cross-links between lysines of a single subunit and are not being considered at this time.

The maps of DmSNAPc incubated with wildtype and mutant DNAs exhibit similar, yet partially different, cross-linking patterns. Out of a total of 27 inter-subunit cross-links, 14 were shared when DmSNAPc was incubated with the two different DNAs (Table 2.1). The single cross-link between DmSNAP190 and DmSNAP50, 2 out of 4 of the cross-links between DmSNAP50 and DmSNAP43, and 11 out of 22 of the cross-links between DmSNAP190 and DmSNAP43 were shared between the wildtype and mutant maps (Table 2.1 and Figure 2.3B compared to C). Interestingly, looking from the converse perspective, 50% of the cross-links between DmSNAP190 and DmSNAP43 and between DmSNAP43 and DmSNAP50 were different depending upon whether the included DNA was wildtype or mutant.

Because the three subunits of DmSNAPc are pre-assembled prior to binding to DNA, it is likely that many of the same cross-links would be maintained in both the free and bound states. Therefore, the unshared cross-links may reflect a change in DmSNAPc conformation

upon binding to a wildtype U6 promoter. If it is assumed that DmSNAPc is free in solution in the presence of the mutant DNA (which should be a safe assumption considering that a PSEA is required for DmSNAPc to bind to DNA), then the number of shared and distinct cross-links seems reasonable.

Table 2.1: Comparison of cross-links of DmSNAPc in the presence of wildtype and mutant U6 promoter DNA. Cross-links between subunits of DmSNAPc in the presence of wildtype U6 DNA (diagrammed in Figure 2.3B) are listed in the left column and cross-links in the presence of mutant U6 DNA (diagrammed in Figure 2.3C) are listed in the right column. The subunits cross-linked are listed in bold at the beginning of each section which is preceded by a gray bar. Each number indicates the position of the lysine cross-linked from each subunit. Shared cross-links are listed in both columns. If a cross-link is absent, it is represented by red shading.

CROSS-LINKS BETWEEN DmSNAPc SUBUNITS AND WILDTYPE U6 PROMOTER DNA	CROSS-LINKS BETWEEN DmSNAPc SUBUNITS AND MUTANT U6 PROMOTER DNA
DmSNAP190:DmSNAP43	**DmSNAP190:DmSNAP43**
DmSNAP190:322---DmSNAP43:284	
DmSNAP190:618---DmSNAP43:259	DmSNAP190:618---DmSNAP43:259
DmSNAP190:618---DmSNAP43:177	DmSNAP190:618---DmSNAP43:177
	DmSNAP190:618---DmSNAP43:126
DmSNAP190:496---DmSNAP43:177	
	DmSNAP190:673---DmSNAP43:259
	DmSNAP190:282---DmSNAP43:126
DmSNAP190:282---DmSNAP43:259	
	DmSNAP190:282---DmSNAP43:284
	DmSNAP190:447---DmSNAP43:284
DmSNAP190:438---DmSNAP43:259	DmSNAP190:438---DmSNAP43:259
	DmSNAP190:438---DmSNAP43:284
DmSNAP190:437---DmSNAP43:259	DmSNAP190:437---DmSNAP43:259
DmSNAP190:172---DmSNAP43:259	DmSNAP190:172---DmSNAP43:259
	DmSNAP190:172---DmSNAP43:187
DmSNAP190:172---DmSNAP43:126	DmSNAP190:172---DmSNAP43:126
DmSNAP190:172---DmSNAP43:177	DmSNAP190:172---DmSNAP43:177
DmSNAP190:174---DmSNAP43:177	DmSNAP190:174---DmSNAP43:177
DmSNAP190:174---DmSNAP43:284	DmSNAP190:174---DmSNAP43:284
	DmSNAP190:93---DmSNAP43:284
DmSNAP190:93---DmSNAP43:187	DmSNAP190:93---DmSNAP43:187
DmSNAP190:93---DmSNAP43:177	DmSNAP190:93---DmSNAP43:177
50% similarity of cross-links between DmSNAP190 and DmSNAP43	
DmSNAP190:DmSNAP50	**DmSNAP190:DmSNAP50**
DmSNAP190:93---DmSNAP50:353	DmSNAP190:93---DmSNAP50:353
100% similarity of cross-links between DmSNAP190 and DmSNAP50	
DmSNAP43:DmSNAP50	**DmSNAP43:DmSNAP50**
DmSNAP43:187---DmSNAP50:101	
DmSNAP43:284---DmSNAP50:101	
DmSNAP43:177---DmSNAP50:101	DmSNAP43:177---DmSNAP50:101
DmSNAP43:126---DmSNAP50:24	DmSNAP43:126---DmSNAP50:24
50% similarity of cross-links between DmSNAP43 and DmSNAP50	

However, it needs to be noted that these maps have 44% fewer cross-links compared to the map of free DmSNAPc from a previously prepared protein sample (Figure 2.3A compared to B and C). The significantly reduced number of cross-links in samples B and C could be due to the masking of potential cross-links by impurities in the submitted sample or due to a lower amount of DmSNAPc submitted for analysis. Alternatively, they could be real phenomena. Thus, these experiments need to be repeated with a higher amount of purer protein.

Affinity purification of DmSNAPc by using an immobilized template

In the experiments described above, DmSNAPc was purified from cellular lysates. Here, initial experiments are described that use an immobilized template to further purify DmSNAPc. There is also another advantage to this method: if the cross-linking is carried out on the DNA affinity resin, this method would ensure that only DmSNAPc bound to the U6 DNA would be available for cross-linking, as unbound protein would be removed during the resin washing steps.

To test the functionality of the assay, a wildtype U6 DNA template was synthesized by IDT that was conjugated with a biotin label. This template was immobilized on magnetic beads coated with streptavidin and incubated with a sample containing nickel-chelate purified DmSNAPc. The expectation was that only proteins that interacted with the U6 template would be bound and that the contaminating proteins would be washed away. The bound proteins were then eluted using SDS and heat and analyzed on a western blot with detection using antibodies against either the DmSNAP43 or DmSNAP190 subunit of DmSNAPc (Figure 2.4A). A reduced level of both proteins was found in the unbound fraction (FT in

lanes 3 and 9) after incubation with the DNA. The wash fractions (W1 and W2 in lanes 4-5 and 10-11) contained little to no DmSNAP protein. However, a strong band was visible in the eluted fraction (E in lanes 6 and 12). This elution confirmed that these subunits of DmSNAPc were capable of binding to the U6 template and were likely purified from other proteins within the sample that could not bind the template.

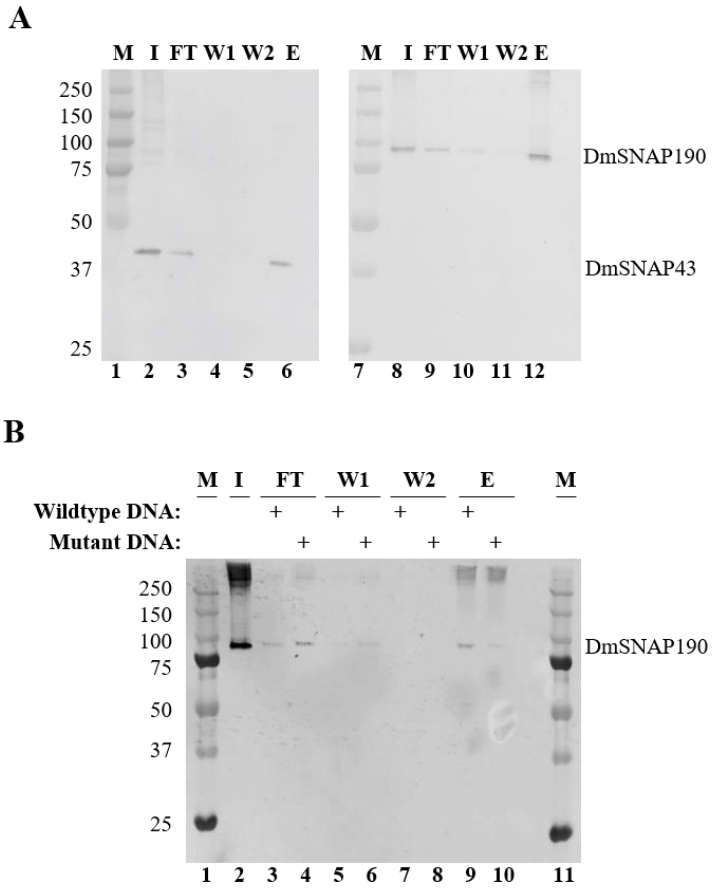

Figure 2.4: DmSNAPc can be pulled down in a specific manner using an immobilized template assay. Western blots testing for the presence of DmSNAPc subunits during steps of a pull down by immobilized template. 4 µg of U6 promoter DNA containing a biotin label was immobilized on 800 µg of magnetic beads coated with streptavidin then incubated with 140 µl of solution containing DmSNAPc. 6 µl of the DmSNAPc input (I) onto the beads was loaded to show the position of the protein. 5 µl of unbound protein (flow through – FT), 7.5 µl of loosely bound protein that was washed off (W1 and W2), and 10 µl of protein eluted with SDS and heat (E) were analyzed on a 10% denaturing polyacrylamide gel. The molecular weights of the protein marker (M) are indicated to the left of the blots. (**A**) DmSNAPc was incubated with wildtype immobilized U6 promoter DNA. Western blots were detected with antibodies against the DmSNAP43 (left panel) and DmSNAP190 (right panel) subunits. (**B**) DmSNAPc was incubated with both wildtype (odd lanes from 3-9) and mutant (even lanes from 4-10) immobilized U6 promoter DNA. The western blot was detected with an antibody against the DmSNAP190 subunit.

Next, binding of DmSNAPc to immobilized wildtype U6 promoter DNA and mutant U6 promoter DNA (which had a fully mutated PSEA and TATA box) was assessed. Binding of DmSNAPc to the wildtype template appeared to be more efficient than to the mutant template due to more DmSNAP190 appearing in the flow-through and first wash fraction from the mutant template than from the wildtype template (compare lane 4 with lane 3 and lane 6 with lane 5). Furthermore, following the washes, significantly more DmSNAP190 was eluted from the wildtype template in comparison to the mutant template (compare lanes 9 and 10). This established that DmSNAPc was binding to the U6 template in a specific manner. These results were also confirmed by similar experiments carried out in the Ranish lab [unpublished results]. Future work will involve utilizing this technique to produce a protein sample that should be more compatible with CXMS analysis.

Due to the preliminary nature of these data, decisive conclusions cannot be reached at the present time. Therefore, future research will involve comparing a cross-linking map of DmSNAPc bound to the U1 PSEA to a map of DmSNAPc bound to the U6 PSEA. It is proposed that this information will provide further evidence for our mechanistic model for RNA polymerase specificity established through protein-DNA photo-cross-linking data [Wang and Stumph, 1998, Li et al., 2004, Lai et al., 2005] that suggests DmSNAPc takes on a different conformation depending on whether it is bound to a U1 or a U6 PSEA.

Additional work will look at specific contacts between DmSNAPc with Bdp1 and TBP to follow up the work presented in Chapter 1 of this dissertation. In the end, this work aims to further show that the conformation of DmSNAPc promotes the recruitment of different sets of transcription factors that then recruit specific polymerases to *Drosophila* snRNA gene promoters.

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CONCLUDING REMARKS

A specific region of Bdp1 required for interaction with DmSNAPc on U6 snRNA gene promoters

Although advances have been made, there is still much to be learned about the mechanism of pre-initiation complex formation on U6 snRNA gene promoters. Therefore, this research aimed to understand the proteins involved, how they interact with each other and the DNA, and how they are recruited into a pre-initiation complex that allows transcription to occur. Chapter 1 describes a pathway for Bdp1 and TBP recruitment by DmSNAPc. Furthermore, specific regions of Bdp1 and TBP were identified that are required for their efficient recruitment. Upon assessing the binding ability of TBP, Bdp1 and Brf1, it was clear that while TBP has a weak association with DmSNAPc, Bdp1 strongly interacts to form a stable complex with DmSNAPc, independent of the other components of TFIIIB.

N- and C-terminal truncations of Bdp1 revealed a small region just C-terminal to the conserved SANT domain that was sufficient for Bdp1 interaction with DmSNAPc. Though the results appear clear, they are not in complete agreement with other reported work. For instance, work in the human system showed that the region N-terminal of the SANT domain is more important for the Bdp1-DmSNAPc interaction as removal of the N-terminal, but not the C-terminal, region of Bdp1 reduced its interaction with SNAPc [Gouge et al., 2017]. This difference could be due to species-specific variations, assay conditions, or other unknown factors. We are hoping that these protein-protein interactions between DmSNAPc and Bdp1 can be verified in future experiments involving cross-linking mass spectrometry (CXMS).

The DmSNAPc-Bdp1 complex is capable of recruiting TBP

Further work presented in Chapter 1 showed that DmSNAPc and Bdp1 together could recruit TBP to the U6 promoter. Since DmSNAPc and TBP only weakly interacted on their own, it was the addition of Bdp1 that allowed for the stable incorporation of TBP. It was discovered that the same region of Bdp1 sufficient for Bdp1 recruitment by DmSNAPc was also required for TBP recruitment by the DmSNAPc-Bdp1 complex. However, it was observed that in truncations that did not contain the SANT domain, the interaction was weaker than in the truncations that did contain the SANT domain. This could be connected to a finding in yeast that the SANT domain confers protease resistance [Saida, 2008], which when absent could alter the stability between the interaction of Bdp1 and TBP. Overall, the most important conclusion from this part of the study is that a region of Bdp1 between amino acids 424 and 510 acts as a bridging module between DmSNAPc and TBP in the formation of the pre-initiation complex on the *Drosophila* U6 snRNA gene promoter.

Furthermore, to understand the TBP requirement for complex formation, truncations of TBP were used to assess the role of the non-conserved N-terminal tail. In general, not much is known about the function of the non-conserved N-terminal tail of TBP though in humans it has interestingly been found to increase U6 transcription efficiency and to be required for SNAPc interaction with TBP [Mittal and Hernandez, 1997]. Similarly, our *Drosophila* TBP truncations that removed the N-terminal region resulted in much weaker interactions with the DmSNAPc-Bdp1 complex. This result suggests that the N-terminal tail, though not essential for complex formation, does contribute to the stability of the complex. Thus, our results reflect the earlier results in the human system and suggest that an important function of the TBP N-terminal tail is for TBP recruitment to U6 and U6-like promoters, and

that this function has been conserved at least since the evolutionary divergence of flies and humans.

Future experiments will involve using the N-terminal tail of TBP fused with the conserved C-terminal region of TRF1, which is similar to the conserved region of TBP but cannot be effectively recruited to the U6 snRNA promoter *in vivo* [Verma et al, 2013] or *in vitro* by EMSAs [unpublished results]. Our belief is that the TBP N-terminal tail will confer upon TRF1 the ability to be efficiently recruited by DmSNAPc-Bdp1. If so, this will provide very strong positive evidence for a role of the N-terminal tail in TBP recruitment. Furthermore, specific contacts between the N-terminal region of TBP with either DmSNAPc and/or Bdp1 will be investigated using CXMS.

The U6 PSEA, but not the U1 PSEA, is required for the recruitment of Bdp1

The experiments in this work support the mechanistic model of snRNA transcription developed by our lab. This model indicates that DmSNAPc takes on different conformations when bound to a U1 PSEA compared to when bound to a U6 PSEA [Wang and Stumph, 1998, Li et al., 2004, Lai et al., 2005]. The different conformations then recruit different sets of transcription factors, leading to a difference in polymerase recruitment. Work in Chapter 1 showed that when a U6 PSEA is exchanged for a U1 PSEA, Bdp1 can no longer be recruited to the U6 promoter by DmSNAPc. On the other hand, alteration of the TATA box had no effect on Bdp1's ability to bind. This provides further evidence that the PSEA is required for conformational changes in DmSNAPc that enable DmSNAPc bound to a U1 PSEA to recruit factors such as TBP, TFIIA and TFIIB, but when bound to a U6 PSEA, to recruit factors such as TBP, Bdp1 and Brf1.

TFIIIB subunits are selectively recruited to genes transcribed by Pol III

Work in Chapter 2 reconfirms the recruitment of Brf1 to *Drosophila* U6 promoters. Chromatin immunoprecipitation assays (ChIPs) showed that Brf1 was present on the U6 DNA, a result that the lab could not confirm using *in vitro* assays in the presence of DmSNAPc. In addition to Brf1, Bdp1 was also found by ChIPs to be recruited to *Drosophila* U6 gene promoters. Although this was expected, it was nevertheless important to confirm this since it had not been previously demonstrated.

Though the different gene classes recruit different sets of transcription factors, many of the factors share structures and/or sequences and often bind to similar regions of the promoter DNA [Colbert and Hahn, 1992; Wang and Roeder, 1995; Colbert et al., 1998; Cabart and Murphy, 2001; Gouge et al, 2017; Vorlander et al., 2018]. This suggests that at one time, all these genes may have been transcribed by the same polymerase, but evolutionary pressures and possible regulatory needs drove the pre-initiation complex to evolve over time.

Future work involving cross-linking mass spectrometry

Overall, the work presented in this dissertation has identified specific molecular interactions involved in the formation of the pre-initiation complex on U6 snRNA gene promoters and has provided information on molecular mechanisms of RNA polymerase specificity (Pol III versus Pol II) on snRNA gene promoters. There are at least five specific requirements to form a stable complex on a *Drosophila* U6 snRNA gene promoter: a Pol III-specific PSEA, DmSNAPc bound to a Pol III-specific PSEA, Bdp1, TBP, and a TATA box.

Interactions between the DmSNAPc subunits were examined in preliminary CXMS analyses described in Chapter 2. Cross-linking of DmSNAPc free of DNA produced a map

displaying interactions between the three subunits that were compatible with co-immunoprecipitation data that mapped domains of the DmSNAPc subunits that interact with each other in solution [Hung et al., 2009]. In more recent CXMS experiments, interactions of the DmSNAP subunits when incubated with wildtype and mutant U6 DNA were carried out. The results, though intriguing, were inconclusive at this stage due to the low abundance of specific protein in the submitted sample. It will be important to examine the reproducibility of these data using higher amounts of DmSNAPc and more pure samples.

Towards this end, an additional step of purification was attempted using an immobilized template, but this protocol has not yet been scaled up for CXMS. In addition to comparing bound and unbound DmSNAPc, further experiments will involve examining the specific cross-links that occur between specific transcription factors on the U6 promoter such as cross-links between DmSNAPc and Bdp1, between Bdp1 and TBP, and perhaps between DmSNAPc and TBP. Such studies should help us better understand the interactions between these proteins.

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APPENDIX

A. Detailed protocol for chromatin immunoprecipitation assay (ChIP)

B. Detailed protocol for electrophoretic mobility shift assay (EMSA)

C. Detailed protocol for nickel-chelate chromatography

D. Detailed protocol for high-salt FLAG purification

E. Detailed protocol for polyacrylamide gel electrophoresis (PAGE) purification of DNA oligos

F. Detailed protocol for protein cross-linking with BS3

G. Developing conditions for SNAPc purification on an immbolized template for BS3 cross-linking

Appendix A: Detailed protocol for chromatin immuno-precipitation assay

I. Solutions and materials

Formaldehyde cross-linking solution

	original solution				final solution	
Chemical	conc.	unit	add	unit	conc.	unit
Tris-HCl (pH8.0)	1.0	M	1.400	ml	50.0	mM
EDTA (pH8.0)	0.5	M	0.056	ml	1.0	mM
EGTA	0.1	M	0.140	ml	0.5	mM
NaCl	5.0	M	0.560	ml	100.0	mM
Formaldehyde	37.0	%	0.757	ml	1.0	%
final solution volume	28.0	ml	* final volume is from all chemicals + 25ml S2 cell culture.			

Note: Add each individual chemical directly to the 25 ml S2 cells in the order shown.

2M glycine

Dissolve 7.5g glycine in 50 ml sterile d.d. water. Store at 4°C.

Sonication buffer

	original solution				final solution	
Chemical	conc.	unit	add	unit	conc.	Unit
Tris-HCl (pH8.0)	1.0	M	0.50	ml	10.0	mM
EDTA (pH8.0)	0.5	M	0.10	ml	1.0	mM
EGTA	0.1	M	0.25	ml	0.5	mM
PMSF	0.2	M	0.125	ml	0.5	mM
final solution volume	50.0	ml	(add d.d. water to bring to the final volume. Store at 4°C.)			

Notes: PMSF is inactivated in aqueous solutions, so stock solution should be made in ethanol or isopropanol and only added to aqueous solutions immediately before use. Add 2.5 μl 0.2M PMSF per 1ml sonication buffer.
Also, add 10 μl protease inhibitor cocktail (SIGMA) per 1 ml sonication buffer right before use.

6M Urea

Dissolve 18.02 g Urea in 30 ml d.d. water. Adjust the volume to 50 ml with d.d. water. Store at room temperature. Use within 1-2 weeks.

ChIP buffer

	original solution				final solution	
chemical	conc.	unit	add	unit	conc.	unit
Tris-HCl (pH8.0)	1.0	M	10.0	ml	10.0	mM
EDTA (pH 8.0)	0.5	M	2.0	ml	1.0	mM
EGTA	0.1	M	5.0	ml	0.5	mM
PMSF	0.2	M	2.5	ml	0.5	mM
glycerol	100.0	%	100.0	ml	10.0	%
Triton-X100	100.0	%	10.0	ml	1.0	%
sodium deoxycholate (powder)	100.0	%	1.0	g	0.1	%
final solution volume	1000.0	ml	(add water to bring to the final volume. Store at 4°C.)			

Notes: PMSF is inactivated in aqueous solutions, so stock solution should be made in ethanol or isopropanol and only add to aqueous solutions immediately before use.

Usually only 200 ml ChIP buffer is needed per dialysis, so add 500 μl 0.2M PMSF for 200 ml ChIP buffer.

Appropriate antiserum and pre-immune serum
Anti-DmSNAP43 Ab (DmSNAP43 (03978) antibody 12-6-04 Nermeen) and Anti-FLAG polyclonal Ab (SIGMA, product code: F7425) are used in this case.

Immobilized Protein A sepharose (PIERCE, product code: 20333)

Note: Binding specificities and affinities of different antibody-binding proteins (protein A, G, A/G, and L) differ between source species and antibody subclass. In this case, protein A is selected because of its high affinity to rabbit IgG (anti-DmSNAP43 Ab and anti-FLAG polyclonal Ab). You may need to use other antibody-binding proteins if other antibodies are used in your application.

TE buffer
Add 1 ml of 1M Tris-HCl pH 8.0 and 200 μl of 0.5 M EDTA pH 8.0 in a 100 ml cylinder. Fill d.d. water to 100 ml graduation. Filter to sterilize. Store at 4°C.

10 mg/ml BSA (NEB, product code: B9001S)
Directly use the NEB 100X BSA (10 mg/ml) that comes with restriction enzymes.

Low-salt wash buffer

	original solution				final solution	
chemical	conc.	unit	add	unit	conc.	unit
Tris-HCl (pH8.1)	1.0	M	4.0	ml	20.0	mM
EDTA (pH 8.0)	0.5	M	0.8	ml	2.0	mM
NaCl	5.0	M	6.0	ml	150.0	mM
SDS	10.0	%	2.0	ml	0.1	%
Triton-X100	100.0	%	2.0	ml	1.0	%
final solution volume	200.0	ml	(add d.d. water to bring to the final volume. Store at 4°C.)			

High-salt wash buffer

	original solution				final solution	
Chemical	conc.	unit	add	unit	conc.	unit
Tris-HCl (pH8.1)	1.0	M	4.0	ml	20.0	mM
EDTA (pH 8.0)	0.5	M	0.8	ml	2.0	mM
NaCl	5.0	M	20.0	ml	500.0	mM
SDS	10.0	%	2.0	ml	0.1	%
Triton-X100	100.0	%	2.0	ml	1.0	%
final solution volume	200.0	ml	(add d.d. water to bring to the final volume. Store at 4°C.)			

Lithium wash buffer

	original solution				final solution	
Chemical	conc.	unit	add	unit	conc.	unit
Tris-HCl (pH8.1)	1.0	M	1.0	ml	10.0	mM
EDTA (pH 8.0)	0.5	M	0.2	ml	1.0	mM
LiCl	10.0	M	2.5	ml	250.0	mM
NP-40	100.0	%	1.0	ml	1.0	%
sodium deoxycholate (powder)	100.0	%	1.0	g	1.0	%
final solution volume	100.0	ml	(add d.d. water to bring to the final volume. Store at 4°C.)			

ChIP elution buffer (freshly made)

	original solution				final solution	
Chemical	conc.	unit	add	unit	conc.	unit
sodium bicarbonate (NaHCO3)	1.0	M	10.0	ml	100.0	mM
SDS	10.0	%	10.0	ml	1.0	%
final solution volume	100.0	ml	(add water to bring to the final volume. Do not chill.)			

DPBS (Invitrogen, product code 14190) (Dulbecco's PBS)
Note: You can use any other 1X PBS from other vendors.

Protease Inhibitor Cocktail (SIGMA, product code P8340)

Sonicator (Branson sonifier 250 Analog, with microtip) (in Huxford lab)

Spectra/Por 2 dialysis tubing (MWCO 12-14000 Da, nominal flat width 10 mm, 0.32 ml/cm) (SpectrumLab, product code 132676)

Dialysis tubing clamps

Tris-HCl (pH6.5)

Proteinase K (2mg/ml)

QIAquick PCR purification kit (QIAGEN, product code 28104)

Platinum PCR SuperMix (Invitrogen, product code: 11306-016)

Appropriate forward and reverse primers for PCR reactions (200 ng/reaction)
U1Forward (5'-GTGTGGCATACTTATAGGGGTGCT-3') and U1Backward (5'-GCTTTTCGATGCTCGGCAGCAG-3') primers that amplify the promoter region of the U1:95Ca gene from -1 to -107 relative to the transcription start site are used in this case.

PCR machine (BioRad iCycler)

10X TBE

10X ChIP loading dye
Add 2.1 ml 1% bromophenol blue to 2.5 ml glycerol in a 15 ml Falcon tube. Add water to bring to 5 ml. Vortex to mix. Store at room temperature.

II. Preparation of dialysis tubing

1. Cut the dialysis tubing into 15 cm pieces (~2 ml capacity/piece).
2. Boil the tubing in 800 ml buffer I in a glass beaker for 10 minutes with stirring.
3. Rinse the tubing thoroughly in d.d. water.
4. Boil the tubing in 800 ml buffer II in a glass beaker for 10 minutes with stirring.
5. Allow the tubing to cool, and then store it in cold room overnight (cover the beaker with aluminum foil). Make sure the tubing is always submerged.
   Note: From now on, always wear gloves to handle the tubing.
6. Before use, wash the tubing inside and out with d.d. water.

III. Formaldehyde cross-linking, sonication, and dialysis

Day 1

1. Grow 3 plates (Corning 100 x 20 mm tissue culture plate) of *Drosophila* S2 cells to 90 % confluency.
2. Harvest cells:
   a. To remove cells adhering to the dish, pipet the medium over the cells gently several times.
   b. Pool cells from all plates and transfer 25 ml of cells into a 50 ml Falcon tube.
3. Add chemicals of formaldehyde cross-linking solution into the 25 ml cells for cross-linking. Mix well. Incubate at room temperature for 10 minutes on a rotating wheel.
4. Add 3.8 ml 2M glycine to final 240 mM to quench the cross-linking reaction.
5. Spin the cells at 700 g, 4°C for 10 minutes (SORVALL, Legend RT). Discard the supernatant.
   Note: The supernatant contains formaldehyde, which is a carcinogen. So it is important NOT to directly drain the supernatant into the sink or trash can. Instead, collect the supernatant into a 50 ml Falcon tube and toss it into the chemical hazard container.
6. Resuspend the cells with 10 ml ice-cold DPBS. Centrifuge at 700 g, 4°C for 10 minutes (SORVALL, Legend RT). Discard the supernatant.
7. Resuspend the cells with 1 ml sonication buffer with 2.5 μl 0.2M PMSF and 10 μl protease inhibitor cocktail. Transfer the suspension to a chilled 15 ml conical-bottom Falcon tube.
8. Sonicate the suspension on ice with the following condition: microtip, 60% duty cycle, 1.5 output, 30 seconds on/1 minute off, 10 cycles (14 minutes total).
9. Transfer sonicated solution to 2 chilled 1.5 ml screw-cap tubes (500 μl/tube). Centrifuge at 13,000 rpm, 4°C for 10 minutes (EPPENDORF, Centrifuge 5415D. In the cold room).
10. Pool supernatant from both tubes to a chilled 15 ml Falcon tube. Mix the solution with equal amount (~1ml) of 6M Urea.
    Note: Mix well. Save a 50 μl aliquot separately in a 1.5 ml screw-cap tube at -20°C in case it is necessary to determine DNA size.

11. Working in the cold room, take out the prepared dialysis tubing (Step II-6). Use a dialysis clamp to close one end of the tubing. Pipet the solution from Step III-10 into the tubing. Close the other end with another clamp.
12. Prepare a beaker containing 200 ml of ice-cold ChIP buffer (prepared the day before). Add 500 µl 0.2M PMSF per 200 ml ChIP buffer right before dialysis. Put tubing into the buffer and stir overnight in the cold room for dialysis.

IV. Preparation of 50% protein A sepharose

Day 2

1. Gently vortex to thoroughly suspend the immobilized protein A sepharose in the vial. **Note:** Ratio of volume of suspension to packed gel is 2 to 1 in the vial.
2. Using a P-1000 with ~2 mm cut-off end of the tip, immediately transfer 200 µl suspended resin (100 µl packed resin) from the vial to a chilled 1.5 ml screw-cap tube. **Notes:** Always use tips cut off at the end to handle the resin.
   More than 200 µl resin may need to be prepared depending upon the number of samples to be done.
3. Centrifuge at 2500 g, 4°C for 3 minutes (EPPENDORF, Centrifuge 5415D. In the cold room). Pipet off the supernatant.
4. Continue working with EPPENDORF Centrifuge 5415D. Wash resin twice with 1 ml sterile d.d. water.
   a. Resuspend beads with 1 ml sterile d.d. water.
   b. Centrifuge at 2500 g, 4°C for 3 minutes.
   c. Pipet off the supernatant.
   d. Repeat steps a-c.
5. Wash resin twice with 1 ml TE buffer.
   a. Resuspend beads with 1 ml TE buffer.
   b. Centrifuge at 2500 g, 4°C for 3 minutes.
   c. Pipet off the supernatant.
   d. Repeat steps a-c.
6. Wash resin once with 1 ml TE+BSA (900 µl TE buffer +100 µl 10 mg/ml BSA).
   a. Resuspend beads with 1 ml TE+BSA.
   b. Centrifuge at 2500 g, 4°C for 3 minutes.
   c. Pipet off the supernatant.
7. Resuspend beads with 100 µl TE+BSA (90 µl TE buffer +10 µl 10 mg/ml BSA). Now you have 200 µl of prepared 50% protein A resin. Store at 4°C.
   Note: Among 200 µl prepared 50% resin, 80 µl is for pre-clearing, 35 µl is for each pre-immune serum and each anti-serum treated samples.

V. Pre-clearing and immunoprecipitation

1. Remove a clamp from one end of the dialysis tubing (Step III-12). Pipet out the solution into a chilled 1.5 ml conical screw-cap tube. Centrifuge at 13,000 rpm, 4°C for 10 minutes (EPPENDORF, Centrifuge 5415D. In the cold room).
2. Transfer the supernatant (~1 ml) into a chilled 1.5 ml screw-cap tube. This is the chromatin solution.
3. Add 80 μl of suspended protein A resin (Step IV-7) to the chromatin solution for pre-clearing. End-over-end rotate at 4°C for 30 minutes.
   Note: This step (pre-clearing) is important to reduce the background signals.
4. Centrifuge at 13,000 rpm, 4°C for 5 minutes (EPPENDORF, Centrifuge 5415D. In the cold room).
5. Aliquot the supernatant into chilled 1.5 ml screw-cap tubes (200 μl for input; 150 μl for each pre-immune serum and each antiserum). Save the remainder in a chilled 1.5 ml screw-cap tube and store it along with the input tube at -80°C.
   Note: Input sample serves as positive PCR control. Pre-immune serum serves as negative immunoprecipitation control.
6. Add 4 μl of antiserum or pre-immune serum into each corresponding tube containing pre-cleared chromatin solution. End-over-end rotate at 4°C overnight.
   Note: The amount of antiserum added may need to be optimized according to what antiserum you use. In this case, the antiserum used are anti-DmSNAP43 Ab and anti-FLAG polyclonal Ab (SIGMA, product code: F7425).

Day 3

7. Using a P-100 with the bottom of the tip cut off, add 35 μl prepared 50% protein A resin (Step IV-7) into each pre-immune tube and each antiserum tube. End-over-end rotate at 4°C for 2 hours.
8. Spin down the resin at 2500 g, 4°C for 3 minutes (EPPENDORF, Centrifuge 5415D. In the cold room). The resin is the important fraction but save the supernatant (as "Flow through") in a 1.5 ml screw-cap tube at -80°C in case it should be needed.
9. Continue working in the cold room and with EPPENDORF Centrifuge 5415D. Wash resin 3 times with 1 ml ice-cold low-salt wash buffer.
   a. Resuspend beads with 1 ml low-salt wash buffer.
   b. End-over-end rotate at 4°C for 5-10 minutes.
   c. Centrifuge at 2500 g, 4°C for 3 minutes.
   d. Pipet off the supernatant.
   e. Repeat steps a-d for 5 more times.
10. Wash resin 3 times with 1 ml ice-cold high-salt wash buffer.
   a. Resuspend beads with 1 ml high-salt wash buffer.
   b. End-over-end rotate at 4°C for 5-10 minutes.
   c. Centrifuge at 2500 g, 4°C for 3 minutes.
   d. Pipet off the supernatant.
   e. Repeat steps a-d for 2 more times.

11. Wash resin twice with 1 ml ice-cold lithium wash buffer.
    a. Resuspend beads with 1 ml lithium wash buffer.
    b. End-over-end rotate at 4°C for 2 hours.
    c. Centrifuge at 2500 g, 4°C for 3 minutes.
    d. Pipet off the supernatant.
    e. Resuspend beads with 1 ml lithium wash buffer.
    f. End-over-end rotate at 4°C overnight (the overnight wash is believed to be important).
    g. Centrifuge at 2500 g, 4°C for 3 minutes.
    h. Pipet off the supernatant.

Day 4

12. Wash resin with 1 ml ice-cold TE buffer.
    a. Resuspend beads with 1 ml TE buffer.
    b. End-over-end rotate at 4°C for 5 minutes.
    c. Centrifuge at 2500 g, 4°C for 3 minutes.
    d. Pipet off the supernatant.
13. Resuspend resin with 1 ml TE buffer. Transfer the resin to another chilled 1.5 ml screw-cap tube to eliminate non-specific DNA bound on the tube wall.
14. Centrifuge at 2500 g, 4°C for 3 minutes. Pipet off the supernatant.

VI. Elution, reverse cross-linking, and DNA purification

1. Add 250 µl of freshly made elution buffer to the resin (Step V-14) to elute the immunoprecipitated protein-DNA complexes. Vortex briefly to mix well.
2. End-over-end rotate at room temperature for 15 minutes. Centrifuge at 2500 g, room temperature for 3 minutes (EPPENDORF, Centrifuge 5424).
3. Transfer the supernatant (eluate) to a 1.5 ml screw-cap tube.
4. Add another 250 µl of elution buffer to the resin to elute again. Repeat Step VI-2. Pool eluate from both elutions together in a 1.5 ml screw-cap tube (~500 µl total). Also, prepare input DNA by adding 300 µl elution buffer to 200 µl thawed input sample (Step V-5) to make final volume 500 µl.
5. Add 20 µl of 5M NaCl to eluate and input DNA. Mix well and incubate at 65°C for 4 hours to reverse crosslinks.
6. Add 10 µl of 0.5M EDTA pH8.0, 20 µl of 1M Tris-HCl pH6.5, and 10 µl of 2 mg/ml proteinase K. Mix well and incubate at 45°C for 1 hour to digest proteins.
7. Using QIAquick PCR purification kit (QIAGEN), follow the manufacturer's instructions to purify the immunoprecipitated DNA and input DNA. Store the purified DNA at -20°C.
   Notes: Use 2500 µl of PB buffer (as 5 volumes PB: 1 volume sample) in the first step (binding step); use 30 µl of TE buffer to elute the purified DNA in the last step. 30 µl of purified DNA is enough for 15 PCR reactions.

Now you will have at least 3 purified DNA sample: 1 for input DNA; 1 (or more) for pre-immune serum precipitated DNA; 1 (or more) for antiserum precipitated DNA.

VII. qPCR reaction

Day 5

1. Prepare the qPCR reaction by using purified pre-immune, anti-serum precipitated DNA, and input DNA (Step-VI-7) as instructed below: (reaction/well)

Reagents	amount (µl)
2X SYBR	5
Forward primer (10µM)	0.3
Reverse primer (10µM)	0.3
Nuclease free water	2.4
DNA from ChIPs (diluted)	2
Total	10

Notes: Since qPCR is highly sensitive, for quality control reasons, each sample should be run in triplicate. Input DNA serves as positive control; Extra reactions that use Nuclease free water instead of DNA from ChIPs should be included as negative no-template control.

Usually Input DNA is diluted 12X and other ChIPed DNA is diluted 3X to add into qPCR reaction.

Appendix B: Detailed protocol for electrophoretic mobility shift assay (EMSA)

I. Solutions and materials

Materials for preparation of radioactive DNA oligo probes:

Annealed double stranded DNA oligos (PSEAs) (1 μg/5 μl)

T4 polynucleotide kinase (T4 PNK) (NEB, product code: M0201L)

10x PNK reaction buffer (reagent supplied with T4 PNK)

^{32}P gamma-ATP (3000 Ci/mmol, 10m Ci/μl) (PerkinElmer)

Chloroform/isoamyl alcohol (24:1)

Saturated phenol

Quick Spin Columns for radiolabeled DNA purification Sephadex G-25, fine (ROCHE, product code: 11273949001)

Materials for bandshift/supershift reactions:

Radioactive oligo probes

0.1M DTT (dithiothreitol)
Diluted from 1M DTT. Preparation of 1M DTT is described two pages ahead.

Poly(deoxyguanylic-deoxycytidylic) acid sodium salt [Poly (dG-dC)] (SIGMA, product code: P9389)
Dissolved in HEMG-100 buffer to obtain final concentration of 1μg/μl.

Poly(deoxyinosinic-deoxycytidylic) acid sodium salt [Poly (dI-dC)] (SIGMA, product code: P4929)
Dissolved in HEMG-100 buffer to obtain final concentration of 1μg/μl.

Glycerol

Purified proteins (DmSNAPs)

Appropriate antibodies (for supershift reactions)

HEMG-100 buffer

	original solution				final solution	
Chemical	conc.	unit	add	unit	conc.	Unit
HEPES K^+ (pH7.6)	1.0	M	500.0	µl	25.00	mM
MgCl2	1.0	M	250.0	µl	12.50	mM
ZnCl2	10.0	mM	20.0	µl	0.01	mM
EDTA (pH8.0)	0.5	M	4.0	µl	0.10	mM
KCl	4.0	M	500.0	µl	100.00	mM
Glycerol	100.0	%	2.0	ml	10.00	%
Sterile d.d. water			16726.0	µl		
final volume			20.0	ml		

Note: Add everything into a 50 ml Falcon tube. Vortex to mix. Store at 4°C

Chemicals for preparation of HEMG-100 buffer:

1M HEPES K^+ (pH 7.6)
Dissolve 119.15 g HEPES (MW 238.3) in 500 ml d.d. water to make 1M HEPES. Titrate with 10N KOH until the pH reaches 7.6. Store at 4°C.

1M $MgCl_2$
Dissolve 101.65 g $MgCl_2$·6H2O in 400 ml d.d. water. Adjust the volume to 500 ml with d.d. water. Filter or autoclave to sterilize. Store at 4°C.
Note: $MgCl_2$ is extremely hygroscopic. Buy small bottles and do not store opened bottles for long periods of time. Once the crystals become saturated with water, dispose of the chemical properly.

10mM $ZnCl_2$
Dissolve 681.4 mg $ZnCl_2$ in 500 ml sterile d.d. water. Filter to sterilize. Store at room temperature.

0.5M EDTA (pH 8.0)
Add 90.8 g of $Na_2EDTA·2H_2O$ to about 400 ml of d.d. water. Stir and adjusted the pH to 8.0 with NaOH (~20g of NaOH pellet). Bring the volume to 500 ml with d.d. water if necessary. Sterilize by autoclaving. Store at room temperature.
Note: $Na_2EDTA·2H_2O$ will not be dissolved until the pH of the solution is adjusted to approximately 8.0 by the addition of NaOH.

4M KCl
Dissolve 149.1 g KCl in 400 ml of d.d. water. Bring the volume to 500 ml with d.d. water. Sterilize by autoclaving. Store at room temperature.

1 M DTT (dithiothreitol)
Add 1.54 g DTT into 10 ml d.d water in a 15 ml Falcon tube. Vortex to dissolve. Wrap with aluminum foil and store at -20°C.

100 mM PMSF (phenylmethylsulfonyl fluoride)
Add 696 mg PMSF in a 50 ml Falcon tube. Add 30 ml 100% ethanol. Vortex to dissolve. Fill with 100% ethanol to 40ml. Invert to mix. Wrap the tube with aluminum foil and store at -20°C.
Note: PMSF is very toxic so handle with care. Aqueous solutions of PMSF are hydrolyzed very rapidly, so the stock solution needs to be made with absolute ethanol or 2-propanol and only add PMSF to aqueous solutions immediately before their use.

Materials for preparation of non-denaturing polyacrylamide gel:

40% non-denaturing acrylamide stock solution (30:1)
Dissolve 38.71 g of electrophoresis-grade acrylamide, 1.29 g electrophoresis-grade bis-acrylamide in 100 ml d.d. water with stirring. Sterilize by passage through a 0.22-μm filter. Wrap the bottle with foil and store at 4°C. Discard the solution if the color turns yellow during storage.

10x non-circulation buffer
Dissolve 60.58 g of Tris, 285.28 g of glycine, and 7.44 g of EDTA in 1.6 L d.d. water with stirring. Stir and adjust the pH to 8.3 with HCl. Bring the volume to 2 L with d.d. water. Store at 4°C (indefinitely). Discard the solution if the color turns yellow during storage.

1x non-circulation buffer (gel-running buffer)
Dilute from 10x non-circulation buffer with d.d. water. Around 800 ml is required for each gel-running apparatus.

10% Ammonia Persulfate (APS)
Add 1 g APS in a 15 ml Falcon tube. Add 10 ml sterile d.d. water. Vortex to dissolve. Aliquot the solution into microfuge tubes (1ml/tube). Store at -20°C (indefinitely).
Note: APS provides the free radicals that drive polymerization of acrylamide and bis-acrylamide. APS decomposes gradually (it will last only a week at 4°C). Thus, once leave the freezer and get thawed, the 10% APS must stay on ice all the time and put back to the freezer right after use.

TEMED
Store at 4°C. Keep on ice when in use.
Note: TEMED serves as the catalyst for the polymerization of acrylamide and bis-acrylamide.

Large and small glass plates, spacers, vacuum glue, metal clamps, 20-well combs, non-stick reagent, pieces of sponge, gel-running apparatus, power supply and wires, food wrap, intensifier screen, film cassette

II. Preparation of Quick Spin columns

You will need to prepare two columns at the same time following the instruction below.

1. Place a collection tube with the end cut-off in a 15 ml Falcon tube. Do another set for the 2nd column.
2. Thoroughly suspend the G-25 resin in columns to make a uniform suspension of the resin by ticking/inverting the column.
3. Remove the cap and tip from columns. Place the two columns into each 15 ml Falcon tubes prepared in step III-1.
4. Centrifuge in the RT-Legend at 1000 g for 3 min, 4°C.
5. Use forceps to take out the column for a while. Remove the flow through and the end-cut-off collection tube from the 15 ml Falcon tube. Replace the column and the end-cut-off collection tube back to the 15 ml Falcon tube.
6. Centrifuge in the RT-Legend at 1000 g for 3 min, 4°C.
7. Use forceps to take out the column for a while. Remove the flow through and the end-cut-off collection tube from the 15 ml Falcon tube. Replace the column and an intact collection tube back to the 15 ml Falcon tube.
   Note: label one intact collection tube with "1", and label the other with the name of your oligos and the date of radiolabeling. Put the column label with "1" into the 15 ml Falcon tube containing "1" collection tube, put the "2" column into the 15 ml Falcon tube containing the collection tube labeled with the detailed information of your oligos.

III. Radiolabeling of annealed DNA oligos

1. Prepare the following reaction in a 1.5 ml conical screw-cap tube:

1 μg annealed oligos (PSEAs)	5 μl
10x T4 PNK reaction buffer	5 μl
T4 PNK (10 U/μl)	1 μl
^{32}P gamma-ATP	8 μl
Sterile d.d. water	31 μl
final volume	50 μl

   Note: use long P10 tips for transferring the gamma-ATP to avoid potential contamination.
2. Incubate the tube in a 37°C water bath for 30 mins.
3. While waiting, prepare the Quick Spin column as described in section II.
4. Take out the oligo tube from the water bath. Add 25 μl of chloroform/isoamyl alcohol (24:1) into the tube.
5. Add 25 μl of saturated phenol (get the lower layer) into the tube.

6. Vigorously vortex for 30 sec.
7. Centrifuge at 12000 rpm or maximum speed for 3 minutes at room temperature.
8. Transfer the aqueous top layer (about 50 μl) from the tube into the center of the prepared column labeled with "1" atop the "1" collection tube inside the 15 ml Falcon tube.
9. Centrifuge in the RT-Legend at 1000 g for 3 min, 4°C.
10. Use forceps to remove the column and transfer the collection tube containing radiolabeled oligos to a rack.
11. Transfer the radiolabeled oligos (about 50 μl) into the center of the prepared column labeled with "2" atop the collection tube labeled with the detailed oligo information inside the 15 ml Falcon tube.
12. Centrifuge in the RT-Legend at 1000 g for 3 min, 4°C.
13. Use forceps to remove the column and transfer the collection tube containing radiolabeled oligos to a rack. Insert the removable cap to the tube. Now this is your radiolabeled and purified DNA oligos. Store your purified oligos on ice if using immediately, otherwise store at -20°C.
14. Prepare two 1.5 ml conical screw-cap tubes each containing 2 μl of purified radioactive oligos. Examine the radioactivity of oligos in these two tubes in the scintillation counter (use #6 for ^{32}P). Store these two tubes in the -20°C freezer for future use.
    Note: to calculate the radioactivity (cpm) of your probes, if the result from the counting is "A" cpm from tube 1 and "B" cpm from tube 2, then the cpm/μl of your probe is (A cpm + B cpm)/(2 μl x 2) = (A+B)/4 cpm/μl.

IV. Preparation of non-denaturing polyacrylamide gel

1. Apply non-stick reagents on a small glass plate. Use kimwipes to spread the reagent evenly. Assemble the treated small glass plate with a large glass plate and 3 spacers into a "sandwich" with vacuum glue applied at the joint of the spacers. Clamp the edge of the sandwich with metal clamps (2 on lower part of each right and left side, 3 on the bottom side. Total 7 clamps are used in this step). Lay the sandwich on a tip box on the bench so the sandwich is tilted with the bottom side touching the benchtop.
2. Prepare the 5% non-denaturing acrylamide solution as follows:
   a. Add 7.5 ml 40% non-denaturing acrylamide, 6 ml 10x non-circulation buffer, and 46.5 ml d.d. water into a 250 ml flask. Swirl to mix. Remove 5 ml from the gel solution and discard.
   b. Add 400 μl of 10% APS and 40 μl of TEMED into the flask. Swirl to mix (avoid bubbles). Use a transfer pipet to remove bubbles if necessary.
   Note: if you need to run supershift reactions on the gel, then you might need to prepare a 4% gel instead: mix together 5 ml of 40% non-denaturing acrylamide, 5ml of 10x non-circulation buffer, 39.56 ml of d.d. water, 400 μl of 10% APS and 40 μl TEMED in the flask. You don't need to discard any gel solution in this case.
3. Immediately (but slowly and steady) pour the gel solution into the middle space of the assembled sandwich. Avoid any bubbles that may occur. Insert a 20-well comb and

immediately clamp two extra metal clamps (one for each upper part of right and left side) to fix the comb.

4. Wait for 30 minutes allowing the gel to completely polymerize.
5. Once the gel is solidified, hook up the gel sandwich onto a gel-running apparatus connected to a power supply with wires.
6. Remove the comb. Pour 1x non-circulation buffer into the upper tank and the lower tank of the gel-running apparatus so the wells of the gel are completely immersed in the buffer. Use a syringe with needle to remove unpolymerized acrylamide and bubbles inside wells, and to remove bubbles from the space in the bottom of the gel.
7. Run to warm up the gel at 100 V for 30 minutes.
   Note: Do not start this warm-up step until your bandshift/supershift reactions are ready for the 30 minutes incubation.

V. Preparation of bandshift/supershift reaction

1. Calculate how much radioactive probe you need according to the number of your reactions and the radioactive strength of the probe. Each reaction requires 1 μl of 50000 cpm/μl probe. For example, if you need 20 reactions and the radioactivity of your probes measured from step III-14 is 400000 cpm/μl, then:
   (50000 cpm/μl x 20 μl)/ (400000 cpm/μl) = 2.5 μl
   Thus, you need 2.5 μl of the 400000 cpm/μl probe to dilute with (20-2.5=17.5) μl of d.d. water to make 20 μl of 50000 cpm/μl probe for your bandshift reaction. You can also use 22 μl instead of 20 μl in the equation to make sure you have enough probe to use.
2. Prepare the probe-mix by mixing 1 μl of the 50000 cpm/μl probe, 2 μl of 1 μg/μl poly (dI-dC) or poly (dG-dC), and 1 μl of 0.1 M DTT in a 1.5 ml conical screw-cap tube <u>for each reaction</u> (so 4 μl of probe-mix per reaction). Multiple by the number of your total reactions to see how much of each reagent you really need.
3. Prepare each bandshift reaction as follow:

HEMG-100	(15-X) μl
Sterile d.d. water	2 μl
Probe-mix	4 μl
Proteins (DmSNAPs)	X μl
Final volume	21 μl

   Note: the final salt concentration should be around 80 mM, and the final glycerol concentration should be around 8%.
4. Incubate the reactions in a 20°C water bath for 30 mins. If a supershift reaction is included, add antibodies in the middle of the incubation (15 minutes after incubation). Start this step with step IV-7 (gel warm-up) at the same time.

VI. Gel running and autoradiography

1. Load each well of the gel with each of your bandshift/supershift reactions. Load an empty well on the side with the non-denaturing dye. Run the gel at 100 V until the fast dye is approximately 3/4 through the gel (it will take around 3 hours and 20 minutes). **Note:** you might need to run the gel longer to allow the dye close the bottom of the gel if you have supershift reactions. This will allow the protein-DNA bands to separate further then it will be much easier to observe supershift bands.
2. Detached the gel sandwich from the gel-running apparatus. Dissemble the sandwich to allow the gel to separate from the small glass plate but stay on the large plate.
3. Tilt the plate with the gel on it to allow the buffer remained on the gel to run away from the gel. Use kimwipes to absorb the buffer.
4. Immediately lay a piece of food wrap on the surface of the gel. That piece of food wrap needs to be large enough to cover the whole gel and the large glass plate to allow full wrapping of the gel.
5. In a dark room, place your wrapped gel/glass plate in a film cassette. Place a film on top of the gel. Place an intensifier screen on top of the film. Close and tightly fasten the cassette. Put the cassette into a -80°C freezer to allow the exposure of the film up to 18 hrs.
6. Develop the film in the darkroom to see the result.

Appendix C: Nickel-chelate chromatography

I. Solutions and materials

O.1M CuSO4
Add 0.25 g $CuSO_4 \cdot 5H_2O$ into 9.91 ml sterile d.d. water in a 15 ml Falcon tube. Vortex to dissolve. Working in the cell culture hood, use 0.22 μm pore-size syringe filter to filter/aliquot $CuSO_4$ solution into 10 of 1.5ml conical screw-cap tubes (~1ml/tube). Store at 4°C.

Stock solution A (for making 5X Native purification buffer)
250 mM NaH_2PO_4 (monobasic sodium phosphate), 2.5 M NaCl
Dissolve 7.8 g $NaH_2PO_4 \cdot 2H2O$ (MW 155.99) and 29.2 g NaCl in 200 ml d.d. water. Filter to sterilize. Store at 4°C.

Stock solution B (for making 5X Native purification buffer)
250 mM Na_2HPO_4 (dibasic sodium phosphate), 2.5 M NaCl
Dissolve 7.1 g Na_2HPO_4 (MW 141.96) and 29.2 g NaCl in 200 ml d.d. water. Filter to sterilize. Store at 4°C.

3M Imidazole
Add 10.2 g imidazole into 30 ml sterile d.d. water in a 50 ml Falcon tube. Vortex to dissolve. Fill with sterile d.d. water to 50 ml. Invert to mix. Store at 4°C.

5X Native purification buffer
Add 45 ml stock solution B in a 100 ml beaker with a stirring stir bar. Titrate with stock solution A (drop by drop, only very small amount is needed) until the pH reaches 8.0. Transfer the solution to a 50 ml Falcon tube and store at 4°C.

1X Native purification buffer (for making Native binding buffer and Native wash buffer) (100 ml/purification)
Add 20 ml 5X Native purification buffer and 75 ml sterile d.d. water in a 150 ml beaker with a stirring stir bar. Titrate with HCl or NaOH until the pH reaches 8.0 (~11 μl of 12M HCl). Bring the volume to 100 ml with sterile d.d. water. Store at 4°C.

Native binding buffer w/ 10 mM Imidazole (12 ml/purification)
Add 30 ml 1X Native purification buffer and 100 μl 3M Imidazole in a 100 ml beaker with a stirring stir bar. Titrate with HCl or NaOH until the pH reaches 8.0 (~3 μl of 12M HCl). Transfer the solution to a 50 ml Falcon tube and store at 4°C.

Native wash buffer w/ 20 mM Imidazole (12 ml/purification)
Add 50 ml 1X Native purification buffer and 335 µl 3M Imidazole in a 100 ml beaker with a stirring stir bar. Titrate with HCl or NaOH until the pH reaches 8.0 (~15 µl of 12M HCl). Transfer the solution to a 50 ml Falcon tube and store at 4°C.

Chemicals for preparation of HEMG-100 buffer:

1M HEPES K^+ (pH 7.6)
Dissolve 119.15 g HEPES (MW 238.3) in 500 ml d.d. water to make 1M HEPES. Titrate with 10N KOH until the pH reaches 7.6. Store at 4°C.

1M $MgCl_2$
Dissolve 101.65 g $MgCl_2$·6H2O in 400 ml d.d. water. Adjust the volume to 500 ml with d.d. water. Filter or autoclave to sterilize. Store at 4°C.
Note: $MgCl_2$ is extremely hygroscopic. Buy small bottles and do not store opened bottles for long periods of time. Once the crystals become saturated with water, dispose of the chemical properly.

10mM $ZnCl_2$
Dissolve 681.4 mg $ZnCl_2$ in 500 ml sterile d.d. water. Filter to sterilize. Store at room temperature.

0.5M EDTA (pH 8.0)
Add 90.8 g of $Na_2EDTA{\cdot}2H_2O$ to about 400 ml of d.d. water. Stir and adjust the pH to 8.0 with NaOH (~20g of NaOH pellet). Bring the volume to 500 ml with d.d. water if necessary. Sterilize by autoclaving. Store at room temperature.
Note: $Na_2EDTA{\cdot}2H_2O$ will not be dissolved until the pH of the solution is adjusted to approximately 8.0 by the addition of NaOH.

4M KCl
Dissolve 149.1 g KCl in 400 ml of d.d. water. Bring the volume to 500 ml with d.d. water. Sterilize by autoclaving. Store at room temperature.

1 M DTT (dithiothreitol)
Add 1.54 g DTT into 10 ml d.d water in a 15 ml Falcon tube. Vortex to dissolve. Wrap with aluminum foil and store at -20°C.

100 mM PMSF (phenylmethylsulfonyl fluoride)
Add 696 mg PMSF in a 50 ml Falcon tube. Add 30 ml 100% ethanol. Vortex to dissolve. Fill with 100% ethanol to 40ml. Invert to mix. Wrap the tube with aluminum foil and store at -20°C.
Note: PMSF is very toxic so handle with care. Aqueous solutions of PMSF are hydrolyzed very rapidly, so the stock solution needs to be made with absolute ethanol or 2-propanol and only add PMSF to aqueous solutions immediately before their use.

HEMG-100 buffer (w/ 20mM Imidazole) (4 ml/purification)

	original solution				final solution	
Chemical	conc.	unit	add	unit	conc.	unit
HEPES K^+ (pH7.6)	1.0	M	500.0	µl	25.00	mM
MgCl2	1.0	M	250.0	µl	12.50	mM
ZnCl2	10.0	mM	20.0	µl	0.01	mM
EDTA (pH8.0)	0.5	M	4.0	µl	0.10	mM
KCl	4.0	M	500.0	µl	100.00	mM
Glycerol	100.0	%	2.0	ml	10.00	%
Imidazole	3.0	M	133.2	µl	20.00	mM
DTT	1.0	M	60.0	µl	3.00	mM
PMSF	0.1	M	100.0	µl	0.50	mM
Sterile d.d. water			16432.8	µl		
final volume			20.0	ml		

Note: Add everything except DTT and PMSF into a 50 ml Falcon tube. Vortex to mix. Store at 4°C. Add DTT and PMSF right before use.

Elution buffer (HEMG-100 buffer w/ 750mM Imidazole) (3 ml/purification)

	original solution				final solution	
Chemical	conc.	unit	add	unit	conc.	unit
HEPES K^+ (pH7.6)	1.0	M	500.0	µl	25.00	mM
MgCl2	1.0	M	250.0	µl	12.50	mM
ZnCl2	10.0	mM	20.0	µl	0.01	mM
EDTA (pH8.0)	0.5	M	4.0	µl	0.10	mM
KCl	4.0	M	500.0	µl	100.00	mM
Glycerol	100.0	%	2.0	ml	10.00	%
Imidazole	3.0	M	5.0	ml	20.00	mM
DTT	1.0	M	60.0	µl	3.00	mM
PMSF	0.1	M	100.0	µl	0.50	mM
Sterile d.d. water			11566.0	µl		
final volume			20.0	ml		

Note: Add everything except DTT and PMSF into a 50 ml Falcon tube. Vortex to mix. Store at 4°C. Add DTT and PMSF right before use.

Dialysis buffer (HEMG-100 buffer W/O imidazole)

	original solution				final solution	
Chemical	conc.	unit	add	unit	conc.	unit
HEPES K^+ (pH7.6)	1.0	M	50.0	ml	25.00	mM
MgCl2	1.0	M	25.0	ml	12.50	mM
ZnCl2	10.0	mM	2.0	ml	0.01	mM
EDTA (pH8.0)	0.5	M	400.0	µl	0.10	mM
KCl	4.0	M	50.0	ml	100.00	mM
Glycerol	100.0	%	200.0	ml	10.00	%
DTT	1.0	M	6.0	ml	3.00	mM
PMSF	0.1	M	10.0	ml	0.50	mM
Sterile d.d. water			to 2L			
final volume			2.0	L		

Note: Add everything except DTT and PMSF into a 4L plastic beaker with 1.5 L d.d. water. Stir to mix. Bring the volume to 2L with d.d. water. Stir to mix. Store at 4°C. Add DTT and PMSF right before use.

DPBS (Invitrogen, product code 14190) (Dulbecco's PBS)
Note: You can use any other 1X PBS from other vendors.

Protease Inhibitor Cocktail (SIGMA, product code P8340)

CelLytic M Lysis buffer (SIGMA, product code C2978)

ProBond Nickel-Chelating Resin (Invitrogen, product code 46-0019)

Poly-Prep Chromatography columns (BioRad, product code 731-1550)

Spectra/Por 2 dialysis tubing (MWCO 12-14000 Da, nominal flat width 10 mm, 0.32 ml/cm) (SpectrumLab, product code 132676)

Dialysis tubing clamps

II. Expression of *Dm*SNAPs in S2 cells

1. Grow 4 big plates (Corning 100 x 20 mm tissue culture plates) of cells in 20 ml selective medium to 70-80% confluency.
2. Induce cells with copper sulfate. Add 100 µl 0.1M $CuSO_4$ into each plate (to final 0.5 mM).
3. Incubate cells for ~24 hours at 22-25°C.

III. Preparation of ProBond column

1. Put a poly-prep chromatography column in a 50ml Falcon tube.
2. Thoroughly suspend the ProBond resin in the vial in order to make a uniform suspension of the resin. Ratio of volume of suspension to packed gel is 2 to 1.
3. Using a 10 ml serological pipet. Immediately transfer 2 ml of suspended resin (containing 1ml packed resin) into the chromatography column.
4. Cap the column. Centrifuge the resin for 1 minute at 420 g.
5. Remove the supernatant. Be careful not to remove any resin.
6. Wash the resin once in sterile d.d. water.
    a. Add 6 ml sterile d.d. water to the column. Resuspend the resin thoroughly by inverting/tapping the column.
    b. Cap the column. Centrifuge at 420 g for 1 minute.
    c. Remove the supernatant.
7. Wash the resin twice with Native binding buffer.
    a. Add 6 ml Native binding buffer to the column. Resuspend the resin thoroughly by inverting/tapping the column.
    b. Cap the column. Centrifuge at 420 g for 1 minute.
    c. Remove the supernatant.
    d. Repeat steps a-c.

    Note: For the <u>last wash</u> with Native binding buffer, if the resin will not incubate with cell lysates immediately, stop at Step a, leave the resin suspended in Native binding buffer and keep the column in the cold room. Centrifuge to remove the supernatant right before use.

IV. Preparation of dialysis tubing

1. Cut the dialysis tubing into 8 cm pieces (the capacity is ~ 1 ml/piece).
2. Put the tubing pieces one-by-one into a beaker containing d.d. water with magnetic stir bar stirring. Allow it to stir for 30 minutes. Make sure all pieces completely immersed in the water during stirring.
3. Transfer the tubing pieces into another beaker containing d.d. water. Make sure all pieces completely immersed in the water. Cover the beaker with aluminum foil and keep it in the cold room until used.

V. Procedures of His_6-tagged protein purification

A. Cell lysis

1. Add and mix 70 µl protease inhibitor cocktail (to final 1%) and 23.3 µl 3M imidazole (to final 10 mM) to 7 ml of chilled CelLytic M lysis buffer in a 15 ml Falcon tube. Keep the tube on ice.
2. Harvest cells: (from 4 big plates)
    a. To remove cells adhering to the dish, pipet the medium over the cells to wash cells off the plate for several times.

b. Collect the cells and medium from every 2 plates to one 50 ml centrifuge tube. (So for each cell line you will need 2 tubes for the 4 big plates)
c. Centrifuge in the RT-Legend at 420 g for 5 minutes.
d. Suck out the supernatant and discard.
e. Wash the cells by resuspending the pellet in one 50 ml tube with 10 ml of DPBS. Transfer the suspended cells to another pellet-containing 50 ml tube. Pipet up and down to resuspend the pellet thoroughly. Now all our cells are in a single 50 ml tube.
f. Centrifuge in the RT-Legend at 420 g for 5 minutes (use balance tube if necessary).
g. Suck out the supernatant and discard.

3. Lyse cells:
   a. Transfer all 7ml of CelLytic M lysis buffer supplemented with 1% protease inhibitor and 10mM imidazole (Step IV-1) to the 50 ml tube containing washed cell pellet (Step V-2-g). Pipet up and down until all cells are lysed.
   b. Transfer the lysed cells back to the 15 ml tube used for CelLytic M lysis buffer storage. Keep the 15 ml tube on ice and move to the cold room.
4. In the cold room, rotate the 15 ml tube containing lysed cells end-over-end for 15 mins to ensure complete lysis.
   Note: From now on, you need to handle the lysed cells <u>in the cold room.</u>
5. After the 15 minute incubation, aliquot the lysed cells into 7 of chilled 1.5 ml conical screw-cap tube (~1ml/tube). Centrifuge the cell lysate 12,000g for 10 mins in the microfuge in the cold room (EPPENDORF, Centrifuge 5415D).
6. Pool the supernatant from the 7 tubes (~7 ml total) into a new chilled 15 ml Falcon tube. Place the tube on ice.
7. If the lysate is viscous, shear the DNA by passing it through an 18-gauge needle four times.
   a. Put an 18-gauge needle on a 10 ml syringe.
   b. Suck the lysate into the syringe and expel it back into the tube slowly four times.
8. Remove 100 μl of the lysate into a chilled 1.5 ml conical screw-cap tube labeled as "lysates" and freeze in liquid N_2. Keep the remainder of lysates on ice.
9. Measure the total amount of the remaining lysate. Add calculated amount of 5M NaCl into lysate to give a final concentration of 500 mM NaCl.
   Note: Add 105.1 μl of 5M NaCl per ml of lysate to get final concentration of 500mM NaCl. For example, if the volume of lysate is 7 ml, add 735.7 μl of 5M NaCl.

B. Resin binding

1. In the cold room, add the cell lysates (~7 ml) to the prepared column containing packed ProBond resin (Step III-7). Resuspend the resin thoroughly by inverting/tapping the column.
2. Incubate the lysates and resin for 2 hours in the cold room on the rocker.
   Note: The incubation time may need to be optimized if proteins other than DmSNAPc are purified.

3. Centrifuge the resin for 1minute at 420 g in the RT-Legend. Transfer the supernatant into a chilled 15 ml Falcon tube. Remove 100 μl of the supernatant from the tube into a chilled 1.5 ml conical screw-cap tube and store frozen in the liquid nitrogen as "Flow through". Store the remainder in -80°C.
4. Wash the resin three times with Native wash buffer.
    a. Add 4 ml Native wash buffer to the column. Resuspend the resin thoroughly by inverting/tapping the column.
    b. Cap the column. Centrifuge at 420 g for 1 minute.
    c. Remove the supernatant.
    a. Repeat steps a-c for two more times.
5. Wash the resin once with HEMG-100 buffer (w/ 20 mM Imidazole).
    a. Add 4 ml HEMG-100 buffer (w/ 20mM Imidazole) to the column. Resuspend the resin thoroughly by inverting/tapping the column.
    b. Cap the column. Centrifuge at 420 g for 1 minute.
    c. Remove the supernatant.

C. Elution of the His_6-tagged protein

1. Elute the bound His_6-tagged protein with three 1ml volumes of Elution buffer (HEMG-100 buffer w/ 750 mM Imidazole).
    a. Clamp the column in a vertical position and snap off the cap on the lower end. Allow the remainder of the buffer in the column flow out and discard (will be just few drops).
    b. Place a chilled 15 ml Falcon tube under the column. Position the tube to ensure that in next step, every drop of the elution from the column will be collected by the 15 ml tube.
    c. Add 1ml of the elution buffer to the column drop by drop. Allow the eluted proteins to come out into the 15 ml tube underneath the column until completely drained (about 5mins).
    d. Remove 50 μl of the elution fraction into a chilled 1.5 ml conical screw-cap tube and store frozen in the liquid nitrogen as "Elution 1". Save the remainder in the tube (~950 μl) on ice.
    e. Repeat steps b-d twice to collect fractions 2 and 3.
2. Now you should have three 15 ml Falcon tubes sitting on ice as Elution 1, 2, and 3.

VI. Dialysis to remove imidazole in elution fractions

1. Prepare a 2L beaker containing 1L of ice-cold dialysis buffer (HEMG-100 buffer W/O imidazole) with stirring. Add 3ml of 1M DTT and 5ml of 100mM PMSF. Stirring.
2. Take out a prepared dialysis tubing (Step IV-3). Remove all the water remained inside/outside of the tubing. Use a dialysis clamp to close one end of the tubing. Pipet the Elution 1 from Step V-C-2 into the tubing. Close the other end with another clamp. Put the clamped tubing into the beaker containing dialysis buffer with stirring. Make sure the tubing is completely submerged.

3. Repeat Step 1 and 2 twice to transfer Elution 2 and 3 into individual dialysis tubing. Allow samples to dialyze for 2 hours.
4. Exchange the dialysis buffer in the beaker with another 1L of ice-cold dialysis buffer supplemented with DTT and PMSF. Dialyze for another 2 hr.
5. Take out the tubing containing Elution 1. Unclamp one end of the tubing. Transfer the solution into a chilled 1.5 ml conical screw-cap tube labeled with “Elution 1 dialyzed”. Store frozen in the liquid nitrogen.
6. Repeat Step 5 twice to transfer Elution 2 and 3 into individual 1.5 ml conical screw-cap tubes as “Elution 2 dialyzed” and “Elution 3 dialyzed”. Store frozen in the liquid nitrogen.

Appendix D: High-salt FLAG purification

I. Solutions and materials

5 mg/ml 3x FLAG peptide solution (SIGMA, product code F 4799)
The 3x FLAG peptide (N-Met-Asp-Tyr-Lys-Asp-His-Asp-Gly-Asp-Tyr-Lys-Asp-His-Asp-Ile-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C) is acidic. In order to dissolve it properly, add 160 µl of 10x Wash Buffer to 4 mg of 3x FLAG peptide.
After the peptide is completely dissolved, add 640 µl of distilled water to the sample. Mix well and store aliquots of 75 µl at -20°C.

10x Wash Buffer
0.5M Tris-HCl, pH7.4, with 1.5M NaCl

1x Wash Buffer
Add 2 ml of 10x Wash Buffer to 18 ml sterile distilled water and mix well.

1x Wash Buffer with 351 mM NaCl
Add 11.7 ml 10x wash buffer (with 1.5 M NaCl conc.) in a 50 ml Falcon tube. Fill to 50 ml marker with sterile distilled water to get a final concentration of 351 mM NaCl. Store at 4°C.

Elution Buffer (for column preparation only)
0.1M Glycine, pH3.5

HEMG-100 buffer

	original solution				final solution	
Chemical	conc.	unit	add	unit	conc.	Unit
HEPES K^+ (pH7.6)	1.0	M	75.0	ml	25.00	mM
MgCl2	1.0	M	15.0	ml	5.00	mM
ZnCl2	10.0	mM	3.0	ml	0.01	mM
EDTA (pH8.0)	0.5	M	600.0	µl	0.10	mM
KCl	4.0	M	75.0	ml	100.00	mM
Glycerol	100.0	%	300.0	ml	10.00	%
Sterile d.d. water			16726.0	µl		
final volume			3.0	L		

CelLytic M Lysis Buffer (SIGMA, product code C 2978)

ANTI-FLAG M2-Agarose Affinity Gel (SIGMA, product code A 2220)

Protease Inhibitor Cocktail (SIGMA, product code P 8340)

Spectra Por 2 Dialysis Tubing (Fisher, product code 132676)

II. Expression of *Dm*SNAPs in S2 cells

1. Grow 8 plates (Corning 100 x 20 mm tissue culture plates) of cells in selective medium to 70-80% confluency.
2. Induce cells with copper sulfate (add to a final concentration of 0.5 mM).
    a. Prepare 0.1 M sterile $CuSO_4$ (add 0.2497 g of $CuSO_4 \bullet 5H_2O$ into 9.91 ml sterile water and mix well, and then use the 0.2 μm pore size syringe filter and syringe to make the copper sulfate solution sterile).
    b. Add 50 μl $CuSO_4$ into 10 ml cells. Swirl the plate to mix it well.
3. Incubate cells for ~24 hours at 22-25°C.

III. Preparation of FLAG resin

1. Thoroughly suspend the ANTI-FLAG M2 affinity gel in the vial in order to make a uniform suspension of the resin. Ratio of volume of suspension to packed gel is 2 to 1.
2. Using a P1000 with ~2 mm cut-off end of the tip. Immediately transfer 320 μl of suspended resin (160 μl packed resin) into a new pre-cooled 1.5 ml tube.
3. Centrifuge the resin for 4 minutes at 2000 g.
4. Remove the supernatant. Be careful not to remove any beads.
5. Wash the beads twice in 1x Wash Buffer.
    a. Resuspend the beads in 0.5 ml of 1x Wash Buffer.
    b. Centrifuge resin 30 seconds at 2000 g.
    c. Remove the supernatant.
    d. Repeat steps a-c.
6. Wash the resin twice with Elution Buffer.
    a. Resuspend the resin in 0.5 ml Elution Buffer.
    b. Centrifuge at 2000 g for 30 seconds.
    c. Immediately remove the supernatant. Do not leave the resin in Elution Buffer for more than 2 minutes.
    d. Repeat steps a-c.
7. Wash the beads four times in 0.5 ml of 1x Wash Buffer each wash.
    a. a. Resuspend the beads in 0.5 ml of 1x Wash Buffer.
    b. Centrifuge resin 30 seconds at 2000 g.
    c. Remove the supernatant.
    d. Repeat steps a-c 3 times.
    e. Leave the resin suspended in 1x Wash Buffer.

IV. Procedures of FLAG-tagged protein purification

A. Cell lysis

1. Add 70 μl protease inhibitor cocktail to 7 ml CelLytic M lysis buffer.

2. Wash cells (for each plate):
    a. To remove cells adhering to the dish, pipet the medium over the cells gently several times.
    b. Collect the cells and medium into a 15 ml centrifuge tube.
    c. Spin for 5 minutes in the RT-6000 at 1600 RPM (420 g).
    d. Decant the supernatant and discard.
    e. Wash the cells by resuspending the pellet in 10 ml of PBS (Phosphate Buffered Saline). Centrifuge for 5 minutes at 1600 RPM in the RT-6000.
    f. Decant the supernatant and discard, then pipet liquid off thoroughly. Touch the mouth of the tube with a Kimwipe.
    g. Resuspend the cell pellet in 840 µl of CelLytic M lysis buffer.
    h. Remove cells and buffer to a 1.5 ml conical screw-cap Eppendorf tube.
3. Incubate the cells end over end for 15 minutes in the cold room.
4. Centrifuge the cell lysate for 10 minutes at 12,000 g (11,400 RPM) in the microfuge in the cold room.
5. Pool the supernatant from the 8 tubes (~7 ml total) into a new chilled 15 ml tube. Place the tube on ice.
6. If the lysate is viscous, shear the DNA by passing it through an 18-gauge needle four times.
    a. Put an 18-gauge needle on a 10 ml syringe.
    b. Suck the lysate into the syringe and expel it back into the tube slowly four times.
7. Remove 200 µl of the lysate into a screw-cap tube and store frozen in the liquid N_2. Keep the remainder on ice.
8. Measure the total amount of the lysate remaining. Add 4 M NaCl into lysate to give a final concentration of 350 mM.
    a. Add 92 µl of 4M NaCl per ml. of lysate (giving a final concentration of 350mM NaCl.)
    b. For example, if the volume of lysate is 7 ml, add 643.8 µl of 4M NaCl.

B. Beads binding

1. Add the washed resin to the cell extract (~7 ml).
    a. Remove the 1x wash buffer from the resin beads.
    b. Resuspend the beads very gently in 500 µl of the lysate.
    c. Transfer the resuspended beads to the 15 ml tube containing the lysate.
    d. Rinse the 1.5 tube (which was containing the beads) with 500 µl of lysate and transfer that to the 15 ml tube containing lysate.
2. Incubate the lysate and beads O/N in cold room on rocker or with end over end rotation.
   Note: The incubation time could be shorter, depends on what cell lines used.
3. Centrifuge the resin for 2 minutes at 1600 RPM in the RT-6000. Remove the supernatant and save it in a 15 ml tube and store in -80°C. Also, remove 200 µl of the supernatant into a screw-cap tube and store frozen in the liquid nitrogen as “Flow through”.

4. Wash the resin TWICE with wash buffer with 350 mM NaCl concentration.
    a. Add 1 ml wash buffer into the tube containing the resin and then resuspend the beads.
    b. Remove beads and wash buffer to a new 1.5 ml tube.
    c. Centrifuge for 30 seconds at 2000 g.
    d. Repeat steps a-c one more time.
5. Wash the resin THREE times with 1.0 ml HEMG wash buffer.
    a. Add 1 ml 1x wash buffer into the tube containing the resin and then resuspend the beads.
    b. Remove beads and wash buffer to a new 1.5 ml tube.
    c. Centrifuge for 30 seconds at 2000 g.
    d. Repeat steps a-c two more times.

C. Elution of the FLAG-fusion protein with 3x FLAG peptide

1. Add 50 μl of 3x FLAG peptide (5 mg/ml) to 1200 μl of HEMG wash buffer so that the final concentration of FLAG peptide would be 200 μg/ml.
2. Elute the bound FLAG-fusion protein with five 230μl volumes of 3x FLAG peptide (200 μg/ml).
    a. Add 230 μl of the 3x FLAG elution buffer to the resin.
    b. Resuspend the resin and let the sample sit on ice in the cold room for 4-5 minutes.
    c. Centrifuge at 2000 g for 30 seconds.
    d. Remove and save the supernatant into a chilled eppendorf tube. This is elution fraction 1.
    e. Repeat steps a-d to collect fractions 2, 3, and 4.
    f. Collect elution fraction 5 by adding 230 μl of the FLAG elution buffer and incubating for 10 minutes at room temperature.
    g. Keep each fraction separate.
    h. Remove 50 μl from each fraction and store each as a separate aliquot in liquid nitrogen.
3. The elution fractions will be assayed by immunoblot, bandshifts, and transcription assays.
4. Combine the elutions with the best activity.

D. Recycle and store the resin immediately

1. Add 500 μl of elution buffer (0.1M glycine, pH3.5) to the resin and centrifuge at 2000 g for 30 seconds.
2. Repeat previous step two more times.
3. Immediately wash the resin with 1 ml of 1x wash buffer and remove the supernatant.
4. Repeat step c four more times.
5. Add 1 ml of 1x wash buffer containing 50% glycerol and 0.02% sodium azide.
6. Store the resin at 4°C.

VII. Dialysis to remove FLAG peptide in elution fractions

1. Cut dialysis tubing into 8 cm pieces (the capacity is ~ 1 ml/piece).
2. Put the tubing pieces one-by-one into a beaker containing d.d. water with magnetic stir bar stirring. Allow it to stir for 30 minutes. Make sure all pieces completely immersed in the water during stirring.
3. Transfer the tubing pieces into another beaker containing d.d. water. Make sure all pieces completely immersed in the water. Cover the beaker with aluminum foil and keep it in the cold room until used.
4. Prepare a 2 L beaker containing 1 L of ice-cold HEMG-100 buffer with stirring. Add 1 ml of 1M DTT (final concentration of 1 mM) right before use.
5. Take out a prepared dialysis tubing. Squeeze to remove all the water inside/outside of the tubing. Use a dialysis clamp to close one end of the tubing.
6. Pipet the combined elutions from step IV-C4 into the tubing. Close the other end with another clamp.
7. Put the clamped tubing into the beaker containing dialysis buffer with stirring. Make sure the tubing is completely submerged.
8. Repeat step 5-7 for any additional elutions. Allow samples to dialyze for 3 hours.
9. Exchange the HEMG-100 buffer in the beaker with another 1 L of ice-cold HEMG-100 buffer supplemented with DTT. Dialyze for another 3 hours.
10. Take out the tubing, unclamp one end and transfer the solution into a labeled, chilled 1.5 ml conical screw-cap tube. Store in the liquid nitrogen.
11. Repeat step 10 for any additional elutions.

Appendix E: Detailed protocol for polyacrylamide gel electrophoresis (PAGE) purification of DNA oligos

I. Solutions and materials

20% Denaturing Acrylamide (19:1) with Urea
Note: Prepare only enough that will be used within 3 weeks. Downsize the volumes and amounts accordingly.
Add 95 g of acrylamide (neurotoxic), 5 g bis-acrylamide, and 100 ml 10x TBE buffer to 150 ml distilled water. Stir to dissolve. Slowly add 210 g urea. Moderately heat to facilitate the dissolving of urea. After dissolving, the final volume should be around 500 ml. Add distilled water to make 500 ml if necessary. Sterilize by passage through 0.22-μm filter. Store at 4°C.
Note: Wear protective clothing/mask/gloves during the preparation. Heating in the chemical hood and DO NOT allow the temperature of the solution over 50°C or acrylamide will evaporate.

10x TBE Buffer
Add 108 g Tris base, 55 g boric acid, and 40 ml 0.5 M EDTA pH8.0 to 800 ml distilled water. Stir to dissolve. Bring to 1 liter with distilled water. Store at room temperature.
Note: The 10x stock buffer will precipitate during storage. To avoid this, after making 20% denaturing acrylamide solution, dilute the 10x stock buffer to 5x stock buffer with distilled water for storage.

10% Ammonia Persulfate (APS)
Add 1 g APS in a 15 ml Falcon tube. Add 10 ml sterile d.d. water. Vortex to dissolve. Aliquote the solution into microfuge tubes (1ml/tube). Store at -20°C.

2x denaturing polyacrylamide loading dye
Add 6 mg bromophenol blue, 6 mg xylene cyanol FF, and 0.15 g EDTA into 20 ml deionized formamide. Stir to dissolve.
Notes: Formamide is used for denaturing oligos. To make 20 ml deionized formamide, add 1.5 g AG501-X8 ion exchange resin into 25 ml formamide in a 50 ml Falcon tube. Rock for 30 minutes. Spin down to pellet the resin. The supernatant is the deionized formamide.
Bromophnol blue (or fast dye, blue color) migrates as a 8mer oligo on a 20% polyacrylamide gel (PAGE). On the other hand, xylene cyanol FF (or slow dye, green color) migrates as a 28mer oligo on a 20% PAGE. You may want to exclude xylene canol FF from the loading dye to avoid the interference if your oligo is 20-40mer.

Ethidium Bromide (EtBr) Stock Solution (5 mg/ml)
Add 1 g ethidium bromide (strong mutagen!) to 200 ml distilled water. Stir for several hours to ensure it has dissolved. Transfer the solution to a dark bottle. Store at room temperature.

20x Saline Sodium Citrate (SSC)
Dissolve 17.53 g NaCl and 8.82 g sodium citrate in 80 ml distilled water. Adjust the pH to 7.0 with a few drops of a 4M NaOH solution if necessary. Bring the volume to 100 ml with distilled water. Sterilize by autoclaving. Dilute to working concentration (0.1x) with sterile distilled water. Store at room temperature.

3M Sodium Acetate (NaOAc) pH5.2
Dissolve 40.81 g sodium acetate·$3H_2O$ (m.w. 136) in 70 ml distilled water. Adjust the pH to 5.2 with glacial acetic acid. Adjust the volume to 100 ml with distilled water. Sterilize by autoclaving. Store at room temperature.

II. Electrophoresis of DNA oligos

1. If your DNA oligos are delivered lyophilized (freeze-dried) in tubes(e.g. oligos ordered from IDT), you will need to dissolve them with sterile distilled water to get oligo concentration 5 mg/ml. Calculate how much sterile distilled water you need to add as follows:
   Sterile distilled water (ul) = [oligo amount (mg)/5 mg/ml] X 1000 μl/ml
2. Prepare a 1:400 dilution of the oligos by adding 2 μl oligo sample into 798 μl sterile distilled water. Measure the optical density at 260 nm of the dilution with the spectrophotometer (Beckman DU-50). Use the OD_{260} value to calculate the real concentration of your DNA oligos:
   Concentration (μg/μl) = [OD_{260} X 30 ug/ml X 400 (dilution factor)]/1000 μl/ml
3. Prepare a 20% PAGE as follows: Add 60 ml 20% denaturing acrylamide, 400 μl 10% APS, and 40 μl TEMED in a 125 ml flask. Swirl to mix. Pour the solution in to an assembled gel apparatus. Insert a 4 well comb. Allow the gel to polymerize for 30 minutes.
4. Pre-run the gel without samples at 40 mA for 30 minutes in 2x TBE buffer.
5. Calculate the volume needed to get 300 μg oligo sample. Mix the equal volume of 2x denaturing polyacrylamide loading dye with the oligo sample.
   Note: Add loading dye to oligo samples only right before loading into the gel.
6. Wash wells. Load oligo samples. Run the gel at 45 mA until bromophenol blue migrating 2/3 of way along the gel.
7. After the run, pour the gel running buffer from the gel apparatus into a metal pan. Submerge the gel into the buffer in the pan. Add EtBr stock solution into the pan to get final EtBr concentration 0.5 μg/ml (10 μl stock solution/100 ml buffer). Rock to stain for 10 minutes.
8. Destain the gel in d.d. water for 20 minutes. Carefully place the gel on plastic wrap on the UV light box. Turn on the UV light to observe the gel. If the most of the oligos are appeared as a single band and migrate at the appropriate position, use a clean razor

blade to cut the band out and chop into several pieces. Immediately transfer these pieces into a 14 ml polypropylene round-bottom tube (BD Falcon, cat # 352059) containing 3.5 ml 0.1X SSC. Tightly close the cap (2 clicks) and tap the tube to a rocker. Wrap the tube with aluminum foil to avoid the light. Rock overnight at room temperature.

II. Purification of DNA oligos

1. Transfer the 0.1X SSC (~3 ml) to a new round-bottom tube. Add equal amount (3 ml) of isoamyl alcohol. Vortex for 10 seconds. Centrifuge for 3 minutes at 1000 rpm. Discard the upper layer solution (isoaml alcohol + EtBr).
2. Add 1/10 volume (300 μl) 3M NaOAc pH5.2 and 2.5 volume (7.5 ml) ice-cold 100% ethanol to the tube. Swirl to mix. Sealed with parafilm and incubate at -80°C overnight.
3. Place the tube (with rubber adaptor) in HB-6 rotor in Sorvall RC5C PLUS. Centrifuge for 1 hour at 10,000 rpm, 4°C.
4. Carefully pour off the supernatant. Keep the supernatant in a 50 ml Falcon tube in case the precipitation doesn't work. Air-dry the pellet for 15 minutes. Resuspend the pellet in 100 μl sterile distilled water. Transfer the solution to a 1.5 ml screw-cap tube. Centrifuge for 2 minutes at max speed to pellet any acrylamide residues.
5. Transfer the supernantant to a new 1.5 ml screw-cap tube. This is your purified DNA oligos. Prepare a 1:100 dilution of your ologo sample. Check OD_{260} and calculate the concentration of the oligo sample.

III. Check the purity of purified DNA oligos

1. Prepare a 20% PAGE as describe in Section II, step 3. Use a <u>20 well</u> comb.
2. Pre-run the gel without samples at 40 mA for 30 minutes in 2x TBE buffer.
3. Calculate the volume needed to get 2.5 μg purified oligo sample. Mix the equal volume of 2x denaturing polyacrylamide loading dye with the sample.
4. <u>Wash the well.</u> Run, stain and destain the gel as previously described. Oligos should migrate as a single band. Take a photo for your record.

Appendix F: Protein cross-linking with BS3

I. Solutions and Materials

A. Materials

NaCl (Fisher #S271-500) ACS certified

HEPES (Fisher #BP310-1) Molecular Biology grade

$MgCl_2$·6H2O (Fisher #BP214-500) For preparation of buffer solutions

Ammonium bicarbonate (MP Biomedicals #150107)

PCR machine (BioRad iCycler)

DNA oligos from integrated DNA technologies
Upon receiving, purify by polyacrylamide gel electrophoresis (PAGE) (refer to Appendix E)
Upper strand U6:96Ab from sequence -75 to -8 (MW 20748.5). Lower strand U6:96Ab from sequence -74 to -7 (MW 21123.8).

```
TGCCAATTCTTATAATTCTCAACTGCTCTTTCCTGATGTTGATCATTTATATAGGTATGTTTTCCTCA
 CGGTTAAGAATATTAAGAGTTGACGAGAAAGGACTACAACTAGTAAATATATCCATACAAAAGGAGTT
```

Mutant U6:96Ab DNA contains fully mutated PSEA and TATA box (upper strand MW 20933.6, lower strand 20947.6).

```
TGCCAATTCTTACCTGATAGGTGACCAGGACTATGATGTTGATCAGGGACCTCGGTATGTTTTCCTCA
 CGGTTAAGAATGGACTATCCACTGGTCCTGATACTACAACTAGTCCCTGGAGCCATACAAAAGGAGTT
```

Amino-terminal Met-FLAG-BAP fusion protein 0.39 μg/μl (Sigma #P-5975)

Monoclonal anti-FLAG M2 alkaline phosphatase antibody produced in mouse (Sigma #A9469-1MG)

B. Solutions

4M NaCl
Dissolve 2.33 g of NaCl (MW 58.44) in a total volume of 10 ml of d.d. water. Store at 4°C.

1M HEPES K^+ (pH 7.6)
Dissolve 119.15 g HEPES (MW 238.3) in ~450 ml d.d. water. Titrate with 10N KOH to pH 7.6. Adjust the volume to 500 ml with d.d. water. Store at 4°C.

1M $MgCl_2$
Dissolve 101.65 g $MgCl_2 \cdot 6H2O$ (MW 203.31) in 400 ml d.d. water. Adjust the volume to 500 ml with d.d. water. Store at 4°C.
Note: $MgCl_2$ is extremely hygroscopic. Buy small bottles and do not store opened bottles for long periods of time. Once the crystals become saturated with water, dispose of the chemical properly.

5X annealing buffer

	original solution				final solution	
Chemical	conc.	unit	add	unit	conc.	unit
HEPES K^+ (pH7.6)	1.0	M	100.0	µl	100.0	mM
MgCl2	1.0	M	25.0	µl	25.0	mM
NaCl	4.0	M	500.0	µl	2.00	M
Sterile d.d. water			375.0	µl		
final volume			1.0	ml	Store at 4°C.	

1M ammonium bicarbonate
Dissolve 0.79 g of ammonium bicarbonate (MW 79.1) in a final volume of 10 ml of d.d. water. Store at room temperature.

bis(sulfosuccinimidyl)suberate (BS3) crosslinker (Thermo Fisher #21585): Five 2 mg vials (MW 572.43)
Prepare immediately after the protein-DNA incubation in step III-B.3. BS3 hydrolyzes rapidly in aqueous solution.
Carefully separate one tube of dry BS3. Cut the plastic connecting the tubes then cut the foil that caps the tubes without uncapping the other tubes.
Add 35 µl of double deionized water for a final concentration of 100mM of BS3. Dissolve completely and use immediately. Discard any remaining solution.

II. Annealing of DNA oligos

1. In a PCR tube, mix together the following: 15 µg of upper stand and 15 µg of lower strand DNA oligos with 1X annealing buffer in a 60 µl reaction.

	original solution			final solution	
Component	conc.	add	unit	conc.	unit
Oligo upper strand		15.0	µg	0.25	µg/µl
Oligo lower strand		15.0	µg	0.25	µg/µl
Annealing buffer	5X	12.0	µl	1X	
Add d.d. water to final volume of		60.0	µl	Store at 4°C.	

2. Use the following program to run the annealing reaction:
   Step 1 95°C 5:00 min
   Step 2 65°C 15:00 min
   Step 3 37°C 15:00 min
   Step 4 25°C 15:00 min
3. This produces 60 µl of 0.5 µg/µl double stranded DNA.
   a. To calculate the molarity of the double-stranded DNA, take the sum of the molecular weight of the upper and lower oligo strands:
   (20748.5 g/mol + 21123.8 g/mol) = 41869.3 g/mol
   b. Calculate the moles in 30 µg of double-stranded DNA:
   $3.0x10^{-5}$ g / (41869.3 g/mol) = $7.17x10^{-10}$ mol
   c. Then determine the molarity in a 60 µl reaction:
   $7.17x10^{-10}$ mol / $6x10^{-5}$ L = 0.012 mM double stranded DNA

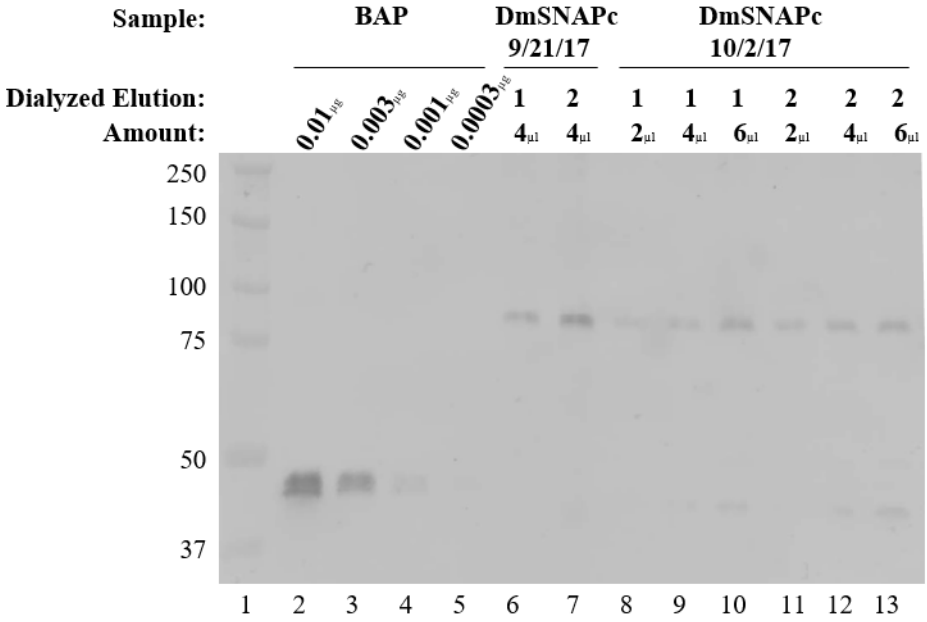
III. Cross-linking of proteins on DNA

A. Approximating DmSNAPc concentration

1. Run increasing amounts of FLAG purified DmSNAPc (505 cell line with N-terminal His_6-FLAG- tagged DmSNAP190) from about 2-6 µl on a 10% SDS-PAGE gel.
2. Also run 0.01, 0.003, 0.001 and 0.0003 µg of FLAG-BAP fusion protein. Prepare as follows:
   Dilute 2 µl of 0.39 µg/µl BAP into 600 µl water to create a 0.0013 µg/µl solution (Solution 1). Add 2.3 µl of Solution 1 to 3 µl of 4X SDS-gel loading dye and 6.7 µl of water for a final amount of 0.003 µg of BAP. Add 7.7 µl of Solution 1 to 3 µl of 4X SDS-gel loading dye and 1.3 µl of water for a final amount of 0.01 µg of BAP. Dilute 10 µl of Solution 1 (0.0013 µg/µl BAP) into 90 µl water to create a 0.00014 µg/µl solution (Solution 2). Add 7.1 µl of Solution 2 to 3 µl of 4X SDS-gel loading dye and 1.9 µl of water for a final amount of 0.001 µg of BAP. Add 2.1 µl of Solution

2 to 3 µl of 4X SDS-gel loading dye and 6.9 µl of water for a final amount of 0.0003 µg of BAP.

3. Transfer the proteins to a membrane.
4. Detect FLAG-tagged DmSNAP190 using an antibody against the FLAG tag epitope.
5. Visually determine which of the DmSNAPc bands is most similar to which of the BAP bands.
    a. Example blot from experiments used in this dissertation:



    b. Example Sample Comparison Chart:
    For the first row, 6 µl of dialyzed elution 1 from DmSNAPc prepared on 10/4/17 had a band intensity between that of 0.001 µg and 0.003 µg of BAP protein, or approximately 0.0016 µg or $3.3x10^{-14}$ moles. Therefore, for 6 µl of DmSNAPc, there is $5.5x10^{-15}$ moles per µl. This sample has a total purified volume of 700 µl therefore, the total moles in the sample are $3.9x10^{-12}$ moles. The remaining samples were analyzed in the same manner.

SNAP190 band	Similar to BAP band	SNAP190 mol/µl		Purified Volume (µl)		Total moles
6 µl 10/4 DE1 =	0.0016 µg; $3.3x10^{-14}$ mol	$5.5x10^{-15}$	x	700	=	$3.9x10^{-12}$
4 µl 10/4 DE2 =	0.0016 µg; $3.3x10^{-14}$ mol	$8.3x10^{-15}$	x	650	=	$5.4x10^{-12}$
4 µl 9/21 DE1 =	0.0016 µg; $3.3x10^{-14}$ mol	$8.3x10^{-15}$	x	350	=	$2.9x10^{-12}$
4 µl 9/21 DE2 =	0.0020 µg; $4.1x10^{-14}$ mol	$1x10^{-14}$	x	300	=	$3.0x10^{-12}$

*The four samples in this chart were combined. Adding each sample's total moles and purified volume gives a combined total of $1.5x10^{-11}$ moles in 2000 µl or $7.6x10^{-15}$ moles per µl

Note: The concentration of the DmSNAPc complex can be no higher than the concentration of DmSNAP190 because the FLAG purification could purify DmSNAP190 that is not complex with DmSNAP43 and DmSNAP50.

B. Cross-linking reaction

1. Before beginning the next steps, set up a 10% SDS-PAGE gel and tubes for each reaction containing 3 µl of 4X SDS-gel loading dye.
2. For each reaction, obtain 5-10 picomoles (about 500 µl) of FLAG purified DmSNAPc (505 cell line) and a 10-35 molar excess of U6 (or U1 or mutant) promoter DNA (annealed in step II-3). Also required is the same amount of protein for a reaction without any DNA.
   Note: An excess amount of DNA should be added to promote protein binding considering that: K_a = [protein-DNA] / [protein] [DNA]
   Re-writing the above equation: [protein-DNA] / [protein] = K_a x [DNA]
   a. For the work in this dissertation, we will assume a conservative K_a of 10^8.
   b. If the DNA is in large excess, the concentration of the DNA will not change significantly during the course of the binding reaction.
3. For the work in this dissertation the following reactions were prepared:

Reaction #	Flag Purified 505 (µl)	U6 DNA (µl)	Total Volume
1 (with WT DNA)	500	WT: 10.5	510.5
2 (with Mut DNA)	500	Mut: 10.5	510.5
3 (without DNA)	500	Anneal Buffer: 10.5	510.5
Moles	$3.8x10^{-12}$	$1.25x10^{-10}$	
Molarity in 510.5 µl	$7x10^{-9}$	$2.5x10^{-7}$	

   Thus, [protein-DNA] / [protein] = K_a x [DNA] = 10^8 x ($2.5x10^{-7}$) = 25
   This indicates that the ratio of the [protein-DNA complex]:[free protein] would be 25 under the stated conditions.
4. Incubate the protein-DNA reactions set up in step B3 in a 25°C water bath for 30 minutes.
5. Immediately after the protein-DNA incubation, remove 9 µl of each sample and add directly into the prepared tubes containing 4x SDS-gel loading dye. This will serve as a non-cross-linked control.
6. To the remaining reaction, immediately add 100 mM BS3 to a final concentration of 2 mM.
   a. For the work in this dissertation, 10.4 µl of 100 mM BS3 were added to the 510.5 µl reaction.
7. Incubate the reactions containing BS3 in a 25°C water bath for 30 minutes.
8. To the reaction, add 1 M ammonium bicarbonate quenching reagent to a final concentration of 20 mM.
   a. For the work in this dissertation, 10.4 µl of 1 M ammonium bicarbonate was added.
9. Quench in a 25°C water bath for 5 minutes.

10. After quenching, immediately load 9 µl of each cross-linked sample to the prepared tubes containing 4X SDS-gel loading dye.
11. Store the remainder at -80^{o}C until samples are sent to the Ranish Lab on dry ice.
12. Detect the desired protein with an antibody specific to an epitope it contains.

Appendix G: Developing conditions for SNAPc purification on an immobilized template for BS3 cross-linking

I. Solutions and Materials

A. Materials

NaCl (Fisher #S271-500) ACS certified

HEPES (Fisher #BP310-1) Molecular Biology grade

$MgCl_2$·6H2O (Fisher #BP214-500) for preparation of buffer solutions

$ZnCl_2$ (Fisher #Z33-100) ACS certified

EDTA (Acros Organics #139-33-3) Molecular Biology grade

KCl (Fisher #BP366-500) for molecular biology and tissue culture use

Tris Base (Fisher #BP154-1) RNase, DNase, and protease free

DTT (Fisher #BP172-25) Electrophoresis grade

Non-stick RNase-free tubes (Fisher #50-591-363)

Metal particle concentrator

360-degree rotator

Nanodrop

DNA oligos from integrated DNA technologies (IDT)

Upper strand and lower strand of U6:96Ab from sequence -121 to +10. Mutant U6:96Ab DNA contains fully mutated PSEA and TATA box. Upper strands are conjugated with a biotin label on the N-terminal end.

Wildtype U6 promoter DNA (-121 to +10)

5′ AAAGGAAATTTTGAAAATCTTAAGCAGAGGGTTCTTAAGACCATTTGCCAATTCTTATAATTCTCAACTGCTCTTTCCTGATGTTGATCATTTATATAGGTATGTTTTCCTCAATACTTCGTTCTTGCTT 3′

3′ TTTCCTTTAAAACTTTTAGAATTCGTCTCCCAAGAATTCTGGTAAACGGTTAAGAATATTAAGAGTTGACGAGAAAGGACTACAACTAGTAAATATATCCATACAAAAGGAGTTATGAAGCAAGAACGAA 5′

Mutant U6 promoter DNA (-121 to +10)

5′ AAAGGAAATTTTGAAAATCTTAAGCAGAGGGTTCTTAAGACCATTTGCCAATTCTTACCTGATAGGTGACCAGGACTATGATGTTGATCAGGGACCTCGGTATGTTTTCCTCAATACTTCGTTCTTGCTT 3′

3′ TTTCCTTTAAAACTTTTAGAATTCGTCTCCCAAGAATTCTGGTAAACGGTTAAGAATGGACTATCCACTGGTCCTGATACTACAACTAGTCCCTGGAGCCATACAAAAGGAGTTATGAAGCAAGAACGAA 5′

M-280 Streptavidin Dynabeads (Thermo Fisher #11205D)

Bovine serum albumen (Fisher # 501003330)

Poly(deoxyguanylic-deoxycytidylic) acid sodium salt [Poly (dG-dC)] (Sigma # P9389)
Dissolved in HEMG-100 buffer to a final concentration of 1μg/μl.

B. Solutions

4M NaCl
Dissolve 2.33 g of NaCl (MW 58.44) in a total volume of 10 ml of d.d. water. Store at 4°C.

1M HEPES K^+ (pH 7.6)
Dissolve 119.15 g HEPES (MW 238.3) in ~450 ml d.d. water. Titrate with 10N KOH to pH 7.6. Adjust the volume to 500 ml with d.d. water. Store at 4°C.

1M $MgCl_2$
Dissolve 101.65 g $MgCl_2$·6H2O (MW 203.31) in 400 ml d.d. water. Adjust the volume to 500 ml with d.d. water. Store at 4°C.
Note: $MgCl_2$ is extremely hygroscopic. Buy small bottles and do not store opened bottles for long periods of time. Once the crystals become saturated with water, dispose of the chemical properly.

10mM $ZnCl_2$
Dissolve 1.36 g $ZnCl_2$ (MW 136.3) in 1 L d.d. water. Store at 4°C

0.5M EDTA (pH 8.0)
Add 90.8 g of $Na_2EDTA·2H_2O$ (MW 336.21) to about 400 ml of d.d. water. Stir and adjust the pH to 8.0 with NaOH (~20g of NaOH pellet). Bring the volume to 500 ml with d.d. water. Store at 4°C.
Note: $Na_2EDTA·2H_2O$ will not be dissolved until the pH of the solution is adjusted to approximately 8.0 by the addition of NaOH.

4M KCl
Dissolve 149.1 g KCl (MW 74.55) in 400 ml of d.d. water. Bring the volume to 500 ml with d.d. water. Store at 4°C.

1M Tris-HCl (pH 7.5)
Dissolve 121.1g Tris Base (MW 121.14) in about 700 ml d.d. water. Adjust the pH to 7.5 with 12M HCl. Bring the volume to 1 L with d.d. water. Store at 4°C.

0.1 M DTT (dithiothreitol)

Dilute 100 µl of 1 M DTT (lab stock) to 900 µl of d.d. water. Wrap with aluminum foil and store at -20°C.

2X annealing buffer

	original solution				final solution	
Chemical	conc.	unit	add	unit	conc.	unit
Tris-HCl (pH7.5)	1.0	M	40.0	µl	20.00	mM
EDTA	0.5	M	8.0	µl	2.00	mM
NaCl	4.0	M	250.0	µl	0.50	M
Sterile d.d. water			1.702	ml		
final volume			2.0	ml	Store at 4°C.	

Adjusting buffer

	original solution				final solution	
Chemical	conc.	unit	add	unit	conc.	unit
Tris-HCl (pH7.5)	1.0	M	20.0	µl	10.00	mM
EDTA	0.5	M	4.0	µl	1.00	mM
NaCl	4.0	M	1.875	ml	3.75	M
Sterile d.d. water			101.0	µl		
final volume			2.0	ml	Store at 4°C.	

1X binding and wash (B&W) buffer

	original solution				final solution	
Chemical	conc.	unit	add	unit	conc.	unit
Tris-HCl (pH7.5)	1.0	M	500.0	µl	10.00	mM
EDTA	0.5	M	100.0	µl	1.00	mM
NaCl	4.0	M	25.0	ml	2.00	M
Sterile d.d. water			24.4	ml		
final volume			50.0	ml	Store at 4°C.	

HEMG-100 buffer

	original solution				final solution	
Chemical	conc.	unit	add	unit	conc.	Unit
HEPES K^+ (pH7.6)	1.0	M	500.0	µl	25.00	mM
$MgCl_2$	1.0	M	250.0	µl	12.50	mM
$ZnCl_2$	10.0	mM	20.0	µl	0.01	mM
EDTA (pH8.0)	0.5	M	4.0	µl	0.10	mM
KCl	4.0	M	500.0	µl	100.00	mM
Glycerol	100.0	%	2.0	ml	10.00	%
Sterile d.d. water			16.7	ml		
final volume			20.0	ml	Store at 4°C	

4X SDS gel loading buffer

	original solution				final solution	
Chemical	conc.	unit	add	unit	conc.	Unit
Tris-HCl pH 7.8	1.0	M	1.5	ml	0.20	M
DTT	1.0	M	3.0	ml	0.40	M
SDS	288.4	g/mol	0.6	g	0.28	M
Bromophenol blue	670	g/mol	0.03	g	5.97	mM
Glycerol	100.0	%	2.4	ml	32.00	%
Bring up to final volume			7.5	ml		

II. Annealing of DNA oligos

1. Upon arrival from IDT, prepare an aliquot of each DNA oligo at a concentration of 2 µg/µl in d.d water.
2. In a 1.5 ml screw cap tube, mix 25 µg (12.5 µl) of upper stand and 25 µg (12.5 µl) of lower strand DNA oligos.
3. Add 25 µl of 2X oligo annealing buffer (for a final volume of 50 µl).
4. To anneal, float the tube in boiling water in a half-filled 1 L beaker. Remove beaker from heat and allow to slowly cool down to room temperature.
5. To the annealed DNA, add 50 µl of Adjustment Buffer. This is to adjust the final concentration of the annealed DNA solution to that of the 1X B&W Buffer.
6. Determine the final DNA concentration using the Nanodrop. The concentration should be approximately 0.5 µg/µl. Store the DNA at 4°C.

III. Washing of Dynabeads

Note: Any rotation of the beads should be done by taping the tube horizontally (on its side) to the 360° rotator. Washes should include 3 minutes of rotation and 3 minutes of concentration on a magnet.

1. Resuspend the Dynabeads (stock 10 mg/ml) in the vial by vortexing.
2. Use a cut pipette tip to transfer 80 µl of beads (800 µg) into two separate 1.5 ml non-stick tubes. One will be for the wildtype experiment, one will be for the negative control (beads without DNA or mutant template).
3. Add 500 µl of 1X B&W Buffer and keep rotating for 3 minutes.
4. Place the tube on a magnet for 3 minutes and discard the supernatant.
5. Repeat steps 3 and 4 to wash a second time.
6. Resuspend the washed beads in 80 µl of 1X B&W Buffer (which is the same as the initial volume of beads).

IV. Immobilization of Nucleic Acids and Bead Blocking

1. Dilute 5 µg of annealed DNA from step II-6 into 100 µl of 1X B&W Buffer. Given the DNA should be about 0.5 µg/ µl, the dilution should be about 10 µl of DNA in 90 µl buffer. Determine the concentration using the Nanodrop. Calculate the amount of DNA in 80 µl. This will be the actual amount of DNA added to the reaction (~ 4 µl).
2. Take the prepared beads from step III-6 and pipet off all the liquid after placing on the magnet for 3 minutes.
3. To the beads add 4 µg of the biotinylated DNA (80 µl of the dilution from step IV-1. Note: This is 4 µg of DNA and 800 µg of beads. 1 mg of beads has a binding capacity of 10 µg of DNA, so all the DNA should be able to bind, though previous tests have shown about 57% retention of input DNA.
4. Incubate the DNA with the beads for 30 minutes at room temperature using gentle rotation.
5. Place the immobilized DNA on the magnet for 3 minutes to pull down the biotinylated DNA coated beads. Remove and save the supernatant at -20°C.
   Note: Later on, determine the DNA concentration in the saved supernatant using the Nanodrop. Estimate the amount of bound DNA by comparing the concentration before and after incubation with the beads.
6. Add 500 µl of 1X B&W Buffer to the beads and keep rotating for 3 minutes.
7. Place the tube on a magnet for 3 minutes. Remove and discard the supernatant.
8. Repeat steps 6 and 7 two more times for a total of 3 washes.
9. Add 500 µl of 5 mg/ml bovine serum albumin (BSA) and rotate for 15 minutes to block the beads.
10. Place the tube on a magnet for 3 minutes and discard the supernatant.
11. Wash 3 times with 500 µl of HEMG-100 buffer. Wait to perform the final wash until you are ready to proceed to the next step.

V. Binding of proteins to immobilized DNA

1. Make sure the prepared beads from step IV-11 have all the supernatant removed.
2. To the beads, add the following 140 µl of pre-mixed solution: 60 µl of nickel purified DmSNAPc (302 cell line with N-terminal His_6-FLAG-tagged DmSNAP43; could also use 505 cell line), 4.2 µl of 0.1 M DTT (final concentration 3 mM), 14 µl of 1 µg/µl dGdC (final concentration 100 ng/µl). Bring up to 140 µl with HEMG-100 buffer (final concentration 100 mM KCl).
3. Rotate the mixture for 30 minutes at room temperature.
4. Collect the beads with the magnet. Save the "flow through" (FT) (solution containing the proteins that did not bind to the DNA) at -20°C.
5. Wash 3 times with 75 µl of HEMG-100 buffer. Save the washes (W1, W2, and W3) at -20°C.
   For the final wash, use a cut tip to transfer the wash and beads to a new non-stick tube.
6. Elute the proteins off the beads with 100 µl of 1X SDS gel loading buffer by placing in a boiling water bath for 5 minutes.
7. Concentrate the beads on the magnet for 3 minutes and save the elution supernatant (E). This is your eluted proteins. Keep on ice to load immediately onto a gel or store at -20°C.

VI. Determination of assay efficiency using SDS-PAGE western blot

1. Prepare a 10% SDS-PAGE gel.
2. Load each lane with sample containing 10% of the protein input into the reaction (control), and 10% of the supernatant from either FT, W1, W2, and E from each reaction, 2.5 µl of 4X SDS gel loading buffer, and water up to 10 µl.
3. Detect the desired protein with an antibody specific to an epitope it contains.