Gene Expression Control by Chromatin Binding Factors

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Gene Expression Control by Chromatin Binding Factors

Gen expressie controle door chromatine bindende factoren

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Chapter 1

Introduction

Structure of DNA and chromatin

In nature, the hereditary material that contains the instructions for making all living matter is known as deoxyribonucleic acid (DNA). Almost all the cells in our body, except mature red blood cells, have DNA of which the vast majority is located in the nucleus. DNA is composed of long stretches of nucleotides, which themselves are build up of sugar (deoxyribose), phosphate, and two kinds of nucleobases, i.e. purines and pyrimidines. The purine bases comprise adenine (A) and guanine (G), whereas the pyrimidines consist of cytosine (C) and thymine (T). In general, DNA is formed by two nucleotide strands which together assemble into a double helix structure (Watson and Crick, 1953). Within this double-stranded helix, the nucleobases present in opposite strands form hydrogen bonds with each other in such a way that adenine forms base pairs with thymine (A-T) and cytosine pairs up with guanine (C-G). As a consequence of this base pairing, the nucleobases adenine and thymine and cytosine and guanine are present in approximately equal amounts, with A and T each accounting for roughly 30%, whereas C and G each make up 20% of the four bases in human (Chargaff et al., 1950). The relative amounts of A, G, C, and T and, therefore, the composition of DNA vary from one species to another though.

The DNA can be divided into several functional domains, such as genic and non-genic regions. The genic regions contain genes, which are pieces of DNA encoding either protein or non-coding ribonucleic acid (ncRNA). In case of the protein-coding genes, information embedded within these genes is transmitted by an intermediate molecule, the messenger RNA (mRNA). This occurs through a process called transcription, which will be explained in more detail later on. The non-genic regions often fulfill regulatory functions which are important for gene transcription.

Within living cells DNA does not simply exist as a double helix of intertwined nucleotide strands, since the total amount of DNA (or genome) in humans is almost 2 meter in length which would be impossible to fit into the cell nucleus with a diameter of only 6 μm. To overcome this problem, DNA is packed in a nucleoprotein structure known as chromatin. Chromatin is made up of a repeating unit called the nucleosome, in which about 147 base pairs of DNA is wrapped in two superhelical turns around an octamer of the core histone proteins H2A, H2B, H3, and H4 (Kornberg, 1974; Luger et al., 1997). This results in a five- to ten-fold compaction of the DNA. The resulting structure can be visualized as "beads on a string", which is further compacted by the association of another histone protein, namely the linker histone also known as histone H1, with the DNA in between the nucleosomes to form the 30-nm fiber. The basic histone proteins stabilize this chromatin structure in such a way that their positively charged amino acid residues contact the negatively charged DNA phosphate backbone. Further compaction of the chromatin to obtain higher-order chromatin is achieved by mechanisms which are not yet completely understood. The highest level of compaction is seen during mitosis, where DNA is visible as chromosomes (Felsenfeld and Groudine, 2003).

Positioning of the nucleosomes along the DNA is thought to be determined by several factors, one of them being the underlying DNA sequence (Segal et al., 2006). In this case, nucleosomes can have a preference for binding certain DNA sequences, such as sequences that have the ability to bend sharply, which is required for the formation of the nucleosome structure. Other non-mutually exclusive factors contributing to nucleosome positioning are DNA binding

proteins, ATP-dependent chromatin remodelers, and the transcription machinery (Jiang and Pugh, 2009; Kal et al., 2000; Moshkin et al., 2012; Struhl and Segal, 2013). Similar to DNA, chromatin can be divided into several distinct regions. In general, two types of chromatin are recognized, i.e. euchromatin and heterochromatin. The euchromatin consists of loosely packed, open chromatin which tends to be actively transcribed, whereas heterochromatin is mostly tightly packed and transcriptionally silent in nature (Gilbert et al., 2004; Simonis et al., 2006). In the context of chromatin nucleosomes act as a natural barrier for factors that mediate processes taking place on the DNA, including DNA replication, repair, and transcription. Although chromatin is considered to be rigid in its structure, it is found to be surprisingly dynamic. This dynamic character is determined by various classes of proteins, like histone chaperones, chromatin remodelers, and histone-modifying enzymes.

Post-translational modification of histones

Already since long time it is known that the cells in our body possess the same DNA, but yet have different morphological characteristics. In the 1950s it was postulated by the developmental biologist Conrad Waddington that this phenotypic variation must result from heritable changes which do not affect the DNA sequence, something which he referred to as "epigenetics". Nowadays, we consider an epigenetic trait to be a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence (Berger et al., 2009). One of these chromosomal changes involves the post-translational modification of histone proteins, which occur predominantly at histone tails that extend from the nucleosome surface (Strahl and Allis, 2000; Suganuma and Workman, 2011). Most modifications take place on lysine (K) residues, which can be methylated, acetylated, ubiquitylated or sumoylated. Other residues appear to be modified more selectively, as is the case for arginine (R), which can be methylated, while serine (S) and threonine (T) residues can become phosphorylated. Most, if not all, of these post-translational modifications form binding platforms for a variety of chromatin interacting factors, which can recognize certain modifications through specialized domains.

The contribution of each single histone modification to DNA-dependent processes is a heavily studied subject at the moment. Most studies link certain histone modifications to either gene activation or repression (Table 1). For example, histone acetylation is generally associated with elevated gene activity, whereas the opposite process, i.e. deacetylation, is considered to have a repressive function. The mechanism by which this occurs could be explained by the fact that the negatively charged acetyl moiety is able to disrupt histone-DNA contacts, resulting in either an open or closed chromatin structure depending on the acetylation status (Fig. 1) (Cheung et al., 2000). However, acetylation can also play a role in gene activation by directly recruiting transcriptional activators to chromatin. In this case, activator proteins can associate with acetylated histone tails via specialized domains, such as bromodomains (Zeng and Zhou, 2002). The contribution of histone methylation in controlling gene activity appears to be more complex, as certain modified lysine residues are associated with gene activation, whereas others are implicated in gene silencing. For instance, methylation of histone H3 at K4, K36, and K79 is generally linked to actively transcribed genes (Greer and Shi, 2012). In contrast, methylated K9 and K27 correlate with gene repression. Another layer of complexity is provided by the methyl-

Table 1. Examples of histone modifications found in *Drosophila*.

Histone residue	Modification	Catalyzed by	Associated with
H2A K118	Ubiquitylation	dRing, Psc	Gene repression
H2B K120	Ubiquitylation	Bre1	Gene activation
H3 R2	Methylation	DART8	Gene repression
H3 K4	Methylation	dSet1, Trx, Trr	Gene activation
H3 K9	Methylation	Su(var3-9, G9a	Gene repression
H3 K9	Acetylation	Gcn5/PCAF	Gene activation
H3 S10	Phosphorylation	JIL-1	Gene activation
H3 K14	Acetylation	Gcn5/PCAF	Gene activation
H3 R17	Methylation	DART4/CARM1	Gene activation
H3 K27	Methylation	E(z)	Gene repression
H3 K27	Acetylation	Nejire/CBP	Gene activation
H3 K36	Methylation	Set2, Mes-4	Gene activation
H3 K79	Methylation	Grappa/Dot1	Gene activation
H4 K16	Acetylation	MOF	Gene activation
H4 K20	Methylation	PR-Set7, Su(var)4-20	Gene repression

ation status of the affected lysine residue, which can be mono-, di-, or tri-methylated. These methylated histone tails might be recognized by factors that harbor domains like the chromodomain (Eissenberg, 2001). Ubiquitylation of histones has been reported mainly for histones H2A and H2B, which occurs at the C-terminal tail, rather than the N-terminus, as is the case for most other marks (Weake and Workman, 2008). Interestingly, both modified histones have been associated with different outcomes. In case of H2A monoubiquitylation, modification of K119 in humans (and K118 in *Drosophila*) correlates well with gene silencing. Conversely, monoubiquitylation of H2B on K120 has been linked to gene activation. Most likely, the final outcome is determined by the factors attracted by each of these histone modifications, which probably involves binding of proteins that contain an ubiquitin-recognition module. It should be noted, however, that many histone modifications seem to be established in a sequential order. This has been reported for ubiquitylation of histone H2B, which is required for subsequent methylation of histone H3 on K4 and K79, and is also referred to as the trans-histone pathway (Briggs et al., 2002; Sun and Allis, 2002).

Histone modifiers contributing to changes in chromatin structure

The chemical modifications found on histone proteins are established by a class of proteins known as the histone-modifying enzymes (Kouzarides, 2007b). These enzymes, which are often called "writers", possess the catalytic activity to attach a certain group, i.e. methyl, acetyl, phosphate, or ubiquitin, to a target protein, which comprise histone, but probably also non-histone proteins. Histone methylation is usually carried out by proteins harboring a SET domain, such as Set1 which methylates H3 on K4, Set2 which methylates H3 on K36, and E(z) which methylates H3 on K27 (Mohan et al., 2012). However, there are also methylases which are distinct from SET-domain histone methyltransferases. Dot1 is such an enzyme, which is responsible for methylation of H3 on K79 (van Leeuwen et al., 2002). Some factors might even

contribute to only one particular methylation state, e.g. mono-, di-, or trimethylation. This appears to be the case for *Drosophila* Trithorax-related (Trr), which selectively targets histone H3 for monomethylation on K4 (H3K4me1), whereas Set1, a closely related protein, mainly affects H3K4 di- and trimethylation (H3K4me2 and H3K4me3, respectively) (Herz et al., 2012). This does not appear to be restricted to the fruit fly, as the mammalian homolog of Trr, Mll3, was shown to perform a similar function. However, in yeast only one protein, namely Set1/COMPASS, regulates all successive methylation steps of H3K4 (Briggs et al., 2001; Miller et al., 2001; Roguev et al., 2001). Since there are enzymes that can methylate histone tails, other proteins must be present that can remove these post-translational modifications. This group of factors is represented by the histone demethylases, which usually carry a characteristic Jumonji C (JmjC) domain, with the exception of LSD1/KDM1A (Tsukada et al., 2006).

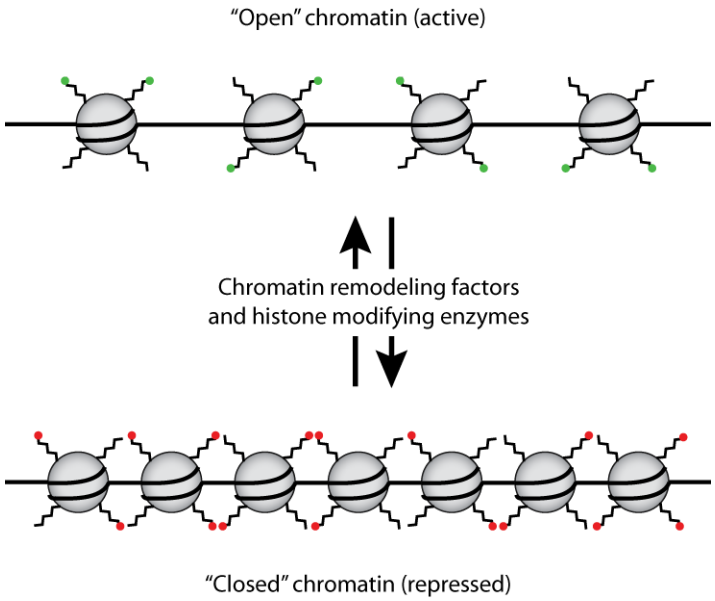

Figure 1. Dynamic changes in the chromatin structure regulate DNA accessibility. Histones (gray circles) are highly modified at their tails by histone modifying enzymes resulting in e.g. methylation, acetylation, phosphorylation, or ubiquitlyation. These modifications are thought to have a stimulatory (green dots) or inhibitory (red dots) effect on transcription by affecting the chromatin structure. Apart from histone modifying enzymes, ATP-dependent chromatin remodeling factors exist which can change the chromatin structure by actively moving nucleosomes along the DNA, thereby opening or closing the chromatin, resulting in activation or repression of transcription, respectively.

Histone modifications are not static, but tend to be rather dynamic. For instance, some developmental genes are silenced early on during embryogenesis and this status is maintained throughout development by factors known as the Polycomb group (PcG) proteins, which can selectively modify histones through methylation of H3K27 and ubiquitylation of H2A (Muller et al., 2002; Wang et al., 2004). Although at first sight it would seem that these modifications are static, they need to be re-established after each cell division in order to maintain proper gene

expression patterns of the genes controlled by PcG factors. Furthermore, modification of histone H2A is thought to occur through cycles of ubiquitylation by the PcG factors dRing and Psc followed by deubiquitylation by the PcG factors Calypso and Asx, which together contributes to gene silencing (Lagarou et al., 2008; Scheuermann et al., 2010; Wang et al., 2004). This parallels findings reported for histone H2B ubiquitylation, where H2B is dynamically modified via ubiquitylation by Bre1 and subsequent deubiquitylation by Ubp8, both of which are necessary for optimal transcriptional activation (Weake and Workman, 2008).

Although chemical modification of histones could in principle affect gene activity directly by causing certain steric changes in the chromatin structure, they also mediate binding of factors that control transcription. These factors, which are also known as the "readers", recognize modified histone tails by specialized domains they possess. A number of well documented domains found in chromatin binding proteins capable of binding post-translationally modified histones are bromodomains, chromodomains, PHD zinc finger (Znf) domains, and Tudor domains (Eissenberg, 2001; Kutateladze, 2011; Zeng and Zhou, 2002). The readers are represented by many different proteins, including factors that are part of the transcription machinery, factors that activate or repress transcription, DNA damage signaling proteins, and ATP-dependent chromatin remodeling enzymes. The latter proteins can actively shape chromatin by using the energy obtained from ATP hydrolysis to disrupt histone-DNA contacts, leading to repositioning of nucleosomes, or alternatively, ejection or exchange of nucleosomes (Clapier and Cairns, 2009; Lorch et al., 2010). By changing the chromatin structure, chromatin remodeling factors (hereafter referred to as remodelers) can have profound effects on processes ranging from DNA repair to gene transcription. Thus, writers and readers collaborate to regulate downstream processes.

Transcription of protein-coding genes

As mentioned earlier, DNA contains the instructions for making proteins. However, in order to produce protein, genes first need to be transcribed into mRNA, which is followed by translation into protein. Transcription is a highly coordinated process which involves a DNA-dependent RNA polymerase that catalyzes formation of the mRNA by reading the DNA sequence of the gene. Since this occurs in the context of chromatin, several steps are required for opening up the chromatin structure to make sure that RNA polymerase II (Pol II) can access the gene that needs to be transcribed. Transcription is often initiated by the recognition of specific DNA sequences by transcription factors, which can be in close proximity to the target gene and are therefore called proximal sites, but can also be located relatively far away from the gene they regulate, in which case they are referred to as enhancer elements (Fuda et al., 2009; Weake and Workman, 2010) (Fig. 2). These transcription factors can either positively or negatively contribute to the transcription process, depending on the factors they bring in. Binding of transcriptional activators to the DNA subsequently results in recruitment of transcriptional co-activators, such as the Mediator complex, and components of the transcription machinery. In addition, activators can recruit certain histone-modifying enzymes and remodelers to open up the chromatin structure in order to facilitate binding of co-activators and the transcription machinery. The co-activator Mediator is thought to interact with transcription factor II D (TFIID), which is one of the gene-

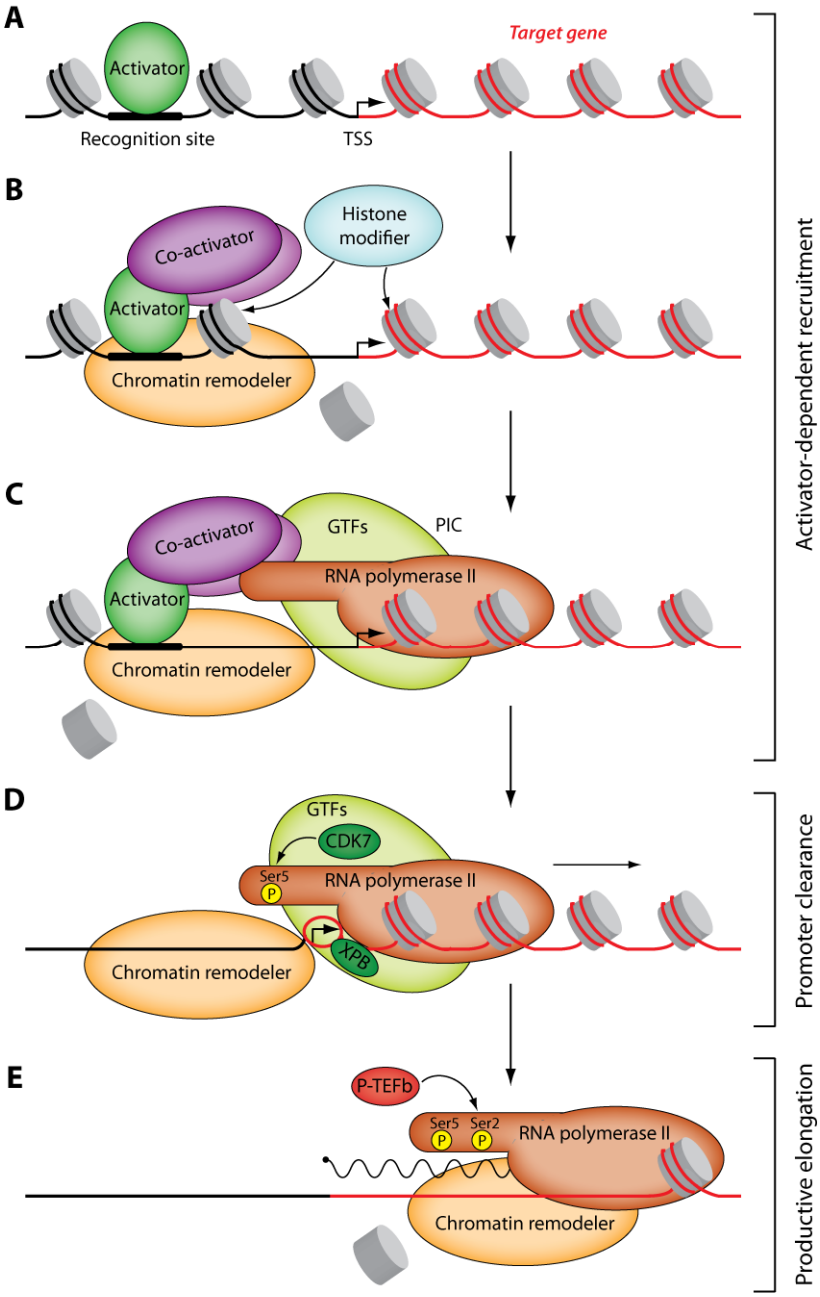

Figure 2. Early steps in the transcription cycle. (A) Promoter selection is determined by the interaction of one or more transcriptional activator(s) with specific DNA sequences (recognition sites) near target genes. Activators subsequently recruit components of the transcription machinery to these genes through protein-protein interactions. (B) Activation of gene expression is provoked by the sequential recruitment of large multi-subunit protein co-activator complexes (shown in shades of purple) through binding to activators. Activators, and possibly histone modifiers which modify the histone tails, also recruit ATP-dependent chromatin remodeling complexes, which move or displace nucleosomes at the promoter, facilitating the rapid recruitment and assembly of co-activators and the general transcription machinery. (C) Co-activators and remodelers cooperate to facilitate the rapid recruitment of Pol II (RNA polymerase II) and the general transcription factors (GTFs) TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH to form the pre-initiation complex (PIC) on the core promoter. These first three steps (A-C) constitute activator-dependent recruitment. (D) Following PIC assembly, the Cdk7 subunit of TFIIH phosphorylates serine residue 5 (Ser5) of the Pol II carboxy-terminal domain (CTD). At the same time, the DNA helicase XPB subunit of TFIIH remodels the PIC, and 11-15 bases of DNA at the transcription start site (TSS) is unwound to establish a single-stranded DNA template into the active site of Pol II. Pol II then dissociates from some of the GTFs, which characterizes an early elongation stage often referred to as promoter clearance. (E) After promoter clearance, Pol II transcribes 20-40 nucleotides into the gene and halts at the promoter-proximal pause site. Efficient elongation by Pol II requires a second

phosphorylation event at serine 2 (Ser2) of the Pol II CTD by Cdk9, which is a subunit of P-TEFb. This creates binding sites for proteins that play important roles in transcription, such as mRNA processing factors and histone modifiers like Set2. Remodelers also facilitate passage of Pol II during the elongation phase of transcription. The transcription cycle continues with elongation of the transcript by Pol II, followed by termination and re-initiation of a new round of transcription (not shown).

ral transcription factors (GTFs), and Pol II, by which it assists in the formation of a functional pre-initiation complex (PIC) on the gene promoter (Johnson et al., 2002). PICs are characterized by the presence of Pol II, Mediator, the GTFs, which include TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH, and numerous chromatin-modifying and -remodeling complexes (Lin et al., 2011; Thomas and Chiang, 2006). The GTFs, with TFIID in particular, help binding of Pol II to the promoter, as it is unable to recognize promoter DNA by itself. The initial step involves binding of the TATA-binding protein (TBP) subunit of TFIID to specific sequences present in a number of core promoters, known as the TATA-box, which is followed by binding of the other GTFs and Pol II to the core promoter region. TFIID can also directly bind to H3K4me3 modified histones via the PHD Znf domain of TAF3 (Vermeulen et al., 2007). Since this modification is often found in the vicinity of active promoters, this provides another way of targeting TFIID to the core promoter (Heintzman et al., 2007).

The first phase of the transcription process described here is potentially rate-limiting where formation of a functional PIC is essential. Subsequent steps are thought to be regulated at the level of Pol II phosphorylation (Fig. 2 and 3). These phosphorylation events take place on the carboxy-terminal domain (CTD) of the largest subunit of Pol II, Rpb1, which contains multiple repeats of the heptamer sequence Tyr_1-Ser_2-Pro_3-Thr_4-Ser_5-Pro_6-Ser_7. Although multiple residues within this repeat can be phosphorylated, the most well documented ones with a role in transcription are serines 2 and 5 (Ser2 and Ser5, respectively). At first, the Cdk7 kinase subunit of TFIIH phosphorylates the Pol II CTD at Ser5. Since this occurs on multiple repeats within the CTD, Pol II becomes hyperphosphorylated at Ser5. This phosphorylation event appears to be stimulated by the presence of the Mediator complex (Kim et al., 1994). Hyperphosphorylation of Ser5, together with unwinding of the DNA at the transcription start site (TSS) by XPB, another TFIIH subunit, triggers the escape of Pol II from the promoter. This promoter clearance causes dissociation of Pol II from some of the GTFs and Mediator, and is followed by transition into an early elongation stage of transcription. Mediator and some of the GTFs may, however, remain associated with the promoter as part of the scaffold complex to facilitate subsequent rounds of Pol II recruitment and re-initiation (Yudkovsky et al., 2000).

During early elongation, Pol II transcribes 20-40 nucleotides into the gene and halts at the promoter-proximal pause site, where it is stalled by the concerted actions of two negative elongation factors, DSIF and NELF (Wada et al., 1998; Yamaguchi et al., 1999). This checkpoint is thought to ensure the proper capping of newly synthesized pre-mRNA, since capping enzymes are recruited to the nascent transcript through the Ser5-phosphorylated CTD (Rodriguez et al., 2000; Schroeder et al., 2000). Productive elongation by Pol II involves subsequent

hyperphosphorylation of the CTD at Ser2 by the positive transcription elongation factor b (P-TEFb), which is recruited to chromatin by direct interaction with BRD4, a bromodomain protein (Jang et al., 2005; Yang et al., 2005). P-TEFb consists of a heterodimer of Cyclin T and the Cyclin-dependent kinase CDK9. In addition to the CTD, P-TEFb phosphorylates both NELF and DSIF, leading to either their release from Pol II or conversion into a positive elongation factor, respectively (Ivanov et al., 2000). Similar to Ser5 phosphorylation, hyperphosphorylation of the Pol II CTD on Ser2 creates binding sites for proteins important for mRNA processing, as well as factors that regulate directionality of transcription, such as the H3K36 methyltransferase Set2, which modifies histones throughout the gene body (Weake and Workman, 2010). Remodelers might also help passage of Pol II through the remainder of the gene to stimulate elongation. Once Pol II reaches the end of the gene, transcription is terminated by cleavage of the nascent transcript to release it from Pol II, followed by polyadenylation of the messenger. Pol II can then be recycled by the action of certain phosphatases, such as FCP1, that remove the phosphate groups placed on the CTD by Cdk7 and P-TEFb, resulting in the recovery of the hypophosphorylated form of Pol II that can participate in a new round of transcription (Cho et al., 1999).

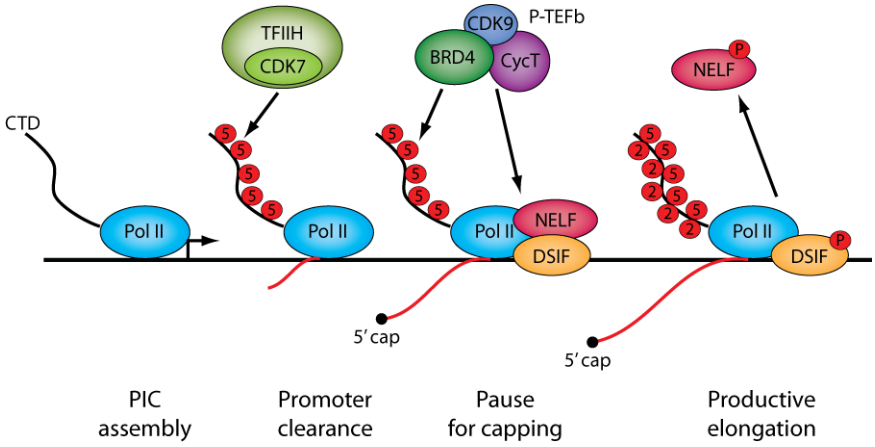

Figure 3. Processive elongation by controlling Pol II CTD phosphorylation. At the start of the transcription cycle, Pol II with its hypophosphorylated CTD is assembled into a PIC at the promoter region. To facilitate promoter clearance and stimulate initiation, Ser5 residues of the CTD heptapeptide repeats are phosphorylated by Cdk7. However, shortly after initiation, the progression of Pol II is stalled by the concerted actions of two negative elongation factors, DSIF and NELF. This checkpoint facilitates the recruitment of capping enzymes to ensure proper 5' capping of the nascent pre-mRNA. To overcome this checkpoint, BRD4 recruits P-TEFb to the transcription template in the vicinity of stalled polymerase, resulting in phosphorylation of DISF, NELF, and the CTD repeats at the Ser2 positions. These phosphorylation events promote the dissociation of NELF and convert DSIF into a positive elongation factor, thereby allowing Pol II to engage in productive elongation to produce full-length transcripts.

Transcription of non-coding RNA genes

The end product of a protein-coding gene is obviously protein, which is produced by a process involving translation of the mRNA by a complex of proteins called the ribosome. Apart from protein-coding genes, the genome also contains genes which do not encode functional protein. These genes are transcribed into RNA, but the resulting RNA is not translated by the ribosome. Instead, these RNAs fulfill crucial roles within the cell, ranging from mRNA translation control to post-transcriptional RNA processing. The cell has divided the task of synthesizing different classes of RNA molecules over three types of DNA-dependent RNA polymerases, i.e. Pol I, II, and III (Roeder and Rutter, 1969) (Fig. 4). All three eukaryotic polymerases are highly similar in structure and even share a number of subunits. However, the total amount of subunits varies, as Pol I has 14 subunits, whereas Pol II and Pol III have 12 and 17 subunits, respectively. As discussed earlier, Pol II is the enzyme responsible for transcription of protein-coding genes. It is estimated, however, that Pol II-dependent transcription accounts for only about 20% of all nuclear transcription, whereas Pol I and Pol III contribute to roughly 70% and 10%, respectively (White, 2005). While Pol II and III are found within the nucleus, Pol I is present in a nuclear sub-

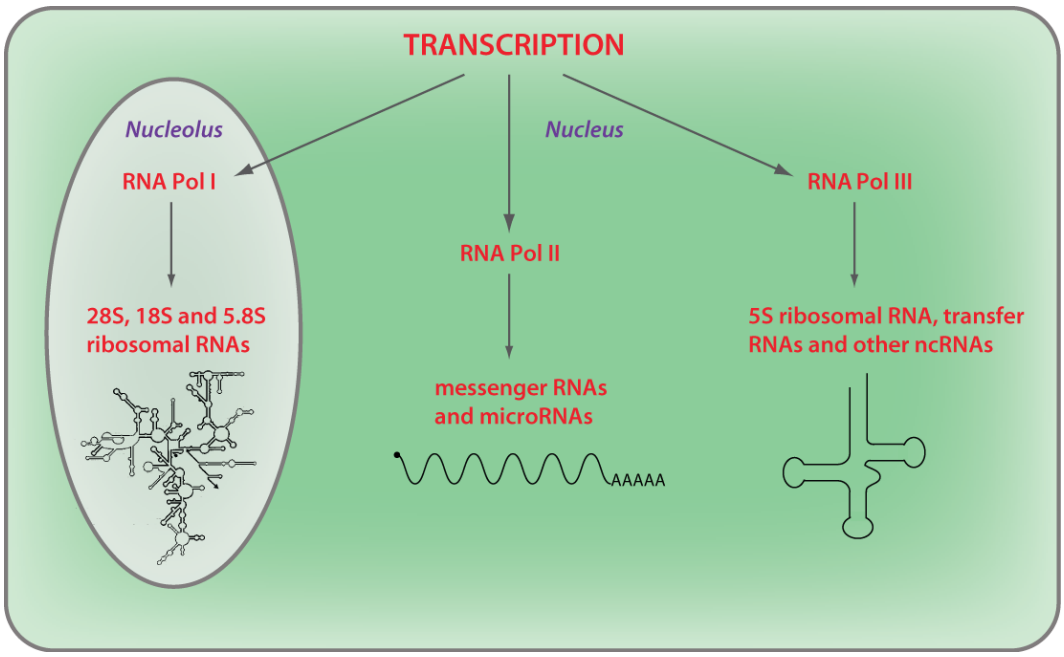

Figure 4. Types of DNA-dependent RNA polymerases. Within the cell, three types of DNA-dependent RNA polymerase enzymes can be distinguished, i.e. RNA polymerase I (Pol I), Pol II, and Pol III. Pol II and III are present inside the nucleus, whereas Pol I is localized in the nucleolar compartment. Pol I is responsible for transcription of mostly long non-coding RNAs (ncRNAs), including the 28S, 18S, and 5.8S ribosomal RNAs (rRNAs). Pol II regulates the synthesis of mRNA by transcribing protein-coding genes, but also contributes to the production of micro RNAs (miRNAs). Pol III synthesizes primarily short ncRNAs, but also some longer ones, including transfer RNAs (tRNAs) and 5S rRNA. All three polymerases together regulate the total amount of protein within the cell.

compartment known as the nucleolus. Inside the nucleolus, Pol I transcribes several ribosomal RNAs (rRNAs), including 28S, 18S, and 5.8S rRNA. These are long ncRNAs that carry out essential structural and catalytic roles within the ribosome. As Pol I takes care of mostof the transcription, it is not surprising that the majority of RNA found in the cell constitutes rRNA. This high content of rRNA is necessary to sustain mRNA translation by the ribosome. Pol III, on the other hand, transcribes a variety of mostly short ncRNAs (Table 2). These include the small 5S rRNA, which is incorporated into ribosomes as well. Apart from that, Pol III produces transfer RNAs (tRNAs), which play key roles in protein synthesis during mRNA translation, U6 small nuclear RNA (snRNA), which regulates mRNA processing, 7SL RNA, which regulates trafficking of nascent polypeptides to membranes, 7SK RNA, which represses Pol II transcription elongation by binding to P-TEFb, and various other ncRNAs.

Transcription carried out by Pol I and III occurs through similar steps as has been described for Pol II. For example, Pol III-dependent transcription requires the action of a number of basal factors, such as TFIIIB, which is reminiscent of TFIID in that it contains TBP, and is necessary for recruiting the polymerase to the promoter (Schramm and Hernandez, 2002). Apart from TBP, TFIIIB also contains either of the TFIIB-related factors BRF1 and BRF2, and BDP1. BRF1 and BRF2 appear to be required for transcription of different types of promoters, where the majority of Pol III templates use BRF1, which have key promoter elements located internally within the transcribed region, such as tRNA and 5S rRNA genes, whereas BRF2 is used instead by promoters located upstream of the initiation site, such as U6 snRNA genes (White, 2011). Genes transcribed by BRF1-containing TFIIIB also require an additional basal factor, TFIIIC, and in particular cases TFIIIA, which is true for 5S rRNA transcription, while the remainder of Pol III targets require SNAPc, which binds proximal sequence elements within the upstream promoter region.

Table 2. Various transcripts synthesized by RNA Polymerase III.

Target	Function
tRNA	Transfer RNA; required for translation of mRNA into protein
5S rRNA	Small ribosomal RNA; found in the large subunit of the ribosome
U6 snRNA	Component of splicesomes; required for pre-mRNA splicing
7SK RNA	Binds and represses P-TEFb, a factor that stimulates transcript elongation by RNA polymerase II
7SL RNA	Acts as scaffold in the signal recognition particle (SRP) which inserts nascent polypeptides into membranes
MRP RNA	Mitochondrial RNA processing RNA; part of an RNP particle that processes pre-rRNA and mitochondrial DNA replication primers
Vault RNA	Part of a very large RNP particle that is implicated in drug resistance and intracellular transport
hY RNA	Human Y RNA; has putative roles in DNA replication and quality control of ncRNAs
H1 RNA	RNA component of RNaseP; processes 5' end of tRNAs
SINE	Short interspersed nuclear element

Recent studies have provided information suggesting more overlap between Pol II and Pol III transcription than previously anticipated. For example, most histone modifications associated with Pol II transcription also appear to be relevant for Pol III-dependent transcription (Barski et al., 2010). Surprisingly, however, genome-wide localization analyses of both human RNA polymerases suggest that Pol II binding exceeds that of protein-coding genes, since it is often found in the vicinity of actively transcribed Pol III target genes as well, where its binding correlates strongly with Pol III occupancy (Barski et al., 2010; Moqtaderi et al., 2010; Oler et al., 2010; Raha et al., 2010). Pol II could also be detected at Pol III transcribed genes in mouse and *Drosophila* cell lines, but not in yeast (Barski et al., 2010). Interestingly, Pol II binding peaks at around 200 base pairs (bp) upstream of Pol III promoters, but these sites often lack open reading frames known to be transcribed by Pol II. Basal factors of the Pol II transcription machinery, including TFIIB, TFIIE, and TFIIH, can also be found at these regions as well as histone modifications associated with active gene promoters, namely H3K4me3. Since Pol II and Pol III share a number of transcription factors which are needed to carry out their function, such as c-Myc, Jun, Fos, and the basal factor TBP, this might explain some of the overlap observed in the genomic distribution (Raha et al., 2010). The functional relevance of this Pol II occupancy near Pol III genes is not exactly known, but it has been suggested that Pol II stimulates transcription performed by Pol III at these sites based on the observation that selective inhibition of Pol II with low doses of α-amanitin reduces expression of a number of Pol III targets (Barski et al., 2010; Raha et al., 2010). However, whether this observed effect is direct or indirect is difficult to reconcile *in vivo*.

Chromatin remodeling complexes

Gene transcription requires that certain regulatory DNA sequences are accessible to the transcription machinery. However, since chromosomal DNA is packaged by nucleosomes, many regulatory DNA elements are hidden and, therefore, cannot be recognized by transcription factors. To overcome this problem, cells are equipped with a set of specialized ATP-dependent chromatin remodeling complexes. Currently, four different families of remodelers can be distinguished based on their enzymatic ATPase subunit, which are conserved from yeast to human. These include the SWI/SNF, ISWI, CHD, and INO80 families (Clapier and Cairns, 2009) (Fig. 5). All these remodelers share some basic properties, such as affinity for the nucleosome, domains that recognize covalent histone modifications, a similar DNA-dependent ATPase domain, domains or proteins that regulate the ATPase domain, and domains or proteins for interaction with transcription factors or other chromatin binding factors. The ATPase domain is absolutely required for remodeling and serves as a DNA-translocating motor to break histone-DNA contacts. Despite these similarities, there are also some obvious differences between the four remodeler families.

The switching defective/sucrose nonfermenting, or shortly SWI/SNF, remodeler family comprises two related complexes, which are referred to as BAP and PBAP or BAF and PBAF in *Drosophila* and humans, respectively (Clapier and Cairns, 2009; Mohrmann and Verrijzer, 2005). Both complexes share a number of subunits, including their ATPase, which is Brahma (Brm) in flies and Brm or Brg1 in humans. Apart from its ATPase domain, Brm contains several other

conserved domains, such as a helicase-SANT (HSA) domain, a Gln-Leu-Gln (QLQ) motif, a BRK domain, and a C-terminal bromodomain. The QLQ motif is thought to mediate protein-protein interactions, whereas the bromodomain might be involved in binding acetylated lysine residues which are present in e.g. histone tails (Zeng and Zhou, 2002). The function of the HSA domain might be to mediate histone binding (Watanabe and Peterson, 2010). The BRK domain, however, has no assigned function yet. Both SWI/SNF complexes are implicated in sliding and ejection of nucleosomes whereby they regulate various processes, including transcription and DNA repair. However, SWI/SNF-type remodelers lack roles in chromatin assembly. Although these remodelers bind to a large number of sites in the genome, there seems to be some degree of specificity which might be provided by additional subunits specific for each complex (Chalkley et al., 2008; Moshkin et al., 2012).

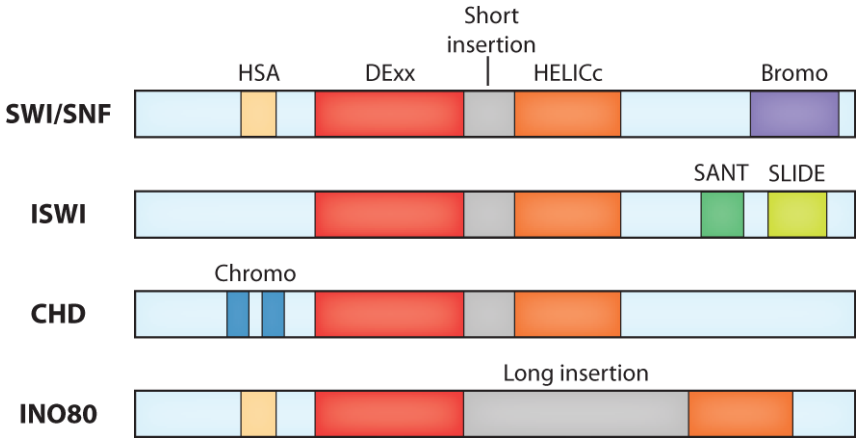

Figure 5. Classification of remodelers by their ATPase subunit. All remodeler families contain a SWI2/SNF2-family ATPase subunit characterized by an ATPase domain that consists of two parts, namely DExx (shown in red) and HELICc (shown in orange). What distinguishes each family are the unique domains residing within, or adjacent to, the ATPase domain. Remodelers of the SWI/SNF, ISWI, and CHD families each have a distinctive short insertion (shown in gray) within the ATPase domain, whereas remodelers of INO80 family contain a long insertion. Each family is further defined by distinct combinations of flanking domains, including the bromodomain (shown in purple) and HSA (helicase-SANT) domain (shown in beige) for the SWI/SNF family, a SANT-SLIDE module (shown in dark and light green) for the ISWI family, tandem chromodomains (shown in blue) for the CHD family, and a HSA domain for the INO80 family.

The imitation switch (ISWI) family of remodelers is characterized by the presence of ISWI as the core ATPase subunit. In *Drosophila* only one gene encodes ISWI, while other organisms often have two genes coding for ISWI, such as SNF2h and SNF2l in case of humans. Multiple distinct complexes containing ISWI have been identified in the fly, i.e. ACF, CHRAC, NURF, RSF, and ToRC, all of which seem to be present in humans as well (Emelyanov et al., 2012; Yadon and Tsukiyama, 2011). ISWI, the catalytic subunit of these complexes, contains several conserved domains, like the HAND, SANT, SLIDE, and DBINO domains. The SANT domain is believed to provide binding to unmodified histone tails, while the other three domains

confer DNA binding properties to ISWI and perhaps contribute to nucleosome recognition (Boyer et al., 2004; Grune et al., 2003). The ISWI binding partners Acf1, Nurf301, dRSF-1, and Toutatis (Tou) contain additional domains which might be important for chromatin targeting *in vivo*. These include the bromodomain present in Acf1, Nurf301, and Tou which could mediate binding to acetylated lysine residues, but also the PHD Znf domain(s) present in all four ISWI binding partners, which in case of Nurf301 has been shown to play a role in binding to H3K4me3 modified histone tails (Wysocka et al., 2006; Zeng and Zhou, 2002). Most ISWI-containing remodelers can optimize spacing of nucleosomes by sliding them along the DNA. By this means they promote chromatin assembly, which can be achieved directly, as shown for RSF, or indirectly through cooperation with histone chaperones, as is the case for ACF and CHRAC (Ito et al., 1997; Loyola et al., 2001). In most instances, this is thought to repress transcription. However, NURF is implicated in transcription activation, which is probably achieved by randomizing nucleosome spacing (Badenhorst et al., 2002; Clapier and Cairns, 2009).

The chromodomain helicase DNA-binding (CHD) class of remodelers covers several proteins with similar ATPases, which are further divided into three subgroups based on the presence or absence of additional domains. These subgroups include CHD1-CHD2, CHD3-CHD4, and CHD5-CHD9 (Marfella and Imbalzano, 2007). All CHD remodelers have a one or two chromodomains, a SNF2-like ATPase domain, and a (putative) DNA binding domain. The CHD1-CHD2 subfamily is represented by a single member in *Drosophila*, i.e. CHD1, the CHD3-CHD4 subfamily by CHD3 and Mi-2, and the CHD5-CHD9 subfamily by Kismet (Kis). Apart from the tandem chromodomains and the ATPase domain, CHD3 and Mi-2 contain an additional paired PHD Znf domain, whereas Kis contains a BRK domain also found in Brm, a SWI/SNF-type remodeler. The chromodomains present in CHD remodelers might be involved in recognition of methylated histone tails (Eissenberg, 2001). A similar function might be fulfilled by the PHD Znf domains, which in some cases have been reported to bind H3K4me3 or H3K36me3 modified histones (Pena et al., 2006; Shi et al., 2006; Shi et al., 2007; Wysocka et al., 2006). CHD family members can be found as a monomer, which appears to be the case for CHD1, but might assemble into large multimeric complexes too, as reported for Mi-2/NuRD (Murawsky et al., 2001). CHD remodelers are implicated in sliding or ejection of nucleosomes to promote transcription, but might be involved in repression as well. For instance, the NuRD complex can achieve transcriptional repression by regulating deacetylation of histones through its Rpd3/HDAC1/HDAC2 subunit. CHD1 and Kis, on the other hand, are implicated in activation of gene expression, most likely by regulating transcription elongation by Pol II (Simic et al., 2003; Srinivasan et al., 2005).

The fourth and last class of chromatin remodeling enzymes, the inositol requiring 80 (INO80) family, comprise multiple related ATPases, namely Ino80 and Domino in flies and Ino80, SRCAP, and p400 in humans (Clapier and Cairns, 2009). These enzymes are characterized by an ATPase domain that is splitted in two halves by a long insertion, which forms a binding interface for certain subunits. Several INO80-type chromatin remodeling complexes can be distinguished, such as *Drosophila* Ino80 and Tip60, and human Ino80, SRCAP, and TRRAP/Tip60. These remodelers are associated with diverse functions, including promoting transcriptional activation and DNA repair. Tip60 is believed to contribute to transcriptional

activation and DNA repair by regulating histone acetylation and exchange of histone variant H2Av (Kusch et al., 2004).

Transcriptional regulation by BRD4

Chromatin regulatory proteins can change the chromatin structure by different means, such as covalent modification of histones or ATP-dependent remodeling of nucleosomes. However, some proteins might not be actively involved in changing the chromatin environment, but rather function by "reading" the histone code in order to recruit other factors involved in transcription to the chromatin. The bromodomains and extraterminal (BET) family of proteins appears to fulfill such a function (Florence and Faller, 2001). Proteins belonging to the BET family are characterized by the presence of tandem bromodomains, an extraterminal domain (ET), and sometimes additional domains, such as the C-terminal motif (CTM), A, B, and SEED (Ser/Glu/Asp-rich) region (Wu and Chiang, 2007). Members of this family include yeast Bdf1 and Bdf2, *Drosophila* Fs(1)h/dBRD4, and mammalian BRD2, BRD3, BRD4, and BRDT. Most of these factors have been found to bind acetylated histones, which can be expected from the fact that they possess two bromodomains (Dey et al., 2003; Kanno et al., 2004; Pivot-Pajot, 2003; Zeng and Zhou, 2002). A unique feature of BET proteins is their persistent association with chromatin during interphase and mitosis, while other bromodomain-containing proteins normally associate only with interphase chromatin. Most of these factors are also widely expressed, with the exception of BRDT, whose expression appears to be restricted to the testes (Paillisson et al., 2007; Shang et al., 2004). Apart from their tandem bromodomains, BET proteins contain an ET domain which is thought to be involved in mediating protein-protein interactions (Rahman et al., 2011; Wu and Chiang, 2007).

Mutations in different BET members causing complete loss of function of the respective protein are often lethal, demonstrating the importance of these factors (Belkina and Denis, 2012). Of all family members, BRD4 is the most studied and well characterized one. BRD4 has been implicated in regulating cell cycle progression, since injection of proliferating cells with anti-BRD4 antibodies leads to G_2/M arrest (Dey et al., 2003). Surprisingly, overexpression of BRD4 in cultured cells results in a G_1/S arrest (Maruyama et al., 2002). Thus, BRD4 might regulate the cell cycle at different stages. Apart from this, BRD4 has been linked to transcriptional control by Pol II. The first indications for such a role came from a study in mouse that found BRD4 to be associated physically with the Mediator co-activator complex (Houzelstein et al., 2002; Jiang et al., 1998). Later on, similar findings were reported for human BRD4 (Wu and Chiang, 2007). It is thought that BRD4 does not interact with the complete 30-subunit Mediator complex, but rather associates with a subcomplex by binding a subunit not present in the CDK module (Fig. 6). BRD4 can also be detected at many genomic sites occupied by Mediator, consisting of promoters and enhancers associated with active genes, suggesting that both factors interact on chromatin as well (Loven et al., 2013). The functional relevance of this is demonstrated by the fact that loss of BRD4 binding to chromatin by treatment of cells with JQ1, a BRD4 inhibitor that disrupts binding of the bromodomains to acetylated histones, results in loss of Mediator binding as well which is accompanied by loss of transcription (Filippakopoulos et al., 2010; Loven et al., 2013).

Since Mediator is crucial for the transcription initiation phase by regulating PIC assembly, BRD4 therefore appears to regulate an early step of the transcription cycle.

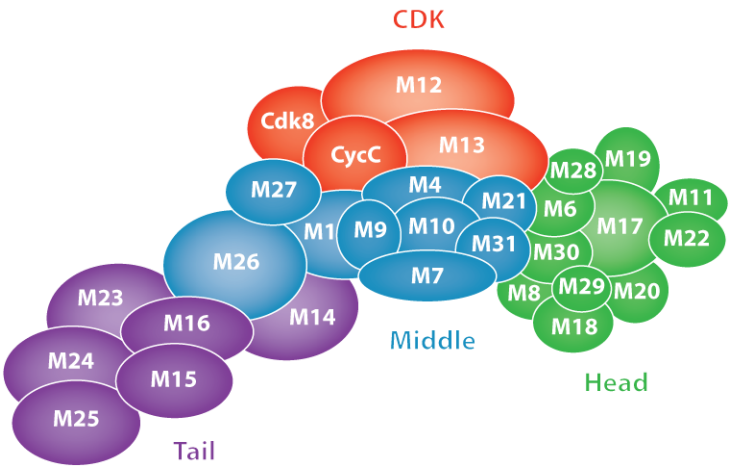

Figure 6. Subunit composition of the Mediator complex. The Mediator co-activator comprises 30 subunits which assemble into four different modules known as the head (shown in green), middle (shown in blue), tail (shown in purple), and kinase or CDK (shown in orange) modules. The head and middle modules are thought to contact Pol II and the GTFs, while the tail module interacts with transcriptional activators bound at regulatory elements. The Mediator complex can exist with or without the presence of the CDK module. This part consisting of the Cdk8 kinase, Cyclin C, MED12 and MED13 has been implicated to function in transcriptional repression, although it might also play a role in gene activation by mechanisms that are not well understood.

Its connection with Mediator does not seem to be the complete story, as several studies have reported BRD4 to be associated with the transcription elongation factor P-TEFb (Jang et al., 2005; Yang et al., 2005). As mentioned earlier, P-TEFb regulates elongation by Pol II through phosphorylation of the CTD on Ser2 and phosphorylation of NELF and DSIF (Ivanov et al., 2000). In this respect, BRD4 probably associates with P-TEFb to bring it to actively transcribed genes where it is able to perform its function. Indeed, loss of BRD4 binding to chromatin also leads to loss of P-TEFb at a number of sites and reduced binding of Pol II to the gene body of the respective genes, indicating that transcription elongation is perturbed (Loven et al., 2013). Although the interaction surface on BRD4 involved in binding to the Mediator complex is not known, association with P-TEFb seems to require the CTM (Bisgrove et al., 2007). This domain can be found only in mammalian BRD4, BRDT, and *Drosophila* BRD4, all of which were able to bind P-TEFb when introduced into mammalian cells. It is estimated that about half of the cellular pool of P-TEFb exists in a complex with BRD4 and is, therefore, active, whereas the other half of P-TEFb is present in a small nuclear ribonucleoprotein (snRNP) particle consisting of the Pol III-transcribed 7SK RNA and HEXIM1/2, which inhibits the kinase activity of the Cdk9 subunit of P-TEFb (Nguyen et al., 2001; Yang et al., 2005; Yang et al., 2001). BRD4 has also been shown to directly associate with some transcriptional activators, such as NF-κB and

p53, which appears to be regulated in an acetylation- or phosphorylation-dependent manner, respectively (Huang et al., 2009; Wu et al., 2013). Thus, BRD4 can regulate transcription at many steps by mediating binding of the activator, Mediator, and P-TEFb to acetylated chromatin.

BRD4 might also play a more direct role in transcription by Pol II, as it appears to possess intrinsic kinase activity by which it can contribute to phosphorylation of the CTD at Ser2 (Devaiah et al., 2012). Apart from its ability to phosphorylate the CTD, BRD4 can also phosphorylate Cdk9 at two different threonine residues, which either repress or activate the kinase activity of the P-TEFb subunit (Devaiah and Singer, 2012). P-TEFb, on the other hand, can enhance the activity of BRD4 by phosphorylating it, while Cdk7 phosphorylates BRD4 resulting in potent inhibition of BRD4's kinase activity. Therefore, it seems there is complex cross-talk among the different kinases to regulate phosphorylation of Pol II. In this case, BRD4 can both act as a scaffold to recruit Mediator and P-TEFb to chromatin and, in addition, might contribute directly to Pol II phosphorylation to coordinate transcription.

Polycomb and trithorax group proteins

The spatial and temporal control of gene expression is essential for normal growth and development of organisms. Epigenetic regulators play an important role in these aspects. This is illustrated by the fact that mutations in certain chromatin modifiers cause abnormal expression of homeotic (Hox) genes in the fly embryo, resulting in phenotypes characterized by a malformed appearance. Based on mutant phenotypes and genetic interactions, these chromatin modifiers have been classified in two separate groups: the Polycomb group (PcG) and trithorax group (trxG), respectively (Muller and Verrijzer, 2009; Schuettengruber et al., 2011). The PcG proteins function to repress transcription, whereas proteins belonging to the trxG activate transcription. However, they do not initiate either of these events, as this is achieved by the action of gap and pair-rule gene products early on, but rather maintain their initial expression pattern. One of their targets are the Hox genes, which encode transcription factors that regulate cell fate along the anteroposterior axis of segmented organisms. As mentioned, mutations in genes belonging to the PcG or trxG which compromise the function of their gene product cause misexpression of Hox genes, resulting in improper specification of cell identity in which one body part can be transformed into another, a process which is known as homeotic transformation. Apart from regulating Hox gene expression, PcG and trxG genes also control transcription of genes involved in development, cell proliferation, stem cell identity and cancer, and are, therefore, involved in many cellular processes (Schuettengruber et al., 2011).

PcG and trxG proteins regulate gene expression by changing the chromatin structure, either through covalent histone modifications or chromatin remodeling. The PcG class is represented by the founding member Polycomb (Pc), which assembles into a complex with proteins Polyhomeotic (Ph), Posterior sex combs (Psc) and Sex combs extra (Sce or dRing), known as Polycomb repressive complex 1 (PRC1) (Shao et al., 1999). PRC1 represses transcription by teaming up with multiple other complexes, including PRC2, Pho-repressive complex (PhoRC), dRing-associated factors (dRAF), and Polycomb repressive deubiquitinase (PR-DUB) (Klymenko et al., 2006; Lagarou et al., 2008; Muller et al., 2002; Scheuermann et al., 2010). Polycomb-mediated gene repression is accompanied by several different covalent histone

modifications, i.e. trimethylation of H3K27 by the E(z) component of PRC2, monoubiquitylation of H2A by dRing/Psc within dRAF, and demethylation of H3K36me2 by the dKDM2 subunit of dRAF (Lagarou et al., 2008; Muller et al., 2002). Deubiquitylation of H2A also appears to be necessary for Hox gene repression, which is carried out by the Calypso protein residing in the PR-DUB complex (Scheuermann et al., 2010). Specificity is achieved by recruitment of PcG complexes to chromatin by particular DNA sequences, known as Polycomb response elements (PREs). This recruitment is thought to be mediated by PhoRC, a complex consisting of Pleiohomeotic (Pho) and dSfmbt, the first being a sequence-specific DNA binding protein (Klymenko et al., 2006). Although the aforementioned histone modifications are clearly correlated with PcG-mediated gene silencing, it is not known exactly how they contribute to transcriptional repression. One possibility is that PcG factors modify histones in order to compact the chromatin structure (Simon and Kingston, 2013). PcG proteins might, however, also directly associate with components of the transcription machinery, such as TFIID, to regulate gene expression (Breiling et al., 2001; Saurin et al., 2001). A recent study suggests that PcG factors, in particular PRC1, might function to block access of Mediator and Pol II to the template DNA, thereby impairing PIC assembly (Lehmann et al., 2012). Taking this into account, it seems plausible that multiple mechanisms are used to obtain gene silencing.

The function of PcG proteins is antagonized by trxG proteins *in vivo*. The latter are represented by trithorax as well as factors that produce a similar phenotype when mutated. *Drosophila* trithorax itself is part of a family known as COMPASS (complex of proteins associated with Set 1), which regulate methylation of H3K4 to activate gene expression (Mohan et al., 2011). Three similar but distinct COMPASS complexes can be found in the fruitfly which are characterized by their core histone methyltransferase, i.e. dSet1, trithorax (Trx), and trithorax-related (Trr). The dSet1 complex appears to be the major methylase responsible for bulk di- and trimethylation of H3K4, while the Trr complex specifically regulates monomethylation of H3K4 and erases the PcG repressive H3K27me3 mark through demethylation by Utx (Ardehali et al., 2011; Herz et al., 2012; Lee et al., 2007; Mohan et al., 2011). The Trx protein has also been reported to be part of another complex called TAC1 (for trithorax acetylation complex) (Petruk et al., 2001). Within this complex Trx promotes acetylation of H3K27 (H3K27ac) by the histone acetyltransferase CBP (Tie et al., 2009). Since acetylation and methylation of H3K27 are mutually exclusive, H3K27ac by CBP is thought to antagonize Polycomb silencing. Furthermore, H3K27ac and H3K4me3 often coincide, both of which are deposited by trxG proteins and contribute to gene activation. Ash1, another histone-modifying enzyme, has been implicated in methylation of H3K4, H3K9, and H4K20 in *Drosophila* by which it can activate Hox gene expression (Beisel et al., 2002). The human homolog of Ash1 appears to be involved in the modification of other histone lysine residues, namely H3K36me2, which demonstrates that different specificities of this enzyme could have evolved over time (Tanaka et al., 2007; Yuan et al., 2011). Similar as what has been observed for H3K27ac, H3K36me2 rarely co-exists with trimethylated H3K27, which suggests that Ash1 prevents the spread of H3K27me3 by PRC2 to antagonize gene silencing by PcG factors (Yuan et al., 2011).

Modifications placed on nucleosomes by histone-modifying enzymes of the trxG class are recognized by other factors that can change the chromatin environment to regulate gene

expression. Several components of chromatin remodeling complexes have been identified as trxG genes (Kennison and Tamkun, 1988). Among these are Brm, Mor, and Osa, which are part of the SWI/SNF-type remodeler. Brm and Mor are core subunits of both BAP and PBAP, whereas Osa is a BAP-specific subunit (Mohrmann et al., 2004). Either of the two remodelers appears to counteract Polycomb silencing *in vivo* as mutations in Sayp, a PBAP-specific component, suppress homeotic transformations caused by Polycomb mutations similar to what has been observed for Brm, Mor, and Osa (Chalkley et al., 2008). The interplay between trxG proteins is demonstrated by a recent report showing that Brm is required for CBP-mediated acetylation of H3K27 (Tie et al., 2012). The demethylase Utx also appears to be essential in this case by erasing the PcG repressive H3K27me3 mark, which is a prerequisite for acetylation on lysine 27. Other chromatin remodelers have been grouped as trxG genes as well based on their ability to maintain expression of homeotic genes or by suppression of PcG mutant phenotypes, such as Nurf301 and Tou, which are part of the ISWI-type remodeling complexes NURF and ToRC, respectively (Badenhorst et al., 2002; Fauvarque et al., 2001). The CHD-type remodeler Kis was also found to suppress Pc mutations in genetic assays and to be required for expression of certain Hox genes (Daubresse et al., 1999; Kennison and Tamkun, 1988). Like most remodelers, Kis appears to be part of a multi-subunit complex, but the identity of its composition is not known at the moment (Srinivasan et al., 2005). Remodelers like Kis and NURF might open up the chromatin structure and mediate gene activation to antagonize Polycomb silencing by associating with H3K4 trimethylated histone tails, either through their chromodomains or PHD Znf, respectively (Schnetz et al., 2009; Wysocka et al., 2006).

Members of the trxG other than histone-modifying enzymes and chromatin remodelers cover DNA binding proteins and general transcriptional (co-)activators. A few examples are Trithorax-like (Trl), Pipsqueak (Psq), dBRD4, components of the Mediator complex, and subunits of cohesin (Gans et al., 1980; Hallson et al., 2008; Kennison and Tamkun, 1988; Schwendemann and Lehmann, 2002). Some of these, like dBRD4 and Mediator, probably play more broad roles in control of gene expression, but might be important during early development for correct expression of Hox genes. Others seem to play dual roles, as they can both activate and repress certain Hox genes or interact genetically with PcG and trxG factors. This is the case for Trl and Psq, but also for the Ino80 chromatin remodeler (Bhatia et al., 2010; Schwendemann and Lehmann, 2002). To distinguish these from "classical" PcG and trxG proteins, they are sometimes referred to as the ETP (enhancers of trithorax and Polycomb mutations) group (Gildea et al., 2000). The mechanism behind their dual function is not exactly known though.

Involvement of the 26S proteasome in gene regulation

Epigenetic control of gene expression involves the concerted action of DNA binding proteins, histone modifiers, chromatin remodelers, and the transcription machinery. However, other factors which at first sight might seem irrelevant could also play an important role in this aspect. One such factor is the 26S proteasome, which is a dedicated protein destruction complex (Fig. 7). Proteins destined to be degraded are marked by polyubiquitylation, a process involving sequential action of ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-ligase (E3) enzymes

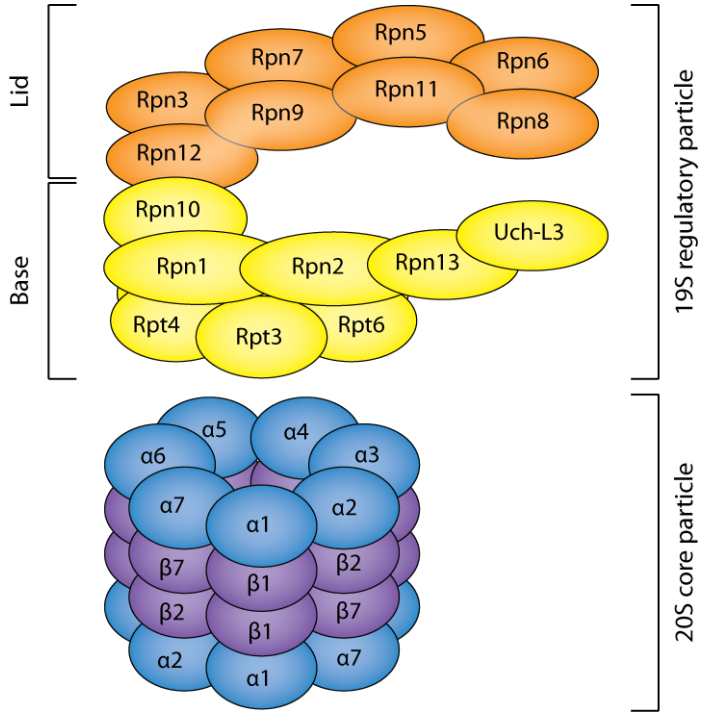

Figure 7. Structure and composition of the 26S proteasome complex. The 26S proteasome is a protein degradation machinery which comprises two major subcomplexes: the 19S regulatory particle (19S RP) and the 20S core particle (20S CP). The 19S RP itself can be divided into two part, i.e. a lid and a base structure. The lid contains eight regulatory particle non-ATPase (Rpn) subunits, including Rpn3, Rpn5-Rpn9, Rpn11, and Rpn12, whereas the base contains five Rpn and six regulatory particle ATPase (Rpt) subunits, which include Rpn1, Rpn2, Rpn10, Rpn13, Uch-L3, and Rpt1-Rpt6. Each component within the 19S RP has a specific function. For instance, Rpn10 and Rpn13 function as ubiquitin receptors, Rpn11 and Uch-L3 control deubiquitylation and the six ATPase subunits unfold the protein substrate. The 20S CP is a barrel shaped structure composed of two rings of α- and β-type subunits. The three β-type subunits β1, β2, and β5 harbor different kinds of proteolytic activities, namely caspase-like, trypsin-like, and chymotrypsin-like activities, respectively. These proteolytic activities are required for degradation of protein substrates into small peptides, which can then be used in presentation to the immune system by major histocompatibility (MHC) class I molecules. The 20S CP is often capped on either side by a 19S RP.

(Pickart, 2001). These enzymes cooperate to attach ubiquitin, a polypeptide consisting of 76 amino acids, to their target protein. Monoubiquitylation, such as reported for histones H2A and H2B, is not sufficient to trigger degradation. Instead, a substrate needs to carry at least four ubiquitin molecules which are attached to each other via lysine residue 48 of ubiquitin (Finley, 2009). The 26S proteasome can recognize these polyubiquitylated proteins because it contains subunits which harbor ubiquitin binding domains. These subunits are located within the 19S regulatory particle (19S RP) of the 26S proteasome, which comprises a lid and base module. Apart from substrate recognition, the 19S RP removes the polyubiquitin chain from the target protein through its intrinsic deubiquitylating activity to recycle ubiquitin, followed by substrate

unfolding in an ATP-dependent manner. The latter is provided by a ring of six ATPase subunits that are part of the 19S base subcomplex, which unfold and translocate the linear target protein into the 20S core particle (20S CP) of the 26S proteasome. This barrel shaped structure contains the proteolytic activity needed for protein degradation. Three types of proteolytic activities have been assigned to the 20S CP, i.e. caspase-like, trypsin-like, and chymotrypsin-like activities. Together, these enzymatic activities are responsible for degradation of proteins into small peptides.

The ubiquitin-proteasome system (UPS) regulates many processes, including cell cycle control, apoptosis, inflammation, signal transduction, protein quality control, and transcription (Finley, 2009). The latter can be affected by breakdown of transcription factors or other components of the transcription machinery, such as Pol II. However, the 26S proteasome, or subcomplexes of this, might also regulate transcription in a non-proteolytic manner. For example, yeast proteasome components have been shown to play a role in the trans-histone pathway (Ezhkova and Tansey, 2004). In this case the two proteasomal ATPases, Rpt4 and Rpt6, were shown to be recruited to chromatin by monoubiquitylated histone H2B after which they regulated the methylation status of H3K4 and H3K79, which was suggested to be important for gene activation and telomeric gene silencing. Several other reports have implied a more direct role for the proteasome in transcription as well, as proteasome components can be found in association with chromatin at actively transcribed genes (Auld et al., 2006; Gillette et al., 2004; Gonzalez et al., 2002; Sikder et al., 2006). The proteasome has been connected to all major steps of the transcription process, namely initiation, elongation, and termination. Its role in initiation was shown by a study reporting that the 19S complex can drive stable complex formation between the transcriptional activator Gal4 and the SAGA histone acetyltransferase complex in an ATP-dependent manner (Lee et al., 2005). Since SAGA acts very early during gene activation by regulating PIC assembly, these results indicate a role for the proteasome in transcription initiation. Hints for a role in elongation came from a study showing that mutations in Rpt6 cause sensitivity to 6-azauracil (6-AU), which is considered to be a hallmark of defects in transcription elongation by Pol II (Ferdous et al., 2001). Moreover, immunodepletion of 19S complexes from *in vitro* transcription reactions significantly influences the ability of initiated Pol II complexes to synthesize long transcripts. The role of the proteasome in transcription termination is illustrated by data showing association of the proteasome with 3' end of genes and the fact that inhibition of the proteolytic activity results in read through of a transcription termination site by Pol II (Gillette et al., 2004).

Whether the complete 26S proteasome or particular subcomplexes of it are involved in transcription is still controversial. Likewise, it is not entirely clear whether the proteasome requires its proteolytic activity to control gene expression. A recent study has provided insight in these aspects by showing that subunits of all major proteasome subassemblies, i.e. 19S lid, base, and 20S CP, associate with chromatin in a virtually identical manner (Geng and Tansey, 2012). Furthermore, the proteolytic activity of the proteasome was shown to be required for full GAL gene activation. The association with chromatin is not limited to Pol II transcribed genes, as proteasome components can also be detected on Pol III target genes and subtelomeric DNA regions. Interestingly, chromatin binding appears to be dependent on active transcription, as

chemical or genetic inhibition of RNA polymerase II or III impairs binding of the proteasome (Geng and Tansey, 2012; Sikder et al., 2006). How the proteasome regulates transcription is an important question. One possibility is that the proteasome mediates turnover of transcriptional regulators on the chromatin, which is supported by the finding that increased binding of transcription factors and Pol II is seen after depletion or chemical inhibition of the proteasome (Szutorisz et al., 2006). Another option is that the proteasome uses its ATPase activity to unfold or refold substrates to modulate their activity on the chromatin. It is, however, unlikely that these substrates are histones, since the proteasome cannot remodel chromatin by itself (Ransom et al., 2009). Nonetheless, the proteasome might control the activity of other chromatin modulators thereby contributing to gene regulation. Ubiquitin could also play an important role in UPS-controlled gene expression by recruiting the proteasome to chromatin, e.g. in the form of monoubiquitylated H2B. Most likely the complete 26S proteasome associates with chromatin to coordinate transcription at multiple stages of the transcription cycle in which it makes use of its proteolytic and non-proteolytic functions depending on the specific need.

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Scope of the thesis

Gene expression control is one of the key processes contributing to cell proliferation, differentiation, and development of living organisms. Many factors converge to regulate transcription which occurs in the context of chromatin, rather than naked DNA. This compacted structure forms a barrier that needs to be overcome in order to control transcription of genes. One class of factors capable of actively changing the chromatin structure is represented by the ATP-dependent chromatin remodeling complexes. Understanding the subunit composition of these enzymes is important for addressing their biochemical activities by which they shape the chromatin. The aim of Chapter 2 is to characterize the Kis-L complex in *Drosophila* starting out with an unbiased proteomics approach. Kis-L was shown to interact with another remodeler, the SWI/SNF-type (P)BAP complex, and colocalizes extensively with this complex on polytene chromosomes in a genome-wide fashion. Kis-L and (P)BAP co-regulate many genes, which in some cases turns out to be cooperative, but in other cases can function in an antagonistic manner. Thus, Kis-L and (P)BAP physically associate to fine-tune gene expression.

Another class of proteins that play crucial roles in transcriptional control is formed by the PcG and trxG epigenetic regulators. How these factors contribute to gene regulation in a mechanistic way is not well understood. Chapter 3 aims to address the mechanism by which PRC1 inhibits transcriptional output. PRC1 specifically blocks binding of the transcriptional co-activator Mediator and Pol II to enhancer and promoter regions, but has only modest effects on binding of most trxG factors. Therefore, PRC1 interferes with PIC assembly by blocking access of Mediator and Pol II to the chromatin, resulting in transcriptional repression.

Gene expression can be modulated at the level of chromatin, but also at other levels. Protein degradation by the 26S proteasome could be another means to regulate transcription. However, the proteasome might directly affect the chromatin environment, but it is not known whether this involves the complete 26S proteasome or subcomplexes consisting of 19S or 20S components. Chapter 4 describes the presence of subunits of both major proteasome subassemblies on polytene chromosomes in *Drosophila*, indicating the association of the full 26S complex with chromatin. Detailed analysis shows that the proteasome colocalizes predominantly with Pol III, rather than Pol II, on chromatin. Furthermore, binding of 19S, but not 20S, is mediated by RNA, whereas monoubiquitylation of histone H2B is not required. Both 19S and 20S function as repressors in controlling Pol III-dependent transcription which does not depend on the proteolytic activity of the complex.

Proteins belonging to the BET family are widely expressed transcriptional regulators. These factors can associate with active chromatin by binding to acetylated histone tails through their tandem bromodomains. This tethers BET proteins to chromatin by which they can recruit various transcriptional activators to promote gene expression. Unlike mammals, *Drosophila* possesses only one BET family member whose role in gene expression control has not been addressed in detail. Chapter 5 describes the mass spectrometric analysis of dBRD4 interacting proteins. The major interacting partner of dBRD4 is the Mediator complex, which also shows

widespread overlap with the BET family member on chromatin. These results suggest a role for dBRD4 in transcription initiation.

Chromatin remodeling complexes are fairly diverse in nature, but contain a similar enzymatic ATPase subunit. ISWI can exist in a number of different complexes which have been identified over time, but whether these represent the full scope of ISWI-containing complexes is not clear. The main goal of Chapter 6 is to reveal all ISWI complexes present in *Drosophila* through proteomic analysis of ISWI associated proteins. At least four distinct complexes containing ISWI are formed: ACF/CHRAC, NURF, RSF, and ToRC. In addition, composition of the NURF has been refined by the identification of two novel subunits, i.e. dBap18 and dHMBXB4. Each ISWI-containing complex shows a particular genome-wide distribution on polytene chromosomes, which suggests that each complex regulates a distinct set of genes.

Chapter 2

Differential Transcriptional Control by Kis and (P)BAP Chromatin Remodeling Complexes

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Abstract

ATP-dependent chromatin-remodeling factors (remodelers) play critical roles in regulating chromatin structure and gene expression. Often remodelers are part of multimeric complexes consisting of several protein subunits which assist in regulating the ATPase activity, complex stability, or in achieving specificity. Here, we focused on *Drosophila* Kismet, which is homologous to mammalian CHD7, to clarify its role in regulating gene transcription. We first established a genetic interaction between Kis and Bap170, a subunit of the PBAP remodeler. Next, we examined the distribution of Kis on larval salivary gland polytene chromosomes and found that it colocalizes with both BAP and PBAP remodeling factors, but not with ISWI. Since the composition of the Kismet complex was unknown, we purified both Kismet-Long (Kis-L) and Kismet-Short (Kis-S) from embryos and found that Kis-L, but not Kis-S, physically associates with the BAP complex and a subset of PBAP. To address whether Kis-L is targeted to chromatin by (P)BAP, we performed RNAi-mediated knockdown in the fly and stained polytene chromosomes to localize the protein on chromatin. By this means we show that Kis-L does not require Brm, the catalytic subunit of (P)BAP, for its recruitment to chromatin and vice versa. Furthermore, we show that knockdown of either Kis-L or Brm does not alter the levels of elongating RNA polymerase II (Pol II) and we also find no changes in H3K27me3 levels after Kis-L RNAi, which is in contrast with an earlier report. Finally, we performed genome-wide expression analysis after Kis-L and Brm knockdown and observe many overlapping target genes. More in-depth analysis of a subset of target genes revealed that Kis-L and Brm can either regulate these genes in the same or opposite direction and we do observe cooperation of both factors in regulating expression of a subset of genes. Thus, we conclude that Kis-L interacts with (P)BAP to fine-tune gene expression.

Introduction

In eukaryotes, genomic DNA is packaged by nucleosomes, which consist of the core histone proteins H2A, H2B, H3 and H4, to ensure that the genetic material is compact enough to fit into the cell nucleus (Kornberg, 1977). These nucleosomal structures make up the chromatin, which forms a natural barrier to all processes that take place on the DNA template, including DNA replication, DNA repair and transcription. Chromatin-remodeling factors (remodelers) play important functions in changing the chromatin structure. They do so by utilizing the energy of ATP hydrolysis to assemble, slide, restructure, or eject nucleosomes (Clapier and Cairns, 2009; Lorch et al., 2010; Zhang et al., 2011). The importance of remodelers is exemplified by the fact that there is no basal transcription on chromatin templates *in vitro*, suggesting an absolute requirement for nucleosome ejection or repositioning (Henikoff, 2008). However, nucleosomes can also facilitate gene transcription in a context-dependent manner (Kristjuhan and Svejstrup,

2004). Thus, nucleosome dynamics offers a potent level of gene regulation, presumably by modulating the accessibility of target DNA elements.

Currently, four major families of remodelers are recognized, each named after its central ATPase and accessory domains (Clapier and Cairns, 2009). These families comprise the SWI/SNF, ISWI, CHD/Mi2, and INO80 remodelers. The *Drosophila* protein Kismet (Kis), which is homologous to CHD6, CHD7, CHD8 and CHD9 in higher eukaryotes, falls into the class of the CHD family. The *kis* gene encodes two major protein isoforms, a large protein of about 574 kDa named Kismet-Long (Kis-L) and a smaller isoform of 225 kDa termed Kismet-Short (Kis-S). The Kis-L protein contains a tandem chromodomain and an ATPase domain typically found in CHD family members. The Kis-S transcript is expressed from an alternative promoter, resulting in a protein that shares the 2105 C-terminal amino acid residues with Kis-L, but lacks the N-terminal domains and is therefore unlikely to have chromatin-remodeling activity. Both Kis-L and Kis-S contain a BRK domain with unknown function, which is also found in Brahma (Brm), the ATPase subunit of the BAP and PBAP chromatin-remodeling complexes and part of the SWI/SNF family (Daubresse et al., 1999). Chromodomains, which are present in Kis-L, can mediate protein-protein or protein-RNA interactions and might also be involved in the selective recognition of methylated histone tails (Brehm et al., 2004). For CHD7, the human counterpart of Kis-L, *in vitro* binding assays revealed that its chromodomains were able to bind mono-, di- and tri-methylated forms of the H3K4 peptide (Schnetz et al., 2009). Therefore, it is likely that Kis-L can bind methylated histone H3K4 tails as well. The ATPase domain of Kis-L shows high homology to ATPase structures of other CHD family members and to that of the SWI/SNF member Brm. Although there is significant sequence identity between Kis-L and Brm, they fall into different classes based on the presence of an additional bromodomain which is present in Brm, but not Kis-L. Potential orthologs of *kis* are present in many organisms, including worms, mice and humans, but not yeast, suggesting that it may have a unique role in transcription or development in higher eukaryotes.

The *kis* gene was originally discovered in a random mutagenesis screen for dominant modifiers of *Polycomb* (*Pc*) mutations (Kennison and Tamkun, 1988). It was shown that mutations in *kis* suppressed mutations in *Pc* based on the extra sex combs phenotype, suggesting that Kis antagonizes Pc. Based on this and the fact that loss of zygotic *kis* function causes homeotic transformations, *kis* is classified as a member of the trithorax group (trxG) of homeotic gene activators which antagonize the Polycomb group (PcG) of transcriptional repressors (Daubresse et al., 1999). Further characterization of *kis* showed that embryos homozygous for *kis* mutations die as first or second instar larvae, indicating that *kis* is an essential gene. Mutations in CHD7, the human ortholog of *kis*, have been implicated in a complex disease called CHARGE syndrome (CS) (Vissers et al., 2004). This syndrome is a sporadic, autosomal dominant disorder characterized by malformations of craniofacial structures, the pheripheral nervous system, ears, eyes and heart. CS occurs due to abnormal development of a multipotent cell population, called the neural crest (NC), which is controlled by CHD7 (Bajpai et al., 2010). Mutations in CHD7 observed in CHARGE patients have been reported to affect the remodeling activity of the ATPase, ranging from subtle to complete inactivation of the catalytic activity (Bouazoune and Kingston, 2012). Flies with reduced Kis expression show defects in the developing central

nervous system, as well as defects in memory and motor function, which is consistent with symptoms observed in CS patients (Melicharek et al., 2010). These data illustrate the conserved function of Kis during evolution.

Transcription is a highly coordinated process that is regulated at multiple different steps, including initiation, elongation and termination. Transcription initiation comprises the formation of functional preinitiation complexes (PICs) in which RNA polymerase II (Pol II) is recruited to gene promoters assisted by sequence-specific transcription factors, co-activators and the general transcription factors (GTFs). During elongation, hypophosphorylated Pol II (Pol IIa) first becomes hyperphosphorylated at serine 5 (Pol IIo^{ser5}) and later on at serine 2 (Pol IIo^{ser2}) of its heptapeptide YSPTSPS repeat located in its C-terminal domain (CTD) (Buratowski, 2009). The last step, transcription termination, involves the recognition of DNA encoded terminator signals, polyadenylation at the 3' end of the transcript and cleavage of the nascent mRNA. It has been proposed that Kis-L plays a global role in the elongation phase, since levels of Pol IIo^{ser2} and elongation factors Spt6 and Chd1 are severely reduced on polytene chromosomes from *kis* mutant larvae (Srinivasan et al., 2005). The same lab concluded that Kis-L functions downstream of positive transcription elongation factor b (P-TEFb), the kinase that phosphorylates Pol II on serine 2, but upstream of the H3K4 methyltransferases Ash1 and Trx (Srinivasan et al., 2008). Furthermore, they demonstrated that loss of *kis* function resulted in increased H3K27me3 levels, a mark associated with PcG-mediated repression, but did not affect global H3K4me2 and -me3 levels. Therefore, it was suggested that Kis-L promotes early elongation and counteracts PcG repression by recruiting the Ash1 and Trx to chromatin. The mechanism by which Kis-L achieves this, however, remains enigmatic.

Most remodelers described to date execute their function as part of multimeric protein complexes. For instance, the ATPase subunit Brm is part of the BAP and PBAP chromatin-remodeling complex (Chalkley et al., 2008; Mohrmann et al., 2004; Moshkin et al., 2012). These two complexes share their catalytic core subunit, together with 6 additional polypeptides, but differ in a number of proteins which are referred to as signature subunits. Previously, the native molecular mass of Kis-L had been determined by size-exclusion chromatography of *Drosophila* nuclear extract and was shown to be around 1 MDa (Srinivasan et al., 2005). This is larger than Kis-L alone, which is almost 0.6 MDa, but smaller than the observed 2 MDa for the BAP/PBAP complexes. This suggested that Kis-L might interact with other proteins which are able to form a complex together. The same study also showed that Kis-L does not physically interact with Pol II or other remodelers like Brm, Mi2 or ISWI. However, the identity of these putative Kis-L binding proteins has not been determined. Analysis of the human Kis/CHD7 interacting partners in NC cells identified the PBAF complex, which is the human counterpart of fly PBAP (Bajpai et al., 2010). It was also shown that CHD7 and PBAF co-occupied several genomic loci and that they cooperated in NC gene expression and cell migration. Therefore, it seemed surprising that although Kis-L is relatively well conserved, its binding partners are not.

In order to elucidate the role of Kis-L in transcriptional regulation, we first analyzed its distribution on larval salivary gland polytene chromosomes. Kis-L was shown to bind many sites and showed striking colocalization with both BAP and PBAP on the chromatin. This appeared to be specific, as little or no overlap was observed with ISWI or Brd8. Next, we attempted to

identify interacting partners of Kis by proteomic analysis of Kis-L and Kis-S purified from *Drosophila* embryo nuclear extract. Our results indicate BAP and a subset of PBAP as the major interacting proteins for Kis-L, but not Kis-S. Apart from (P)BAP, we also find *Drosophila* BRD4 (dBRD4) and Mediator associated with Kis-L and show overlapping patterns on polytene chromosomes. Knockdown of either Kis-L or Brm in the salivary glands of third instar larvae demonstrated that Kis-L is recruited to chromatin independently of Brm and vice versa. Unlike earlier published work, we show that knockdown of either Kis-L or Brm does not alter the levels of chromatin-associated Pol IIo^{ser2}, nor do we detect any changes in H3K27me3 levels on polytene chromosomes after Kis-L knockdown. Finally, by performing genome-wide expression profiling we find that Kis-L and Brm have many overlapping target genes. Although both proteins co-occupy many genes, they can act as an activator or repressor or have antagonistic functions. At some genes, we detect cooperation between Kis-L and Brm in regulating transcription. Based on these results, we propose that Kis-L interacts with BAP and a subset of PBAP to fine-tune gene expression.

Results

Functional relationship between Kis and PBAP

To date, it is not entirely clear if Kis functions on its own or whether it requires the action of additional proteins to control gene expression. Experiments in flies have not led to the identification of such proteins. However, a recent study in humans revealed a functional relationship between CHD7, which is an ortholog of *Drosophila* Kis, and the PBAF remodeler (Bajpai et al., 2010). Since the PBAF remodeling factor also exists in flies (where it is known as PBAP), we were interested to see if Kis could also functionally interact with PBAP. To test this hypothesis, we made use of the genetic tools available in the fly. In our setup, we used conditions were we depleted either Kis or a PBAP subunit, Bap170, controlled by an *sd[638]-Gal4* or *sal-Gal4* driver, followed by phenotypic analysis of the developing wing. Knockdown of Kis using either of the two Gal4 drivers resulted in clear wing defects, including reduced wing size, truncation of L2 vein, and netting of L3 and L4 (Fig. 1A). Depletion of Bap170 on the other hand produced very mild phenotypes, such as slightly reduced wing size and minor irregularities in the wing veins. In order to establish a functional relationship between the two factors, we used a combination of Kis and Bap170 knockdown after which we analyzed the resulting wing phenotype. Strikingly, double knockdown of Kis and Bap170 led to enhanced wing defects, such as severely reduced wing size and increased vein netting, suggesting that both genes act in parallel in the same functional processes.

To explore whether similar results could be obtained in tissues other than the wing, we used a genetic combination that made it possible to label all of the cells in the stem cells lineage of the adult gut by GFP at the time of Kis or Bap170 ablation. This way we were able to monitor the proliferation of the gut stem cells under conditions where Kis or Bap170 were depleted by RNAi. In the control situation, large GFP labeled clones were detected in the adult mid-gut (Fig. 1B). Knockdown of either Kis or Bap170 resulted in lineages with with fewer progeny, which in-

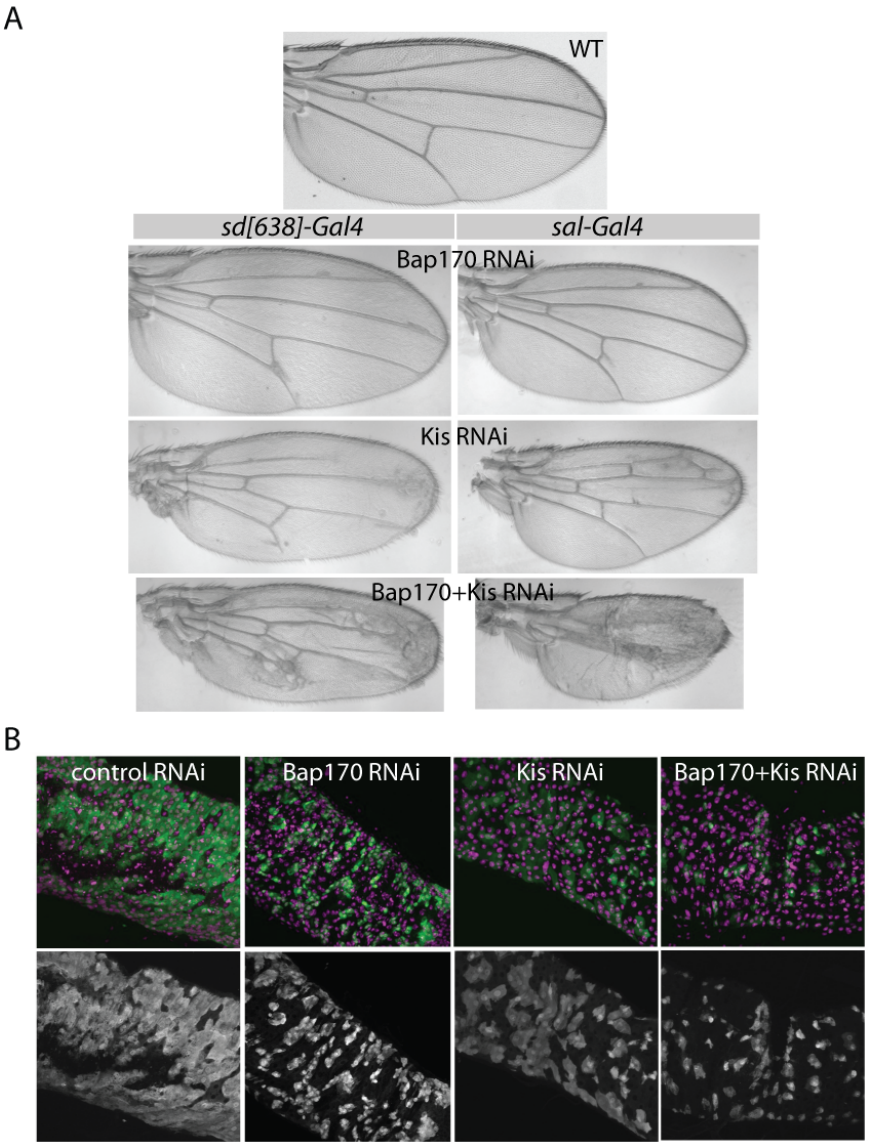

Figure 1. Kis and PBAP interact genetically. (A) Reduction of Kis or Bap170 expression by tissue-specific knockdown using either the *sd[638]-Gal4* (left panels) or *sal-Gal4* (right panels) driver causes defects observed in the developing wing. Phenotypes observed in the Bap170 knockdown were mild, with reduced wing size and slight irregularities in the wing veins being the most noticeable ones. Kis RNAi, however, resulted in clearly reduced wing size and a range of venation defects, including truncations of L2 vein and netting of L3 and L4. Combined knockdown of Kis and Bap170 enhanced the defects associated with the single knockdowns considerably, leading to a dramatic reduction in wing size and increased vein netting. An example of a wild-type (WT) wing is shown on the top. (B) RNAi-mediated knockdown of Kis and Bap170 in adult gut stem cell lineages perturbs their proliferation. Adult gut stem cells were labeled with GFP (in green) at the time of ablation using escargot-Gal4 with a flip-out cassette. Remaining cells were visualized by DAPI staining (in purple). Knockdown of either Kis or Bap170 resulted in stem cell lineages with fewer progeny. Similar to the result in the wing, this defect was enhanced when both genes were ablated simultaneously, suggesting that the two proteins function independently to regulate the same process.

dicates that the renewal divisions have been perturbed. Double knockdown of Kis and Bap170 together resulted in lineages with even fewer progeny, where most clones had less than four cells. Thus, as with the wing, the combined knockdown of Kis and Bap170 results in a worsened phenotype which appears to be additive and suggests that the two proteins function independently to regulate the same processes.

Kis-L colocalizes with BAP and PBAP on chromatin

Since *kis* encodes two major protein isoforms, a large polypeptide of 574 kDa (Kis-L) and a smaller isoform of 225 kDa (Kis-S), with different domain structures (Fig. 2A), we wanted to be able to discriminate between the two proteins. To this end we generated polyclonal antibodies raised against the N-terminus specific for Kis-L and against the 46 unique amino acids of Kis-S. We also raised an antibody against the common C-terminus that recognized both Kis-L and Kis-S (data not shown). Next, we analyzed the expression of both protein isoforms in embryo nuclear extract and salivary gland extracts using our α-Kis-L and α-Kis-S antibodies (Fig. 2B). Both isoforms could be detected in embryo nuclear extracts, but only Kis-L was expressed in salivary gland tissue, which is in agreement with an earlier report (Srinivasan et al., 2005).

Given the fact that Kis-L has all the features of a chromatin-remodeling enzyme, we were interested in determining its genome-wide binding profile in *Drosophila*. We also wanted to compare its distribution on the chromatin with other remodelers, including PBAP. Therefore, we co-stained larval salivary gland polytene chromosomes with our α-Kis-L antibody and antibodies against other remodeling enzymes such as Brm, which is the enzymatic core subunit of BAP and PBAP (Fig. 2C). Our first impression was that Kis-L binds many sites and is primarily associated with the interband regions on polytene chromosomes. Binding of Kis-S could not be determined as it was not expressed in salivary gland tissue. Interestingly, we noted striking overlap between Kis-L and Brm binding sites, suggesting that both remodelers bind similar genomic loci. Since Brm is the ATPase subunit of the BAP and PBAP chromatin-remodeling complexes, we wondered whether Kis-L would also colocalize with signature subunits of both complexes. Co-staining of Kis-L with Osa, a signature subunit of the BAP complex, revealed a high degree of overlap between the two proteins (Fig. 2D). Likewise, co-staining of Kis-L with Polybromo, a signature subunit of the PBAP complex, showed a similar degree of overlap (Fig. 2E). To rule out the possibility that Kis-L simply colocalized with any remodeler, we also performed immunolocalization of Kis-L together with the ISWI and Tip60 remodelers on polytene chromosomes. In general, we hardly detected overlap between Kis-L and ISWI on chromatin (Fig. 2F). Similarly, little overlap was observed between Kis-L and Brd8, a subunit of the Tip60 remodeling complex (Fig. 2G). This demonstrates that Kis-L does not just bind any site on the chromatin, as there seems to be some specificity.

Previously, it was suggested that Kis-L is associated with sites of active transcription based on highly overlapping distributions of Kis-L with Pol II on polytene chromosomes (Srinivasan et al., 2005). We also determined the overlap between Kis-L and Pol II using an antibody raised against the N-terminal and middle parts of Rpb1, the largest subunit of Pol II. This antibody recognizes all major forms of Pol II, including the Pol IIa (initiation), Pol IIo^{ser5} (early elongation), and Pol IIo^{ser2} (late elongation) forms. To our surprise, stainings using this antibody revealed a relatively low degree of overlap with Kis-L (Fig. 2H). As pointed out by Tamkun and colleagues, the relative levels of Kis-L and Pol II varied from site to site, which therefore complicates the interpretation of these results. Nonetheless, taken together, we conclude that Kis-L, BAP and PBAP bind similar sites on the chromatin.

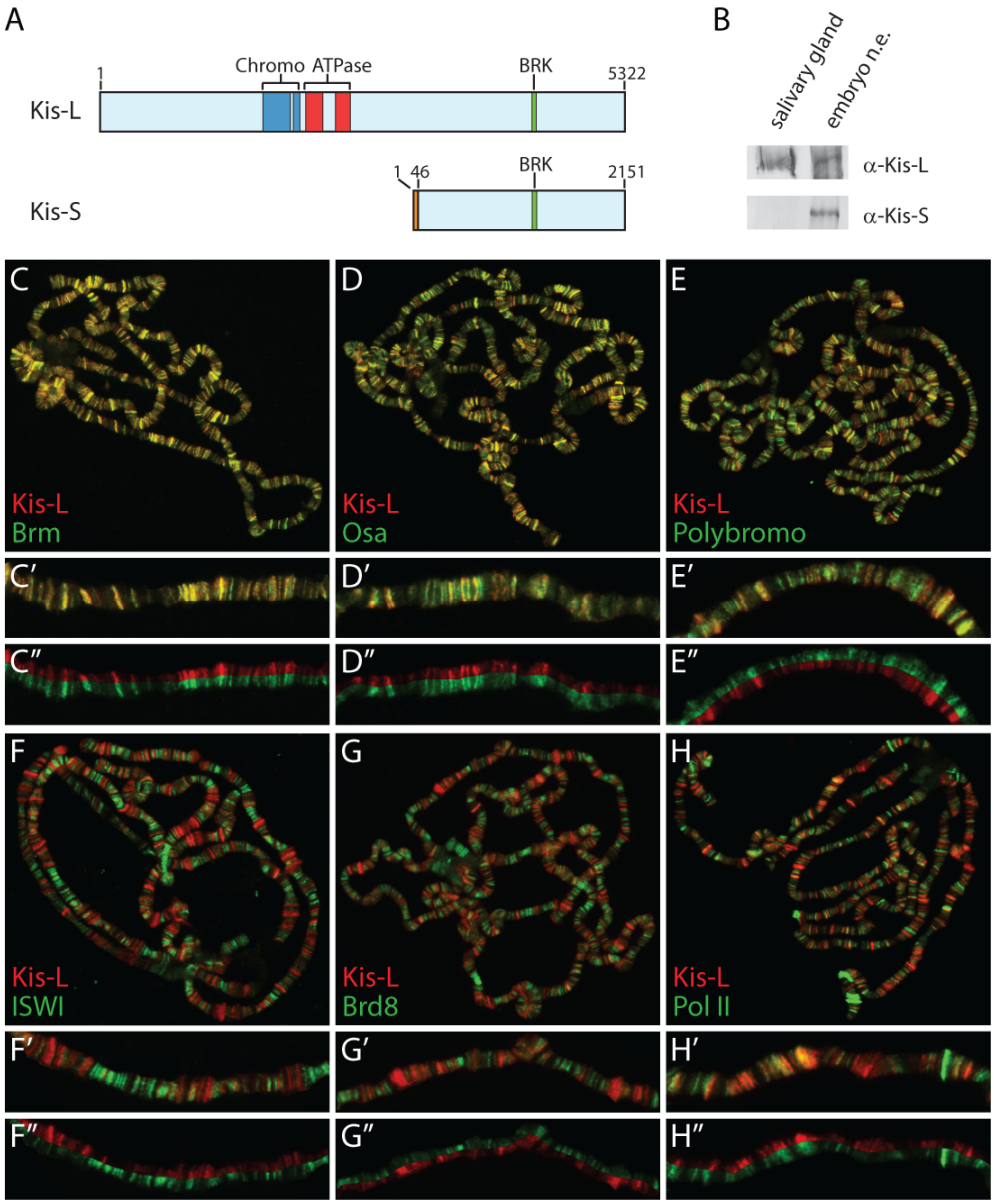

Figure 2. Kis-L colocalizes with BAP and PBAP on polytene chromosomes. (A) Schematic diagram of Kis-L and Kis-S isoforms with conserved protein domains. Kis-L contains a tandem chromodomain and ATPase domain and has the C-terminal 2105 amino acid residues in common with Kis-S, which contains a BRK domain. Kis-S has a unique N-terminal portion comprising amino acids 1-46. Isoforms and domain structures are drawn to scale. (B) Analysis of expression patterns of Kis isoforms. Kis-L is expressed in both salivary glands and embryo nuclear extract (n.e.), whereas Kis-S is only present in embryo nuclear extract. (C-H) Polytene chromosome co-stainings of Kis-L (in red) with (C) Brm, (D) Osa, (E) Polybromo, (F) ISWI, (G) Brd8, and (H) Pol II (all in green). Representative magnifications are shown below the panels, with either zooms including merged (‘) or splitted (“) channels. Note the striking overlap between Kis-L and Brm, Osa, and Polybromo and the marginal colocalization with ISWI and Brd8. Partial overlap is observed between Kis-L and Pol II.

Kis-L, but not Kis-S, physically interacts with BAP and a subset of PBAP

It had been suggested earlier that Kis-L and Kis-S are likely to be subunits of distinct protein complexes (Srinivasan et al., 2005). This was based on determination of the native molecular mass in embryo extracts, which turned out to be about 1 MDa for Kis-L and 650 kDa for Kis-S. However, this study failed to address the exact nature of the different complex compositions. Therefore, we took an unbiased proteomic approach in order to identify novel interacting partners of Kis-L and Kis-S in *Drosophila* embryo nuclear extracts. We purified Kis-L and Kis-S using our highly specific antibodies followed by mass spectrometric analysis of the immunoprecipitated proteins (Table 1). A first glance at the results showed that Kis-L and Kis-S do not physically interact with each other. More thorough analysis of the mass spectrometry data revealed both the BAP and PBAP complex as potential interacting partners of Kis-L, but not Kis-S. We find all known subunits of BAP and PBAP in our Kis-L purification, including BCL7-like, which was identified recently in humans (Middeljans et al., 2012), and dBrd7, Tth, and D4, which were newly identified subunits in *Drosophila* (Moshkin et al., 2012).

Apart from (P)BAP, we also detected a significant amount of the Mediator complex associated with Kis-L. Although Mediator is relatively abundant in these embryo extracts, we normally do not detect all 30 subunits that comprise the Mediator complex in our various immunopurifications. Moreover, our mass spectrometric analysis revealed dBRD4, a protein of the bromodomains and extraterminal (BET) family, as a potential Kis-L interacting protein. Both human and *Drosophila* BRD4 have been shown to co-purify with the Mediator complex (Jiang et al., 1998; Wu and Chiang, 2007; Wu et al., 2003) Therefore, we believe these data represent true interactions which link Kis-L to transcriptional regulation.

Additional proteins with a role in transcription found to specifically co-immunoprecipitate with Kis-L are Lodestar (Lds) and GAGA/Trithorax-like (Trl). Lds is a protein with DNA-dependent ATPase activity and has been implicated in transcription termination by promoting transcript release (Xie and Price, 1996). Trl is a sequence-specific DNA binding transcription factor thought to play a role both in PcG-mediated repression and in trxG-mediated activation of gene expression (Mahmoudi et al., 2003; Vaquero et al., 2008). None of these factors appeared to interact with Kis-S, since we failed to detect any peptides by mass spectrometry. Therefore, we propose that Kis-L, but not Kis-S, binds many factors that play a role in transcription, including, (P)BAP, Mediator, dBRD4, Lds and Trl.

Since our mass spectrometry data suggested that Kis-L associates with a number of different factors, we were interested in the native molecular mass of Kis-L. To this end, we performed size-exclusion chromatography by loading the heparin-400 mM KCl fraction (H0.4 fraction) of *Drosophila* embryo nuclear extracts onto a Sephacryl S-300 column, followed by elution of the fractions, SDS-PAGE and Western blotting with antibodies for Kis-L and Kis-S. Although previous work has demonstrated a native molecular mass of about 1 MDa for Kis-L and roughly 650 kDa for Kis-S (Srinivasan et al., 2005), our results suggest that both Kis isoforms peak in the same fractions with an apparent molecular mass of approximately 2 MDa (Fig. 3A). Moreover, Brm, Osa and Sayp also seem to peak in the same fractions, indicating that Kis-L might indeed physically associate with Brm-containing complexes. Mediator and dBRD4, two other putative Kis-L interacting proteins, co-eluted with Kis-L in the peak fractions, sugges-

Table 1. List of Kis-L and Kis-S associated proteins identified by mass spectrometry.

Protein identity	M.W. (kDa)	Kis-L			Kis-S			Description
		Mascot	*emPAI*	#	*Mascot*	*emPAI*	#	
Kismet-Long	574	14491	6.2	219				Kis-L
Kismet-Short	225				5892	7.48	94	Kis-S
Brahma	185	5166	8.28	78				(P)BAP core
Moira	131	4209	21.46	58	230	0.12	4	
Bap111	79	2201	17.51	29				
Snr1	42	931	5.75	14				
Bap60	58	2013	14.94	29				
Bap55	47	1231	6.78	17	266	0.46	5	
Tth	45	315	0.68	7				
BCL7-like	17	460	22.42	8				
D4	55	894	2.32	14				BAP complex
Osa	284	3837	2.03	59				
Polybromo	190	2223	1.3	39				PBAP complex
Bap170	183	1107	0.62	25				
Sayp	213	321	0.12	7				
dBrd7	96	653	0.64	12				
MED1	150	2285	2.09	36				Mediator complex
MED4	28	790	8.63	12				
MED6	28	1077	25.64	16				
MED7	26	694	9.21	11				
MED8	28	1015	21.1	14				
MED9	17	157	1.63	3				
MED10	16	624	17.71	8				
MED11	20	441	2.83	6				
MED12/Kto	279	4239	2.22	66				
MED13/Skd	280	3368	1.68	54				
MED14	172	2925	3.09	53				
MED15	81	758	1.21	12				
MED16	90	1642	2.95	27				
MED17	72	1950	7.57	30				
MED18	25	450	4.63	8				
MED19	35	680	6.99	10				
MED20	28	1133	91.21	16				
MED21	16	542	10.15	9				
MED22	17	316	3.84	5				
MED23	167	3024	3.81	48				
MED24	112	1893	2.51	30				
MED25	97	1211	2.05	20				
MED26	166	339	0.13	6				
MED27	34	1273	16.05	21				
MED28	21	417	6.6	7				
MED29/Ix	21	457	9.26	6				
MED30	35	671	2.42	10				
MED31	24	283	1.31	5				
Cdk8	54	474	0.87	7				
Cyclin C	31	692	2.92	11				
dBrd4/Fs(1)h	205	2185	1.14	32				Bromodomain protein
Lodestar	118	3419	7.02	50				Transcription termination factor
Trl/GAGA	55	429	0.95	7				Sequence-specific TF

Listed proteins have Mascot score > 100

ting that these factors might bind Kis-L as well. ISWI appeared to have a different elution profile with a lower molecular mass, suggesting that ISWI and Kis-L are part of distinct complexes. As expected, USP7 and GMPS, two proteins known to form a stable complex, did not overlap with Kis-L.

To further strengthen our findings and to discriminate between Kis isoforms, we performed co-immunoprecipitations (co-IPs) with α-Kis-L and α-Kis-S antibodies followed by immunoblotting for some of our candidate interactors identified by mass spectrometry. We confirmed physical interactions between Kis-L and a number of (P)BAP subunits, including Brm, Mor, Polybromo, Bap170, Osa, and D4, but failed to detect Sayp and dBrd7 in the Kis-L co-IPs (Fig. 3B). These data suggest that Kis-L binds BAP and a subset of PBAP that lacks the subunits Sayp and dBrd7. Kis-S on the other hand, interacted with neither of these proteins. We also verified MED1, a subunit of the Mediator complex, and dBRD4 as Kis-L associated factors, whereas no physical interactions were observed with ISWI, which is consistent with the results

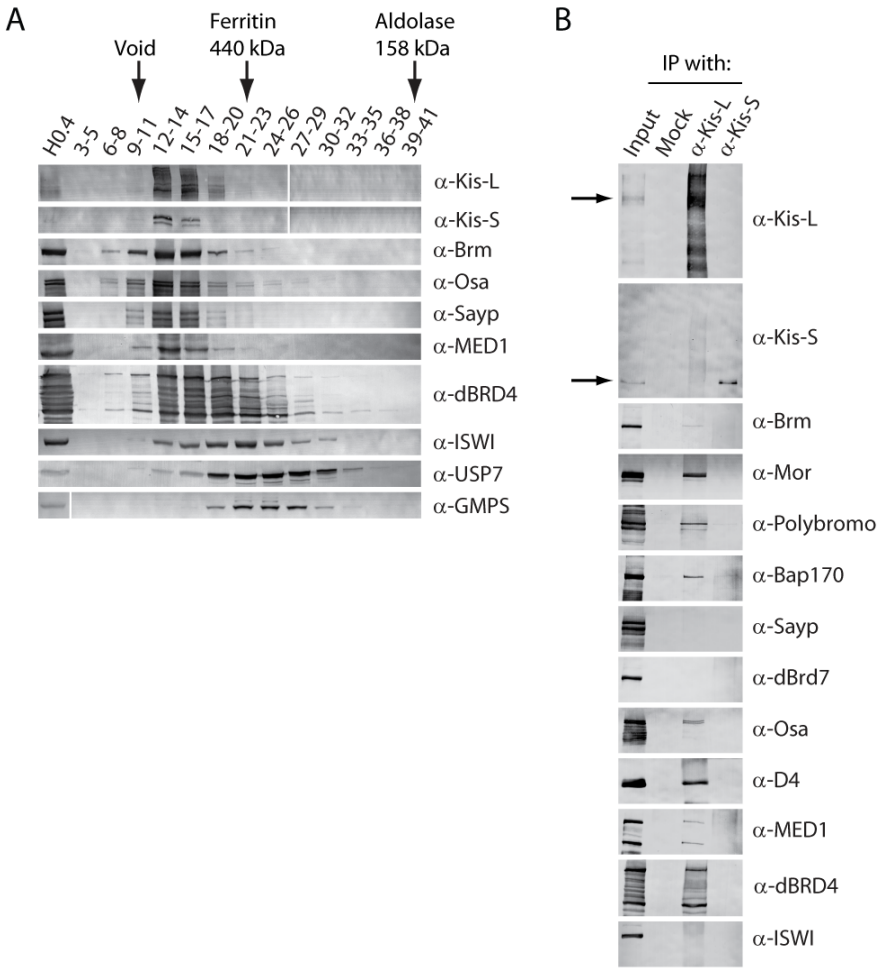

Figure 3. Kis-L, but not Kis-S, physically interacts with a subset of BAP and PBAP. (A) Size-exclusion chromatography from the heparin-400 mM KCl fraction (H0.4 fraction) of embryo nuclear extract followed by SDS-PAGE and immunoblotting with the indicated antibodies. The elution of the voided volume (void) and the elution of the known markers Ferritin (440 kDa) and Aldolase (158 kDa) are indicated. Note that Kis-L and Kis-S both peak in the same fractions (12-17) and co-elute with BAP, PBAP, dBRD4 and Mediator, whereas ISWI, USP7, and GMPS show clearly distinct elution patterns, demonstrating that not all proteins peak in the same fractions. (B) Co-immunoprecipitation of BAP and PBAP subunits with Kis-L, but not Kis-S. Crude *Drosophila* embryo nuclear extracts were incubated with either preimmune serum (mock) or an α-Kis-L or α-Kis-S antibody. Immunopurified proteins were resolved by SDS-PAGE and analyzed by immunoblotting using the indicated antibodies. Two percent of the input material was loaded for reference. Arrows indicate the bands representing either Kis-L or Kis-S. Note that Kis-L, but not Kis-S, interacts with BAP and PBAP subunits as well as Mediator and dBRD4.

obtained by size-exclusion chromatography. Most of the interactions seen by co-IP appeared to be substoichiometric, indicating that Kis-L is not a stable subunit of these complexes, but rather acts as an interacting factor. Taken together, our results strongly suggest that Kis-L, but not Kis-S, physically interacts with (P)BAP, Mediator and dBRD4.

Kis-L and Brm are targeted to chromatin independently

As Kis-L physically associated and colocalized with (P)BAP, we were intrigued by the possibility that Kis-L could be recruited to chromatin by interaction with (P)BAP or vice versa. To explore this *in vivo*, we took advantage of the available Kis and Brm RNAi lines and crossed them with a Sgs3-Gal4 line driving expression of the interfering RNA specifically in the larval salivary gland tissue. By RNAi-mediated knockdown of Kis-L followed by immunostainings on polytene chromosomes, we observe a near complete loss of Kis-L protein in the larval salivary glands (Fig. 4A and B). Knockdown of Kis-L, however, did not affect binding of Brm to chromatin. Since Brm is a core subunit of both BAP and PBAP, this results suggests that (P)BAP does not require Kis-L for its recruitment to chromatin. We then wondered whether the opposite was true, i.e. whether Kis-L requires Brm for its chromatin targeting. Therefore, we performed the same experiment as before, but this time we knocked down Brm instead of Kis-L in the salivary glands. Stainings of polytene chromosomes with α-Brm antibodies reveal a strong reduction in Brm protein levels, without affecting the localization of Kis-L (Fig. 4C and D). Thus, we conclude that Kis-L and Brm can bind chromatin independently. Although not formerly shown, our results also suggest that stability of Kis-L and Brm is unaffected by knockdown of either Brm or Kis-L, respectively.

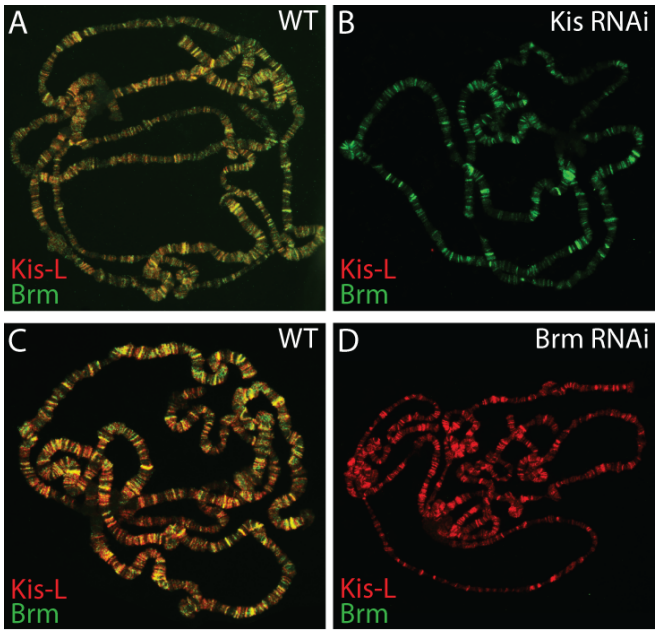

Figure 4. Kis-L does not recruit Brm to chromatin and vice versa. (A) Salivary gland polytene chromosomes from wild-type (WT) larvae were stained for Kis-L (in red) and Brm (in green) to localize the proteins on chromatin. (B) Same as A, but here staining was performed on polytene chromosomes isolated from salivary glands in which Kis-L was depleted by RNAi. Knockdown of Kis-L did not affect the localization of Brm on the polytene chromosomes. (C, D) Same as A and B, but this time RNAi against Brm was performed. Likewise, knockdown of Brm did not affect the distribution of Kis-L on polytene chromosomes. Note that knockdown of both Kis-L and Brm is very efficient, but does not compromise the overall morphology of the chromosomes.

Kis-L and Brm do not regulate global levels of elongating Pol II

Transcription by Pol II is a dynamic process which is regulated at many steps. It requires that Pol II binds to the core promoter of a gene, followed by sequential phosphorylation of the CTD at serine residues 5 and 2 by different kinases to promote transition from abortive initiation to productive elongation. Previous data had implicated Kis-L in early steps of transcription elongation, whereas it was suggested that Brm played a more global role in the transcription process (Armstrong et al., 2002; Srinivasan et al., 2005; Srinivasan et al., 2008). In both cases these conclusions were based on reduced levels of different modified forms of Pol II on polytene chromosomes. For Kis-L a hypomorphic P element-induced *kis* allele (*kis*^{k13416}) was used and it was shown that homozygous *kis*^{k13416} larvae had reduced amounts of Pol IIo^{ser2}, but not Pol IIa or Pol IIo^{ser5} on polytene chromosomes (Srinivasan et al., 2005). As for Brm, by using a dominant-negative *brm* allele (*brm*^{K804R}) which lost its ATPase activity, it was demonstrated that larvae had severely reduced levels of Pol IIa and Pol IIo^{ser2} on polytene chromosomes (Armstrong et al., 2002). However, since we did not observe great overlap between Kis-L and Pol II (Fig. 2H) and Brm and Pol II (data not shown) on larval salivary gland polytene chromosomes, these results seemed unexpected. Therefore, we used tissue-specific expression of Kis-L or Brm RNAi in the larval salivary gland after which we performed immunolocalization experiments of Pol II on polytene chromosomes. Knockdown of Kis-L in the salivary gland was effective as shown earlier and, more importantly, did not affect the levels of Pol IIo^{ser2} on polytene chromosomes (Fig. 5A and B). We observed the same when using an antibody that recognized all major forms of Pol II (data not shown). These results are in sharp contrast to earlier published data. Next, we wondered what would happen to Pol II after knockdown of Brm in the salivary glands. Although we could efficiently deplete Brm, no obvious effects were observed on the levels of chromatin-associated Pol IIo^{ser2} (Fig. 5C and D). Therefore, we conclude that Kis-L and Brm do not regulate the levels of elongating Pol II at the chromatin surface which also questions their role in the regulation of global transcription by Pol II.

Kis-L does not directly counteract trimethylation of H3K27

The gene encoding Kis was originally discovered in a screen for dominant modifiers of *Pc* mutations (Kennison and Tamkun, 1988). Additionally, loss of zygotic *kis* function was shown to cause homeotic transformations, which therefore classified Kis as a trxG family member (Daubresse et al., 1999). It is well appreciated that factors belonging to the trxG class function by antagonizing PcG-mediated gene repression during development. Gene silencing by PcG factors is generally accompanied by covalent modifications on histone tails, with H3K27me3 being the most studied one. A previous report mentioned that levels of H3K27me3 were increased in *kis* mutant larvae without affecting the localization of Pc, suggesting that Kis-L antagonizes PcG repression by counteracting H3K27 methylation (Srinivasan et al., 2008). We attempted to reproduce these results by performing knockdown of Kis-L in the salivary glands followed by stainings for H3K27me3 on polytene chromosomes (Fig. 5E and F). However, we failed to detect changes in the level of H3K27me3, suggesting that Kis-L is not involved in counteracting H3K27 trimethylation by PcG proteins.

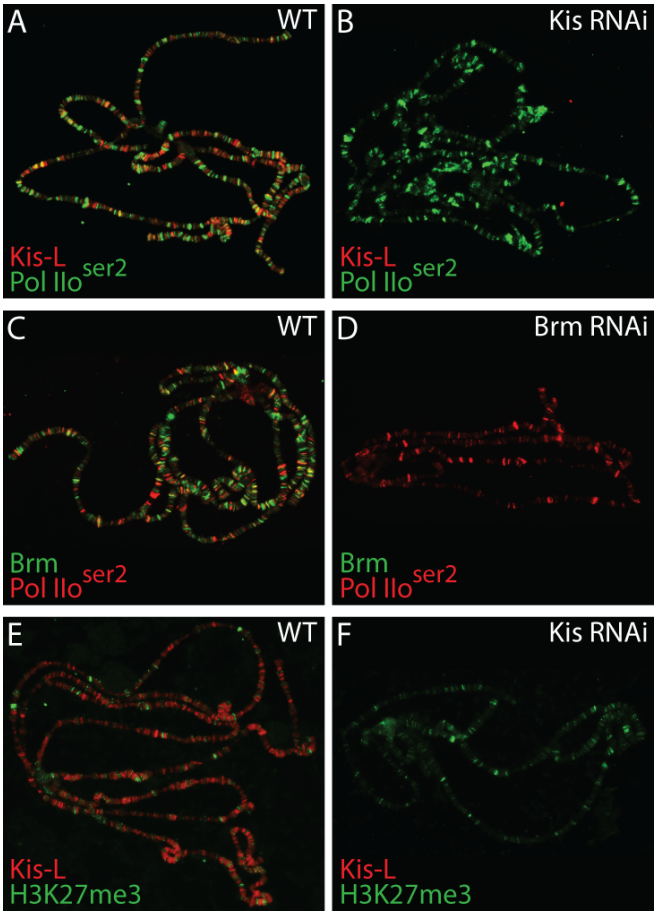

Figure 5. Kis-L and Brm do not regulate levels of chromatin-associated elongating Pol II. (A) Salivary gland polytene chromosomes from wild-type (WT) larvae were stained for Kis-L (in red) and elongating Pol II (Pol IIo^{ser2}, in green). (B) Same as A, but here staining was performed on polytene chromosomes isolated from salivary glands in which Kis-L was depleted by RNAi. Knockdown of Kis-L did not affect the levels of elongating Pol II. (C, D) Same as A and B, but this time RNAi against Brm was performed and polytenes were stained for Brm (in green) and Pol IIo^{ser2} (in red). As with Kis-L RNAi, knockdown of Brm did not affect the levels of elongating Pol II. (E, F) Same as A and B, but this time polytenes were stained for Kis-L (in red) and H3K27me3 (in green). Knockdown of Kis-L did not change global levels of H3K27me3 on polytene chromosomes.

Kis-L partially colocalizes with Mediator and dBRD4 on chromatin

Our proteomics analysis of Kis-L interacting factors not only revealed (P)BAP as a major interactor, but also showed association with the Mediator co-activator and the BET protein dBRD4. Since both Mediator and dBRD4 have a clear link with transcription and are able to bind chromatin, we were interested whether Kis-L, Mediator and dBRD4 would occupy the same sites on chromatin. For this purpose, we generated polyclonal antibodies directed against MED1, a subunit of the head module of the Mediator complex, and dBRD4. Next, we performed immunolocalization of Kis-L together with MED1 and dBRD4 on larval salivary gland polytene chromosomes to assess their genomic distribution. Co-staining of Kis-L and MED1 on polytene chromosome squashes demonstrated a decent amount of overlap (Fig. 6A). However, as noted earlier for Pol II, the relative amounts of Kis-L and MED1 seemed to vary from site to site. We then analyzed the distribution of dBRD4 on chromatin in relation to Kis-L. Similar to what we observed for MED1, Kis-L colocalized with dBRD4 to a large extent (Fig. 6B). Not surprisingly, as dBRD4 can associate with the Mediator complex, we found striking overlap between MED1 and dBRD4 on polytene chromomes (data not shown). Our results demonstrate that Kis-L not only physically interacts, but also partially colocalizes with Mediator and dBRD4 on chromatin, which is reminiscent of shared transcriptional targets between the different factors.

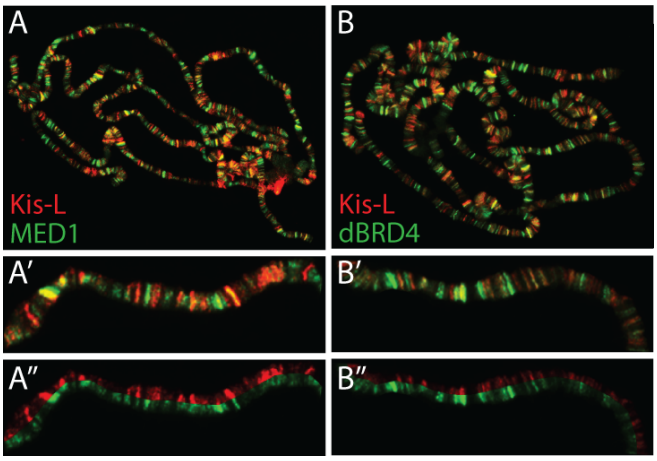

Figure 6. Kis-L overlaps partially with Mediator and dBRD4 on polytene chromosomes. (A) Co-staining of Kis-L (in red) and MED1 (in green) on polytene chromosomes. Partial overlap between the two proteins is observed. (B) Same as A, but this time Kis-L (in red) was co-stained with dBRD4 (in green). Here, partial overlap is observed as well. Representative magnifications are shown below the panels, with either zooms including merged (‘) or splitted (“) channels.

Kis-L and Brm control overlapping transcriptomes

Kis-L is a strictly nuclear protein, it has features typically associated with chromatin-remodeling enzymes and it can bind chromatin. Furthermore, it interacts physically with a number of factors involved in transcriptional control, including (P)BAP, Mediator and dBRD4. We recently identified the genome-wide transcriptional circuitry regulated by BAP and PBAP, which revealed a functional relationship between both complexes (Moshkin et al., 2007). This study also showed that the core subunits Brm and Mor are critical for the structural integrity of (P)BAP, but that functional specificity of BAP and PBAP requires the signature subunits Osa, Polybromo, or Bap170. As we found physical interactions between Kis-L and (P)BAP and observed overlapping distributions on polytene chromosomes, we wondered whether Kis-L could regulate transcription of the same genes as BAP and PBAP. To answer this question experimentally, we employed RNAi-mediated knockdown of Kis-L and Brm in *Drosophila* S2 cells to deplete the protein levels followed by extraction of total RNA and high-throughput sequencing (RNA-seq). Knockdown of Kis-L resulted in 1608 genes being differentially affected, whereas Brm depletion changed the expression level of 3681 genes (Fig. 7A). The majority of transcripts affected by Kis-L RNAi also changed in case of Brm knockdown (>75%), suggesting a high degree of overlap in controlling gene expression. Brm, however, appeared to regulate a relatively large number of genes which did not seem to be controlled by Kis-L, which would suggest a role for Brm beyond Kis-L in transcriptional regulation.

As a confirmation of our results obtained by genome-wide RNA-seq, we performed RT-qPCR analysis on a selection of genes affected by Kis-L and/or Brm knockdown. Genes were splitted into two groups based on their behavior, i.e. upregulated or downregulated (Fig. 7B and C, respectively). For both groups, 8 genes were picked after which their expression level was analyzed by qPCR. We were able to verify the relative abundance of these transcripts observed by RNA-seq after knockdown of Kis-L or Brm. Some genes, such as Fu12 and CG1998, appeared to be upregulated only after knockdown of Kis-L, whereas other genes like Cg25C, Ugt86Da, and Sprt, were upregulated solely as a result of Brm RNAi (Fig. 7B). However, the majority of upregulated Kis-L targets also appeared to be elevated after knocking down Brm, which was confirmed for the transcripts of Ome, Npc2b, and CG9733. Gene expression levels of Rab39

and αTubulin (αTub) were unaffected by either knockdown, which is consistent with our RNA-seq data. Next, we focused on the group of downregulated genes after knockdown of either Kis-L or Brm. The messenger levels of Argk and CG5958 were downregulated by Kis-L knockdown, whereas Ts and Stg levels were decreased only down as a result of Brm depletion (Fig. 7C). A large portion of genes, including Teq, GlcAT-P, Egr, and Tna, showed decreased expression

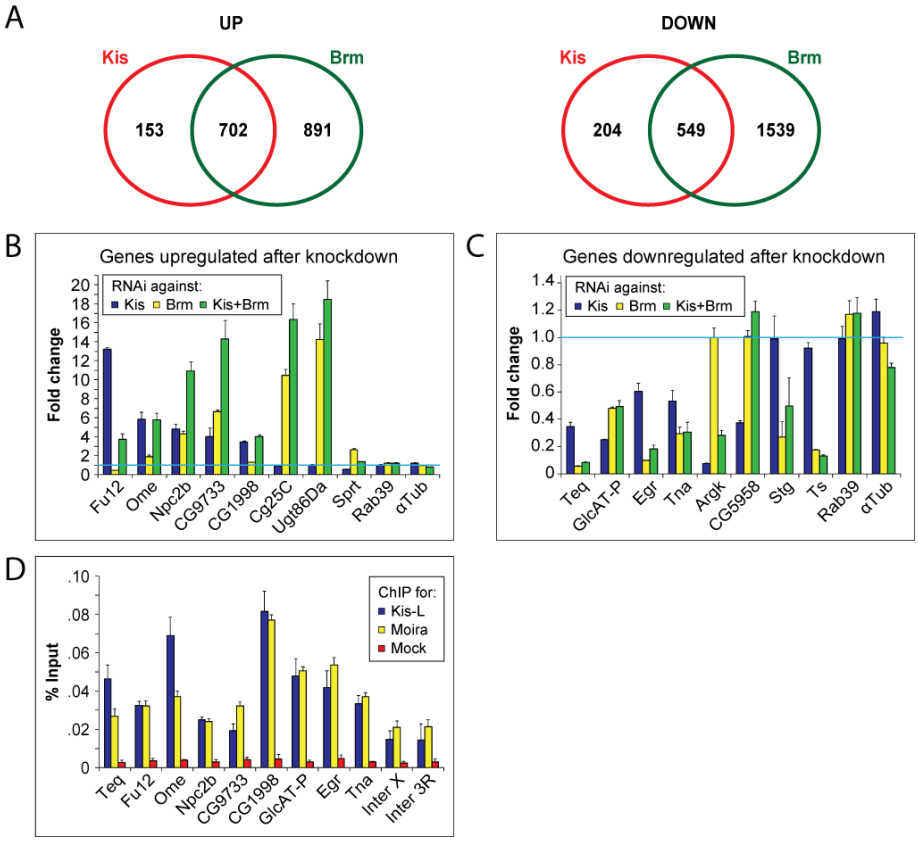

Figure 7. Genome-wide expression profiling reveals that Kis-L and Brm co-regulate many target genes. (A) S2 cells were treated with dsRNA against Kis-L, Brm, or GFP (= mock) after which total RNA was extracted and sequenced by high-throughput sequencing. Analysis of the RNA-seq data revealed a number of genes either up- (UP) or downregulated (DOWN) which is depicted in a Venn diagram to show overlapping target genes for Kis and Brm. (B, C) RNA was extracted from RNAi treated S2 cells which was then converted into cDNA followed by expression analysis of a number of representative genes by qPCR. In this case also double knockdowns of Kis-L and Brm were performed. Messenger RNA levels were normalized against those of CG11306, a gene whose expression did not change under the conditions used here. Data is represented as fold change over the mock RNAi. The blue line indicates normalized gene expression levels of the mock. In panel B genes are shown which are upregulated after knockdown, whereas panel C illustrates downregulated genes. (D) ChIP-qPCR analysis of the binding of Kis-L and Mor, a BAP and PBAP core subunit, to a subset of target genes of which the expression was affected by the knockdown of Kis-L and Brm. For mock ChIPs, preimmune serum was used. Intergenic regions located on chromosome X and 3R (Inter X and Inter 3R, respectively) served as controls. Data is represented as a percentage of the input.

when Kis-L or Brm was depleted by RNAi. Rab39 and αTub transcript levels on the other hand remained unaffected by either knockdown.

To assess whether Kis-L and (P)BAP could cooperate in gene regulation, we performed double knockdown of Kis-L and Brm and analyzed the expression of the genes we verified earlier by qPCR. We did observe signs of cooperation between Kis-L and Brm for Npc2b and CG9733, where double knockdown increased their expression level 2- to 3-fold when compared to Kis-L or Brm knockdown alone (Fig. 7B). For other genes, which were differentially affected by depletion of Kis-L or Brm, more intermediate effects were observed in case of the double knockdowns. For instance, Fu12, which was robustly upregulated after Kis-L RNAi and slightly downregulated by Brm depletion, showed an intermediate expression level when double knockdown was applied. Intermediate effects were also observed for Sprt and Argk (Fig. 7B and C), suggesting that Kis-L and Brm antagonize each other and are both required for expression of these genes. Expression of a number of genes appeared to be dominated by either Kis-L or Brm alone as double knockdown did not change their expression level relative to the single knockdown. For the genes examined, Brm seemed to be dominant over Kis-L most of the time, as was the case for Teq, GlcAT-P, Egr, Tna, and CG5958, whereas Kis-L only seemed to dominate Brm in regulating the expression of the Ome gene. Based on these findings, we conclude that Kis-L and (P)BAP interact to fine-tune gene expression.

Although gene expression profiling studies can be very powerful in identifying gene regulatory networks, there is one major limitation in that one cannot discriminate between direct and indirect effects caused by the RNAi treatment. To examine whether our putative target genes were also bound by both remodelers, we performed ChIP using α-Kis-L and α-Mor antibodies followed by qPCR analysis of a selection of genes. By this means we were able to confirm binding of both Kis-L and Mor, which represents BAP and PBAP, to their putative target genes (Fig. 7C). We used preimmune serum as a control, and showed that Kis-L and Mor specifically bound their target sites, whereas the mock ChIP showed no specific enrichment on any of the sites tested. We did, however, observe some binding of the remodelers on intergenic regions, but this binding appeared to be less relevant compared to the other sites tested, as ChIP enrichment was lower on the intergenic regions. Binding profiles seemed similar for Kis-L and Mor, which is in agreement with the colocalization of Kis-L and (P)BAP observed on polytene chromosomes. Thus, we conclude that putative target genes of Kis-L and (P)BAP identified by RNA-seq and RT-qPCR are also bound by these remodelers which, therefore, likely reflect direct transcriptional targets. Overall, our data suggest that Kis-L and (P)BAP are involved in controlling overlapping transcriptional circuitries.

Discussion

Eukaryotic gene expression is controlled by a wide variety of transcriptional regulators which are often part of larger functional units by forming protein-protein interactions. Most of the chromatin-remodeling enzymes known to date comprise multiple subunits that together define their biochemical properties and are thought to play important architectural or enzymatic roles or

be required for gene-specificity. Here, we examined the protein-interaction network of the remodeler Kis and found that Kis-L, but not Kis-S, associates with known transcriptional regulators, including BAP, a subset of PBAP, Mediator, and dBRD4. The association of Kis-L with (P)BAP seems to be functional *in vivo*, as Kis and Bap170 were found to interact genetically. Analysis of the genome-wide distributions of Kis and its associated factors on polytene chromosomes suggest that Kis-L co-occupies loci bound by (P)BAP and reveals some overlap with Mediator and dBRD4. Kis-L does not appear to be required for targeting of (P)BAP to chromatin and vice versa, suggesting that these remodelers can bind chromatin independently. Furthermore, we present data showing that both Kis-L and Brm do not affect the levels of elongating Pol II and that Kis-L does not change tri-methylation of H3K27, which is in sharp contrast with earlier published work. Lastly, our genome-wide expression profiling together with our more detailed qPCR analysis suggest many shared transcriptional targets for Kis-L and Brm. We propose that Kis-L associates with (P)BAP to fine-tune gene expression.

As CHD7, the human ortholog of Kis, was found to functionally interact with PBAF (Bajpai et al., 2010), we wondered whether this was a conserved interaction. Therefore, we made use of Drosophila genetics to establish a possible link between Kis and PBAP. Our results confirmed the results in humans, as we found a genetic interaction between Kis and Bap170, a PBAP representative subunit. These results encouraged us to explore this relationship in more detail, by using state-of-the-art techniques.

In order to gain a better understanding of the function of Kis in transcriptional control, we employed a proteomics survey in which we purified the two major isoforms encoded by *kis* using highly specific antibodies. By this means we found that Kis-L, but not Kis-S, interacts with the related chromatin-remodeling factors BAP and PBAP, and we also detected Mediator and dBRD4, which are considered to be more general transcriptional regulators. Previous studies concerning *Drosophila* Kis-L, however, failed to address the identity of the Kis-L complex. Based on gel filtration chromatography it was first proposed that Kis-L forms a complex of about 1 MDa distinct from that of the (P)BAP complex (Srinivasan et al., 2005). Immunoprecipitation experiments failed to detect a physical interaction between Kis-L and Brm, which therefore reinforced this proposal. A later study from the same lab using sucrose density gradients demonstrated a native molecular mass of 0.5 MDa for Kis-L, which is lower than the 1 MDa observed earlier (Srinivasan et al., 2008). Since Kis-L itself is 574 kDa, which corresponds more or less with the observed molecular mass of 0.5 MDa, it was suggested that Kis-L is not part of a larger complex but rather functions as a monomer to regulate transcription. Our results, however, contradict these findings and suggest that Kis-L can function as part of a high molecular weight complex. By performing size-exclusion chromatography we show that Kis-L peaks in the same fractions as BAP and PBAP, which is distinct from the elution profile observed for ISWI. Moreover, we were able to confirm our proteomics data by performing immunoprecipitations followed by Western immunoblotting, further strengthening our data. We do not propose that Kis-L is part of a stable complex that comprises BAP and PBAP components, as the results presented here indicate that Kis-L likely forms substoichiometric interactions with BAP and a subset of PBAP. Therefore, we prefer to view Kis-L as a factor capable of associating with (P)BAP, but do not rule out that it can act as a monomer as well.

Interestingly, the interaction of Kis-L with PBAP appears to be conserved, as CHD7 has been shown to physically interact with the PBAF complex in humans (Bajpai et al., 2010).

Remodelers need to be targeted to chromatin in order to execute their function. Genome-wide localization studies on *Drosophila* salivary gland polytene chromosomes presented in this study reveal remarkable overlap between Kis-L and BAP and PBAP components. This appeared to be specific for (P)BAP, as we did not detect colocalization of Kis-L with ISWI or Tip60 chromatin-remodeling factors. These results, combined with our Kis-L mass spectrometric analysis, led us to hypothesize that Kis-L and Brm could bind chromatin in a cooperative manner. To test this experimentally, we performed RNAi-mediated knockdown of Kis-L or Brm in the larval salivary gland followed by immunostainings on polytene chromosomes. We did not detect any changes in the genomic distribution of either Kis-L or Brm after knockdown, suggesting that they can bind chromatin independently. Currently it is not known how Kis-L is targeted to chromatin. Earlier work from our lab has shown that the (P)BAP complex can act as an essential coactivator for Zeste-directed transcription on chromatin templates (Kal et al., 2000). Since Zeste is a sequence-specific DNA binding transcription factor and binds selective subunits of the (P)BAP complex, we proposed that Zeste recruits (P)BAP to specific genes whereby the remodeler functions to create an open chromatin conformation that could facilitate the docking of other regulatory proteins. Similar findings were obtained for *Drosophila* NuRD, where the transcription factor Tramtrack69 (Ttk69) was shown to physically associate with the NuRD remodeler and recruit it to selective genes (Reddy et al., 2010). Although we have not attempted to study the recruitment of Kis-L in a more detailed manner, we did find some transcription factors in our purifications, including Trl, Zeste, and Pipsqueak (Table 1 and data not shown). Therefore, a similar recruitment mechanism might apply to Kis-L as well. It will be interesting to address the role of these transcription factors in their ability to target Kis-L to chromatin.

Another way of tethering remodelers to chromatin could be achieved through the selective recognition of certain covalently modified histone tails. Kis-L contains two chromodomains which in theory might be able to bind methylated histone tails (Brehm et al., 2004). Although *in vitro* binding experiments with the second chromodomain (CD2) of Kis-L failed to detect binding to histone H3K4me2, H3K4me3 or H3K9me2 peptides (Srinivasan et al., 2008), it is possible that both domains are required for recognition of modified histones. Indeed, experiments with both chromodomains from CHD7 revealed binding to mono-, di- and tri-methylated forms of the H3K4 peptide (Schnetz et al., 2009). Binding of CHD7's chromodomains appeared to be specific for H3K4 methylated tails, as no binding was observed to H3K36me3 or H3K27me3 modified histones. However, as stated by the authors, only a subset of methylated H3K4 was bound by CHD7 which, therefore, cannot account for the specificity at particular sites on chromatin. As mentioned before, it is likely that sequence-specific transcription factors play a role in this aspect.

Recently it has become evident that different remodelers execute unique biological functions. Comparison of the genome-wide distribution of remodelers from distinct classes by ChIP-chip revealed unique sets of genomic targets and demonstrated that each remodeler generates distinct chromatin signatures (Moshkin et al., 2012). Regardless of these differences, remodelers do appear to act at a global scale as they bind many sites in the genome. For instance,

Brm has been suggested to facilitate global transcription by RNA polymerase II based on its association with nearly all transcriptionally active chromatin on salivary gland polytene chromosomes (Armstrong et al., 2002). When Brm function was diminished by expression of a dominant-negative form a dramatic reduction in the association of RNA polymerase II with chromatin was observed. Similar observations were made for Kis-L, where it was shown that the levels of elongating Pol II were severely reduced on polytene chromosomes from *kis* mutant larvae (Srinivasan et al., 2005). We have tried to reproduce these results by using *Drosophila* strains expressing RNAi targeting Brm or Kis-L in the salivary gland followed by immunostainings on polytene chromosomes. We did not observe any changes in the levels of chromatin-associated Pol IIo^{ser2} after knocking down either Brm or Kis-L, which therefore questions these earlier published results. Similar results were obtained by using an antibody that recognizes all forms of Pol II (data not shown). Tamkun and colleagues also noted a strong decrease in the binding of Spt6 and Chd1, two elongation factors, to polytene chromosomes in *kis* mutant larvae. Although we did not examine these two factors, we did test NELF-A, which is part of the negative elongation factor (NELF) complex known to be required for transcription elongation by Pol II. Tissue-specific knockdown of Kis-L in the salivary gland did not interfere with the binding of NELF-A to polytene chromosomes (data not shown), suggesting that transcription elongation might be unperturbed under these conditions. Apart from Pol II and elongation factors, we also did not detect any changes in the level of H3K27 trimethylation when Kis-L was depleted by RNAi in the salivary gland, whereas Tamkun and co-workers found increased H3K27me3 in *kis* mutant larvae. It is possible that these discrepancies are the result of a different experimental setup. We have used RNAi-mediated knockdown to deplete expression of Kis-L or Brm, whereas earlier studies have used either expression of a dominant-negative form of Brm or a hypomorphic P element-induced *kis* allele. In the latter two cases, chromosomes generally appear slightly thinner than normal, whereas tissue-specific knockdown of Kis-L or Brm did not alter the chromosome architecture, which might account for some of the differences observed.

Our data presented in this study have revealed a physical link between Kis-L and (P)BAP, but also identified Mediator and dBRD4 as Kis-L associated factors. Given the fact that both human and *Drosophila* BRD4 have been shown to co-purify with selective forms of Mediator (Jiang et al., 1998; Wu and Chiang, 2007; Wu et al., 2003), it appears that Kis-L interacts with a specialized Mediator complex. We also found that dBRD4 physically associates with the Mediator complex (data not shown) and noted a significant overlap in the genomic distribution of Kis-L, Mediator and dBRD4 on polytene chromosomes. In humans, Mediator has been shown to be required for the assembly of a functional PIC by interacting with TFIID and recruiting most of the general transcription factors (GTFs) to the template (Johnson et al., 2002). Furthermore, Mediator directly contacts the Pol II holoenzyme and is considered to be required for global gene expression primarily through regulation of transcription at the initiation phase, but it also appears to play roles at later stages (Taatjes, 2010). The exact role played by BRD4 is not yet fully understood, but its bromodomains might be involved in the recognition of acetylated histone tails which tether it to chromatin. Once arrived, BRD4 could recruit Mediator which then brings in Pol II at the promoter to initiate transcription, whereas BRD4 might recruit P-TEFb (positive transcription elongation factor b) later on to stimulate elongation by phosphorylating serine 2 of

the Pol II CTD (Chiang, 2009). Currently we do not know the functional relevance of the physical interaction observed between Kis-L and Mediator and dBRD4, but it is an interesting lead to follow up on. Since CHD7 has been reported to bind active enhancer elements and Mediator, together with cohesin, can mediate DNA loop formation between enhancers and core promoters (Kagey et al., 2010; Schnetz et al., 2010), it is tempting to speculate that Kis-L plays a role in enhancer-promoter communication to control gene expression.

Even though remodelers appear to have broad functions, they can be involved in regulating gene expression of a specific set of genes. Since Kis-L showed a physical and genetic interaction with (P)BAP and colocalized on the chromatin at a genome-wide scale, we were interested in determining the transcriptional circuitries controlled by Kis-L and Brm, the enzymatic motor of BAP and PBAP. Our gene expression analysis demonstrated a remarkable overlap in the transcriptomes regulated by both ATPases. More detailed examination of their target genes revealed different classes of genes. A large number of genes were upregulated by Kis-L or Brm knockdown, whereas even more genes appeared to be downregulated as a result of the RNAi treatment. This already shows that it is not possible to label Kis-L or (P)BAP as activators or repressors, since their function is likely to be context-dependent. By performing a combined knockdown of Kis-L and Brm, we found genes that require only Kis-L or Brm, or require both factors for their expression. Moreover, Kis-L appeared to overrule Brm in controlling expression of some genes (e.g. Ome), whereas Brm dominated in other cases (e.g. Teq and CG5958). We also observed antagonism between Kis-L and Brm in the regulation of a number of genes, including Fu12 and Sprt, further demonstrating gene-specific activities of both Kis-L and (P)BAP. At last, we identified genes regulated by Kis-L and Brm in a cooperative manner, as double knockdown increased their expression level 2-fold (Npc2b) to 3-fold (CG9733) when compared to Kis-L or Brm knockdown alone. Similar observations were made in higher eukaryotes, where it was demonstrated that CHD7 and PBAF cooperate to promote NC gene expression and cell migration during embryogenesis (Bajpai et al., 2010). Whether the observed cooperation requires the catalytic activity of Kis-L is currently unknown, as no studies have yet reported remodeling activity for Kis-L. Human CHD7, on the other hand, has recently been shown to possess ATP-dependent nucleosome remodeling activity with characteristics distinct from SWI/SNF- and ISWI-type remodelers (Bouazoune and Kingston, 2012). It is tempting to speculate that the different remodeling activities of Kis-L and (P)BAP converge which together determine the effect on gene expression. Taken together, our results suggest that Kis-L and (P)BAP control overlapping transcriptomes and we propose that Kis-L physically associates with (P)BAP in order to fine-tune gene expression.

Experimental procedures

Antibodies and immunological procedures

Polyclonal antibodies were generated by immunizing guinea pigs or rabbits with GST fusion proteins expressed in *Escherichia coli* and were affinity purified as described previously (Chalkley and Verrijzer, 2004). The following antigens were used: Kis-L amino acids (aa) 1-509 and 50-406

(unique part of Kis-L), Kismet aa 4938-5322 (common C-terminal part of Kis-L & Kis-S), Kis-S aa 1-46 (unique part of Kis-S), Rpb1 (Pol II) aa 1-397 and 508-866, Brd8 aa 1-344 and 521-873, dBRD4 aa 1-322, MED1 aa 1-450 and 456-784, Brd7 aa 1-585 and 630-861, and D4 aa 286-497. Other antibodies have been described, including α-OSA (Treisman et al., 1997), α-Mor and α-Polybromo (Mohrmann et al., 2004), α-Brm and α-ISWI (Kal et al., 2000), α-BAP170 and α-SAYP (Chalkley et al., 2008), α-USP7 and α-GMPS (van der Knaap et al., 2010), α-H3K27me3 (07-449, Upstate), and α-RNA polymerase II H5 (Pol IIo^{ser2}) (MMS-129R, Covance).

Polytene chromosome immunostaining

The analysis of polytene chromosomes was performed essentially as described previously (Armstrong et al., 2002) with minor modifications. Briefly, salivary glands from *Drosophila* 3rd instar larvae were dissected in 0.7% NaCl after which the glands were fixed in 45% acetic acid/1.85% formaldehyde for 1 to 2 minutes at room temperature. Fixed tissue was transferred onto coated poly prep slides (Sigma) after which the glands were squashed and slides were frozen in liquid nitrogen. Squashes were incubated with blocking buffer (1% BSA dissolved in PBS/0.1% Triton X-100) overnight at 4°C. Next, squashes were incubated with primary antibody (α-Kis-L, 1:1000; affinity purified α-Brm, 1:50; α-Polybromo, 1:200; α-OSA, 1:50; α-ISWI, 1:200; α-Brd8, 1:100; α-Rpb1, 1:200; α-Pol IIo^{ser2}, 1:200; and α-H3K27me3, 1:50) in blocking buffer for 1 hour at room temperature in a humid chamber. Slides were washed three times with PBS/0.1% Triton X-100 and the squashes were subsequently incubated with appropriate secondary antibodies (Alexafluor, Molecular Probes) diluted 1:500 in blocking buffer for 1 hour at room temperature. Finally, slides were washed again three times with PBS/0.1% Triton X-100 and mounted in Vectashield with DAPI (Vector Laboratories, Inc.). Images were captured by using a Leica DM-RXA microscope and processed by using Photoshop.

Fly stocks

All fly stocks were maintained under standard conditions, and crosses were performed using standard procedures. Wild-type flies were of the following genotype: *FM6/white*. RNA interference (RNAi) lines (Dietzl et al., 2007) that have been used in this study: *Kismet* (strain 46685), *Brm* (strain 37721), and *Bap170* (obtained from the Vienna *Drosophila* RNAi Centre, http://stockcenter.vdrc.at/control/main). Gal4 driver lines that have been used: *Sgs3-Gal4* (strain 6870), *sd[638]-Gal4*, *sal-Gal4*, and *esg-Gal4* (obtained from the Bloomington *Drosophila* Stock Center at Indiana University, http://flystocks.bio.indiana.edu/). All crosses were performed at 25°C and were repeated several times.

Protein purification and mass spectrometry

Nuclear extracts were prepared from 0 to 12 hours-old *Drosophila* embryos as described (Kamakaka and Kadonaga, 1994). Immunopurification procedures using specific antibodies directed against Kis-L (GR689) or Kis-S (GR817) and mass spectrometric analysis were all performed as described previously (Chalkley et al., 2008; Chalkley and Verrijzer, 2004). Briefly, extracts were incubated for 2 hours at 4°C with α-Kis-L or α-Kis-S antibodies coupled to Protein A-Sepharose beads (GE Healthcare). Next, beads were washed twice with HEMG buffer (25

mM HEPES-KOH [pH 7.6], 0.1 mM EDTA, 12.5 mM $MgCl_2$, 10% glycerol, 0.1% NP-40, 1 µg/ml of leupeptin, aprotinin and pepstatin and 0.2 mM AEBSF [(α-aminoethyl)benzenesulfonyl fluoride]) containing 100 mM KCl (HEMG/100), 5 times with HEMG/600, twice with HEMG/100 containing 0.01% NP-40, and finally once with 100 mM KCl. Affinity purified proteins were subsequently eluted with Glycine buffer (100 mM Glycine, 150 mM NaCl) pH 2.5. Eluted proteins were TCA precipitated and dissolved in 1x SDS sample buffer after which the proteins were resolved by SDS-PAGE and visualized by Coomassie blue staining. Polypeptides were identified by mass spectrometry on an LTQ-Orbitrap hybrid mass spectrometer (ThermoFischer). Typical contaminants, also present in immunopurifications using beads coated with preimmune serum or antibodies directed against irrelevant proteins, were omitted from the dataset.

For small scale co-immunoprecipitations, 15 µl of crude serum was coupled to 20 µl of Protein A-Sepharose which was subsequently incubated with ~2 mg *Drosophila* embryo nuclear extract for 2 hours at 4°C. Beads were washed and proteins were eluted similar as described above. Eluted proteins were resolved by SDS-PAGE and visualized by Western immunoblotting with appropriate antibodies.

For analysis of native protein sizes, the crude *Drosophila* embryo nuclear extracts were further fractionated using a Sephacryl S-300 column (Pharmacia) as described previously (Mohrmann et al., 2004). Briefly, nuclear extracts were concentrated by chromatography on a POROS-heparin (PerSeptive Biosystems) column equilibrated with HEMG/100 buffer (25 mM HEPES-KOH [pH 7.6], 0.1 mM EDTA, 12.5 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol [DTT], 0.2 mM AEBSF, 1 µM pepstatin, 0.01% Nonidet P-40 [NP-40], 100 mM KCl), followed by a step elution with HEMG/400 buffer (HEMG/100 buffer with 400 mM KCl instead of 100 mM KCl). The heparin-400 mM KCl fraction (H0.4 fraction) was loaded onto an 800-ml Sephacryl S-300 column (elution volume, 300 ml) equilibrated and developed with HEMG/100 buffer. Fractions were separated by SDS-PAGE and analyzed by immunoblotting with α-Kis-L, α-Kis-S, α-Brm, α-OSA, α-SAYP, α-MED1, α-dBRD4, α-ISWI, α-USP7, and α-GMPS antibodies.

Cell culture, RNA interference, RT-qPCR and RNA-sequencing

Drosophila Schneider's line 2 cells (hereafter S2 cells) were cultured in Schneider's medium (Invitrogen) supplemented with 10% fetal calf serum (Thermo) and 1% Penicillin-Streptomycin. Cells were treated with double-stranded RNA (dsRNA) for 4 days, as described previously (Worby et al., 2001). Double-stranded RNAs were synthesized using the Megascript T7 kit (Ambion). Total RNA was isolated from >1x10^6 cells using TriPure isolation reagent (Roche) and 1 µg of this were used for oligo-dT primed cDNA synthesis by using the SuperScript II Reverse Transcriptase (Invitrogen). Quantitative PCR (qPCR) was performed on a CFX96 real-time PCR detection system (Bio-Rad). Reactions were performed in a total volume of 25 µl containing SYBR Green I (Sigma), platinum Taq polymerase (Invitrogen), 200 µM dNTPs, 1x reaction buffer, 1.5 mM $MgCl_2$, 500 nM of corresponding primers and 1 µl of cDNA. Data analysis was performed by applying the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). CG11306 was used as an internal control mRNA. The data presented are the results of three independent biological

replicate experiments. For genome-wide expression analysis, at least 3 μg of total RNA was used in Illumina Next Generation Sequencing (ServiceXS B.V., Leiden).

Chromatin immunoprecipitation

ChIP experiments were performed as described (Mohd-Sarip et al., 2006) with minor modifications. Briefly, S2 cells were fixed with 1% formaldehyde for 10 minutes at room temperature. Crosslinking was quenched by the addition of glycine at a final concentration of 125 mM after which the plates were placed on a rocker for 5 minutes at room temperature. Next, cells were washed twice with ice cold PBS and resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl [pH 8.1], 1 μg/ml of leupeptin, aprotinin and pepstatin and 0.2 mM AEBSF). Chromatin was sheared to an average length of ~300-500 bp by using the Bioruptor UCD-200 (Diagenode SA). For each ChIP experiment 100 μg of sheared chromatin was used. Immunoprecipitations were performed with the following antibodies: α-Kis-L (GR690), α-Mor (PV127), and preimmune serum for mock ChIPs. Antibody incubations were performed overnight at 4°C after which 20 μl of pre-blocked protein A Sepharose (GE Healthcare) was added. This was incubated for another 90 minutes, followed by extensive washes, overnight de-crosslinking at 65°C and DNA elution with QIAquick PCR purification kit (Qiagen). qPCR was performed using 1.5 μl ChIP DNA per 25 μl reaction. The data presented are the results of three independent biological replicate experiments.

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Supplementary information

Table S1. Primers used for generating dsRNA.

Target	Forward primer	Reverse primer
GFP	ATGGTGAGCAAGGGCGAG	CTTGTACAGCTCGTCCATGC
Kis-L	CCACATCCGCTACAGCAACAG	CTGCGCTGACAAGTGGTGAC
Kis-S+L	GCTATCCAAACGAGCACGTGG	AGCGTACTGGCATCTGCCGA
Kis-S	ATGGAGGTTATTAAACCCATGG	CTCCAAATAAATGCCTTTTCCA
Brm	CCAGCATGCAGGACAACC	TCGTGCACTGAACAAACTCC

Table S2. Primers used for RT-qPCR analysis.

Target	Forward primer	Reverse primer
Ome	TACGCTGACGAGGATCATGGA	ATTCGCCAAAGAAGCGATCC
Fu12	GCCGGTGGTCATATCGAAGT	GACACCTCGGGTAGGATGTG
CG9733	GGACAGTTCCGGAAGGACAG	CAGCCACGTTCGTGTAGACT
Npc2b	CTACCCGACCGTCAGTTTGG	TTTGGCGGGTATCTGGAAGC
Argk	TGTACGATGGCATCACCGAG	GGCACCCGTGGATAGATGTT
Teq	CCTCCGAGGTCGATTCGTTC	CACCACCAGGGCAATATCGT
GlcAT-P	GTTCTTGAAGGAGCGTCCCA	TCGTCAAGTGGTGCCAAACT
CG1998	TTGGCCTATGCCAGCTATCG	TCAGCTCAAAGCAGACCCAG
Stg	CGTTATCTAAGTTTGGGTGTTATCG	TGTGTCTGCGTCGTGTGC
Ugt86Da	TGTGGAAGTTCGAGGACACG	CGTGCGTAATAAAGGCCAGC
Ts	ACATCCCAAAGATGGCCCTG	ATGTTGAAGGGTACGCCCAG
Tna	GCTAGTCCATCGACCATCGG	GCGCTTGATCTTCTGCACAC
Sprt	GGCAGGCACAAGCTCATCTA	CGGTTAGGATCGGTCGAGGA
CG5958	CAGAAGTTCCTCGACCCCTC	GGCTCCACTCCTCCACATAC
Cg25C	AATTACCGACCGACCTGGAC	TGGACATACGACCGAGGCTA
Egr	ATCCCCGGTCAGAAGATGGA	TGCAGTATGCACGATTCCGA
Rab39	TCCGTTCGATCACCAAGTCC	ATCCACAGCGGTATGTGCTC
αTub84B	TCGACAGCTTGCCGTCTCTAGC	ACTGCCGGCTATGAGGCTTGAC
CG11306	AGGGTTCAGCAGCGACAGATG	AAGAGGCGGTAGTAGGCAAGC

Table S3. Primers used for ChIP-qPCR analysis.

Region	Chr	Coordinates	Forward primer	Reverse primer
Teq	3L	9067152-9067270	CGCCAACAATGTGTTCCGTG	GCAGATTTTGAGCAAGGCCA
Fu12	2L	8451355-8451240	TCGTGGAACAGGGGTAGAGA	TGAAGTGCGCCTTACTGTGG
Ome	3L	14699683-14699779	TGTTGACTCAGGCTCTCTGC	GTGCCAATTGCCGATTACCG
Npc2b	3R	9917533-9917666	AAATCGAAGGCCCTAGCTGC	GAGGTCAGCTCCTGGAAGTC
CG9733	3R	26071785-26071673	CCAGACACTCTACTCCGTGC	TGCAGCAAACATAGGGCTGA
CG1998	X	13307301-13307170	ACACGGCGAGACACCAATAC	AGCCTAGGTACTCATCCCGA
GlcAT-P	3L	11178462-11178346	CCAGCTGATAGCCAACCCTT	AGCCAAGACCTCCTAACCCT
Egr	2R	5966516-5966658	TATGACTGCCGAGACCCTCA	CCAGACCGATGAACCCCAAA
Tna	3L	10847249-10847325	CTGTCTCTGTCGCACTGCAT	CGTCGGTCAGTCGGCAAATA
Intergenic X	X	12232899-12233004	AGCAGGGTGGTTGAAAGTCC	ATGGACCGAGCACGCATAAT
Intergenic 3R	3R	11938351-11938461	CACAGCTTCCCAGGAGTCAG	GCTATCCAGCTGTCACTCCC

Chapter 3

PRC1 Blocks Mediator and Pol II Recruitment to Repress Transcription

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Chapter 4

Gene Expression Control by the Ubiquitin-Proteasome System

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Chapter 5

Drosophila BRD4 Interacts With the Mediator Transcriptional Coactivator Complex

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Abstract

Bromodomain-containing protein 4 (BRD4) is a ubiquitously expressed transcriptional coactivator that associates with chromatin by preferentially binding acetylated histone tails and non-histone proteins through its tandem bromodomains. It is a member of the bromodomain and extraterminal (BET) family, which includes BRD2, BRD3, BRD4, and BRDT. BRD4 thought to stimulate transcription by recruiting positive transcription elongation factor b (P-TEFb) to chromatin which then phosphorylates RNA polymerase II (Pol II) at serine 2 of its C-terminal domain (CTD) to promote elongation. Apart from recruiting P-TEFb, BRD4 has also been linked to the general coactivator Mediator, since it was shown to associate with both murine and human Mediator. Here, we focused on *Drosophila* BRD4 (dBRD4), also known as Fs(1)h (female sterile [1] homeotic), which is the only known BET family protein in the fruit fly. By performing an unbiased proteomics approach, we identify the Mediator complex as the major interacting partner of dBRD4. Subsequent genome-wide localization analysis of both factors on larval salivary gland polytene chromosomes revealed a high degree of overlapping binding sites, showing that Mediator and dBRD4 not only physically interact, but also colocalize on chromatin. We are currently assessing the transcriptional circuitries controlled by Mediator and dBRD4 in order to provide a functional link between the two transcriptional regulators.

Introduction

Gene expression is important for many fundamental biological processes such as cell differentiation, development, and biodiversity, and is controlled at multiple different levels by proteins that play roles in e.g. transcription, splicing, RNA export, and translation (Levine and Tjian, 2003). Transcription of protein-coding genes is facilitated by RNA polymerase II (Pol II), which synthesizes messenger RNA (mRNA) with the help of numerous chromatin binding factors. Pol II is unable to recognize promoter DNA on its own and instead requires the action of the general transcription factors (GTFs) TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (Orphanides et al., 1996). Transcription by Pol II can be divided into three main steps, i.e. initiation, elongation, and termination, where the initial phase starts with the recognition of core promoter elements by TFIID and sequential assembly of the other GTFs and Pol II to form the preinitiation complex (PIC). Whether transcription proceeds, however, is determined by the combined action of stimulatory and inhibitory signals derived from sequence-specific DNA bound transcription factors. Large coactivator or corepressor complexes which assemble on the chromatin integrate these regulatory signals from transcription factors to either activate or repress transcription, respectively (Sikorski and Buratowski, 2009). One such coactivator is the Mediator complex, which appears to be a universal requirement for Pol II transcription by stimulating both basal and activated transcription (Jiang et al., 1998; Lee and Young, 2000). When the activating signals overrule the negative ones, transcription continues with the hyperphosphorylation of

Rpb1, the largest subunit of Pol II, at serine (Ser) residue 5 of its C-terminal repeat domain (CTD) by TFIIH, followed by hyperphosphorylation of Ser2 by positive transcription elongation factor b (P-TEFb) (Buratowski, 2009). These successive phosphorylation events promote productive transcription elongation and are thought to attract a number of proteins involved in mRNA processing and export. Termination, the final stage of transcription, involves the recognition of DNA encoded terminator signals, followed by polyadenylation of the transcript at the 3' end and cleavage of the nascent mRNA.

One potent level of gene regulation is provided by the native chromatin structure, which forms a natural barrier to processes that take place on the DNA, including transcription. Post-translational modifications found on nucleosomal histone proteins H2A, H2B, H3, and H4, are thought to play an important role in gene regulation (Strahl and Allis, 2000). Histone tails are heavily modified by histone-modifying enzymes, also known as the "writers", leading to e.g. methylation, phosphorylation, ubiquitylation or acetylation, which can attract other factors to chromatin known as the "readers". Readers comprise a wide variety of proteins which often possess one or more well characterized domains that mediate interaction with modified histone tails. One subgroup comprises the chromatin-remodeling enzymes which can recognize and bind certain histone marks after which they actively change the chromatin structure by assembling, sliding, restructuring, or ejecting nucleosomes (Clapier and Cairns, 2009). Another subgroup of readers is formed by the bromodomain and extraterminal (BET) family of proteins that includes human bromodomain-containing protein 2 (BRD2), BRD3, BRD4 and a testes-specific BET protein called BRDT (Florence and Faller, 2001). These proteins are tethered to chromatin by binding preferentially to acetylated lysine residues found in histone, but also non-histone proteins, via their tandem bromodomain (Zeng and Zhou, 2002). BET family members are also found in other organisms, as *Drosophila* contains the female sterile (1) homeotic (Fs[1]h) protein and *Saccharomyces cerevisiae* expresses two bromodomain factors named Bdf1 and Bdf2. Apart from their two bromodomains (BD1 and BD2), BET proteins are also characterized by the presence of an extraterminal (ET) domain, which appears to function as a protein-protein interaction motif, and sometimes contain additional motifs, like motif A, B, SEED (Ser/Glu/Asp-rich region) and a C-terminal motif (CTM).

BRD4 is a widely expressed transcriptional regulator which has been implicated in activation of transcription. One way by which it can stimulate gene expression appears to be through the recruitment of P-TEFb to chromatin (Jang et al., 2005; Yang et al., 2005). By this means, BRD4-dependent recruitment of P-TEFb promotes CTD phosphorylation of Pol II at Ser2 and stimulates transcription elongation. This was shown to induce transcription of *c-MYC* and *c-JUN* and also enhanced transcription from the HIV-1 promoter (Jang et al., 2005). The interaction with P-TEFb has been attributed to BRD4's CTM, which is also conserved in BRDT and *Drosophila* Fs(1)h, but not in other BET family members (Bisgrove et al., 2007). Interestingly, like BRD4, both factors can specifically interact with P-TEFb as well when introduced into mammalian cells. However, whether this is physiologically relevant *in vivo* is not known. P-TEFb is a cyclin-dependent kinase consisting of the two subunits Cyclin T and Cdk9 and is normally present in two distinct intracellular pools: an active and an inactive one. In its inactive form, P-TEFb is held in a complex with the 7SK small nuclear ribonucleoprotein (snRNP) particle

consisting of the 7SK snRNA and a protein called HEXIM1/2 which inhibits the kinase activity of the Cdk9 subunit (Michels et al., 2004; Nguyen et al., 2001; Yang et al., 2001). The active P-TEFb pool is formed by interaction with BRD4 alone and is characterized by the absence of 7SK snRNA and HEXIM1/2. It is estimated that each pool accounts for about half of the P-TEFb present in the cell (Yang et al., 2005). Besides interacting with P-TEFb, BRD4 has also been found in association with selective Mediator complexes in both mammals and *Drosophila* (Jiang et al., 1998; Wu and Chiang, 2007; Wu et al., 2003). Furthermore, BRD4 can bind certain activators directly, as has been demonstrated for NF-κB and p53 (Huang et al., 2009; Wu et al., 2013). Therefore, it seems plausible that the function of BRD4 is not restricted to one particular step of the transcription process, but rather appears to be intimately involved in gene regulation by controlling binding of the activator, Mediator, and P-TEFb to chromatin (Chiang, 2009).

The interest in BRD4 has recently been raised tremendously by studies demonstrating its involvement in tumorigenesis. For example, the occurrence of a known chromosomal translocation where the N-terminal part of human BRD4 is fused to the almost complete Nut (nuclear protein in testis) protein to generate BRD4-NUT has been linked to squamous cell carcinoma (French et al., 2003). Furthermore, chemical inhibition of BRD4 has been shown to selectively inhibit transcription of key oncogenic drivers, such as *c-MYC*, in a variety of tumors, including multiple myeloma (MM), acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), and Burkitt's lymphoma (BL) (Dawson et al., 2011; Delmore et al., 2011; Mertz et al., 2011; Ott et al., 2012; Zuber et al., 2011). Inhibition of BRD4 can be achieved by treatment of cells with selective bromodomain inhibitors, i.e. I-BET or JQ1, both of which specifically bind to the tandem bromodomain of BRD4 to disrupt chromatin targeting (Filippakopoulos et al., 2010; Nicodeme et al., 2010). Treatment with these selective inhibitors appears to have an anti-tumor effect by shutting down transcription of oncogenes, which causes a cell cycle arrest leading to inhibition of proliferation and, conversely, promotes differentiation in both disease relevant cell lines and patient-derived material (Filippakopoulos et al., 2010; Mertz et al., 2011; Zuber et al., 2011). Although BRD4 appears to be a global regulator of gene expression, its inhibition selectively affects the expression of only a small number of genes in particular cell types. An explanation for this observation is provided by a recent paper, which demonstrated co-occupancy of Mediator and BRD4 on promoters and active enhancers and regions named super-enhancers (Loven et al., 2013). Super-enhancers are large enhancer regions which in a MM-relevant cell line appeared to be associated with genes that play a prominent role in the disease state such as *c-MYC*, *IRF4*, *PRDM1/BLIMP-1*, and *XBP1*. Interestingly, super-enhancers were also found in other tumor types, where they are similarly associated with key oncogenes. At these sites unusual high levels of both Mediator and BRD4 were found to be present and, surprisingly, treatment with JQ1 led to preferential loss of Mediator, BRD4, and P-TEFb at super-enhancers, accompanied by a loss of transcription at super-enhancer associated genes. Since tumor cells are generally addicted to overexpression of oncogenes, this selective inhibition of oncogene expression by blocking the binding of BRD4 and, accordingly, Mediator and P-TEFb, likely forms the basis for the anti-tumor effect observed when treating cells with BET small-molecule inhibitors.

To date, the exact mechanism by which BRD4 regulates transcription is not known. Analysis of BRD4 in humans is further complicated by the existence of additional BET family members, which might be able to substitute for BRD4 in its absence. Since fruit flies contain only one BET protein, we were interested in characterizing *Drosophila* Fs(1)h (hereafter called dBRD4) in more detail. We started out by performing an unbiased proteomics approach to identify the interacting partners of dBRD4 in *Drosophila* embryos. Our results imply that Mediator is the predominant interactor of dBRD4 in the fruit fly. In contrast, we did not find P-TEFb to specifically co-purify with dBRD4. Reciprocal immunoprecipitations (IPs) with antibodies directed against different Mediator subunits confirmed the physical interaction with dBRD4. Next, we determined the native mass of dBRD4 in embryo nuclear extract by size-exclusion chromatography which revealed its presence in similar fractions as Mediator, demonstrating that both factors are capable of forming a complex. Analysis of the genome-wide distribution of dBRD4 and Mediator on larval salivary gland polytene chromosomes revealed a high degree of overlap, suggesting that both transcriptional regulators can physically associate and bind to the same regions on the chromatin. We are currently performing transcriptome analysis to uncover the functional overlap between dBRD4 and Mediator in controlling gene expression. This will hopefully lead to a better understanding of the role of dBRD4 in regulating transcription.

Results

Drosophila BRD4 interacts physically with the Mediator complex

BRD4 has been shown to bind several critical transcriptional regulators in both mice and humans, including the P-TEFb kinase and the Mediator complex (Jang et al., 2005; Jiang et al., 1998; Wu and Chiang, 2007; Wu et al., 2003; Yang et al., 2005). We were interested in the sole BET protein present in *Drosophila*, named Fs(1)h. Alignment of the conserved bromodomains (BD1 and BD2) and the ET and CTM motifs with human BRD2, BRD3, BRD4, and BRDT using hierarchical clustering software (Corpet, 1988) revealed a high degree of similarity between the proteins (Fig. S1). Although BD1, BD2, and the ET motif were present in all proteins, the CTM could only be identified in Fs(1)h, BRD4, and BRDT. For simplicity, Fs(1)h will therefore now be referred to as dBRD4.

One of the aims during this study was to identify the interacting partners of dBRD4. *Drosophila* BRD4 has been purified from S2 cells previously, but the associated proteins had not been identified (Chang et al., 2007). Furthermore, in this case it did not concern endogenous dBRD4, but rather a recombinant FLAG-tagged version of the short isoform of dBRD4 that had been introduced into S2 cells. In order to identify dBRD4-associated proteins in a more natural setting, we immunopurified dBRD4 from embryo nuclear extracts using highly specific antibodies generated against the N-terminus of dBRD4, followed by mass spectrometric analysis of the co-precipitated proteins (Table 1). We identified dBRD4 itself with high confidence, illustrating the specificity of our antibody. Apart from that, we also found the entire 31-subunit Mediator complex, suggesting that *Drosophila* BRD4, similar to its murine and human counterparts, interacts physically with Mediator. However, we failed to identify P-TEFb in our

Table 1. List of dBRD4, MED1 and MED13 associated proteins identified by mass spectrometry

Protein identity	M.W. (kDa)	dBRD4			MED1			MED13/Skd			Description
		Mascot	*emPAI*	#	*Mascot*	*emPAI*	#	*Mascot*	*emPAI*	#	
dBRD4/Fs(1)h	205	5197	11.52	70	768	0.58	13	236	0.14	3	Bromodomain protein
MED1	150	3546	3.86	51	4691	11.74	63	2264	2.2	35	Mediator complex
MED4	28	1071	20.32	15	1639	171.8	22	711	9.89	11	
MED6	28	1292	52.84	19	1483	101.7	20	840	12.86	15	
MED7	26	881	14.04	13	908	26.27	14	692	6.89	11	
MED8	28	1116	49.85	16	1116	65.34	16	858	23	13	
MED9	17	126	1.17	3	259	3.22	5	97	0.85	3	
MED10	16	342	4.33	5	424	6.39	6	275	2.79	4	
MED11	20	235	0.66	3	582	11.23	8	341	3.18	7	
MED12/Kto	279	5730	3.46	85	6774	6.65	99	7296	10.58	112	
MED13/Skd	280	4677	2.7	76	5877	5.67	87	6131	7.1	85	
MED14	172	3951	5.16	64	5146	11.42	73	2112	1.92	39	
MED15	81	1426	2.5	21	1841	4.48	26	824	1.24	16	
MED16	90	2006	3.42	31	2704	12.33	41	1491	3.2	28	
MED17	72	2765	17.96	40	3476	66.13	47	2072	9.01	34	
MED18	25	419	3.32	8	774	29.12	12	376	3.13	7	
MED19	35	729	6.27	11	1119	17.72	17	607	2.36	10	
MED20	28	1103	63.52	16	1202	235.5	18	784	26.25	14	
MED21	16	307	3.99	6	473	9.48	8	175	1.91	5	
MED22	17	223	2.26	4	381	5.59	6	96	1.31	2	
MED23	167	2959	2.49	46	4113	6.16	60	2069	1.65	39	
MED24	112	2222	3.07	32	2914	9.39	42	1981	3.1	35	
MED25	97	1731	3.32	27	1923	5.98	29	1275	1.96	22	
MED26	166	557	0.25	10	2400	1.98	45	54	0.04	2	
MED27	34	1852	48.99	27	1299	22.02	21	1179	17.68	20	
MED28	21	296	1.98	6	522	9.25	10	144	0.94	3	
MED29/Ix	21	529	7.78	7	643	10.95	9	372	3.43	6	
MED30	35	806	2.75	12	916	4.57	12	457	1.48	8	
MED31	24	539	4.34	9	710	9.84	10	436	4.15	9	
Cdk8	54	1263	5.5	22	2110	54.12	36	2014	57.93	34	
Cyclin C	31	566	2.92	11	878	7.46	15	945	10.85	14	
Cdk9	47	47	0.07	1							P-TEFb subunit
Rpb1/RpII215	209	140	0.05	3	2164	1.33	46				RNA pol II complex
Rpb2/RpII140	134				559	0.42	12				
Rpb3/RpII33	31	188	0.37	3	370	1.21	6				
Rpb4	16				143	0.91	3				
Rpb5	24	74	0.31	1	109	0.33	2	115	0.54	2	
Rpb7	19				316	1.48	5				
Rpb8	17				236	3.11	4				
Rpb9/RpII15	15				156	0.95	3				
CG18619	11				257	2.45	3				DNA binding protein
CG4709	59				508	0.96	11				Zn finger protein
Sbf	223				2629	1.29	48				Chromatin binding
REG	28	985	20.32	18	521	2.51	10	364	2.98	8	Proteasome activator
Dorsal	75	891	1.78	14							Sequence-specific TF
Hsf	82	1419	2.18	22	852	0.86	14	1987	6.04	30	
dDP	50	400	0.6	7	44	0.07	1				

dBRD4 purification, as we did not detect Cyclin T, whereas only 1 peptide was counted for Cdk9. Therefore, it appeared that dBRD4 did not interact with P-TEFb under the conditions used here. We did find a number of sequence-specific DNA binding transcription factors associated with dBRD4, including Dorsal (Dl), heat shock factor (Hsf), and dimerization partner (Dp). Interestingly, the Dl transcription factor contains domains also found in p53 and NF-κB, which are proteins that have been reported to interact with BRD4 in mammals (Huang et al., 2009; Wu et al., 2013).

Since Mediator seemed to be the major interacting for dBRD4 in *Drosophila* embryo nuclear extract, we also performed reciprocal IPs with specific antibodies directed against the MED1 and MED13 subunits of the Mediator complex (Table 1). Analysis of Mediator associated proteins by mass spectrometry consistently revealed the presence of dBRD4, thereby confirming the interaction of dBRD4 with Mediator. We also picked up Pol II in the MED1 purification, but not in the MED13 sample, which is consistent with the notion that the Mediator middle part that contains MED1 can contact Pol II (Dotson et al., 2000). We could not detect Pol II with high confidence in the dBRD4 purification, suggesting that dBRD4 and Pol II do not interact physically. However, they are likely to interact indirectly in which case the Mediator complex functions as an adaptor between dBRD4 and Pol II.

In order to investigate the association of dBRD4 with Mediator further, we performed size-exclusion chromatography to fractionate the heparin-400 mM KCl (H0.4) fraction of embryo nuclear extract, after which the eluate was analyzed by Western immunoblotting to reveal the presence of dBRD4 and Mediator components. The antibodies raised against dBRD4 recognized two major bands in the embryo nuclear extract, which represent the long and short isoforms (Fig. 1A and B). They did migrate slower than what would be expected from their theoretical size, which is approximately 210 kDa for the long and 120 kDa for the short isoform, respectively. More importantly, however, dBRD4 co-eluted with MED1, MED12, and MED13 subunits, demonstrating the presence of a large multiprotein complex. As expected, all three Mediator components co-eluted in similar fractions, which is a requirement for the formation of a stable complex. The elution profile of dBRD4 was comparable to that observed for Pol II, although we did not detect physical interaction with Pol II. The metabolic enzyme IMPDH served as a reference and showed a clearly distinct elution profile from dBRD4, Mediator and Pol II with a smaller apparent molecular mass. Both dBRD4 isoforms seemed to be present in mostly the same fractions, although their relative amounts appeared to vary between fractions. Nevertheless, it does suggest that dBRD4-long and -short are capable of interacting with the Mediator complex.

To verify the interactions observed between dBRD4 and Mediator by our proteomics analysis we performed IPs for both factors followed by Western immunoblotting with antibodies directed against dBRD4 and Mediator components. Immunoprecipitation of dBRD4 followed by Western blotting revealed the presence of all tested Mediator subunits, i.e. MED1, MED12, and MED13 (Fig. 1C). By contrast, Pol II and the chromatin remodeler ISWI did not co-precipitate with dBRD4, demonstrating the specificity of the observed interactions. We then performed IP for the different Mediator subunits and again identified dBRD4 as an associated factor. The IP-Western results also provided additional information concerning the two dBRD4 isoforms. IP-Western let us to distinguish between dBRD4-short and -long, while the mass spectrometer could not do this with certainty, since the short isoform is identical to the long one, with the latter containing a unique C-terminal extension that includes the CTM. Our IP-Western results revealed interaction of both isoforms with MED1, whereas mostly dBRD4-long alone seemed to interact with MED12 and MED13. Therefore, some interactions might be attributed specifically to one of the dBRD4 isoforms. Pol II, on the other hand, was again only found in association with MED1, and not MED12 or MED13, which is consistent with our mass spectrometry data

of MED1 and MED13. ISWI, on the other hand, interacted with none of the Mediator proteins. To summarize, we have identified Mediator as the major interacting partner of *Drosophila* BRD4 in embryo nuclear extract.

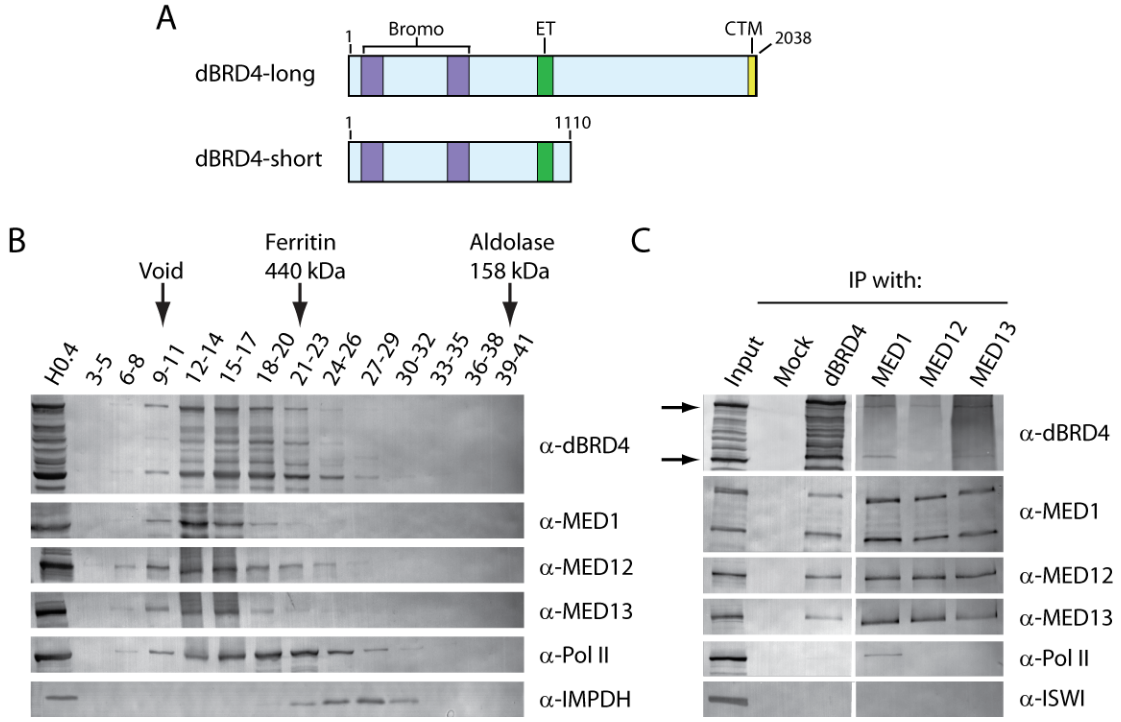

Figure 1. dBRD4 interacts physically with the Mediator complex. (A) Schematic of dBRD4 isoforms with conserved protein domains. Both dBRD4 isoforms contain a tandem bromodomain and an extraterminal (ET) domain, but only dBRD4-long contains a conserved C-terminal motif (CTM) in its extended C-terminus. (B) Size-exclusion chromatography from the heparin-400 mM KCl fraction (H0.4 fraction) of embryo nuclear extract followed by SDS-PAGE and immunoblotting with the indicated antibodies. The elution of the voided volume (void) and the elution of the known markers Ferritin (440 kDa) and Aldolase (158 kDa) are indicated. Note that both dBRD4 isoforms co-elute with Mediator and Pol II. IMPDH served as a control as it shows a clearly distinct elution pattern. (C) Co-immunoprecipitation of dBRD4 with Mediator subunits MED1, MED12, and MED13. Crude *Drosophila* embryo nuclear extracts were incubated with preimmune serum (mock) or α-dBRD4, α-MED1, α-MED12, or α-MED13 antibodies. Immunopurified proteins were resolved by SDS-PAGE and analyzed by immunoblotting using the indicated antibodies. Two percent of the input material was loaded for reference. Arrows mark the two different dBRD4 isoforms.

dBRD4 and Mediator colocalize on chromatin

Given the fact that we established that dBRD4 and Mediator can interact physically, we wondered whether both factors could be found at similar sites on the chromatin as well. To investigate their genome-wide distribution on chromatin we stained polytene chromosomes isolated from third instar larval salivary glands with our antibodies directed against dBRD4 and MED1 (Fig. 2). Comparison of the localization of dBRD4 and MED1 revealed a striking overlap

in their genomic distribution, as demonstrated by the similar patterns in the individual channels and yellow staining in the overlay (Fig. 2A-C). A magnified portion made this even more clearly visible (Fig. 2D and E). The vast majority of bands stained for dBRD4 colocalized with MED1, whereas MED1 could also be detected on additional sites which seemed to lack presence of dBRD4.

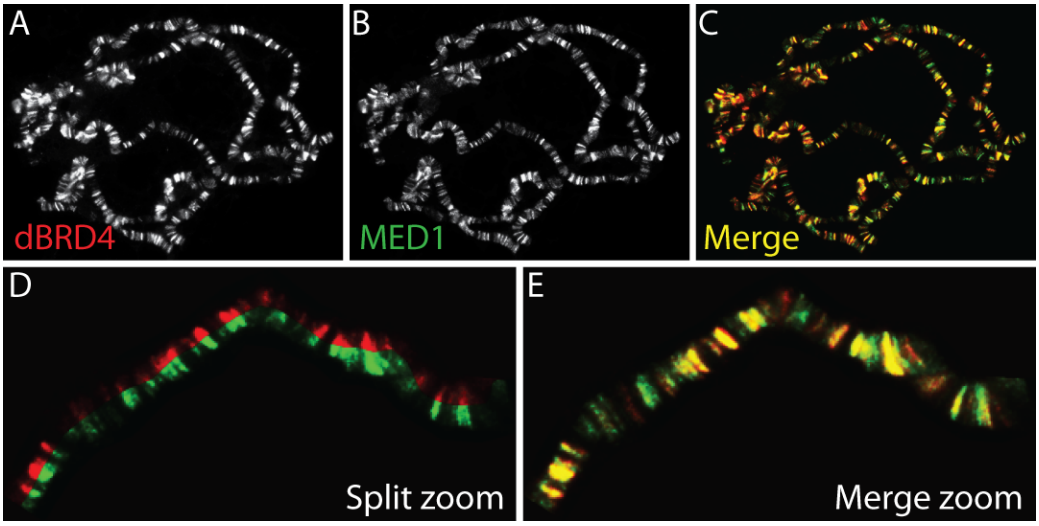

Figure 2. dBRD4 colocalizes with Mediator on polytene chromosomes. (A-C) Co-staining of dBRD4 (A, in red) and MED1 (B, in green) on polytene chromosomes. The colocalization of dBRD4 and MED1 on many loci is demonstrated by the yellow staining in the merge panel (C) and the similar patterns in the separate images. (D, E) A representative magnification of the upper panels is shown with either zooms including splitted (D) or merged (E) channels.

Similar to human BRD4, the *Drosophila* BET protein has been linked to transcriptional activation of certain genes (Chang et al., 2007). Although dBRD4 does not seem to interact with Pol II directly, we were interested in comparing their genome-wide localization pattern on chromatin. Therefore, we stained polytene chromosomes for dBRD4 and Pol II which uncovered a significant amount of overlap (Fig. 3A-E). The colocalization of dBRD4 and Pol II was not as striking as what we observed for dBRD4 and Mediator, but we did find many dBRD4-stained bands to be co-occupied by Pol II. The relative levels of both factors seemed to vary from site to site, which explains why a number of sites appear either green or red in the overlay, while both proteins do localize to these regions. Pol II did seem to bind more loci than dBRD4, which might be explained by the fact that dBRD4 colocalizes predominantly with some forms of Pol II (e.g. Ser2 or Ser5 phosphorylated Pol II). We have not addressed this issue in more detail though. As anticipated, Mediator also colocalized with Pol II to some extent (Fig. 3F-J). Mediator is thought to co-occupy mostly sites bound by the hypophosphorylated form of Pol II, as CTD phosphorylation of Ser5 releases Pol II from Mediator to promote transition from initiation to elongation. Taken together, our data reveal the co-occupancy of dBRD4 and Mediator at many sites on the chromatin and, in addition, demonstrate colocalization of dBRD4 with Pol II.

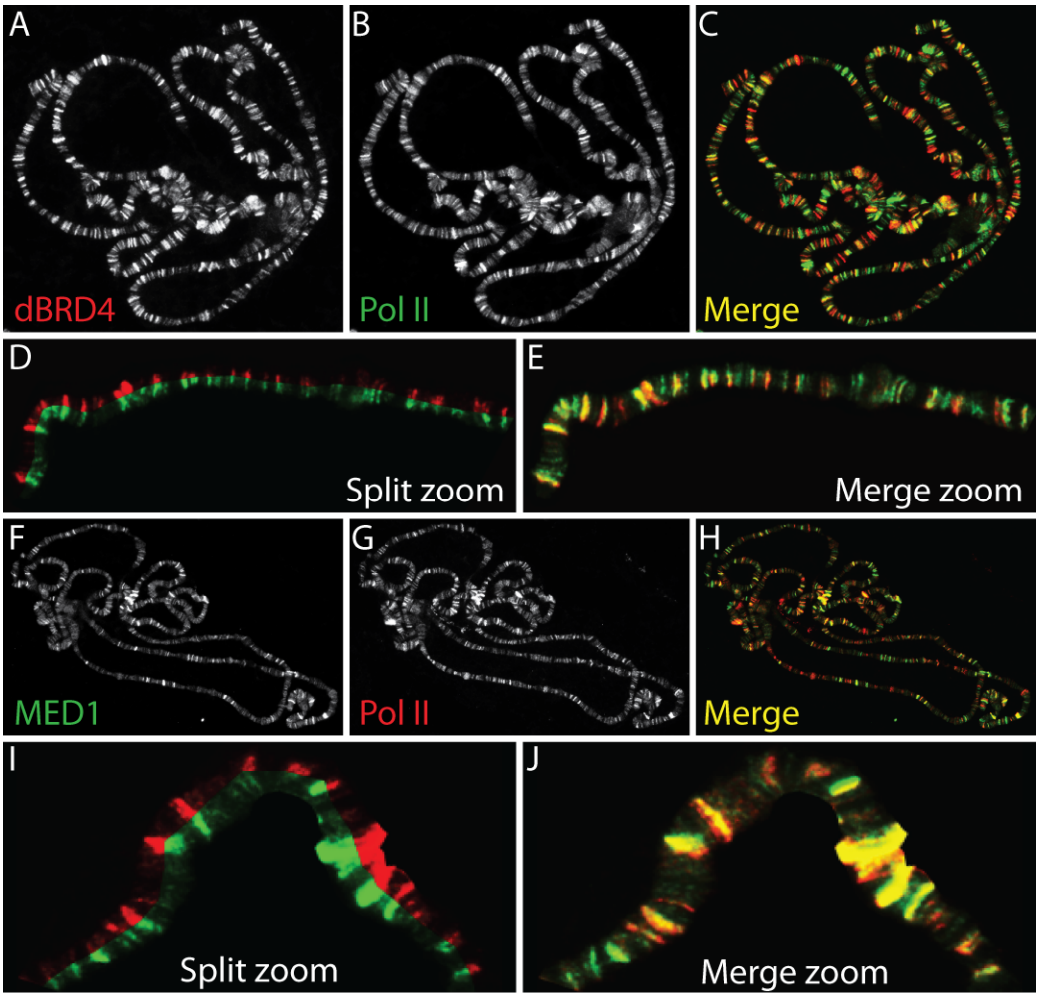

Figure 3. Colocalization of dBRD4 and Mediator with Pol II on polytene chromosomes. (A-C) Co-staining of dBRD4 (A, in red) and Pol II (B, in green) on polytene chromosomes. The colocalization of dBRD4 and Pol II on many loci is demonstrated by the yellow staining in the merge panel (C) and the similar patterns in the separate images. (D, E) A representative magnification of the upper panels is shown with either zooms including splitted (D) or merged (E) channels. (F-H) Co-staining of MED1 (F, in green) and Pol II (G, in red) on polytene chromosomes. The colocalization of MED1 and Pol II on many loci is demonstrated by the yellow staining in the merge panel (H) and the similar patterns in the separate images. (I, J) A representative magnification of the upper panels is shown with either zooms including splitted (I) or merged (J) channels.

Gene expression profiling for dBRD4 and Mediator

The observed physical interaction of dBRD4 with Mediator together with the high degree of overlapping binding sites on chromatin implies an intimate relationship between both factors. In order to assess the functional significance of these observations we performed RNAi-mediated knockdown of dBRD4 and MED1 in S2 cells followed by extraction of total RNA and high-throughput sequencing (RNA-seq) to identify the global transcriptional circuitries controlled by

dBRD4 and Mediator. Knockdown of dBRD4 and MED1 was efficient, as demonstrated by Western immunoblot analysis (Fig. 4A and B). We are currently in the process of analyzing the RNA-seq data obtained after knocking down either dBRD4 or MED1.

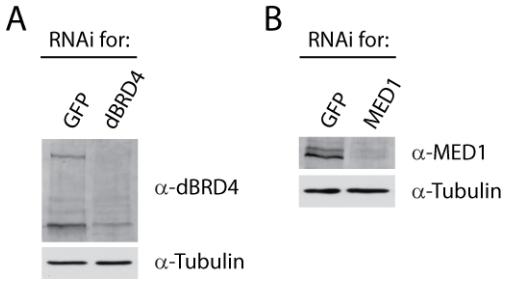

Figure 4. Knockdown of dBRD4 and MED1 for expression analysis. (A) S2 cells were treated with dsRNA against dBRD4 or GFP (= mock) after which whole cell lysates were prepared and analyzed for knockdown efficiency by Western immunoblotting using the indicated antibodies. Tubulin served as a loading control and indicates equal loading. (B) Same as A, but here knockdown was performed for MED1 and GFP followed by Western immunoblotting with the indicated antibodies.

Discussion

In this study we have aimed to identify the interacting proteins of *Drosophila* BRD4. As a result of a proteomics screen for dBRD4 interactors in embryo nuclear extract, we identified the Mediator coactivator as a major dBRD4 associated protein complex. Likewise, we successfully picked up dBRD4 in several independent Mediator purifications, demonstrating the reliability of our data. These results were further supported by IP-Westerns, which also showed the interaction of dBRD4 with Mediator. Genome-wide localization analysis of dBRD4 and Mediator on polytene chromosomes suggests that most of the sites bound by dBRD4 are bound by Mediator as well. In addition, we show a significant colocalization of dBRD4 with Pol II on chromatin. In summary, these observations link dBRD4 to the Mediator complex which suggests a critical role for dBRD4 in transcriptional control by Pol II.

The BET family comprises a small subset of proteins with features reminiscent of chromatin binding factors. In mammals, BRD2, BRD3, BRD4, and BRDT are the representatives of this family, which are generally widely expressed proteins with the exception of BRDT, whose expression seems to be restricted to the testis (Shang et al., 2004). All of these four BET members have been reported to bind acetylated histone tails, thereby providing a link with chromatin-mediated processes (Dey et al., 2003; Kanno et al., 2004; Leroy et al., 2008; Pivot-Pajot, 2003). In *Drosophila*, dBRD4 is the only known BET family member, which was originally shown to cause homeotic transformations in predominantly female flies when the gene was mutated (Gans et al., 1980). Later on, *dbrd4* was demonstrated to interact genetically with mutations in *ash1*, *ash2*, *trx*, and *Ubx*, which therefore classified it as a trithorax group (trxG) gene (Digan et al., 1986; Shearn, 1989). Genes belonging to the trxG usually encode transcriptional activators, which seemed to be true for dBRD4 as well as it was shown to activate transcription of *Ubx* (Chang et al., 2007). The same lab had purified dBRD4-short from S2 cells and observed consistent copurification of another protein, which they referred to as FAP56 (for FSH-

associated protein of 56 kDa). The identity of this protein had not been determined, but since we uncovered a physical interaction between dBRD4 and Mediator, we suspect this FAP56 protein could in fact be the Cdk8 protein kinase subunit of the Mediator complex. We believe so because Cdk8 has a similar size (54 kDa) and possesses kinase activity. The latter is an important point, as Chang et al. demonstrated the presence of a kinase activity in dBRD4-short preparations. This activity was not observed for bacterially expressed dBRD4-short, which could indicate that the kinase activity of dBRD4-short preparations is the result of other copurifying factors, such as Cdk8.

Initial work on human BRD2 (also known as RING3) characterized this protein as a novel serine/threonine (Ser/Thr) kinase with scrambled subdomains (Denis and Green, 1996). On the contrary, murine BRD2 (also called Fsrg1) did not seem to possess kinase activity despite over 90% sequence homology to the human protein, which therefore questioned the data of this initial study (Rhee et al., 1998). Interestingly, however, a more resent study has also reported a kinase activity for human BRD4 (Devaiah et al., 2012). Here, it was shown that BRD4 could phosphorylate itself as well as the Pol II CTD on Ser2 both *in vitro* and *in vivo*, an activity that is normally attributed to P-TEFb. BRD4 was shown to contain several regions with homology to kinase subdomain motifs scattered across its N-terminal 699 amino acids. Therefore, it was classified as an atypical kinase, similar as to what had been reported for BRD2 (Denis and Green, 1996). Since the kinase subdomains seemed to be located in the N-terminus of BRD4, both the long and the short isoform contain these domains and thus might possess kinase activity. Hence, we cannot rule out the presence of an intrinsic kinase activity in *Drosophila* BRD4. More experiments will need to be performed in order to address this issue.

Our results described here provide a strong link between dBRD4 and the Mediator transcriptional coactivator, which parallel observations made for the mouse and human BRD4 proteins (Jiang et al., 1998; Wu and Chiang, 2007; Wu et al., 2003). First of all, we have demonstrated a physical interaction of the two factors by performing IP for dBRD4 as well as Mediator followed by mass spectrometric analysis. These results were confirmed subsequently by IP-Westerns with antibodies directed against dBRD4 and Mediator subunits. Furthermore, size-exclusion chromatography experiments with the H0.4 fraction of *Drosophila* embryo nuclear extract showed co-elution of dBRD4 and Mediator, suggesting that these factors can form a multiprotein complex. Apart from a physical interaction, we also detected extensive colocalization of dBRD4 with Mediator on larval salivary gland polytene chromosomes, which indicates that the two transcriptional regulators likely interact on chromatin as well. In mammals, BRD4 has also been reported to interact with the transcription elongation factor P-TEFb (Jang et al., 2005; Yang et al., 2005). Our proteomic analysis failed to identify P-TEFb as a dBRD4-associated factor though. It is possible that BRD4 in *Drosophila* simply does not interact with P-TEFb. However, another possibility is that we do not pick up this heterodimer of Cyclin T and Cdk9 because the antibody we used interferes with the binding of dBRD4 to P-TEFb. Our antibody is raised against the N-terminal part comprising amino acid residues 1-322, which includes the first bromodomain of dBRD4. Using this antibody to IP dBRD4 therefore might block access to BD1. Since murine BRD4 has been reported to bind P-TEFb through both of its bromodomains, immunoprecipitation of dBRD4 with an antibody that binds the N-terminal part,

including BD1, might, therefore, reduce or abolish binding to P-TEFb (Jang et al., 2005). However, a more recent study demonstrated that binding of BRD4 to P-TEFb is mediated by a C-terminal portion of BRD4 which includes the CTM (Bisgrove et al., 2007). This conserved domain is also present in BRDT and *Drosophila* BRD4, and experiments using recombinant C-terminal portions of these proteins demonstrated specific interaction with P-TEFb. Thus, there should be another explanation for the fact that we do not observe a physical interaction of dBRD4 with P-TEFb. It is possible, however, that binding of our polyclonal antibodies to dBRD4 block access of P-TEFb indirectly through steric hindrance. Alternatively, the interaction with P-TEFb could be of weak nature which might be lost due to our stringent IP conditions. Therefore, it might be useful to purify dBRD4 with multiple antibodies directed against various regions of the protein and under several different conditions in order to obtain a complete picture of its interaction partners.

The association of Mediator with dBRD4 and the colocalization on chromatin suggests a role for dBRD4 in transcription initiation. The observed overlapping genomic distributions of dBRD4 and MED1 in *Drosophila* salivary gland tissue are supported by recent experiments in human multiple myeloma cells where chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) of BRD4 and MED1 showed very similar binding profiles for the two proteins (Loven et al., 2013). Mediator and BRD4 appeared to bind active enhancers and promoters and so-called super-enhancers, where co-occupancy with Pol II at active transcription start sites (TSSs) was observed. Furthermore, when BRD4 was inhibited with the small-molecule JQ1, not only binding of BRD4 at these sites was severely reduced, but binding of MED1 was decreased as well. Chromatin binding seemed to be reduced preferentially at super-enhancers which, therefore, could explain the difference in sensitivity of certain genes to respond to BRD4 inhibition. A similar observation was made for Cdk9, the kinase subunit of P-TEFb, which also showed a decrease in the amount of protein associated with genomic regions distal to the TSS. The presence of Pol II in the gene body of active genes was strongly reduced after JQ1 treatment, whereas binding to the promoter seemed largely unaffected, suggesting that transcription elongation is impaired specifically when BRD4 binding is inhibited. Taking this into account, it is likely that BRD4 plays multiple roles in the transcription process (Chiang, 2009). BRD4 might initially bind transcriptional activators, such as p53 or NF-κB, and associate with acetylated histone tails to promote its targeting to (super-)enhancers and promoters. Subsequent steps could involve BRD4-mediated recruitment of the Mediator complex to allow PIC assembly and entry of Pol II into the promoter region through phosphorylation of the CTD on Ser5, followed by recruitment of P-TEFb to promote phosphorylation on Ser2 and productive transcription elongation by Pol II. BRD4 might also directly contribute to CTD phosphorylation, as it happens to possess intrinsic kinase activity (Devaiah et al., 2012). A recent study suggested that cross-talk among the CTD kinases Cdk7, Cdk9, and BRD4, could be crucial for efficient elongation by Pol II (Devaiah and Singer, 2012). In *Drosophila*, dBRD4 has not yet been implicated in Ser2 phosphorylation of the Pol II CTD. It will be interesting to address its role in this aspect in a more detailed manner.

In summary, parallel to the observations made in mammals, *Drosophila* BRD4 seems to interact specifically with the Mediator complex. Additionally, we find that dBRD4 and Mediator

overlap at the majority of their genomic binding sites in a global fashion. Thus, we propose an intimate relation between Mediator and dBRD4 in transcriptional control.

Experimental procedures

Antibodies and immunological procedures

Polyclonal antibodies were generated by immunizing guinea pigs or rabbits with GST fusion proteins expressed in *Escherichia coli* and were affinity purified as described previously (Chalkley and Verrijzer, 2004). The following antigens were used: dBRD4 amino acids (aa) 1-322, MED1 aa 1-450 and 456-784, and Rpb1 aa 1-397. Other antibodies have been described, including α-Kto and α-Skd (Janody et al., 2003), α-ISWI (Kal et al., 2000), α-IMPDH (Kozhevnikova et al., 2012), and anti-α-Tubulin (T8203, Sigma).

Polytene chromosome immunostaining

The analysis of polytene chromosomes was performed essentially as described previously (Armstrong et al., 2002) with minor modifications. Briefly, salivary glands from *Drosophila* 3rd instar larvae were dissected in 0.7% NaCl after which the glands were fixed in 45% acetic acid/1.85% formaldehyde for 1 to 2 minutes at room temperature. Fixed tissue was transferred onto coated poly prep slides (Sigma) after which the glands were squashed and slides were frozen in liquid nitrogen. Squashes were incubated with blocking buffer (1% BSA dissolved in PBS/0.1% Triton X-100) overnight at 4°C. Next, squashes were incubated with primary antibody (α-dBRD4, 1:200; α-MED1, 1:100; and α-Rpb1, 1:200) in blocking buffer for 1 hour at room temperature in a humid chamber. Slides were washed three times with PBS/0.1% Triton X-100 and the squashes were subsequently incubated with appropriate secondary antibodies (Alexafluor, Molecular Probes) diluted 1:500 in blocking buffer for 1 hour at room temperature. Finally, slides were washed again three times with PBS/0.1% Triton X-100 and mounted in Vectashield with DAPI (Vector Laboratories, Inc.). Images were captured by using a Leica DM-RXA microscope and processed by using Photoshop.

Protein purification and mass spectrometry

Nuclear extracts were prepared from 0 to 12 hours-old *Drosophila* embryos as described (Kamakaka and Kadonaga, 1994). Immunopurification procedures using specific antibodies directed against dBRD4 (GR808), MED1 (GR868), and Skd and mass spectrometric analysis were all performed as described previously (Chalkley et al., 2008; Chalkley and Verrijzer, 2004). Briefly, extracts were incubated for 2 hours at 4°C with α-dBRD4, α-MED1, α-Kto, or α-Skd antibodies coupled to Protein A-Sepharose beads (GE Healthcare). Next, beads were washed twice with HEMG buffer (25 mM HEPES-KOH [pH 7.6], 0.1 mM EDTA, 12.5 mM $MgCl_2$, 10% glycerol, 0.1% NP-40, 1 μg/ml of leupeptin, aprotinin and pepstatin and 0.2 mM AEBSF [(α-aminoethyl)benzenesulfonyl fluoride]) containing 100 mM KCl (HEMG/100), 5 times with HEMG/600, twice with HEMG/100 containing 0.01% NP-40, and finally once with 100 mM KCl. Affinity purified proteins were subsequently eluted with Glycine buffer (100 mM glycine,

150 mM NaCl) pH 2.5. Eluted proteins were TCA precipitated and dissolved in 1x SDS sample buffer after which the proteins were resolved by SDS-PAGE and visualized by Coomassie blue staining. Polypeptides were identified by mass spectrometry on an LTQ-Orbitrap hybrid mass spectrometer (ThermoFischer). Typical contaminants, also present in immunopurifications using beads coated with pre-immune serum or antibodies directed against irrelevant proteins, were omitted from the dataset. For small scale co-immunoprecipitations, 15 µl of crude serum was coupled to 20 µl of Protein A-Sepharose which was subsequently incubated with ~2 mg *Drosophila* embryo nuclear extract for 2 hours at 4°C. Beads were washed and proteins were eluted similar as described above. Eluted proteins were resolved by SDS-PAGE and visualized by Western immunoblotting with appropriate antibodies. For analysis of native protein sizes, the crude *Drosophila* embryo nuclear extracts were further fractionated using a Sephacryl S-300 column (Pharmacia) as described previously (Mohrmann et al., 2004). Briefly, nuclear extracts were concentrated by chromatography on a POROS-heparin (PerSeptive Biosystems) column equilibrated with HEMG/100 buffer (25 mM HEPES-KOH [pH 7.6], 0.1 mM EDTA, 12.5 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol [DTT], 0.2 mM AEBSF, 1 µM pepstatin, 0.01% Nonidet P-40 [NP-40], 100 mM KCl), followed by a step elution with HEMG/400 buffer (HEMG/100 buffer with 400 mM KCl instead of 100 mM KCl). The heparin-400 mM KCl fraction (H0.4 fraction) was loaded onto an 800-ml Sephacryl S-300 column (elution volume, 300 ml) equilibrated and developed with HEMG/100 buffer. Fractions were separated by SDS-PAGE and analyzed by immunoblotting with α-dBRD4, α-MED1, α-Kto, α-Skd, α-Rpb1, and α-IMPDH antibodies.

Cell culture, RNA interference and RNA-sequencing

Drosophila Schneider's line 2 cells (hereafter S2 cells) were cultured in Schneider's medium (Invitrogen) supplemented with 10% fetal calf serum (Thermo) and 1% Penicillin-Streptomycin. Cells were treated with double-stranded RNA (dsRNA) for 4 days, as described previously (Worby et al., 2001). Double-stranded RNAs were synthesized using the Megascript T7 kit (Ambion). Total RNA was isolated from $>1x10^6$ cells using TriPure isolation reagent (Roche). For genome-wide expression analysis, at least 3 µg of total RNA was used in Illumina Next Generation Sequencing (ServiceXS B.V., Leiden).

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Supplementary information

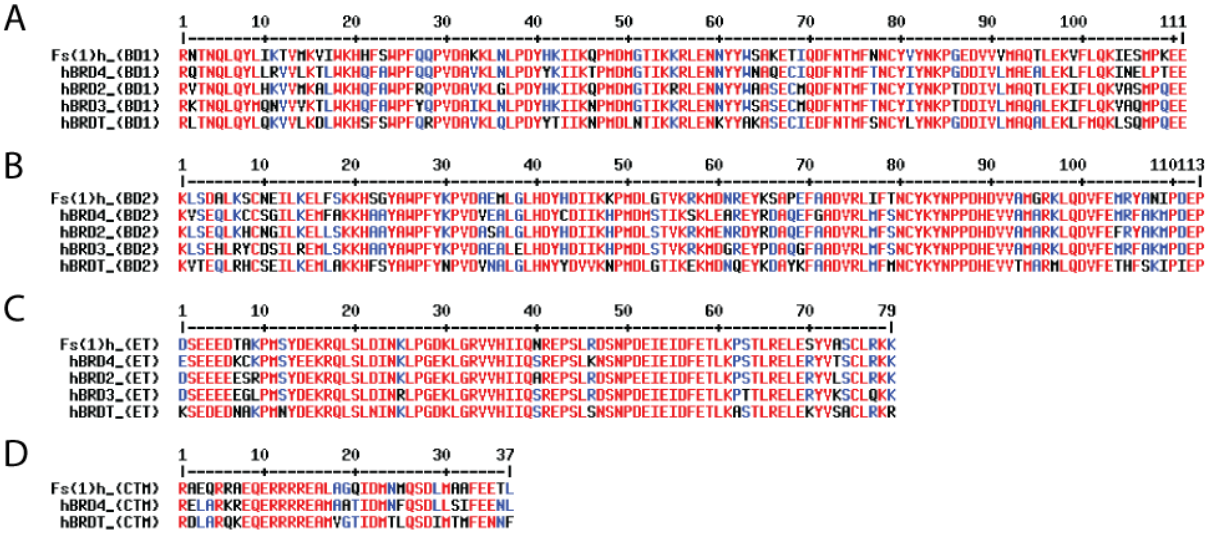

Figure S1. *Drosophila* Fs(1)h shows homology to human BET family members. (A-D) Protein domains of the long isoform of Fs(1)h were compared to those present in human BET proteins BRD4, BRD2, BRD3, and BRDT using multiple sequence alignment with hierarchical clustering (http://multalin.toulouse.inra.fr/multalin/). (A) Alignment of bromodomain 1 (BD1). (B) Alignment of bromodomain 2 (BD2). (C) Alignment of the extraterminal (ET) domain. (D) Alignment of the C-terminal motif (CTM). Note that the CTM is absent from BRD2 and BRD3. Red letters indicate amino acid residues which are present in all proteins, blue letters represent highly conserved amino acids and black letters mark poor or non-conserved amino acids.

Table S1. Primers used to generate dsRNA.

Target	Forward primer	Reverse primer
GFP	ATGGTGAGCAAGGGCGAG	CTTGTACAGCTCGTCCATGC
dBRD4	ATGTCGTCCAGTGAGCCAC	GCTGAGTTCCCGGTGTACT
MED1	AGCGGATCCAATGCAAAGTCC	AGCCTCATAGGATGGCCACC

Chapter 6

Functional Dissection of *Drosophila* ISWI-Containing Chromatin Remodeling Complexes

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Abstract

The structure and activity of chromosomes can be modified through mobilization of nucleosomes by ATP-dependent chromatin remodeling factors (remodelers). One class of remodelers comprises the ISWI subfamily, where nucleosome remodeling activity is dictated by the ISWI ATPase. However, ISWI does not appear to act on its own as it has been found to be part of several distinct multisubunit complexes. In order to address the composition of these different protein complexes, we performed a proteomics approach in which we purified ISWI from *Drosophila* embryo nuclear extract. We identified at least four separate complexes that contain ISWI, including ACF/CHRAC, NURF, RSF, and ToRC. Furthermore, we discovered two novel NURF subunits that contain either a SANT domain or an HMG box, which we named dBap18 and dHMGXB4, respectively. Comparison of the domain structure of the ISWI-interacting proteins Acf1, Nurf301, dRSF-1, and Toutatis (Tou) reveals the presence of highly similar domains, suggesting parallel biochemical activities of the different complexes. Analysis of their *in vivo* localization on larval salivary gland polytene chromosomes, however, uncovered a specific genomic distribution of these factors. Nurf301 appeared to colocalize predominantly with ISWI, whereas Acf1, dRSF-1, and Tou only partially overlapped with the ATPase. Apart from ISWI, dRSF-1 was shown to interact physically with the BAP remodeler that includes the Brm enzymatic motor, while Tou was found to associate with GMPS and USP7. Interestingly, dRSF-1 also colocalized extensively with Brm on polytene chromosomes. Likewise, Tou specifically co-occupied several loci that were bound by GMPS and USP7. Our data thus provides a concise analysis of the ISWI-containing complexes in *Drosophila* which identified four major assemblies, i.e. ACF/CHRAC, NURF, RSF, and ToRC.

Introduction

The eukaryotic genome is organized in a highly dynamic structure called chromatin. In the context of chromatin, about 146 base pairs of DNA is wrapped around an octamer of histone proteins in a repeated fashion, better known as the nucleosome (Kornberg, 1977). On the one hand, this packaging of the genetic material provides a clear advantage of organizing a large and complex genome in the nucleus, but on the other hand, it can also block access of proteins to DNA. To counteract this intrinsic barrier the cell is equipped with a variety of factors that can modulate the chromatin structure, such as chromatin-modifying enzymes and ATP-dependent chromatin remodeling factors (remodelers). Chromatin modifiers catalyze the formation of specific post-translational modifications on the amino- and carboxy-terminal tails of histone proteins, while remodelers use the energy of ATP hydrolysis to change nucleosome positions or to incorporate histone variants into chromatin (Clapier and Cairns, 2009; Kouzarides, 2007a; Lorch et al., 2010). These alterations ultimately lead to different functional chromatin states affecting local chromatin structure and, consequently, the expression of specific genes.

Chromatin remodeling factors often reside in large multimeric complexes and exist in different flavors. In general, four major subfamilies of remodelers are known, which include SWI/SNF, CHD/Mi2, INO80, and ISWI (Clapier and Cairns, 2009). These four classes can be distinguished from each other by the presence of their central ATPase and accessory domains. In the case of the Imitation Switch (ISWI) protein, the ATPase domain is present at the N-terminus, whereas its C-terminus harbors HAND, SANT and SLIDE domains which are important for nucleosome recognition and remodeling *in vitro* (Grune et al., 2003). In higher eukaryotes, ISWI is an abundant and ubiquitously expressed protein that is essential for cell viability (Deuring et al., 2000; Stopka and Skoultchi, 2003). Furthermore, the loss of ISWI function in specific context in humans is associated with a developmental disorder called Williams syndrome (Bozhenok et al., 2002; Lu et al., 1998). Thus, ISWI appears to fulfill an important role *in vivo*.

In *Drosophila*, ISWI is the ATPase component of several distinct protein complexes, including ACF (ATP-utilizing chromatin assembly and remodeling factor), CHRAC (chromatin accessibility complex), NURF (nucleosome remodeling factor), RSF (remodeling and spacing factor), and ToRC (Toutatis-containing chromatin remodeling complex) (Emelyanov et al., 2012; Hanai et al., 2008; Ito et al., 1997; Tsukiyama et al., 1995; Varga-Weisz et al., 1997). Although ISWI is able to carry out nucleosome remodeling, nucleosome rearrangement, and chromatin assembly reactions by itself *in vitro*, its association with a number of other proteins may function to regulate the ATPase activity or to confer specificity (Corona et al., 1999). In case of ACF, its two subunits Acf1 and ISWI function synergistically in the assembly of chromatin (Ito et al., 1999). Likewise, Chrac-14 and Chrac-16, the two additional subunits that distinguish CHRAC from ACF, have reported to facilitate ISWI/Acf1-mediated nucleosome sliding *in vitro* (Hartlepp et al., 2005; Kukimoto et al., 2004). In the NURF complex, ISWI is associated with three other cofactors, i.e. Nurf301, Nurf55, and Nurf38, where Nurf301 plays an important role in ISWI-mediated nucleosome sliding (Xiao et al., 2001). ToRC appears to require all three subunits, Toutatis (Tou), CtBP, and ISWI, for its maximal biochemical activity (Emelyanov et al., 2012). Finally, in the context of RSF, the p325/Rsf-1 subunit has been proposed to act as a histone chaperone by allowing nucleosome formation, while ISWI provides the energy for the nucleosome spacing activity (Loyola et al., 2003). Interestingly, overexpression of Rsf-1 has been associated with several types of cancer, including ovarian and breast carcinoma (Mao et al., 2006; Shih Ie et al., 2005). The contribution of Rsf-1 upregulation in this aspect could be the induction of DNA damage and promotion of genomic instability (Sheu et al., 2010).

The ISWI protein is well conserved in eukaryotes, as yeast contains two homologs, Isw1 and Isw2, while the mammalian genome also encodes two homologs known as SNF2h and SNF2l. Studies in humans and mice have revealed the presence of SNF2h in a number of distinct complexes similar to the ones reported in *Drosophila*, including ACF or WCRF (for Williams syndrome transcription factor-related chromatin remodeling factor), CHRAC, RSF, and the nucleolar remodeling complex NoRC, which resembles ToRC (Bochar et al., 2000; LeRoy et al., 2000; LeRoy et al., 1998; Poot et al., 2000; Strohner et al., 2001). The other ISWI homolog, SNF2l, has been reported to be the ATPase subunit of the human NURF complex (Barak et al., 2003). Apart from these, other complexes have been characterized such as WICH (WSTF-ISWI chromatin remodeling complex), which contains SNF2h and WSTF, and CERF (CECR2-

containing remodeling factor), which harbors SNF2l and CECR2 (Banting et al., 2005; Bozhenok et al., 2002). Thus, all described *Drosophila* ISWI-containing complexes are also found in higher eukaryotes, suggesting a key role for each of these complexes in the regulation of chromatin-mediated processes. It is interesting to note that so many different chromatin-remodeling complexes have evolved that contain an identical ATPase subunit. Functional diversification of these complexes could be attributed to the non-enzymatic subunits, which might have the ability to sense DNA adjacent to the nucleosome thereby providing distinct regulation that contributes to distinct cellular function (He et al., 2008).

The final outcome of the remodeling activities governed by ISWI-type complexes is the regulation of gene expression. ISWI has been implicated in both transcriptional activation and repression, where the final result is most likely determined by the proteins associated with ISWI. Initial genetic and biochemical studies have supported a role for ISWI in promoting transcription (Corona and Tamkun, 2004). However, the preferential localization of ISWI at transcriptionally silent chromatin together with microarray gene expression studies has suggested that ISWI also has an important role in transcriptional repression (Corona et al., 2007; Deuring et al., 2000). The mechanism used by ISWI to regulate gene expression is most likely determined by its ability to form regularly spaced nucleosome arrays on chromatin (Corona et al., 1999). However, how ISWI is targeted to chromatin to regulate expression of specific genes is not well known. Some data suggests a role for covalent histone modifications in tethering NURF to the template (Wysocka et al., 2006). Transcription factors, such as GAGA, might also be important for the recruitment of ISWI-containing complexes (Tsukiyama et al., 1995). Alternatively, subunits that are part of the complex including ISWI, like CtBP, could confer binding of the complex to chromatin *in vivo* (Emelyanov et al., 2012). However, chances are that multiple mechanisms exist which could be context-dependent.

Since ISWI has been found to be part of several different complexes, we were wondering whether we could identify additional ISWI-containing complexes and novel subunits characteristic for certain complexes. To test this in an unbiased way, we purified ISWI from *Drosophila* embryo nuclear extract followed by mass spectrometric analysis to identify the associated polypeptides. We identified at least four distinct complexes previously described by others, i.e. ACF/CHRAC, NURF, RSF, and ToRC. Furthermore, we unmasked two novel subunits of NURF, named dBap18 and dHMGXB4, and we also found association of *Drosophila* Rsf-1 (dRSF-1) with the BAP chromatin remodeler and physical interaction of Tou with the metabolic enzyme GMPS and its partner protein USP7. By comparing the genome-wide localization of the different ISWI-binding factors, we reveal colocalization of ISWI with primarily Nurf301, and only partial overlap with Acf1, dRSF-1 or Tou on larval salivary gland polytene chromosomes. Further characterization of their genomic distribution showed that dRSF-1 overlapped with Brm, a core BAP subunit, at the majority of the sites, whereas Tou specifically colocalized with GMPS and USP7 on several distinct loci. Taken together, our data confirms the presence of several distinct ISWI-containing complexes and shows differences in their genomic distribution, which suggests that each complex regulates the expression of a specific set of genes.

Results

ISWI is present in at least four distinct complexes

Drosophila ISWI had previously been shown to be part of a number of different complexes, which included ACF, CHRAC, NURF, RSF, and ToRC (Emelyanov et al., 2012; Hanai et al., 2008; Ito et al., 1997; Tsukiyama et al., 1995; Varga-Weisz et al., 1997). We were interested if there were still complexes or subunits that were missed in these previous studies. Therefore, we set out an unbiased proteomics screen in which we purified ISWI from *Drosophila* embryo nuclear extract followed by mass spectrometric analysis of the associated proteins. We identified a number of factors that are thought to be core components of different complexes, such as Acf1, Chrac-14, Nurf301, dRSF-1, and Tou which define ACF, CHRAC, NURF, RSF, and ToRC, respectively (Fig. 1A and Table S1). In order to establish whether these complexes are indeed separated, we also purified Acf1, Nurf301, dRSF-1, and Tou using highly specific antibodies. Mass spectrometric analysis of Acf1-associated proteins revealed the presence of ISWI, but not Nurf301, dRSF-1, or Tou, which demonstrates that ACF is distinct from other ISWI-containing complexes. In addition, we also found Chrac-14 and Chrac-16 to co-purify with Acf1, which are subunits of the CHRAC complex. However, we did not identify topoisomerase II (topo II), another reported CHRAC subunit, in our Acf1 purification (Varga-Weisz et al., 1997). ACF and CHRAC are, therefore, very similar in composition with the exception that CHRAC contains two extra subunits, i.e. Chrac-14 and Chrac-16. Given their related nature, ACF and CHRAC will hereafter be referred to as ACF/CHRAC. As expected, purification of Nurf301 revealed the association of ISWI and NURF signature subunits Nurf55 and Nurf38, which together define the NURF complex. Interestingly, we also identified two novel proteins, i.e. CG33695 and CG4617, which show homology to human BPTF associated protein of 18 kDa (BAP18) and HMG box domain containing 4 (HMGXB4), respectively. Since these proteins were also present in our ISWI purification and they both have been shown to associate with human NURF components as well we believe they represent novel subunits of the NURF complex (Vermeulen et al., 2010). *Drosophila* RSF-1 appeared to form a complex with ISWI only, as these two factors were the most predominant proteins identified in the dRSF-1 purification. Lastly, Tou associated with ISWI and CtBP, as reported previously (Emelyanov et al., 2012). Some components, such as Nurf38, were found in multiple purifications, but since we observe low amount of these proteins more often, we consider them as contaminants.

Given the fact that ISWI can associate with multiple proteins, we wondered whether their native size would correspond to what can be expected from their theoretical size. Therefore, we examined the native size of each complex by applying size-exclusion chromatography on the heparin-400 mM KCl (H0.4) fraction of *Drosophila* embryo nuclear extract, after which the eluate was resolved by SDS-PAGE followed by Western immunoblotting (Fig. 1B). ISWI seemed to be present in multiple fractions and co-eluted with all the major signature subunits that define the four distinct complexes in a way that would be expected for large multiprotein assemblages. When comparing individual elution profiles of the different proteins, it seemed that Nurf301 and Acf1 were present in a more broad range than dRSF-1 and Tou in a manner that overlapped with

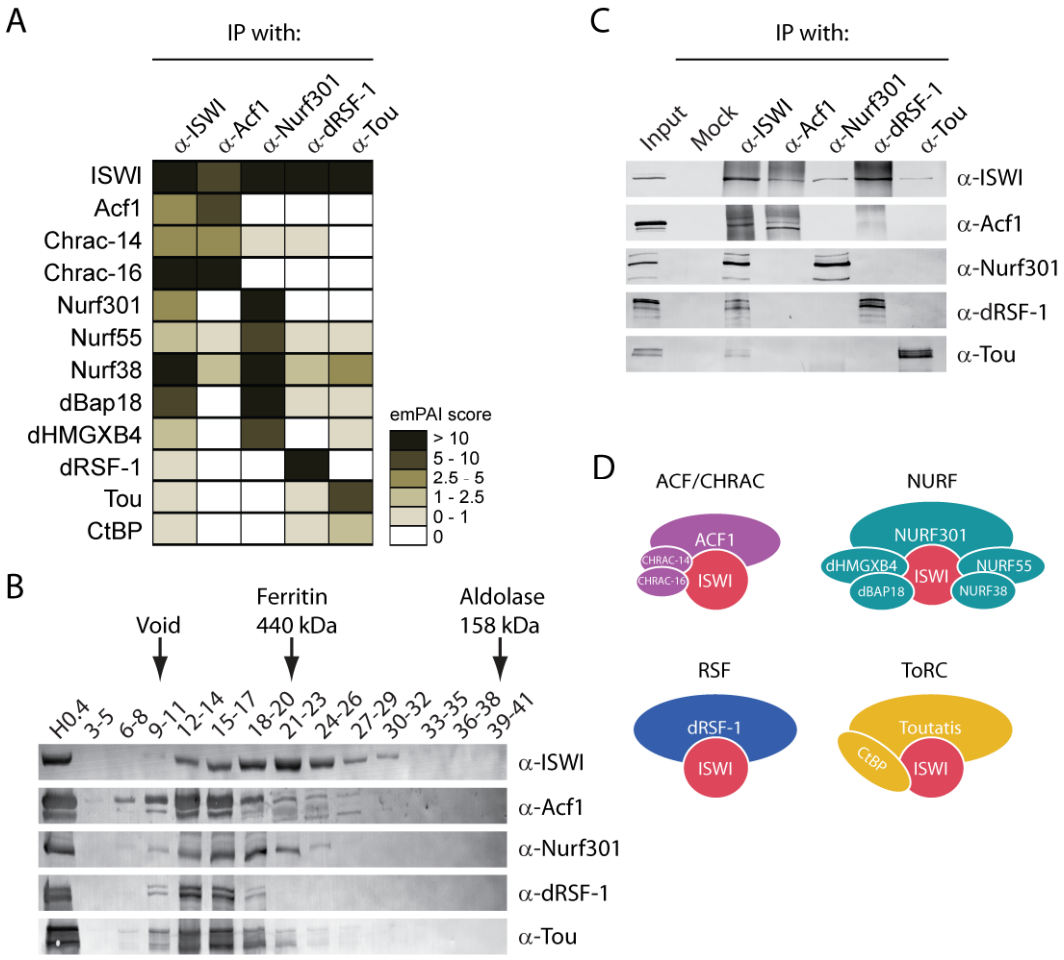

Figure 1. ISWI is present in four distinct complexes. (A) ISWI, Acf1, Nurf301, dRSF-1, and Tou were immunoprecipitated from *Drosophila* 0-12 hours embryo nuclear extracts using highly specific antibodies. Proteins were identified by mass spectrometry on an LTQ-Orbitrap hybrid mass spectrometer. Listed are the most prominent identified proteins which make up the different ISWI-containing complexes. Identified proteins are color-coded based on their assigned emPAI score. (B) Size-exclusion chromatography from the heparin-400 mM KCl fraction (H0.4 fraction) of embryo nuclear extract followed by SDS-PAGE and immunoblotting with the indicated antibodies. The elution of the voided volume (void) and the elution of the known markers Ferritin (440 kDa) and Aldolase (158 kDa) are indicated. Note that ISWI co-elutes with Acf1, Nurf301, dRSF-1, and Tou. (C) Co-immunoprecipitation of ISWI with Acf1, Nurf301, dRSF-1, and Tou. Crude *Drosophila* embryo nuclear extracts were incubated with preimmune serum (mock) or other antibodies as indicated. Immunopurified proteins were resolved by SDS-PAGE and analyzed by immunoblotting using the indicated antibodies. Two percent of the input material was loaded for reference. (D) Cartoon depicting the four different ISWI-containing complexes: ACF/CHRAC, NURF, RSF, and ToRC.

ISWI, suggesting that NURF and ACF are the most abundant complexes in embryo nuclear extract. However, ISWI could be detected in fractions that lacked Acf1 or Nurf301 as well, which indicates that free ISWI protein which is not incorporated in a complex could also exist. To

further confirm our findings, we performed immunoprecipitations (IPs) for ISWI, Acf1, Nurf301, dRSF-1, and Tou followed by identification of a selection of proteins by Western immunoblot analysis. IP for ISWI consistently revealed the association of Acf1, Nurf301, dRSF-1, and Tou (Fig. 1C). In contrast, Acf1, Nurf301, dRSF-1, and Tou co-precipitated ISWI only, which suggests that each of these factors is part of a distinct ISWI-containing complex that is mutually exclusive. Taken together, our data strongly suggest that ISWI can be part of at least four distinct complexes and have provided a more detailed picture of the NURF complex by identification of two novel subunits (Fig. 1D).

ISWI-associated proteins are closely related and share multiple functional domains

As ISWI is able to form several distinct protein complexes, we were interested in the nature of individual subunits of ACF/CHRAC, NURF, RSF, and ToRC. A closer look at the ISWI interacting proteins revealed a highly similar domain structure for Acf1, Nurf301, dRSF-1, and Tou (Fig. 2). All four proteins appeared to contain one or more plant homeodomain (PHD) zinc fingers (ZnFs) and a DDT (DNA binding homeobox and different transcription factors) domain, usually located at the N-terminus. ZnF domains are likely to be involved in binding DNA, RNA, protein or lipid substrates, whereas the DDT domain is predicted to be a DNA binding domain.

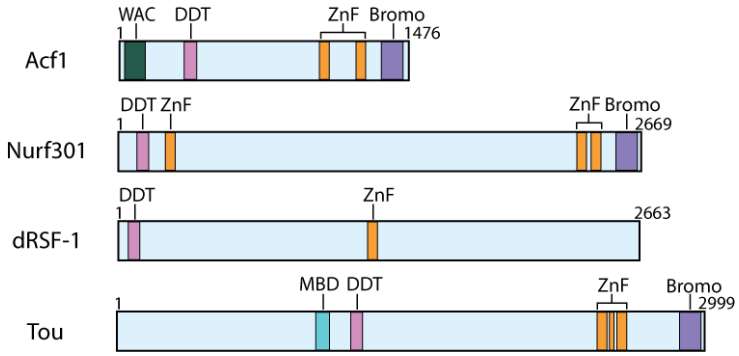

Figure 2. ISWI-interacting factors are proteins with similar domain structures. Schematic diagram depicting the domain structure of Acf1, Nurf301, dRSF-1, and Tou. All four proteins contain an N-terminal DDT domain and one or more Zn-fingers. Each factor, except dRSF-1, contains a C-terminal bromodomain, whereas Acf1 contains an additional WAC (WSTF/Acf1/cbp146) domain and Tou contains an MBD (methyl-CpG-binding domain).

In case of NURF, the PHD ZnF of BPTF, the human homolog of Nurf301, can recognize histone H3 tails trimethylated at lysine 4 (H3K4me3), thereby tethering the complex to chromatin (Wysocka et al., 2006). Except for dRSF-1, all proteins also contain a C-terminal bromodomain, which is thought to bind acetylated lysine residues (Jeanmougin et al., 1997). Apart from these characteristic domains, some proteins contain additional domains which appear to be unique. For example, Acf1 contains a WAC (WSTF/Acf1/cbp146) domain which has been proposed to mediate binding of Acf1 to DNA (Fyodorov and Kadonaga, 2002). Tou, on the other hand, harbors a methyl-CpG-binding domain (MBD) which is thought to be involved in

binding to DNA, preferentially to methylated CpG dinucleotides, but might mediate protein-protein interactions as well. MBD domains are often associated with transcriptional repressors, which is consistent with the fact that ToRC and NoRC, its human counterpart, can repress transcription of proneural and ribosomal RNA genes, respectively (Emelyanov et al., 2012; Santoro et al., 2002).

Since Acf1, Nurf301, dRSF-1, and Tou can all interact physically with ISWI, it is not unlikely that one of their common domains is involved in this interaction. One possible candidate is the DDT domain, as this domain is only found in Acf1, Nurf301, dRSF-1, and Tou in *Drosophila*. Moreover, for Acf1 it has been shown that deletion of a region encompassing the DDT domain severely impaired binding to ISWI (Eberharter et al., 2004; Fyodorov and Kadonaga, 2002). Acf1 appeared to bind to a C-terminal portion of ISWI that included the SANT and SLIDE domains (Eberharter et al., 2004). To our knowledge, the contribution of the DDT domain of Nurf301, dRSF-1, and Tou in mediating binding to ISWI has not been thoroughly investigated.

NURF is the predominant chromatin-associated ISWI complex in the salivary gland

The existence of at least four distinct ISWI complexes suggests that there must be some functional differences between them. One of the questions we wanted to address is how are these different complexes localized on chromatin *in vivo*? To answer this, we performed genome-wide localization analysis of all major ISWI-interacting factors on *Drosophila* larval salivary gland polytene chromosomes. Double fluorescent labeling of ISWI and Nurf301 revealed a striking colocalization where both factors co-occupied the vast majority of sites (Fig. 3A). In contrast, co-staining of ISWI with dRSF-1 showed only a partial overlap in their genomic distribution on polytene chromosomes (Fig. 3B). Likewise, ISWI colocalized with Tou on only a number of sites (Fig. 3C). Unfortunately, we were unable to directly compare the localization of ISWI with Acf1 on chromatin due to antibody issues. However, since ISWI colocalized with Nurf301 at most of its binding sites, we performed co-staining of Nurf301 with Acf1 (Fig. 3D). This revealed very limited colocalization of Acf1 with Nurf301 and, therefore, we do not expect to find much overlap of Acf1 with ISWI either. Thus, it seems that the four distinct ISWI-containing complexes display different genome-wide binding properties on chromatin and, in addition, our data suggests that NURF is the major complex associated with chromatin in the salivary gland.

Since dRSF-1, Tou, and Acf1 showed a different genomic distribution on polytene chromosomes and only partially colocalized with ISWI, we wondered whether they would colocalize with other factors. Our mass spectrometric analysis suggested that dRSF-1 not only interacts with ISWI, but also with the BAP chromatin remodeler (Fig. 4A and Table S2). To investigate whether dRSF-1 bound similar sites as BAP, we performed co-staining of dRSF-1 with Brm, the ATPase subunit of BAP. We observed extensive colocalization of both proteins, suggesting that they co-occupy similar sites (Fig. 4B). In contrast to this result, Tou overlapped with Brm on only a fraction of the bound regions (Fig. 4C). This again illustrates the binding specificity of the different ISWI-containing complexes. Like dRSF-1, Tou also appeared to have more binding partners, as we identified the metabolic enzyme GMPS and the deubiquitylating enzyme USP7 in our Tou purification (Fig. 5A and Table S2). These two proteins have been de-

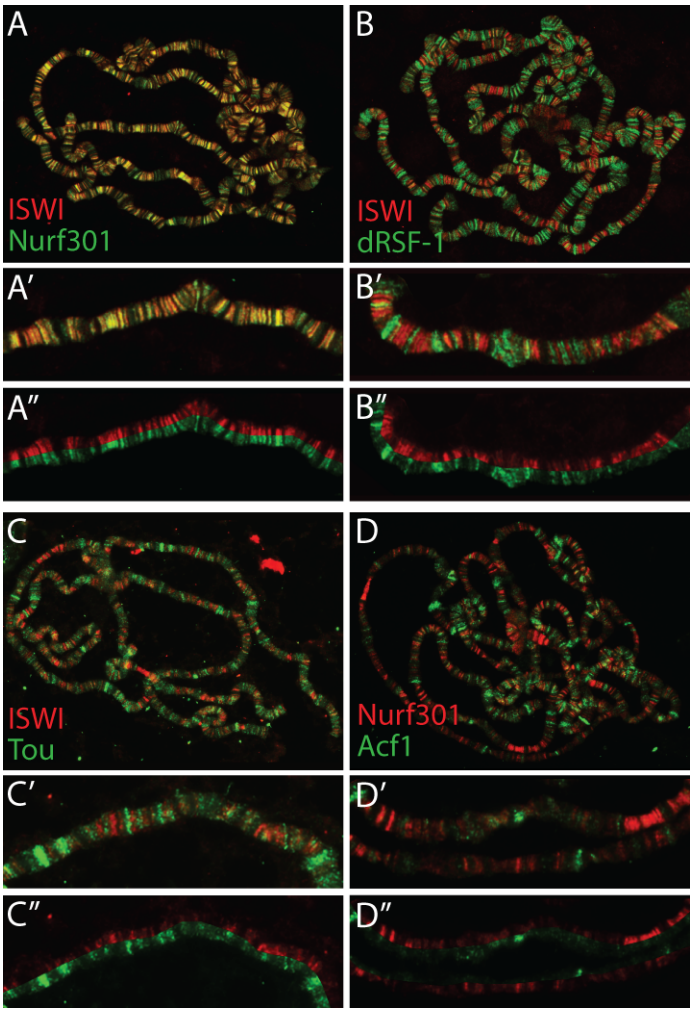

Figure 3. ISWI colocalizes primarily with Nurf301 on polytene chromosomes. (A-C) Double fluorescent labeling of ISWI with Nurf301, dRSF-1, and Tou on polytene chromosomes. (A) Co-staining of ISWI (in red) with Nurf301 (in green). (B) C-staining of ISWI (in red) with dRSF-1 (in green). (C) Co-staining of ISWI (in red) with Tou (in green). (D) Co-staining of Nurf301 (in red) with Acf1 (in green) on polytene chromosomes. Representative magnifications are shown below the panels, with either zooms including merged (‘) or splitted (“) channels. Note that ISWI colocalizes predominantly with Nurf301, but not with dRSF-1 or Tou, which is demonstrated by the yellow staining and overlapping patterns in the magnified sections. Direct comparison of the localization of ISWI and Acf1 could not be performed, but as Acf1 shows little overlap with Nurf301, it most likely does not overlap well with ISWI either.

monstrated to form a stable complex in *Drosophila* and to bind chromatin (van der Knaap et al., 2005). As GMPS and USP7 associated with Tou in embryo nuclear extracts, they could in principle bind similar sites on the chromatin as well. To test this, we co-stained Tou with either GMPS or USP7 on polytene chromosomes (Fig. 5B and C). Although Tou clearly bound sites which were not stained by GMPS or USP7, a number of loci were co-occupied by both Tou and GMPS or USP7. Our results have shown that the genomic localization of Acf1 was quite different from that observed for ISWI. However, we have not studied the localization of Acf1 in more detail. Thus, our results suggest that NURF is the main ISWI-containing complex in the salivary gland that is associated with polytene chromosomes. Furthermore, we detect largely overlapping genomic distributions of dRSF-1 and Brm, whereas Tou specifically colocalizes with GMPS and USP7 on a number of loci.

ISWI-interacting factors remain stable in the absence of ISWI protein

Subunits that are part of multiprotein complexes often fulfill several functions within their complex. For instance, they can have enzymatic activity, contribute to the chromatin binding specificity of the complex or have an architectural role. In this case, ISWI is the enzymatic com-

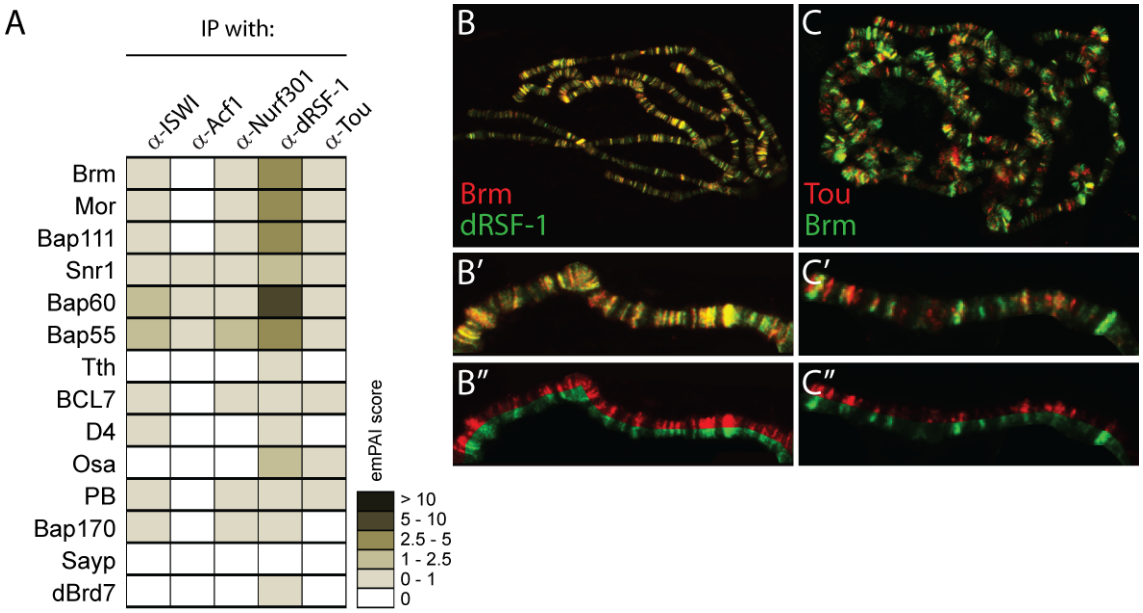

Figure 4. dRSF-1 interacts and colocalizes with Brm. (A) Identification of subunits of the BAP and PBAP complex as additional dRSF-1 interacting factors by mass spectrometric analysis. The way of representation is identical to that shown in Figure 1A. (B) Co-staining of dRSF-1 (in green) and Brm (in red) on polytene chromosomes. A high degree of overlap between the two proteins is observed. (C) Co-staining of Tou (in red) with Brm (in green). Here, only partial overlap is observed. Representative magnifications are shown below the panels, with either zooms including merged (‘) or splitted (“) channels.

ponent that contains the ATPase activity necessary for nucleosome remodeling, whereas other associated factors that define ACF/CHRAC, NURF, RSF, or ToRC could regulate binding specificity as we observed differences in the binding of each distinct ISWI-containing complex to chromatin. However, since ISWI is a core component of each of the four assemblies, we were interested if depletion of ISWI by RNAi-mediated knockdown could affect the stability of its binding partners. Therefore, we knocked down ISWI in S2 cells followed by analysis of the proteins levels of ISWI, Acf1, Nurf301, dRSF-1, and Tou by Western immunoblot. Knockdown of ISWI efficiently depleted its corresponding protein levels (Fig. 6). In contrast, we did not observe any changes in the levels of Acf1, Nurf301, dRSF-1, or Tou. This result suggested that physical association with ISWI does not increase the stability of the respective protein. To investigate the contribution of signature subunits to the stability of other ISWI complex components, we systematically knocked down Acf1, Nurf301, dRSF-1, and Tou. The knockdown of all factors, except Acf1, resulted in a marked reduction in their expression level. However, knockdown of either Nurf301, dRSF-1, or Tou did not affect the protein levels of ISWI nor did it affect expression of other signature subunits. Therefore, it appears that the stability of individual ISWI complex components is not dependent on the presence of subunits from other distinct ISWI complexes. Unfortunately, we could not address the role of Acf1 in this aspect as we failed to efficiently knockdown Acf1. In summary, our results suggest that the stability of

individual ISWI-interacting proteins is not affected by the absence of ISWI or components of other ISWI-containing complexes. In addition, the data presented here suggest that ISWI-binding factors can exist without the presence of ISWI.

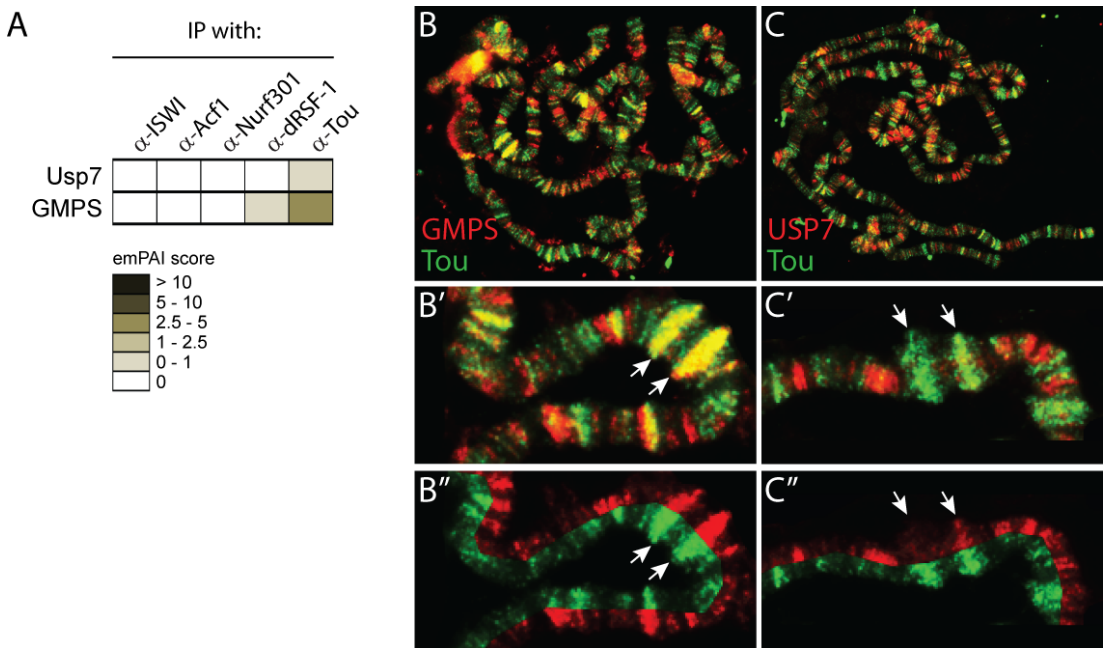

Figure 5. Toutatis interacts and colocalizes with GMPS and USP7 on selective loci. (A) Identification of Usp7 and GMPS as additional Tou interacting factors by mass spectrometric analysis. The way of representation is identical to that shown in Figure 1A. (B) Co-staining of Tou (in green) and GMPS (in red) on polytene chromosomes. Colocalization of the two proteins is observed on selective loci, as indicated by the arrows. (C) Same as B, but this time Tou (in green) is co-stained with USP7 (in red). Here, overlap is observed on specific sites as well, albeit to a lesser extent as compared to Tou and GMPS. Arrows indicate the same regions as in B. Representative magnifications are shown below the panels, with either zooms including merged (‘) or splitted (“) channels.

Discussion

The main goal of this project was to make an inventory of the different ISWI chromatin remodeling complexes in *Drosophila*. In order to do so, we have taken an unbiased proteomics approach in which we purified ISWI from embryo nuclear extract. By this means, we identified a number of ISWI-associated factors which we have verified by reciprocal IPs followed by mass spectrometric analysis. Our results suggest that ISWI can be part of at least four distinct complexes, i.e. ACF/CHRAC, NURF, RSF, and ToRC. In addition, we picked up two novel subunits of the NURF complex, which we named dBap18 and dHMGXB4. To compare the four distinct ISWI-containing complexes, we determined their genome-wide localization on chromatin by staining of larval salivary gland polytene chromosomes. This led to the observation that

Nurf301 predominantly colocalizes with ISWI, whereas dRSF-1, Tou, and Acf1 only partially overlapped with ISWI. Therefore, we propose that the majority of ISWI is incorporated in the NURF complex in salivary gland tissue to regulate nucleosome remodeling and gene expression. *Drosophila* RSF-1 protein colocalized extensively with Brm, which is consistent with the fact that these factors can interact physically. Likewise, Tou appeared to associate with GMPS and USP7 and also co-occupied a number of loci with these two proteins on polytene chromosomes. Lastly, knockdown experiments in S2 cells have shown that depletion of ISWI does not affect the stability of ISWI-interacting factors and vice versa, suggesting that subunits of the different complexes can exist in the absence of ISWI. In summary, *Drosophila* has at least four distinct ISWI-containing complexes that show different binding specificities on chromatin and are likely to regulate a divergent set of genes.

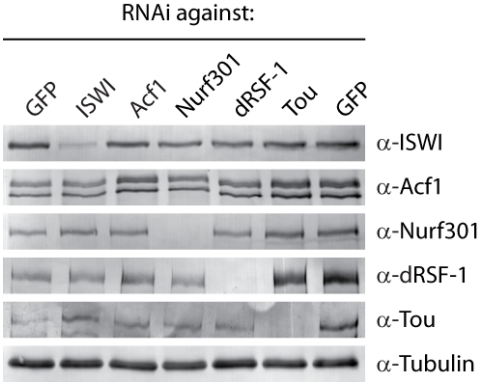

Figure 6. Knockdown of ISWI does not affect the stability of its interacting partners. S2 cells were treated with dsRNA against ISWI, Acf1, Nurf301, dRSF-1, Tou, or GFP (= mock) after which whole cell lysates were prepared and analyzed for knockdown efficiency by Western immunoblotting using the indicated antibodies. Tubulin served as a loading control and indicates equal loading. Note that we failed to deplete Acf1 protein levels efficiently.

Over the past number of years, several complexes have been identified that were reported to contain the ISWI ATPase. These include ACF, CHRAC, NURF, RSF, and ToRC (Emelyanov et al., 2012; Hanai et al., 2008; Ito et al., 1997; Tsukiyama et al., 1995; Varga-Weisz et al., 1997). Our mass spectrometry screen for ISWI-interacting factors is consistent with this earlier work, as we also identified all known complexes. However, our CHRAC complex seemed to comprise the subunits ISWI, Acf1, Chrac-14, and Chrac-16, but lacked topo II, which was initially shown to be a component of CHRAC (Varga-Weisz et al., 1997). Later studies have refined the composition of CHRAC, and it is now generally accepted that CHRAC does not contain topo II (Eberharter et al., 2001). This is also consistent with work on the human complex, which lacked topo II as well (Poot et al., 2000). Apart from ACF/CHRAC, we identified NURF as a major ISWI complex in *Drosophila*. Our data is in agreement with its reported complex composition, where NURF was shown to comprise ISWI, Nurf301, Nurf55, and Nurf38 (Tsukiyama et al., 1995). Interestingly, we also found two novel subunits, a SANT domain protein and an HMG box protein named dBap18 and dHMGXB4, respectively. These two proteins consistently co-purified with both ISWI and Nurf301, but not with Acf1, dRSF-1, or Tou. Furthermore, both proteins have been shown to associate with the human NURF complex as well (Vermeulen et al., 2010). Thus, we present dBap18 and dHMGXB4 as two novel *bona fide* subunits of NURF. Characterization of RSF, yet another ISWI-containing complex, has demonstrated that it is

composed of only ISWI and RSF-1, in both human and *Drosophila* (Hanai et al., 2008; LeRoy et al., 1998). Analysis of dRSF-1 associated proteins by mass spectrometry also revealed the presence of predominantly ISWI which, therefore, confirms these statements. Besides ISWI, we also identified a significant amount of the BAP chromatin-remodeling complex in our dRSF-1 preparations. We do not consider dRSF-1 as a stable component of BAP, but rather suggest that it can associate with BAP under certain conditions. Lastly, investigation of the interacting partners of Tou identified ISWI and CtBP, which are the three subunits that define ToRC (Emelyanov et al., 2012). Although CtBP could be co-purified together with Tou and ISWI, the interaction appeared to be substoichiometric, as judged by the assigned mascot and emPAI scores. Based on this, together with the fact that Tou does not localize properly on chromatin in the absence of CtBP (Emelyanov et al., 2012), we propose that CtBP is not a core component of ToRC, but rather associates with the complex to direct binding to specific regions on the chromatin. Alternatively, there could be two separate Tou-containing complexes as has been proposed by Emelyanov et al., i.e. one that contains both ISWI and CtBP and another that contains only ISWI. To clarify this, more detailed analysis is required though.

The distinct ISWI complexes share their enzymatic motor subunit, but yet appear to have different biological functions which are likely to be attributed to their signature subunits. ACF and CHRAC can both mediate chromatin assembly through the formation of regularly spaced nucleosome arrays *in vitro* (Ito et al., 1997; Varga-Weisz et al., 1997). Acf1 and ISWI function synergistically in this aspect, where Acf1 is thought to promote nucleosome sliding and alter the directionality of nucleosome movement by ISWI, probably by its interaction with core histones through its PHD ZnF domains (Eberharter et al., 2001; Eberharter et al., 2004). Likewise, the histone-fold proteins Chrac-14 and Chrac-16 facilitate nucleosome sliding by ISWI, but appear to do so by weakly interacting with the DNA rather than binding to the histones (Hartlepp et al., 2005). Both ACF and CHRAC are considered to repress transcription *in vivo* by promoting formation of periodic nucleosome arrays (Fyodorov et al., 2004). The remodeling activity of ISWI within the NURF complex is regulated in part by Nurf301, which can stimulate histone octamer sliding carried out by ISWI (Xiao et al., 2001). Furthermore, Nurf301 can associate with histone tails modified by acetylation (H4K16ac) or methylation (H3K4me2/3) through its bromodomain and PHD ZnF domains, respectively, thereby providing a means of binding specificity (Kwon et al., 2009; Wysocka et al., 2006). Another level of specificity could result from interaction of Nurf301 with sequence-specific transcription factors, such as GAGA and heat shock factor (Hsf) (Badenhorst et al., 2002; Okada and Hirose, 1998; Tsukiyama et al., 1995). The role of Nurf55 and Nurf38 within NURF is not exactly understood, although they do not seem to be required for the activity *in vitro* as Nurf301 and ISWI alone are sufficient for ATP-dependent remodeling of reconstituted chromatin (Xiao et al., 2001). The biological function of NURF *in vivo* is thought to be mainly activation of transcription, as has been demonstrated for e.g. heat shock genes and homeotic genes in *nurf301* mutant flies (Badenhorst et al., 2002). The RSF remodeler can both assemble and remodel chromatin in a test tube, thereby creating a periodic array of nucleosomes similar as ACF/CHRAC (LeRoy et al., 1998). However, one major difference between RSF and ACF/CHRAC is that the latter is thought to cooperate with histone chaperone NAP-1 in chromatin assembly, whereas RSF can do the job all by itself (Ito et al.,

1997; Loyola et al., 2001). The large subunit of RSF, dRSF-1, probably acts as a chaperone allowing nucleosome formation while ISWI provides the energy for nucleosome spacing activity (Loyola et al., 2003). Although RSF was initially found to be required for transcription activation *in vitro* its *in vivo* function could be the opposite, as dRSF-1 mutant flies behave as a dominant suppressor of position effect variegation (Hanai et al., 2008; LeRoy et al., 1998). In this case, RSF could regulate silent chromatin formation by directing exchange of histone H2A for the histone variant H2Av by physical interaction with the Tip60 complex (Hanai et al., 2008). Interestingly, a similar function has been assigned to RSF in humans, where RSF has been implicated to actively participate in the incorporation of the histone variant CENP-A in chromatin at the centromere (Perpelescu et al., 2009). In the context of ToRC, chromatin assembly mediated by ISWI appears to be stimulated by the other two subunits, Tou and CtBP (Emelyanov et al., 2012). The mechanism by which this occurs is not exactly known though. The role of ToRC-mediated chromatin assembly *in vivo* could be both activation of transcription and transcriptional repression (Emelyanov et al., 2012; Vanolst et al., 2005). The outcome might depend on interaction of the ISWI-Tou dimer with CtBP, which is a transcriptional corepressor. However, since the current available data is limited to only few genes, more detailed analysis is required to assign a biological function to chromatin remodeling by ToRC. It is noteworthy to mention that the human homolog of ToRC, NoRC, plays a crucial role in silencing of ribosomal RNA genes, probably through recruitment of DNA methyltransferase and histone deacetylase activity to the ribosomal DNA promoter (Santoro et al., 2002). Both TIP5 and its fly homolog, Tou, contain a methyl-CpG-binding domain which links these proteins to DNA methylation. Although the functional significance of DNA methylation in mammals is widely accepted, its contribution in *Drosophila* is highly controversial.

In this study, we have determined the genome-wide localization of ACF/CHRAC, NURF, RSF, and ToRC on polytene chromosomes. Our analysis suggests diverse binding specificities for each complex as we observe differences in their genomic distribution. For instance, Nurf301 colocalized predominantly with ISWI, whereas dRSF-1, Tou, and Acf1 overlapped only partially with ISWI. Furthermore, dRSF-1 shared many binding sites with Brm and could also associate directly with the Brm-containing BAP complex by physical interaction. In contrast, Tou showed restricted overlap with Brm on polytene chromosomes, but instead colocalized with GMPS and USP7 on specific loci. Interestingly, Tou could associate with these two proteins as well since we identified them in our Tou purification. Likewise, reciprocal IPs for GMPS and USP7 could retrieve Tou by mass spectrometry, suggesting their interaction is not simply an artifact (Jan van der Knaap, unpublished data). An important question that arises from these data is why would there be so many different complexes containing an identical ATPase subunit? A possible answer to this could be that every complex affects chromatin structure at different positions in the genome in order to regulate expression of a distinct set of genes. This would be in line with our localization study, where all four complexes show a different distribution on polytene chromosomes. However, our results also imply that a number of sites are occupied by the signature subunits, but not ISWI, as is the case for Acf1, dRSF-1, and Tou. Whether the different subunits can function without ISWI remains to be determined. Alternatively, NURF could be the major ISWI-containing complex in larval salivary glands. This

is in line with genetic data, where mutations in *iswi* or *nurf301* cause a similar phenotype with aberrant condensation of the male X chromosome in salivary gland tissue (Badenhorst et al., 2002; Deuring et al., 2000). Thus, it is also possible that the different remodeling complexes have restricted expression patterns in a tissue-specific manner. Since Acf1, Nurf301, dRSF-1, and Tou are likely to compete for binding to ISWI, e.g. by direct interaction of the DDT domain with the C-terminus of ISWI (Eberharter et al., 2004), the relative abundance of each factor could determine which complex is formed. However, to support this statement, a comprehensive comparison of the expression patterns of each of the complex components in different tissues is required.

In summary, we have systematically analyzed and compared the complexes formed by the DNA-dependent ATPase ISWI in *Drosophila* embryo nuclear extract. We have found ISWI to be present in at least four distinct complexes: ACF/CHRAC, NURF, RSF, and ToRC. Furthermore, we uncovered significant differences in the genome-wide localization of each of these complexes, where Nurf301 mainly colocalized with ISWI, whereas other signature subunits showed only limited overlap with the enzymatic motor. Therefore, our results suggest functional differences between each of the four ISWI-containing complexes. It will be interesting to reveal the transcriptional circuitries controlled by ACF/CHRAC, NURF, RSF, and ToRC, to gain more insight into their biological functions *in vivo*.

Experimental procedures

Antibodies and immunological procedures

Polyclonal antibodies were generated by immunizing guinea pigs or rabbits with GST fusion proteins expressed in *Escherichia coli* and were affinity purified as described previously (Chalkley and Verrijzer, 2004). The following antigens were used: Acf1 amino acids (aa) 1280-1462, Nurf301 aa 361-673 and 2016-2234, dRSF-1 aa 2049-2390, and Toutatis (Tou) aa 60-390 and 2751-2999. Other antibodies have been described, including α-ISWI and α-Brm (Kal et al., 2000), α-USP7 and α-GMPS (van der Knaap et al., 2010), and anti-α-Tubulin (T8203, Sigma).

Polytene chromosome immunostaining

The analysis of polytene chromosomes was performed essentially as described previously (Armstrong et al., 2002) with minor modifications. Briefly, salivary glands from *Drosophila* 3rd instar larvae were dissected in 0.7% NaCl after which the glands were fixed in 45% acetic acid/1.85% formaldehyde for 1 to 2 minutes at room temperature. Fixed tissue was transferred onto coated poly prep slides (Sigma) after which the glands were squashed and slides were frozen in liquid nitrogen. Squashes were incubated with blocking buffer (1% BSA dissolved in PBS/0.1% Triton X-100) overnight at 4°C. Next, squashes were incubated with primary antibody (α-ISWI, 1:100; α-Acf1, 1:50; α-Nurf301, 1:200; α-dRSF-1, 1:200; and α-Tou, 1:100) in blocking buffer for 1 hour at room temperature in a humid chamber. Slides were washed three times with PBS/0.1% Triton X-100 and the squashes were subsequently incubated with appropriate secondary antibodies (Alexafluor, Molecular Probes) diluted 1:500 in blocking buffer for 1 hour

at room temperature. Finally, slides were washed again three times with PBS/0.1% Triton X-100 and mounted in Vectashield with DAPI (Vector Laboratories, Inc.). Images were captured by using a Leica DM-RXA microscope and processed by using Photoshop.

Protein purification and mass spectrometry

Nuclear extracts were prepared from 0 to 12 hours-old *Drosophila* embryos as described (Kamakaka and Kadonaga, 1994). Immunopurification procedures using specific antibodies directed against ISWI (PV14), Acf1 (GR719), Nurf301 (GR710), dRSF-1 (GR773), and Tou (GR824) and mass spectrometric analysis were all performed as described previously (Chalkley et al., 2008; Chalkley and Verrijzer, 2004). Briefly, extracts were incubated for 2 hours at 4°C with α-ISWI, α-Acf1, α-Nurf301, α-dRSF-1, or α-Tou antibodies coupled to Protein A-Sepharose beads (GE Healthcare). Next, beads were washed twice with HEMG buffer (25 mM HEPES-KOH [pH 7.6], 0.1 mM EDTA, 12.5 mM $MgCl_2$, 10% glycerol, 0.1% NP-40, 1 μg/ml of leupeptin, aprotinin and pepstatin and 0.2 mM AEBSF [(α-aminoethyl)benzenesulfonyl fluoride]) containing 100 mM KCl (HEMG/100), 5 times with HEMG/600, twice with HEMG/100 containing 0.01% NP-40, and finally once with 100 mM KCl. Affinity purified proteins were subsequently eluted with Glycine buffer (100 mM glycine, 150 mM NaCl) pH 2.5. Eluted proteins were TCA precipitated and dissolved in 1x SDS sample buffer after which the proteins were resolved by SDS-PAGE and visualized by Coomassie blue staining. Polypeptides were identified by mass spectrometry on an LTQ-Orbitrap hybrid mass spectrometer (ThermoFischer). Typical contaminants, also present in immunopurifications using beads coated with pre-immune serum or antibodies directed against irrelevant proteins, were omitted from the dataset.

For small scale co-immunoprecipitations, 15 μl of crude serum was coupled to 20 μl of Protein A-Sepharose which was subsequently incubated with ~2 mg *Drosophila* embryo nuclear extract for 2 hours at 4°C. Beads were washed and proteins were eluted similar as described above. Eluted proteins were resolved by SDS-PAGE and visualized by Western immunoblotting with appropriate antibodies.

For analysis of native protein sizes, the crude *Drosophila* embryo nuclear extracts were further fractionated using a Sephacryl S-300 column (Pharmacia) as described previously (Mohrmann et al., 2004). Briefly, nuclear extracts were concentrated by chromatography on a POROS-heparin (PerSeptive Biosystems) column equilibrated with HEMG/100 buffer (25 mM HEPES-KOH [pH 7.6], 0.1 mM EDTA, 12.5 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol [DTT], 0.2 mM AEBSF, 1 μM pepstatin, 0.01% Nonidet P-40 [NP-40], 100 mM KCl), followed by a step elution with HEMG/400 buffer (HEMG/100 buffer with 400 mM KCl instead of 100 mM KCl). The heparin-400 mM KCl fraction (H0.4 fraction) was loaded onto an 800-ml Sephacryl S-300 column (elution volume, 300 ml) equilibrated and developed with HEMG/100 buffer. Fractions were separated by SDS-PAGE and analyzed by immunoblotting with α-ISWI, α-Acf1, α-NURF301, α-dRSF-1, α-Tou, α-Brm, α-USP7, and α-GMPS antibodies.

Cell culture and RNA interference

Drosophila Schneider's line 2 cells (hereafter S2 cells) were cultured in Schneider's medium (Invitrogen) supplemented with 10% fetal calf serum (Thermo) and 1% Penicillin-Streptomycin.

Cells were treated with double-stranded RNA (dsRNA) for 4 days, as described previously (Worby et al., 2001). Double-stranded RNAs were synthesized using the Megascript T7 kit (Ambion).

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Supplementary information

Table S1. List of ISWI, Acf1, Nurf301, dRSF-1 and Tou associated proteins identified by mass spectrometry.

Protein identity	M.W. (kDa)	ISWI		Acf1		Nurf301		dRSF-1		Tou	
		Mascot	*emPAI*	*Mascot*	*emPAI*	*Mascot*	*emPAI*	*Mascot*	*emPAI*	*Mascot*	*emPAI*
ISWI	119	4420	15.95	3856	9.42	6184	37.77	5487	35.27	4529	17.01
Acf1	170	3640	4.91	4265	5.04						
Chrac-14	14	488	4.72	388	3.46	59	0.26	80	0.28		
Chrac-16	16	443	24.68	406	12.42						
Nurf301	301	6017	3.95			11636	17.18				
Nurf55	49	903	2.49	74	0.16	1324	5.84	458	0.55	376	0.67
Nurf38	38	1338	25.34	728	2.32	1698	79.4	557	2.32	578	2.7
dBap18	32	735	5.46			988	12.04	77	0.13	167	0.45
dHMGXB4	46	679	1.19			1265	5.72			96	0.17
dRsf-1	304	2323	0.73					12514	30.34		
Toutatis	322	1493	0.42					58	0.01	8163	8.53
CtBP	42	168	0.4					65	0.09	620	1.52

Table S2. Additional ISWI, Acf, Nurf301, dRSF-1 and Tou associated proteins identified by mass spectrometry.

Protein identity	M.W. (kDa)	ISWI		Acf1		Nurf301		dRSF-1		Tou	
		Mascot	*emPAI*	*Mascot*	*emPAI*	*Mascot*	*emPAI*	*Mascot*	*emPAI*	*Mascot*	*emPAI*
Brahma	185	140	0.08			81	0.02	3234	2.87	144	0.06
Moira	131	475	0.28			831	0.43	2680	4.69	435	0.25
Bap111	79	297	0.26			183	0.14	1448	4.24	301	0.38
Snr1	42	318	0.81	51	0.09	154	0.27	698	1.78	470	0.98
Bap60	58	571	1.53	93	0.13	264	0.33	1770	8.85	376	0.64
Bap55	47	533	1.3	397	0.7	745	1.51	974	3.22	613	0.98
Tth	45							146	0.27		
BCL7-like	17	82	0.23			88	0.22	112	0.53	113	0.52
D4	55	49	0.1					500	0.78		
Osa	284							2726	1.05	97	0.03
Polybromo	190	345	0.14			320	0.13	593	0.21	238	0.1
Bap170	183	160	0.08			41	0.02	107	0.04		
Sayp	213										
dBrd7	96							154	0.08		
Usp7	130									779	0.43
GMPS	77							63	0.05	1538	2.54

Table S3. Primers used to generate dsRNA.

Target	Forward primer	Reverse primer
GFP	ATGGTGAGCAAGGGCGAG	CTTGTACAGCTCGTCCATGC
Acf1	GTAAAACCTGCAAGCCGATG	ACATCGATCGATCACGAATCG
Nurf301	CTACTGAGGATTGGCAGTGC	CAGAAGCCGTCGTTAACGAG
dRSF-1	GTTGCCACGAACAGGGAC	CATGGAGCATTGGATCTGC
Tou	ATGCCCGGCGTCTATCCA	TCAGGAGTGCTTGTCAGTCAG

Chapter 7

General Discussion

Transcription of genes by DNA-dependent RNA polymerases is essential for all living organisms. It directly controls the abundance of RNA and contributes to the amount of protein within the cell. Both RNA and protein have a limited life time, which emphasizes the need for continuous synthesis of these two types of molecules. However, tight regulation of transcription is crucial for maintaining a proper balance in gene expression levels at any given time to ensure cellular homeostasis. Competition between activators and repressors is a major determining factor in regulating the final transcriptional output. Many different proteins play a role in transcriptional control, some of which may have a relatively well defined role, while the function of others may be largely unexplored. Obtaining a detailed picture of how each factor contributes to the transcription process is important for understanding the mechanisms of gene expression control and might help in identifying drug targets for the treatment of diseases. This thesis illustrates the role of various chromatin binding factors in regulating transcription of protein-coding and non-coding genes, such as chromatin remodelers Kis-L and (P)BAP, the epigenetic Polycomb group proteins, the 26S proteasome complex, and bromodomain protein dBRD4 and the Mediator co-activator.

Chapter 2 describes the purification and functional characterization of *Drosophila* Kis-L and its interacting proteins. Initial work had identified the *kis* gene to interact genetically with mutations in *Pc* (Kennison and Tamkun, 1988). Specifically, mutations in *kis* obtained by ethyl methanesulfonate (EMS) or γ-rays treatment suppressed mutations in the *Pc* gene. Further characterization of *kis* revealed that mutations in this gene suppressed *Pc* mutant phenotypes by blocking ectopic transcription of homeotic genes (Daubresse et al., 1999). The *kis* gene was shown to encode two isoforms, i.e. Kis-S and Kis-L, both of which share a conserved domain, the BRK domain, with the chromatin remodeling factor Brm. However, only Kis-L contains other functional domains associated with chromatin remodeling enzymes, such as an ATPase domain and two chromodomains. Therefore, Kis-L was placed in the CHD family of chromatin remodelers. Since most of the remodelers known to date function as part of multimeric complexes, investigations were performed to find out whether this was true for Kis-L as well (for an overview see Fig. 1). Indeed, gel filtration chromatography analysis suggested that Kis-L was part of a large complex of about 1 MDa in size (Srinivasan et al., 2005). However, later re-examination of these results using sucrose density gradients revealed a significantly lower native molecular mass of Kis-L which appeared to correspond to the monomeric protein (Srinivasan et al., 2008). Thus, it was concluded that Kis-L does not form a complex, but rather acts alone to regulate transcription. We started out differently by using a proteomics approach to identify possible Kis-L and Kis-S interacting proteins. Our results are indicative for a physical interaction of Kis-L with the Brm-containing BAP and PBAP complexes. These data are further confirmed by performing IP-Westerns and size-exclusion chromatography. The observed interactions seem specific for Kis-L, as Kis-S does not interact with (P)BAP.

It is interesting to point out that Tamkun and colleagues already investigated a possible physical interaction between Kis-L and other remodelers, including Brm, by IP-Western. They did not, however, succeed to find Kis-L to be associated with Brm (Srinivasan et al., 2005). One possible explanation for the discrepancy between these and our own results could be due to a

different experimental setup. We have found that in order to pick up interactions by IP-Westerns, a lot of optimization is required. Furthermore, we also observe relatively weak, substoichiometric, interactions between Kis-L and Brm. However, our mass spectrometric analysis consistently identifies Brm as one of the major proteins present in the Kis-L purifications. Since Brm is a rather large protein, it is possible that it is prone to degradation. We have made similar observations for Kis-L, which seems to be degraded to a certain extent in cell extracts. For Western immunoblot detection this can be a major problem, but for mass spectrometry this does not form a real obstacle, since proteomic analysis relies on the identification of small peptides rather than intact protein. Our own data is further strengthened by the fact that we can identify other (P)BAP components to be associated with Kis-L and that Kis-L colocalizes with (P)BAP on polytene chromosomes in a genome-wide fashion. The physical interaction of Kis-L with (P)BAP appears to be conserved, as CHD7, the human ortholog of Kis-L, was found to interact with the PBAF complex in human neural crest cells (Bajpai et al., 2010). Interestingly, both remodelers seemed to cooperate in regulating gene expression in the neural crest. We have analyzed the transcriptional circuitries controlled by both Kis-L and Brm in *Drosophila* S2 cells. Our results suggest that Kis-L and Brm can cooperate in gene expression control as well. However, we also reveal that both factors can function antagonistically, depending on the gene context. Therefore, we propose that Kis-L and (P)BAP physically associate and co-occupy genomic loci to fine-tune gene expression. We do not believe that Kis-L binds the complete (BAP and) PBAP complex, but rather consider it to interact with a subset of (P)BAP based on our mass spectrometry and IP-Western data. It would be interesting to corroborate this with reciprocal IP-Western or -mass spectrometry experiments using antibodies directed against any of the (P)BAP core and signature subunits. Genome-wide ChIP-seq analysis for Kis-L and (P)BAP would also be very informative to infer direct target genes, which is sometimes difficult when performing only genome-wide expression analysis due to secondary effects caused by the treatment. Lastly, since Kis-L is a putative chromatin remodeling enzyme it would be useful to perform remodeling assays with the protein. Experiments with human CHD7 have shown that it functions as a nucleosome remodeling factor and that mutations in CHD7 associated with CHARGE syndrome patients affect its ability to remodel chromatin (Bouazoune and Kingston, 2012).

Chapter 3 deals with the mechanism of gene silencing by Polycomb repressive complex 1. Although it is known for a long time that PcG factors maintain a silenced gene expression state, it is not well understood how this is accomplished in a mechanistic way. Several different mechanisms have been proposed by which PRC1 represses transcription (Simon and Kingston, 2013). One of these could involve monoubiquitylation of histone H2A on K118. This modification is, however, placed on histone H2A by a PRC1-like complex named dRAF, which contains dRing and Psc, but lacks the Pc and Ph subunits (Lagarou et al., 2008). This complex also contains an additional subunit, dKDM2, which stimulates dRAF's ability to ubiquitylate H2A and functions as a demethylase to erase the active H3K36me2 mark as well. As H3K36me2 modified histones are rarely found to co-exist with the PRC2-induced H3K27me3 mark (Yuan et al., 2011), demethylation of H3K36me2 is likely to contribute to H3K27 trimethylation by PRC2.

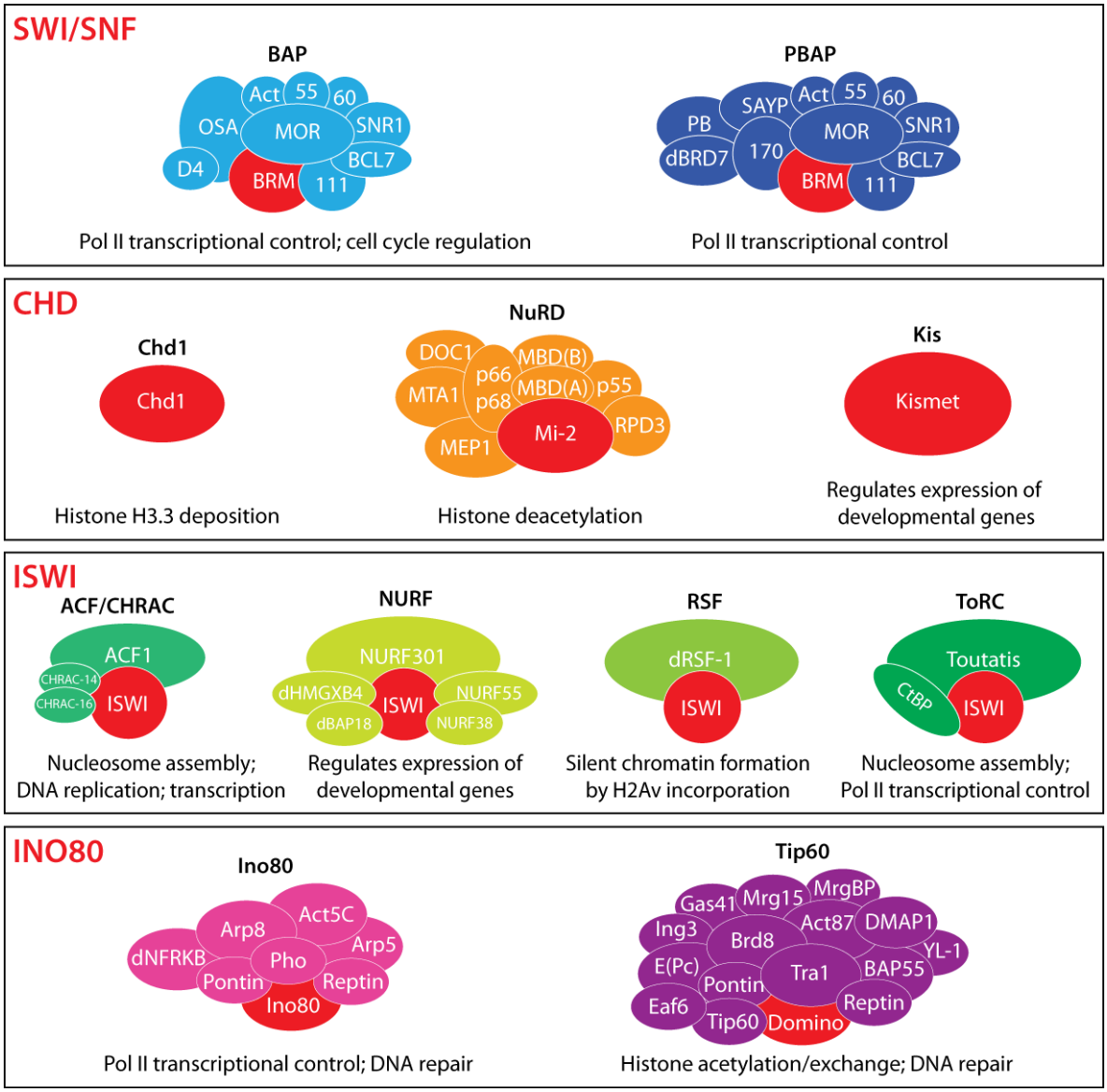

Figure 1. Overview of the chromatin remodelers present in *Drosophila* with their respective function. The ATPase subunit of each remodeler is highlighted in red.

Monoubiquitylation of H2A has been shown to be required for efficient silencing of a subset of Polycomb target genes, but clearly a number of genes do not rely on H2A monoubiquitylation for proper repression (Gutierrez et al., 2012). Therefore, other mechanisms must operate to ensure correct maintenance of gene expression patterns by PRC1. Some evidence points to the direction of chromatin compaction by PRC1 complexes (Simon and Kingston, 2013). Our ChIP results, however, suggest that compaction of the chromatin template does not play a major role in maintaining a repressed state of the Hox genes in cultured *Drosophila* BG3 cells. We observe only subtle changes in histone occupancy and DNA accessibility at Hox gene promoters and PREs after depletion of PRC1 components, which are unlikely to contribute to chromatin compaction. Systematic analysis of ChIP data from PcG factors indicates that PRC1 assists in

binding of the DNA-binding protein Pho, but not the methyltransferase E(z), to Hox gene promoters and PREs. The discovery that PRC1 and Pho bind PREs cooperatively is in agreement with an earlier study from our lab and makes the findings reported in this Chapter more reliable (Mohd-Sarip et al., 2005). The most striking observation we made was actually the fact that TFIID could be found at Hox gene promoters and PREs, while these regions were largely devoid of Mediator and Pol II in the repressed state. Interestingly, RNAi-mediated knockdown of PRC1 components resulted in a strong increase in the binding of Mediator and Pol II to these same genomic sites, suggesting that PRC1 blocks access of both factors to the chromatin template. Since Mediator and Pol II are indispensable for transcription, this likely presents a mechanism by which PRC1 represses transcriptional output.

The finding that PRC1 can interfere with recruitment of Mediator and Pol II parallels data from a recent *in vitro* study which showed that PRC1 can inhibit PIC assembly and promote dissociation of preassembled PICs by blocking the recruitment of Mediator to H3K27 methylated chromatin templates (Lehmann et al., 2012). Since Mediator is a key factor for the formation of a functional PIC, interfering with its recruitment would hamper PIC formation and, therefore, transcriptional activation. An intriguing observation made in this study and by us as well is that TFIID behaved different from Mediator as its binding did not seem to be impaired in the presence of PRC1. However, we did note differences between individual subunits of TFIID, since PRC1 depletion led to a consistent increase in the binding of TAF150 at Hox gene promoters, whereas dTBP binding at Hox gene promoters and PREs was strongly decreased. This could be the result of direct physical interactions of PRC1 with TBP and possibly other TBP-associated factors (Breiling et al., 2001; Saurin et al., 2001). A quite remarkable observation made by Carey and colleagues was that PRC1 bound H3K27 methylated templates better when TFIID alone was present and that PRC1 appeared to stabilize TFIID binding even after dissociating Mediator (Lehmann et al., 2012). Genome-wide analysis of ChIP data obtained from mouse ES cells showed that the majority of PRC1-bound loci are also bound by TBP. Functional analysis of these loci revealed that genes co-occupied by PRC1 and TBP have a significantly lower expression level compared to genes bound by TBP alone, which suggests that these PRC1-bound genes are indeed targets of Polycomb silencing. A possible explanation for this phenomenon could be that TFIID is present at these genes for rapid induction when their gene products are required during development. This is supported by the notion that the analyzed Hox promoters in our study are bivalent, i.e. they carry both H3K27me3 and H3K4me3, which is characteristic of a poised state. The necessity for Mediator is exemplified by the fact that genes occupied by both TBP and MED1 show higher expression levels than genes lacking MED1 (Lehmann et al., 2012). Our results also indicate that combined depletion of PRC1 and MED1 partially restores the initially repressed state of Hox genes. Thus, Mediator appears to be a key factor which is targeted by PRC1 to achieve gene silencing.

Another important finding of our study is the fact that we did not detect Pol II on Hox gene promoters when PRC1 was present. This suggests that these Hox genes do not contain paused polymerase under conditions where these genes are repressed. Furthermore, this places PRC1 binding upstream of Pol II recruitment and, therefore, argues that PRC1 targets an early step of the transcription process. We propose that gene silencing by PRC1 involves blocking of

transcription initiation by impeding recruitment of Mediator and Pol II to the chromatin template which thereby interferes with PIC assembly. Since Mediator does not bind DNA directly, but instead needs to be recruited by transcriptional activators, it will be interesting to identify the factors responsible for this event. A possible candidate is dBRD4, as it can directly associate with Mediator and co-localizes on chromatin genome-wide (shown in Chapter 5). Other candidates might be sequence-specific DNA binding transcription factors known to play a role in epigenetic control by trxG and PcG factors, such as Trl, Zeste, and Psq.

In Chapter 4 we comment on the role of the 26S proteasome in the regulation of Pol III-dependent transcription. We have analyzed the genomic distribution of a 19S and a 20S proteasome subunit on *Drosophila* polytene chromosomes and found that both components colocalized at the majority of bound loci. For some time the debate in the field has been which form of the proteasome is present on the chromatin. Our results are suggestive for the presence of the complete 26S proteasome, since subunits of both major subcomplexes can be found on chromatin in a largely overlapping pattern. This is also proposed by a recent study that systematically analyzed the association of native proteasome components with yeast chromatin (Geng and Tansey, 2012). Here it was found that subunits of the 19S lid, base and 20S core bound chromatin in a virtually indistinguishable manner. Furthermore, chromatin binding of the proteasome appeared to be dependent on active transcription, as proteasome subunits were lost from the *GAL10* gene when transcription was shut down by glucose addition. Similar observations were made when transcription by Pol II or Pol III was inhibited by genetic or chemical inhibition, respectively (Geng and Tansey, 2012; Sikder et al., 2006). The results presented in Chapter 4 provide more insight in this aspect by demonstrating that the presence of RNA species is a key determinant for proteasome binding to chromatin, rather than transcription per se. However, association of 20S with chromatin was still detected under conditions where 19S binding was compromised, suggesting that other mechanisms might operate for 20S recruitment. Another means of recruiting the proteasome to the chromatin template has been suggested to rely on the monoubiquitylation of histone H2B (Ezhkova and Tansey, 2004). We have tested this for the proteasome in *Drosophila* by performing tissue-specific knockdown of Bre1, the enzyme responsible for H2B monoubiquitylation. Overall binding of 19S did not appear to be affected by loss of H2Bub1, suggesting that this modification on H2B does not tether the proteasome to chromatin in the fruit fly. Since we have not analyzed the binding of 20S under these conditions, we cannot rule out that H2Bub1 is involved in some aspects of proteasome recruitment to chromatin.

Most studies dealing with the proteasome have linked it to transcription carried out by RNA polymerase II. Our genome-wide analysis of the distribution of 19S and 20S on *Drosophila* polytene chromosomes, however, revealed only limited colocalization with Pol II on chromatin, whereas widespread overlap with Pol III was observed. Further analysis of Pol III transcripts suggests a functional role for the proteasome in regulating Pol III-dependent transcription where both 19S and 20S repress transcription of a number of Pol III genes. Apart from which form of the proteasome associates with chromatin, it has also been questioned whether the proteolytic activity of the proteasome is required for transcriptional regulation. In our setup, treatment of

cells with proteasome inhibitors did not have a major impact on transcription of Pol III target genes, suggesting that the proteasome fulfills a non-proteolytic role in this aspect. The exact nature of this is unclear at the moment. Right now we can only speculate about this. One hypothesis would be that the proteasome directly inhibits Pol III transcriptional output by changing Pol III's complex composition with the use of its ATPase activity to unfold or extract certain subunits. The proteasome might also simply bind Pol III coding regions to block passage of Pol III and thereby decreasing gene transcription levels. Alternatively, given the RNA-dependent association of 19S with chromatin, the proteasome could bind nascent Pol III transcripts and trigger their degradation. In this case, the proteasome might assist in RNA degradation carried out by intracellular RNases or might even contribute directly as it has been proposed to possess endogenous RNase activity (Kulichkova et al., 2010). Whichever mechanism operates the proteasome most likely functions to restrict Pol III-dependent transcription in order to maintain cellular homeostasis, by ensuring a proper balance between protein production and protein turnover. An important step forward would be to identify the RNA species bound by the proteasome.

The content of Chapter 5 concerns the purification and functional characterization of *Drosophila* BRD4. This protein is part of the BET family that also comprises mammalian BRD2, BRD3, BRD4, and BRDT and yeast Bdf1 and Bdf2. Most of these BET members have been shown to interact with chromatin by binding to acetylated histone tails via their tandem bromodomains. By interacting with modified histones, BET proteins are thought to recruit other factors to the chromatin template in order to regulate transcription and perhaps other processes as well. We were interested in the proteins associated with dBRD4 to gain a better understanding of its role in gene expression control. Our proteomics analysis of immunopurified dBRD4 from *Drosophila* embryo nuclear extracts suggests that the Mediator complex is the major interacting partner of dBRD4. Since mammalian BRD4 has been reported to physically interact with Mediator as well (Jiang et al., 1998; Wu and Chiang, 2007), this appears to be a conserved interaction. Apart from their physical interaction we also find widespread colocalization of dBRD4 and Mediator on larval salivary gland polytene chromosomes, which is paralleled by data from genome-wide ChIP-seq experiments in human cells (Loven et al., 2013). Together, this suggests that both factors are capable of interacting in solution as well as on chromatin. It is possible that dBRD4 recruits Mediator to enhancers and promoters to regulate gene activation. This is supported by the fact that treatment of cells with JQ1, a small molecule that blocks binding of BRD4 to chromatin, also led to a reduction in the binding of Mediator to enhancer and promoter regions (Loven et al., 2013). However, since the reduction in Mediator binding was relatively modest at most promoters and enhancers, with the exception of the so-called super-enhancers, it is likely that BRD4 does not account for bulk Mediator recruitment. This might be achieved by other transcriptional activators, such as sequence-specific DNA binding transcription factors.

Based data from human cells, BRD4 does not seem to interact with the complete 30-subunit Mediator complex, but rather interacts with a selective form of the Mediator complex through interaction with a subunit not present in the CDK module (Wu and Chiang, 2007). Our own mass spectrometric analysis of purified dBRD4 does identify the complete Mediator

complex, although some subunits appear to be overrepresented compared to other subunits. For example, Mediator subunits that are part of the head and middle module seem to be more abundant in the dBRD4 purified material. Since the head and middle structure can contact Pol II (Dotson et al., 2000), it is tempting to speculate these parts of the Mediator co-activator interact with dBRD4 in order to bring the protein in close proximity to Pol II. Once arrived, dBRD4 might contribute to the transcription process by regulating the phosphorylation state of the Pol II CTD. This could be achieved directly, as mammalian BRD4 was shown to possess kinase activity capable of phosphorylating Pol II at Ser2 of the CTD (Devaiah et al., 2012). However, whether *Drosophila* BRD4 possesses kinase activity as well is debatable, although some evidence does point into that direction (Chang et al., 2007). Alternatively, BRD4 could regulate phosphorylation of the CTD on Ser2 by bringing in P-TEFb (Jang et al., 2005; Yang et al., 2005). Unlike mammalian BRD4, we do not have any evidence that *Drosophila* BRD4 interacts with P-TEFb as well. Therefore, this issue awaits further clarification. It will be interesting to address to role of dBRD4 in the regulation of Pol II CTD phosphorylation. Furthermore, it will be important to assess the transcriptional circuitries controlled by both dBRD4 and Mediator to understand the relevance of the observed physical interaction and colocalization on chromatin of these two factors.

The last experimental part of this thesis, Chapter 6, provides an inventory of the ISWI-containing chromatin remodeling complexes present in *Drosophila* (Fig. 1). We identified at least four different complexes that contain ISWI, i.e. ACF/CHRAC, NURF, RSF, and ToRC, which is consistent with findings from other studies (Emelyanov et al., 2012; Hanai et al., 2008; Ito et al., 1997; Tsukiyama et al., 1995; Varga-Weisz et al., 1997). In addition, we discovered two novel subunits, i.e. dBap18 and dHMGXB4, which are part of the NURF complex and were recently identified in humans as well (Vermeulen et al., 2010). By performing immunolocalization analysis on polytene chromosomes we show that the subunits that define each complex occupy different loci genome-wide, suggesting that each ISWI-containing complex has different specificities and regulates distinct gene sets. Furthermore, our results argue that NURF is the predominant chromatin-bound ISWI-containing complex in larval salivary gland tissue, as Nurf301 colocalizes with ISWI at the vast majority of loci on polytene chromosomes.

An important question that arises is why there would be so many different complexes that possess a similar ATPase subunit? Perhaps each distinct ISWI-containing complex plays roles in specific tissues or at particular developmental stages and may regulate a separate set of genes with the help of its signature subunits. It will, therefore, be helpful to determine the expression profile of ISWI and signature subunits in different tissues and at different timings during development. Performing gene expression analysis after depletion of individual ISWI complex components will also provide more insight into the genes regulated by each remodeling complex. Finally, genome-wide ChIP-seq experiments in different cell lines might offer clues as well as to which genes are targeted by each remodeler.

In conclusion, our work on the chromatin remodelers Kis-L, (P)BAP, and ISWI, the epigenetic PcG regulators, the dBRD4 transcriptional activator, and the 26S proteasome complex has opened up new avenues for research, but also raised many more interesting questions which

present a challenge for the future. The focus of future research will lie in addressing these questions in order to increase our understanding of the role of these chromatin binding factors in transcriptional control.

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Summary

Tight control of transcription is vital for maintaining a proper balance in gene expression levels and, consequently, is indispensable for normal growth and development of organisms. Since gene products generally participate in large networks, a slight deviation in the expression of any given gene can have profound downstream effects. Many proteins including chromatin remodelers, histone modifying enzymes, transcription factors and the basal transcription machinery are known to contribute to the total transcriptional output of a cell, but despite considerable progress in the scientific field, much remains to be learned about the transcription process. Therefore, studying proteins involved in transcription is important for understanding the underlying mechanisms of gene regulation and might aid in the treatment of disease by identifying drug targets.

Chromatin remodeling enzymes often execute their task with the help of accessory factors in the context of a complex. Chapter 2 describes the functional characterization of Kis-L associated proteins isolated from *Drosophila* embryo nuclear extracts and identified the (P)BAP remodeler as a major interacting partner. Genome-wide immunolocalization analysis on larval salivary gland polytene chromosomes revealed widespread overlap of Kis-L and (P)BAP on chromatin. Their targeting to chromatin appears to be independent of each other, as Kis-L still localized properly in the absence of Brm, a (P)BAP core subunit, and vice versa. In contrast to earlier work, both Kis-L and Brm did not affect the phosphorylation state of the Pol II CTD at Ser2 and Kis-L did not regulate H3K27me3 levels. Analysis of genome-wide RNA-seq data obtained from Kis-L and Brm depleted S2 cells revealed largely overlapping gene targets of which a number of genes were confirmed by RT-qPCR. Kis-L and Brm regulated gene expression in either the same or opposite direction and cooperated in some cases. These observed effects are likely to be direct as both remodelers were shown to bind the affected genes by ChIP-qPCR. Thus, we propose that Kis-L associates with (P)BAP to fine-tune gene expression.

In Chapter 3 we addressed the mechanism by which PRC1 represses transcription. Depletion of core PRC1 subunits in *Drosophila* BG3 cells resulted in strong de-repression of a selection of Hox genes accompanied by loss of PRC1 and Pho binding, but not E(z), to the PREs and promoters of these genes. De-repression of the Hox genes as a result of PRC1 depletion does not seem to rely on major changes in the chromatin compaction state, as histone density and DNA accessibility remained largely unchanged. Acetylation of H3K27 was increased, whereas H3K27me3 was decreased to some extent at PREs and promoters in the de-repressed condition. Promoter-associated methylation of H3K4 on the other hand was increased upon Hox gene activation, all of which correlates with the expression status of these genes. Most of the trxG factors, which are supposed to antagonize PcG proteins, showed increased binding to Hox gene promoters and PREs after PRC1 depletion, which is consistent with their role in transcriptional activation. Binding of individual TFIID subunits seemed differentially affected by the presence of PRC1, while Mediator and Pol II were practically absent under these conditions. However, knockdown of PRC1 resulted in a striking increase in the binding of Mediator and Pol

II to the promoters and, in the case of Mediator, PREs of the analyzed Hox genes. In conclusion, PRC1 represses transcription by blocking the recruitment of Mediator to chromatin.

Chapter 4 provides novel insights into the role of the 26S proteasome in transcriptional control by Pol III. Assessment of the genome-wide localization of components of the 19S and 20S proteasome revealed a striking overlap in their distribution on polytene chromosomes, which is suggestive for the presence of the complete 26S proteasome on chromatin. Crude mapping of the proteasome binding sites identified the 5S rDNA locus, which is a Pol III transcribed region. Cololcalization with Pol III on chromatin is widespread, since the majority of proteasome bound loci were co-stained by Pol III as well, whereas only little overlap with Pol II was observed. In contrast to reports based on yeast data, monoubiquitylation of H2B is unlikely to play a role in the recruitment of the proteasome to chromatin. Instead chromatin binding of at least 19S is dependent on the presence of RNA, which is demonstrated by the loss of 19S staining after *in vivo* RNase treatment of salivary gland tissue. Both 19S and 20S inhibited expression of Pol III target genes while they had no effect on Pol II transcribed genes. Inhibition of the proteolytic activity of the proteasome had no major impact on transcription of Pol III target genes, which demonstrates that the proteasome plays a non-proteolytic function in the control of Pol III regulated transcription. Taken together, these data link the 26S proteasome to Pol III-dependent transcription of ncRNAs independent of its proteolytic activity and suggest its involvement in cellular homeostasis that exceeds all expectations.

Chapter 5 describes the purification and functional characterization of *Drosophila* BRD4. Mass spectrometric analysis revealed that dBRD4 interacts with the Mediator co-activator in embryo nuclear extracts. This interaction seems to be important as it is conserved among higher eukaryotes. Besides physically interacting in solution, dBRD4 and Mediator also colocalize extensively on larval salivary gland polytene chromosomes. A large degree of overlap on chromatin was observed between dBRD4 and Pol II as well, which implicates this protein in transcription carried out by Pol II. Thus, *Drosophila* BRD4 interacts with the Mediator complex and both factors co-occupy similar sites on the chromatin. A putative working model would be that dBRD4 recruits the Mediator complex to stimulate transcription.

Chapter 6 aims to provide an inventory of the chromatin remodeling complexes that share the ISWI ATPase in *Drosophila*. By using a proteomics approach, we identified at least four distinct ISWI-containing complexes, including ACF/CHRAC, NURF, RSF, and ToRC. Analysis of their genome-wide binding profiles on polytene chromosomes revealed different distributions for the subunits specifying each complex. Nurf301 predominantly colocalized with ISWI, whereas Acf1, dRSF-1 and Tou shared only a selection of sites with ISWI. In addition, dRSF-1 overlapped extensively with Brm, while Tou colocalized on specific loci with GMPS and USP7. These proteins were also found to associate physically with either dRSF-1 or Tou, suggesting that dRSF-1 interacts with a Brm-complex on chromatin and Tou can interact with GMPS and USP7 on some genomic targets. In conclusion, ISWI can be part of at least four different complexes in *Drosophila* which are likely to regulate different sets of genes based on their genome-wide localization on polytene chromosomes.

Nederlandse samenvatting

Strak gecontroleerde transcriptie is cruciaal voor het behouden van een juiste balans in gen expressie niveaus en is als gevolg daarvan onmisbaar voor normale groei en ontwikkeling van organismen. Gezien het feit dat gen producten normaal gesproken onderdeel zijn van grote netwerken kan een kleine afwijking in de expressie van welk gen dan ook enorme gevolgen hebben. Van veel eiwitten, waaronder chromatine remodelleerders, histon veranderende enzymen, transcriptie factoren en de basale transcriptie machinerie is bekend dat zij bijdragen aan de totale transcriptionele uitvoer van een cel, maar ondanks aanzienlijke vooruitgang in het wetenschappelijke veld moet er nog veel geleerd worden over het transcriptie proces. Daarom is het belangrijk om eiwitten te bestuderen die betrokken zijn bij transcriptie om zo de onderliggende mechanismen van gen regulatie te begrijpen, wat ook weer kan bijdragen aan de behandeling van ziekten door het identificeren van therapeutische doelen.

Chromatine remodellerende enzymen vervullen hun taak vaak met de hulp van andere eiwitten in de context van een complex. Hoofdstuk 2 beschrijft de functionele karakterisatie van Kis-L geassocieerde eiwitten geïsoleerd uit celkern extracten van *Drosophila* embryo's en identificeerde de (P)BAP remodelleerder als één van de meest prominente interactie partners. Genoom-wijde immunolokalisatie analyse op polytene chromosomen uit larvale speekselklieren onthulde wijd verspreide overlap van Kis-L en (P)BAP op chromatine. De recrutering van beiden naar chromatine lijkt onafhankelijk te zijn van elkaar, aangezien Kis-L nog steeds goed lokaliseerde in de afwezigheid van Brm, een kern subeenheid van (P)BAP, en vice versa. In tegenstelling tot eerder werk hadden zowel Kis-L als Brm geen invloed op de fosforylatie status van de Pol II CTD op Ser2 en werden H3K27me3 niveaus niet door Kis-L gereguleerd. Analyse van genoom-wijde RNA-seq data verkregen uit Kis-L en Brm uitgeputte S2 cellen onthulde grotendeels overlappende gen doelen waarvan een aantal genen werd bevestigd met RT-qPCR. Kis-L en Brm reguleerden gen expressie ofwel in dezelfde richting of in de tegenovergestelde richting en werkten samen in sommige gevallen. Deze geobserveerde effecten zijn hoogstwaarschijnlijk direct aangezien beide remodelleerders de beïnvloedde genen bonden zoals aangetoond met ChIP-qPCR. Wij stellen dus voor dat Kis-L met (P)BAP associeert om zo de gen expressie verfijnd af te stellen.

In Hoofdstuk 3 richten wij ons op het mechanisme waarbij PRC1 transcriptie remt. Depletie van kern PRC1 subeenheden in *Drosophila* BG3 cellen resulteerde in een sterke de-repressie van een selectie Hox genen en werd vergezeld van een verlies aan PRC1 en Pho binding, maar niet dat van E(z), aan de PRE's en promotors van deze genen. De-repressie van de Hox genen na PRC1 depletie is waarschijnlijk niet het gevolg van veranderingen in de compactie van het chromatine, aangezien de histon dichtheid en DNA bereikbaarheid grotendeels onveranderd bleven. Acetylering van H3K27 was verhoogd, terwijl H3K27me3 enigszins verminderd was op PRE's en promotors in de gederepresseerde conditie. Aan de andere kant, promotor-geassocieerde methylering van H3K4 was verhoogd tijdens Hox gen activatie, wat allemaal correleert met de expressie status van deze genen. De meeste trxG factoren, waarvan gedacht wordt dat zij PcG eiwitten tegenwerken, lieten verhoogde binding zien aan Hox gen

promotors en PRE's na PRC1 depletie, wat consistent is met hun rol in transcriptionele activatie. Binding van individuele TFIID subeenheden leek differentieel beïnvloed door de aanwezigheid van PRC1, terwijl Mediator en Pol II praktisch niet aanwezig waren onder deze omstandigheden. Echter, knockdown van PRC1 resulteerde in een enorme toename in de binding van Mediator en Pol II op promotoren en, in het geval van Mediator, PRE's van de geanalyseerde Hox genen. Kort gezegd blokkeert PRC1 de recrutering van Mediator naar het chromatine om zo transcriptie te remmen.

Hoofdstuk 4 verstrekt nieuwe inzichten betreffende de rol van het 26S proteasoom in transcriptionele controle door Pol III. Bepaling van de genoom-wijde lokalisatie van componenten van het 19S en 20S proteasoom onthulde een opvallende overlap in hun distributie op polytene chromosomen, dat suggestief is voor de aanwezigheid van het complete 26S proteasoom op chromatine. Het grof in kaart brengen van de proteasoom bindingsplekken identificeerde het 5S rDNA locus wat een Pol III afgeschreven regio is. Colokalisatie met Pol III op chromatine is wijd verspreid aangezien de meerderheid van de proteasoom gebonden loci ook aangekleurd werden door Pol III, terwijl slechts weinig overlap met Pol II werd waargenomen. In tegenstelling tot verslagen gebaseerd op gist data is het onwaarschijnlijk dat monoubiquitylering van H2B een rol speelt in de recrutering van het proteasoom naar chromatine. In plaats daarvan is de binding van op zijn minst 19S afhankelijk van de aanwezigheid van RNA, wat gedemonstreerd wordt door het verlies aan 19S kleuring na *in vivo* RNase behandeling van speekselklier weefsel. Zowel 19S als 20S remde de expressie van Pol III doelgenen terwijl zij geen effect hadden op Pol II afgeschreven genen. Remming van de proteolytische activiteit van het proteasoom had geen opvallende gevolgen voor de transcriptie van Pol III doelgenen wat aantoont dat het proteasoom een niet-proteolytische functie vervult in de controle van Pol III gereguleerde transcriptie. Alles bij elkaar genomen verbinden deze data het 26S proteasoom met Pol III afhankelijke transcriptie van ncRNA's onafhankelijk van zijn proteolytische activiteit en suggereert het zijn betrokkenheid bij cellulaire homeostase op een manier die alle verwachtingen overschrijdt.

Hoofdstuk 5 beschrijft de zuivering en functionele karakterisatie van *Drosophila* BRD4. Massaspectrometrie analyse onthulde dat dBRD4 met de Mediator co-activator interacteert in embryo celkern extracten. Deze interactie lijkt belangrijk te zijn gezien het feit dat het geconserveerd is in hogere eukaryoten. Naast het gegeven dat ze interacteren in oplossing vertonen dBRD4 en Mediator opvallende colokalisatie op polytene chromosomen geïsoleerd uit larvale speekselklieren. Een grote hoeveelheid overlap op chromatine werd ook waargenomen voor dBRD4 en Pol II wat het eerstgenoemde eiwit verbindt aan transcriptie uitgevoerd door Pol II. *Drosophila* BRD4 interacteert dus met het Mediator complex en beide factoren bezetten dezelfde plekken op het chromatine. Een mogelijk werkend model zou kunnen zijn dat dBRD4 Mediator recruteert om zo transcriptie te stimuleren.

Hoofdstuk 6 is erop gericht om een inventaris te verschaffen van de chromatin remodellerende complexen die de ISWI ATPase delen in *Drosophila*. Door gebruik te maken van een proteomics aanpak hebben wij tenminste vier verschillende ISWI bevattende complexen geïdentificeerd, waaronder ACF/CHRAC, NURF, RSF en ToRC. Analyse van hun genoom-wijde bindingsprofielen op polytene chromosomen onthulde verschillende distributies van de

subeenheden die elk complex specificeren. Nurf301 colokaliseerde voornamelijk met ISWI, terwijl Acf1, dRSF-1 en Tou slechts enkele plekken deelden met ISWI. Verder overlapte dRSF-1 grotendeels met Brm, terwijl Tou colokaliseerde met GMPS en USP7 op specifieke plekken. Deze eiwitten konden ook fysiek associëren met ofwel dRSF-1 of Tou wat suggereert dat dRSF-1 met een Brm-complex interacteert op chromatine en Tou kan interacteren met GMPS en USP7 op enkele genomische plekken. Kort gezegd kan ISWI onderdeel zijn van tenminste vier verschillende complexen in *Drosophila* die hoogstwaarschijnlijk verschillende sets van genen reguleren afgaande op hun genoom-wijde lokalisatie op polytene chromosomen.

Curriculum Vitae

Personal details

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Education

2006 – 2008	**Master of Science** *Cancer Genomics & Developmental Biology* Faculty of Biomedical Sciences, Utrecht University, Utrecht, the Netherlands
2003 – 2006	**Bachelor of Science** (cum laude) *Biology* Faculty of Biology, Utrecht University, Utrecht, the Netherlands

Work experience

2008 – 2013	**PhD** Dissertation title: *Gene expression control by chromatin binding factors* Lab of Prof. C. Peter Verrijzer Dept. of Biochemistry, Erasmus Medical Center, Rotterdam, the Netherlands
2008 (2 months)	Short internship Title: *Small molecule screening in Danio rerio to identify compounds that affect development* Lab of Dr. Jeroen Bakkers Hubrecht Institute, Utrecht, the Netherlands
2007 – 2008 (6 months)	Minor research project Title: *Generation of a tissue-specific RNAi system in C. elegans to study Wnt secretion* Lab of Dr. Hendrik C. Korswagen Hubrecht Institute, Utrecht, the Netherlands
2006 – 2007 (9 months)	Major research project Title: *The function and localization of Wip1 during recovery from the G_2/M DNA damage checkpoint* Lab of Prof. René H. Medema Experimental Oncology, University Medical Center, Utrecht, the Netherlands

Publications

1. Herz H.M., Mohan M., Garruss A.S., Liang K., Takahashi Y.H., Mickey K., **Voets O.**, Verrijzer C.P. & Shilatifard A. (2012). Enhancer-associated H3K4 monomethylation by Trithorax-related, the Drosophila homolog of mammalian Mll3/Mll4. Genes Dev. 26(23):2604-20.

2. Macurek L., Lindqvist A., **Voets O.**, Kool J., Vos H.R. & Medema R.H. (2010). Wip1 phosphatase is associated with chromatin and dephosphorylates gammaH2AX to promote checkpoint inhibition. Oncogene. 29(15):2281-91.

3. Lindqvist A., de Bruijn M., Macurek L., Brás A., Mensinga A., Bruinsma W., **Voets O.**, Kranenburg O. & Medema R.H. (2009). Wip1 confers G2 checkpoint recovery competence by counteracting p53-dependent transcriptional repression. EMBO J. 28(20):3196-206.

PhD Portfolio

Name PhD student:	Olaf Voets	PhD period:	Sep 2008 - Feb 2013
Erasmus MC Department:	Biochemistry	Promotor:	Prof. C.P. Verrijzer
Research School:	Medisch-Genetisch Centrum (MGC)		

1. PhD training

	Year
General courses	
- Molecular and cell biology (Rotterdam, NL)	2009
Specific courses	
- Instellingsgebonden regelgeving stralingshygiëne op deskundigheidsniveau 5A/B (Rotterdam, NL)	2010
Attended seminars and workshops	
- Training "Promotie in eigen regie" (Utrecht, NL)	2009
- Seminar "Physics of the Genome" (Leiden, NL)	2009
- 16th MGC PhD student workshop (Bruges, BE)	2009
- NPC PhD Day (Delft, NL)	2013
Presentations	
- 17th MGC PhD student workshop, poster presentation (Cologne, GER)	2010
- 18th MGC PhD student workshop, oral presentation (Maastricht, NL)	2011
- Epigenetic Marks and Cancer Drugs, poster presentation (Santa Fe, USA)	2013
(Inter)national conferences	
- 18th MGC symposium (Rotterdam, NL)	2008
- 2nd SCDD symposium "Stem Cells, Development and Regulation" (Amsterdam, NL)	2008
- CGC/CBG meeting "Molecular mechanisms and mouse models in cancer" (Amsterdam, NL)	2008
- 19th MGC symposium (Rotterdam, NL)	2009
- 3rd SCDD symposium "Stem Cells, Development and Regulation" (Amsterdam, NL)	2009
- CGC/CBG meeting "Molecular mechanisms in cancer" (Amsterdam, NL)	2010

2. Teaching

	Year
Supervising undergraduate students	
- Supervision of HBO student (Myrte Jansen)	2009
- Supervision of MBO student (Dennis Schmitz)	2009-2010
- Supervision of University Bachelor student (Marc Solà Ginés)	2010-2011

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