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Investigating the Function of the Preinitiation Complex and the Consequences of

Chromatin Modifications

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy in Molecular Biology

by

Lynn Wayne Lehmann

2013

ABSTRACT OF THE DISSERTATION

Investigating the Function of the Preinitiation Complex and the Consequences of Chromatin

Modifications

By

Lynn Wayne Lehmann

Doctor of Philosophy in Molecular Biology

University of California, Los Angeles, 2012

Professor Michael F. Carey, Chair

Eukaryotic transcription requires the concerted action of numerous proteins including

activators, co-activators, the general transcription factors (GTFs), RNA polymerase II (RNA Pol II),

chromatin remodelers and modifiers, and chromatin- binding effector proteins. Although many

of the components of the preinitiation complex (PIC) have been described, a comprehensive

understanding of all preinitiation components and what affects chromatin modifications have

on PIC formation remains vague. This doctoral thesis investigates the composition and function

of eukaryotic PICs in HeLa and mouse embryonic stem cells. Using Multi Dimensional Protein

Identification Technology (MuDPIT), others in the lab showed that most transcription

components necessary for initiation were present in in vitro formed PICs in both cell types.

Additionally, numerous chromatin remodeling proteins, elongation factors, and chromatin

modifying enzymes were also identified proteomically. Intriguingly, two major co-activator

complexes SAGA and Mediator, ranked among the highest activator-inducible proteins as detected by MuDPIT analysis. I generated HeLa nuclear extracts immuno-depleted of Mediator and SAGA and performed mechanism-based immobilized template experiments on chromatin to show both complexes interact with GAL4-VP16 directly, and do not compete for promoter access. I also used the immobilized template to show that Mediator, but not SAGA, serves as the main scaffold to recruit the PIC machinery. Genome-wide, enrichment patterns of Mediator, RNA Pol II, TATA-binding protein, and mRNA levels strongly correlate suggesting a prominent role for Mediator in PIC assembly. Lastly, I used in vitro transcription assays to show that SAGA is required for activated transcription on chromatin. Overall, these data support a working model where Mediator serves as the main co-activator complex, contributing to PIC formation, stability, and function, while SAGA acts after PIC formation, conferring activity to PICs on chromatin.

Although the role of histone tail modifications and subsequent effector binding proteins is an area of considerable study, the role that histone modifications play in PIC formation and activity is poorly understood. This thesis examines the role of the Chromodomain Helicase DNA-binding protein 1 (CHD1), known to bind histone H3 trimethylated at Lysine 4, in PIC assembly and function. I purified murine CHD1 to near homogeneity using a baculovirus-based insect cell system, and prepared the samples used for identification of CHD1-interacting proteins by MuDPIT of associated proteins from HeLa nuclear extracts. My collaborator on this project, Justin Lin, used purified CHD1 to show that the protein stimulates transcription on chromatin and further activates transcription on H3K4me3 chromatin. Further, mechanistic experiments performed by others in the lab revealed that CHD1 recruitment to immobilized templates is dependent on Mediator and enhanced by H3K4me3. Our in vitro and in vivo experiments

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suggested that Mediator and CHD1 interact to enhance transcription. The summation of these data support a model where Mediator acts to support both PIC assembly and the binding of CHD1 to PICs, with H3K4me3 providing a second interaction platform responsible for the recruitment of CHD1 to active genes.

In an opposing role, chromatin modifications also play a role in establishing and maintaining a silent transcriptional state. In the final chapter of this thesis, I investigate the affects of Polycomb Repressive Complex 1 (PRC1) on PIC formation and activity. I purified PRC1 and investigated the role of the complex in PIC assembly and function on H3K27me3 templates. I show that PRC1 can both block and dissociate the majority of PIC components including Mediator, SAGA, elongation factors and the general transcription factors to silence transcription. Notably, the ability of GAL4-VP16 and TATA-binding protein (TBP) to bind promoters was retained in the presence of PRC1. I again used immobilized template experiments with purified Mediator, TFIID, and PRC1 to show that PRC1 specifically blocks Mediator but not TFIID. Utilizing previously reported genome-wide binding in mouse embryonic stem cells, we showed that Ring1b and TBP displayed enriched binding profiles at developmental genes, and this enrichment significantly correlated with reduced expression as compared to genes enriched for Mediator, TBP, and Ring1b. In summary, the results of this study highlight a possible role for TFIID and PRC1 in co-regulating the expression of developmental genes.

The dissertation of Lynn Wayne Lehmann is approved.

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2013

To My Parents: Wayne John Lehmann and Darlene Virginia Benson

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Chapter 1

Introduction to Gene Regulation in Eukaryotes

This thesis defines the key components and related mechanism(s) that drive the formation of the Pre-Initiation Complex (PIC) on eukaryotic chromatin. Furthermore, this thesis also probes the mechanisms of transcriptional activation by CHD1, and repression by PRC1. The work described herein addresses a number of questions, including: Are there yet undiscovered components of mammalian PICs? If so, what are the mechanisms for recruitment of these complexes and how do they affect PIC function? What are the major bridging factors responsible for PIC formation and function? What is the role of the chromatin remodeler and H3K4me3-binding protein CHD1 in PIC formation? Is the recruitment of this protein a consequence or integral part of PIC formation? Lastly, how does PRC1, a complex known to bind to H3K27me3 and silence gene expression, act on the PIC to silence transcription, and what steps in PIC formation are influenced by this complex? In this dissertation, I will introduce transcription and the general characteristics and components of the transcriptional preinitiation complex. Further, I will discuss chromatin structure and function. I will then explain what is known about each of the PIC components that are relevant to my studies. Lastly, I will introduce the role of transcription in development, and what is known about the role the polycomb complex plays in transcriptional silencing. The following body of work introduces the current knowledge in the field and advances this knowledge by providing detailed answers to the questions posed above.

Transcription in Eukaryotes

Development of a eukaryotic organism relies on numerous transcriptional regulatory events in order to achieve the precise temporal and spatial regulation of gene expression. The process of transcription is carried out by RNA Polymerase (RNA Pol) I, II, and III [1]. In plants, RNA Pol IV and V exist, and have specialized roles [2, 3]. The activity of RNA Polymerase II, which was first discovered in rat liver nuclei preparations, functions to transcribe DNA, resulting in RNAs that mature into mRNAs, which encode for

the cellular proteins [4]. Roeder and colleagues were the first to isolate the active RNA polymerases and used α-amanatin to show that RNA Pol II transcription results in mRNA synthesis[1, 5]. To gain further knowledge about transcription, Matsui and colleagues used fractionated nuclear extract preparations and purified RNA Pol II to show that other proteins and complexes, denoted the general transcription factors (GTFs), were required for robust transcription [6]. In subsequent studies, numerous groups have isolated distinct co-activator complexes involved in the formation of an activator-induced RNA Pol II Preinitiation Complex (PIC) [7-11]. These complexes include transcription factor IID (TFIID) and the Mediator. The recruitment of these and other regulatory complexes to a gene promoter is likely an ordered process that is critical for proper expression of genes [12]. My dissertation studies expand on these important findings, suggesting a much more complex mechanism that involves the recruitment of co-activator, chromatin-remodeling and -modifying complexes that facilitate both stability and function of PICs. Further, these complexes include the machineries necessary not only for initiation of transcription, but also for productive elongation and chromatin modification associated with gene activation.

The General Transcription Factors

The initial biochemical evidence describing how the initiation of RNA Pol II-mediated transcription required numerous co-factors came from an in vitro experiment. Four fractions of nuclear extracts (A, B, C, and D) from a P11 phosphocellulose ion exchange column were incubated with DNA templates in the presence of purified RNA Pol II; subsequent transcription of the adenovirus DNA template was observed in vitro [6, 13]. Further studies showed that three of these fractions were necessary for accurate initiation of transcription (fractions A, C, and D) [6, 14]. The proteins required for transcription in

fractions A and D were named Transcription Factor for Pol II A (TFIIA) and D (TFIID), respectively [15]. The activity in fraction C was fractionated into TFIIB, -E, -F, and –H [16-20].

Following the isolation of the General Transcription Factors (GTFs), a large number of in vitro biochemical studies were performed to determine the role of each complex in transcription [15]. From these studies, it was determined that the GTFs all serve as critical factors in recruiting and positioning RNA Pol II for accurate transcription. As part of a general theme of strong interactions mediated by the summation of individual weaker interactions, all of the GTFs have been shown to bind to RNA Pol II directly [15, 21]. Within the TFIID complex, TATA-binding protein (TBP) has high affinity to the TATA-box and bends DNA at a sharp angle [22]. In addition, TBP interacts with TFIIA and TFIIB, which acts stabilize TBP binding and to position TFIID and TFIIB correctly on the promoter for interaction with RNA Pol II [23-25]. TFIIF is closely associated with RNA Pol II as it often co-purifies with the polymerase [26-28]. The region of interaction between RNA Pol II and TFIIF is thought to be close to the TFIIB interface, as biochemical evidence suggests that both GTFs aid in start site selection and interact with one another once associated with RNA Pol II [24]. Biochemical experiments on TFIIF have also suggested a role in elongation, with supporting evidence from Chromatin Immunoprecipitation (ChIP) studies showing enrichment of TFIIF in the coding regions of genes [29, 30]. The binding of TFIIF aids in the recruitment of TFIIE, a hetero-tetramer that affects the later stages of PIC assembly, specifically the recruitment and activity of TFIIH [21]. Hahn's group has recently shown in yeast that the TFIIEα subunit contacts the RNA Pol II clamp, and that the TFIIEβ encircles promoter DNA [31]. Further, the XPB subunit of TFIIH is adjacent to TFIIE in the PIC, binding to DNA downstream from the transcription start site [31]. TFIIH binding to the PIC is critical, as it plays a role in the melting of the DNA strands via the XPB subunit, and phosphorylating the C-terminal domain (CTD) of RNA Pol II via the CDK7 subunit [29, 32, 33].

In a foundational study, Buratowski used native gel electrophoresis assays to study the binding of the GTFs and RNA Pol II to the adenovirus major late promoter. The results showed that five complexes containing distinct components bound, and were the result of the sequential binding of the GTF's and RNA Pol II [7]. From this study an ordered model of PIC assembly was proposed (Figure 1). In contrast, experiments performed by Young's lab suggested that RNA Pol II co-purified with numerous GTFs and chromatin remodelers [10]. This complex, lacking only TFIIA and TFIID, was designated as the holoenzyme. The subsequent mechanism proposed by Koleske and Young was that the DNA-binding activity of TBP resulted in TFIIA-TFIID complex, a bridging factor responsible for recruiting an intact holoenzyme to promoters containing TATA-boxes (Figure 1) [10, 11]. It is interesting to note that combination RNA Pol II and the GTFs in vitro results in basal transcriptional activity, yet fails to support activator-mediated transcription that is critical for the tight regulation of developmental genes. The integral question of how activator proteins specifically stimulated transcription remained an important outlying question. It is of no surprise that a number of coactivator complexes, namely Mediator, TFIID, and SAGA were identified and found to act as bridging factors between transcription activator proteins with specific DNA binding affinity and the general transcription machinery [34, 35]. These coactivator complexes will be described in depth below.
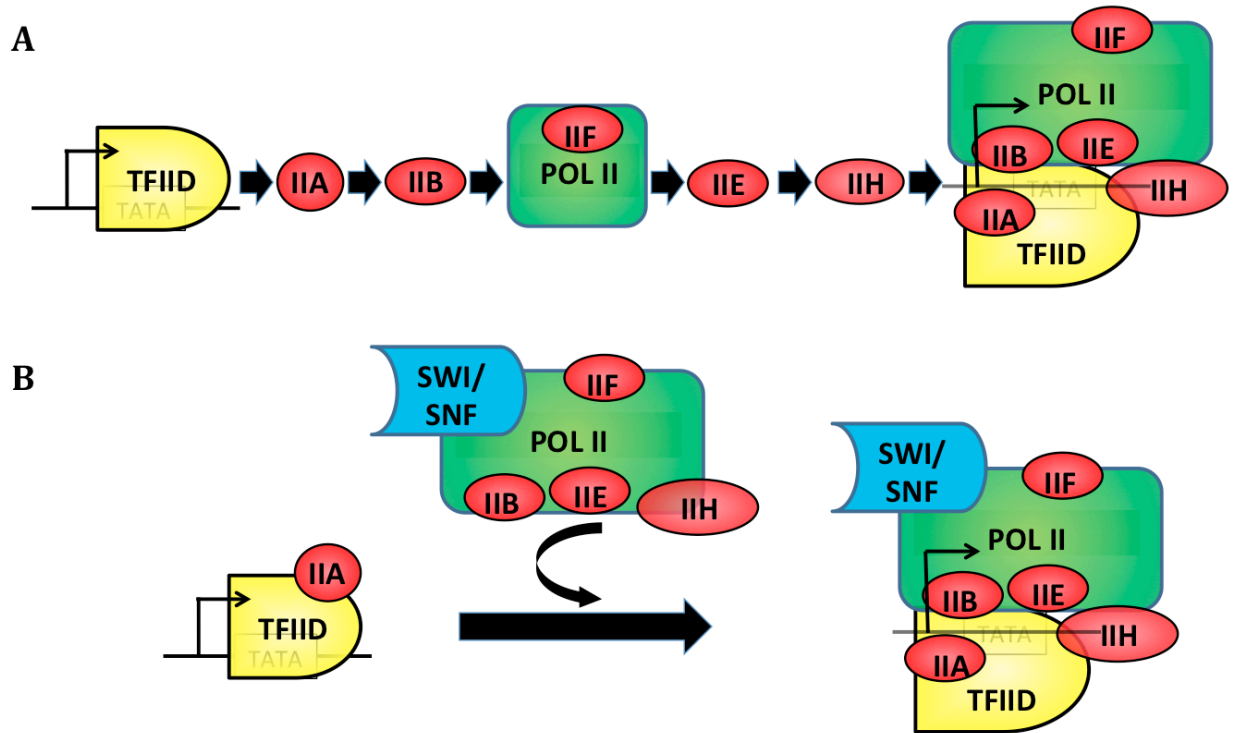
Figure 1. Two opposing models of PIC assembly. (A) The sequential PIC assembly model is shown. (B) The recruitment of the holoenzyme by promoter bound TFIID/TFIIA model is shown.

Chromatin Structure and Function

Eukaryotes compact, protect, and maintain their genomic DNA in a structure called chromatin. The most basic unit of chromatin is a nucleosome, comprised of approximately 147 bp of DNA wrapped 1.7 times around an octamer of histones (two pairs each of histone H2A, H2B, H3, and H4) [36, 37]. This basic nucleosome crystal structure has been solved, and can also be observed by electron microscopy where it appears as "beads on a string" [36, 38]. Within the cell, chromatin exists in two broad categories known as euchromatin and heterochromatin [39]. Euchromatin represents a more relaxed structure, is thought to be more accessible to DNA binding proteins and is associated with active genes. On the other hand, heterochromatin has a more dense structure, is relatively less accessible to DNA binding proteins, and is refractory to gene activation [40]. Importantly, the presence of chromatin in

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general creates an environment that is refractory to gene expression and must be overcome for effective transcription initiation [41, 42].

Chromatin provides a level of transcriptional regulation above the DNA sequence contained within. Nucleosomes can block the accessibility to the underlying DNA sequence, which can affect the binding of transcriptional regulators. Further, the numerous isoforms of histones encoded in humans results in numerous distinct octamers that can be incorporated into nucleosomes [43].

One of the most intensely studied properties of chromatin is the ability of the unstructured histone tails to be post-translationally modified. The tails of all histones in the octamer can be modified by numerous covalent modifications including methylation of arginines, phosphorylation of serines, and acetylation, sumoylation, ubiquitination, and methylation of lysines with over 100 modifications of the histone tails described to date [42, 44, 45]. The Allis group made the original hypothesis that the combination of these modifications could be a "histone code" where the various marks are read by effector proteins and result in distinct, context-dependent downstream consequences on transcription [46]. Although it appears that gene regulation may be more complex than originally thought, there are a number of histone modifications that broadly correlate with euchromatin and gene activation, as well as heterochromatin and transcriptionally silent genomic areas [45, 47]. For example, heterochromatin is generally characterized as having trimethylated H3K27 or H3K9 and hypo-acetylated histone tails [47]. Euchromatin has a larger set of associated marks, with H3K4 and H3K36 trimethylation, and H3K14, H3K18 and H4 acetylation being the most prominent [48, 49]. A large number of biological processes including DNA repair, X-chromosome inactivation, heterochromatin formation, and transcriptional regulation have been shown to be affected by the modification state of the H3 and H4 histone tails [50-

52]. Accordingly, aberrant regulation of these modifications has been implicated in contributing to developmental defects and cancer [53-55].

An area of particular interest is developmental gene promoters that appear to be demarcated for both activation and repression. Such a chromatin state exists in mouse and human pluripotent stem cells, where the positive H3K4me3 and repressive H3K27me3 histone modifications mark the promoters of numerous developmental genes [56]. Genomic regions bearing theses marks are termed "bivalent" and are thought to represent a poised state capable of switching to an active state based when the developmental queue is received.   Indeed, recent reports have shown that the maintenance of H3K4me3 at developmental promoters is critical for full differentiation potential [57].   This thesis focuses on the underlying mechanisms of how the local chromatin environment and presence of either activating or silencing effector proteins effects the formation and resulting function of PICs. Understanding how transcription is regulated at the level of chromatin modification effector proteins is crucial to further our knowledge of complicated developmental programs.

Histone Methyltransferases and Acetyltransferases

Histone methyltransferases (HMTs) are specific enzymes that possess methyltransferase activity on the histone tails. Methyl groups are transferred from S-Adenosyl Methionine to amino acid residues. HMT activity was first isolated in *Drosophila* proteins, and named for the conserved SET domain found in Su(var)3-9, Enhancer of Zeste, and Trithorax which has also been identified in all known HMTs with the exception of Dot1 [50, 58-60]. In particular, lysines can be mono- di- or tri-methylated with various downstream outcomes based on the degree of modification, which residue is modified, and location

relative to the transcriptional start site of a gene [61]. At least five lysines, namely K4, K9, K27, K36, and

K79, within the H3 tail, and one lysine on H4, K20, have been shown to be methylated by histone

methyltransferases (HMTs). These modifications can be separated into two broad categories based on

transcriptional consequence: H3K4, K36 and K79 are associated with active chromatin, and H3K9, K27,

and H4K20 are associated with silent chromatin [44].

Allfrey and Mirsky first reported a description of the enzyme activity that catalyzes the transfer of an

acetyl group from acetyl-Coenzyme-A to lysine residues in histones [62, 63]. Thrity years later, the Allis

group was the first to describe the isolation of an active histone acetyltransferase (HAT), p55, from

Tetrahymena [64]. Further analysis of the p55 protein suggested that it was the homolog of the yeast

transcriptional activator General Control Non-repressible 5 (GCN5) [65, 66]. This discovery was the first

indication that HAT activity may have a direct consequence on transcriptional activity. Based on both

biochemical and genome-wide studies, increasing levels of histone acetylation directly correlated with

increased gene expression [67, 68]. This activation of transcription observed in vivo is most likely due to

the recruitment of chromatin modifying enzymes and histone chaperones (see below).

Active Histone Modifications

H3K4me3, a classic example of a transcription-associated histone tail modification, is broadly conserved

from planarians to humans and is mediated by the SET domain-containing COMPASS complex in yeast

and the Set1 protein in mammals [42, 69-71]. Genome-wide, H3K4me3 strongly correlates with active

genes and peaks at the transcription start site, spreading into the coding regions of active genes [42, 72,

73]. Interestingly, in yeast, a genetic knock out of the SET1 protein specifically abolishes global

H3K4me3 and shows that the lack of this modification results in a slow growth phenotype, indicating that this mark is non-essential and most likely functions to enhance transcription [74].

The enrichment of H3K4me3 at gene promoters suggests a possible role for a chromatin effector protein that binds to the modification. One such protein, Chromodomain Helicase DNA binding protein 1 (CHD1), is an ATP dependent chromatin remodeler that contains a N-terminal tandem chromodomain, shown to specifically recognize H3K4me3, an ATP dependent helicase domain most closely related to SWI/SNF, and a C-terminal A/T hook domain responsible for DNA binding [75-79]. Intriguingly, CHD1 has been shown to interact with SAGA in yeast [80]. Since the original discovery by Delmas and colleagues in 1993, numerous reports have implicated CHD1 as a factor critical in development [76]. Plath and colleagues reported the CHD1 is essential in the maintenance of open chromatin and pluripotency in mouse embryonic stem cells [81]. The authors use siRNA-mediated knockdown of CHD1 to show that lack of the protein results in aberrant differentiation and transcriptional profiles. In Drosophila, genetic knockouts of CHD1 are viable with only a mild notched wing phenotype. However, adults null for CHD1 are sterile underscoring a role for the chromatin remodeler in reproduction [82].

Repressive Histone Modifications

Transcriptional repression via methylation of the N-terminal histone tails is a well-studied process, generally centered on the H3K27 and H3K9 residues [50, 59, 72]. Once methylated by Ezh2 and the Suv39 family of HMTs (Suv39H1, G9a, and GLP), respectively, these residues serve as docking sites for effector proteins, which blocks accessibility to the underlying DNA [50, 59, 83-85]. The H3K27me3 modification is bound by the CBX subunit of Polycomb Repressive Complex 1 (PRC1) and the H3K9me3 modification is bound by the heterochromatin 1 (HP1) family of proteins using a conserved

chromodomain [86, 87]. Both the modifications and binding proteins are critical for the formation and spreading of heterochromatin and proper regulation of developmental genes [52, 58, 88-90].

The classical mark of heterochromatin, H3K9 methylation, is enriched and involved in the forming the silenced state of telomeres, pericentric regions, and X-chromosomes. As mentioned previously, the human pre-SET domain-containing Suv39 family of proteins methylates the H3K9 tail to the mono, di, and trimethylated state. The original discovery of the H3K9me3 HMTs came in *Drosophila*, where screens for suppressors of position effect variegation (PEV) resulted in the isolation of the Su(var)3-9 HMT [84, 91]. This finding indicated that histone methylation plays a direct role in transcriptional silencing, as PEV is an indication of abnormal spreading of heterochromatin into actively expressed regions of the genome. In more recent studies, mice null for Suv39H1 lacked H3K9me3 at pericentric heterochromatin and had abnormal centromere function [92-94]. These results highlight the essential role of the Suv39 HMT in heterochromatin establishment and function.

Recognition of Modified Histone Tails

The activity of methyl- and acetyltransferases on histone tails creates a binding site for chromatin effector proteins. The recognition and specificity of interaction with a methylated lysine residue can be mediated by the interaction of a chromodomain present in some binding proteins with the modified lysine residue and neighboring amino acids. Numerous genetic studies and chromodomain structures have revealed that effector proteins dock to specific methylated lysines. For example, once methylated, H3K9me3 serves as the recruitment platform for the HP1 family of proteins (HP1alpha, beta, and gamma). Chromatin compaction is thought to occur through HP1-HP1 interactions and through HP1-

nucleosome interactions. Trimethylated H3K27 is recognized by the chromodomain-containing PC (CBX in humans) subunit of PRC1. Once docked to the modification, PRC1 may be able to compact chromatin, as suggested by both in vitro and in vivo studies [95, 96]. However, the precise mechanism of PRC1-mediated transcriptional silencing remains elusive. This mechanism will be discussed in detail below and in chapter four.

The acetylation of histone tails can affect the local chromatin environment by serving to recruit factors that harbor a bromodomain (BRD) [97]. Significantly, the bromodomain-containing proteins have been shown to either have ATP-dependent chromatin remodeling or histone chaperone activity. For example, the BRD2 and BRD3 associate with chromatin significantly enriched in H4K5, H4K12, and H3K14 acetylation [98]. Once recruited to promoters, BRD2 acts as a histone chaperone, and is required for cyclin D1 expression in vivo [99]. BRD-containing proteins are conserved from yeast to humans, critical for adipogenesis, gametogenesis, suppression of oncogenesis, and cell specification, and the target of emerging drug candidates to treat disease [99-101].

Mediator

A key co-activator complex for PIC formation, Mediator, was classified as a limiting component of yeast nuclear extract that could counteract activator interference of transcription in vitro [8]. Initial biochemical studies led to the discovery that the Mediator complex augmented activator-induced transcription despite a lack of any DNA binding affinity [8, 9]. While using genetic techniques to investigate complexes involved in transcription in *S. cerevesiae*, similar proteins were contemporaneously identified by the Young group while screening for suppressors of RNA Pol II CTD

deletion mutants, (known as RNA Pol B, and hence named suppressors of RNA Pol B (SRB)) [10, 11, 102]. Other studies consequently showed that Mediator was indeed a multi-subunit complex highly conserved across numerous species and critical for transcription in vivo [103, 104]. Mediator has since been shown to be critical for linking the binding of transcription factor(s) to a promoter with the recruitment of the PIC [105, 106]. Years of challenging biochemical studies by numerous groups have shed light on the structure and function of the Mediator complex.

The Mediator is comprised of multiple modules termed the head, middle, tail and kinase in yeast and humans [107-109]. A list of the modular assignments for each protein in the complex is shown in TABLE 1. Numerous interactions made by the Mediator further support a role played by the complex in PIC formation. First, the tail module has been shown to interact with RNA Pol II, TFIIB, and TBP, as well as affecting basal transcription levels [108, 110]. Second, the middle module is implicated in binding to TBP, TFIIE, and the RNA Pol II CTD [111]. Lastly, the head module is linked to PIC formation and re-initiation of transcription with additional evidence suggesting this module interacts with TFIIE and TFIIH [110, 112, 113].

Meyers and Kornberg stated in 2000 that the convergence of activities and shared subunits of the various Mediator preparation was remarkable and placed the Mediator in a central role as a bridging factor between activators and the transcriptional machinery, an idea first postulated by Young.

Table 1. List of Mediator subunits and modular assignments. Subunits previously crystalized are shown in bold

	H. sapiens				S. cerevisiaea	S. pombe
New name	TRAP/SMCC	ARC/DRIP	CRSP	PC2		
MED1	TRAP220	ARC/DRIP205	CRSP200	TRAP220	Med1	Pmc2
MED1L						
MED2					Med2	
MED3					Pgd1/Hrs1/Med3	
MED4	TRAP36	ARC/DRIP36		TRAP36	Med4	Pmc4/SpMed4
MED5					Nut1	
MED6	hMed6	ARC/DRIP33		hMed6	Med6	Pmc5/SpMed6
MED7	hMed7	ARC/DRIP34	CRSP33	hMed7	Med7	SpMed7
MED8		ARC32			Med8	Sep15/SpMed8
MED9					Cse2/Med9	
MED10	hNut2	hMed10		hNut2	Nut2/Med10	SpNut2
MED11					Med11	
MED12	TRAP230	ARC/DRIP240			Srb8	SpSrb8
MED12L						
MED13	TRAP240	ARC/DRIP250			Ssn2/Srb9	SpTrap240
MED13L						
MED14	TRAP170	ARC/DRIP150	CRSP150	TRAP170	Rgr1	Pmc1/SpRgr1
MED15		ARC105		PCQAP	Gal11	SpGal11*
MED16	TRAP95	DRIP92		TRAP95	Sin4	
MED17	TRAP80	ARC/DRIP77	CRSP77	TRAP80	Srb4	SpSrb4
MED18					Srb5	Pmc6/Sep11
MED19					Rox3	SpRox3
MED20	hTRFP			hTRFP	Srb2	SPAC17G8.05*
MED21	hSrb7	hSrb7		hSrb7	Srb7	SpSrb7
MED22					Srb6	SpSrb6
MED23	TRAP150β	ARC/DRIP130	CRSP130	TRAP150β		
MED24	TRAP100	ARC/DRIP100	CRSP100	TRAP100		
MED25		ARC92				
MED26		ARC70	CRSP70			
MED27	TRAP37		CRSP34	TRAP37		Pmc3
MED28						
MED29	Hintersex					
MED30	TRAP25					
MED31	hSoh1			hSoh1	Soh1*	SpSoh1/Sep10*
CDK8	hSrb10	CDK8			Srb10/Ssn3/Ume5	SpSrb10
CycC	hSrb11	CycC			Srb11/Ssn8/Ume3	SpSrb11

HEAD
MIDDLE
TAIL
UNASSIGNED
KINASE

Human orthologs of the yeast Mediator have since been identified using biochemical and genetic techniques and is further described below [114-117]. Data from multiple laboratories supports that the human Mediator contains up to thirty subunits. The number and stoichiometry of subunits may vary to some degree. To investigate this idea, the Conaway group used MuDPIT analysis of various Mediator preparations isolated from five HeLa cell lines bearing different stably integrated FLAG tagged Mediator subunits and purified by anti-FLAG immuno-affinity [117]. They found a consensus of 30 subunits in all preparations, with varying amounts of individual subunits detected, which were dependent upon which subunit was tagged for purification, supporting the idea that functionally distinct Mediator complexes do not exist.

The first evidence of a mammalian homolog to yeast Mediator, provided by the Roeder group, was the purification of polymerase cofactor 2 (PC2), which fostered activator-stimulated transcription in vitro [118, 119]. Subsequently, the same group, using ligand-activated Thyroid Receptor as bait, reported the first biochemical isolation of mammalian Mediator to homogeneity [120, 121]. The resulting complex was named Thyroid Receptor Associated Proteins (TRAP). Shortly after, numerous groups used various activators as bait with different strategies to isolate the factors necessary for activator-induced transcription. The complexes isolated were named vitamin-D receptor interacting proteins (DRIP), activator recruited cofactor (ARC), rat Med, mouse Med, and E1A associated complex (human Mediator) [122-128]. Importantly, the purified PC2, TRAP, DRIP, ARC, and human Mediator complexes stimulated transcription in the presence of activator on templates in vitro.

Depending on purification strategy and stringency of purification, Mediator can generally be isolated in two major assemblies. The difference between the two complexes is the presence of a dissociable

module with kinase activity, comprised of MED12, MED13, CDK8 and Cyclin C, or the presence of MED26. The binding of each sub-module appears to be mutually exclusive [117]. Of interest, the association with MED26 is thought to correlate with activated transcription, while the association with the MED12-conatining module is thought to decrease the activating function of Mediator in some contexts [129-131].

Numerous activators have been shown to make direct interactions with Mediator subunits resulting in the recruitment of the massive complex to promoters. VP16 interacts with MED25 [132]. MED1, and specifically the LXXLL motif contained within, has been found to serve as docking site for numerous ligand-activated nuclear hormone receptors [122, 133, 134]. E1A recruits the Mediator to promoters through the MED23 subunit [124]. Lastly, STAT2 and SMADS have been shown to interact with MED14 and MED15, respectively [135, 136]. The wide variety of phenotypes observed in Mediator knockouts can be explained at least partially by the large number of surfaces contacted by various activators.

Numerous lines of biochemical evidence have converged to support a role for Mediator as a central cog of PIC formation. In yeast, ChIP for GTFs in Mediator mutant strains underscored that the co-activator complex was critical for the recruitment of TFIIB, TFIIH and TBP to promoters [137, 138]. Further, yeast nuclear extracts depleted of Mediator subunits failed to support activator-dependent PIC formation in vitro [139]. The mammalian Mediator has also been shown to aid in the recruitment of TFIID, RNA Pol II, and the GTFs to activator bound promoters [106, 124, 140, 141]. Mediator and TFIID can bind to promoters cooperatively and the TFIID-TFIIA-Mediator (DAMed) complex acts as a platform to recruit the remaining GTFs and RNA Pol II [106]. The results of a study from the Carey lab underscore the strength of DAMed to recruit PIC components; pre-binding of the complex can negate the need for an

activator protein for PIC function [141].   Importantly, the DAMed complex is somewhat homologous to

the "reinititaion scaffold" described in yeast [142].   This similarity further reinforces the idea that co-

activators play a critical role in gene regulation on chromatin.

In more recent studies, Mediator has been shown to also function to promote transcriptional

elongation. The Conaway group has provided evidence that MED26 interacts with CDK9 and members

of the Super Elongation Complex (SEC) at a site that overlaps with the region responsible for interaction

with TFIID [131].   The consequence of this shared interaction surface is that the recruitment of

elongation factors to the RNA Pol II bound Mediator depends on the abrogation of the interaction

between MED26 and TFIID.   This finding further supports the idea that the PIC components are

regulated temporally in regards to the transcriptional status of RNA Pol II. In another example of the

step-wise nature of PIC assembly, the Carey lab has shown that the initial recruitment of Mediator by

the activator is required for the concomitant recruitment of the p300 histone acetyltransferase [12].

Once present in the PIC, a catalytic switch in p300 is triggered resulting in the acetylation of itself and

chromatin.   This modification stimulates the release of p300 from the PIC and stimulates the

recruitment of TFIID and subsequent PIC transcriptional activity on chromatin. These studies highlight

that Mediator is not merely a central bridging factor in PIC assembly, but also functions to orchestrate

the correct ordered recruitment of factors necessary for transcriptional initiation and elongation.

TFIID

A second co-activator complex, identified in early studies of PIC formation, is the TFIID complex. TFIID,

consisting of fourteen TBP Associated Factors (TAFs) and TBP, acts in both basal and activator stimulated

transcription [143]. Analysis by electron microscopy revealed that TFIID has three major lobes, two of

which contact TFIIA and TFIIB [144]. This structural study also revealed that TFIID has a central cleft,

filled by TBP, that separates the two lobes [144]. The TBP subunit binds to the TATA box and this

binding has long been thought to be an early event in the sequential PIC assembly pathway [7]. In a

pioneering study, Tijan lab discovered that both TAFs and TBP were required to activate transcription via

the NTF-1 activator protein in a purified system [145]. This led to the hypothesis that TFIID was the

scaffold complex recruited by activators to drive PIC formation and thus activated transcription. Further

support for this idea came with the findings that numerous activators required TFIID for transcription,

and that activators recruit and stabilize the binding of TFIID to a promoter in vitro [145-148]. In addition

to the previously mentioned recognition of the TATA box by TBP, TAFs bind to the initiator (Inr),

downstream promoter element (DPE), and downstream core element (DCE). *Drosophila* TAF1 and TAF2

have been implicated in Inr recognition [149]. The human TAF6/TAF9 dimeric complex binds to the DPE

in electro-mobility shift assays and TAF1 contacts the DCE as shown in a crosslinking study [150, 151].

The large size and lobular structure of TFIID creates a large surface area to the complex that allows for

multiple interactions with activators to occur concomitantly. Thus activators and TFIID can cooperatively

bind to promoters to synergies transcriptional activation [152].

An interesting puzzle arose when a study reported the ability of the GAL4-VP16 activator protein to

stimulate transcription in HeLa nuclear extracts that were depleted of the TAFs [153]. This finding

contradicted the evidence that TFIID could support activator-stimulated transcription. However,

subsequent studies resolved this issue. When the Mediator complex was isolated and subjected to in

vitro transcription experiments, it revealed that the complex could functionally substitute for TFIID.

Currently, the consensus in the field is that the Mediator and TFIID bind to promoters in a cooperative manner to enhance transcription [12, 141].

The SAGA Complex

GCN5 was the first transcription-associated HAT identified [64-66]. Previous biochemical and genetic studies implicated GCN5 in associating with ADA2 and ADA3 in yeast [154, 155]. Ensuing purifications of GCN5 complexes resulted in the identification of the first HAT complex, SPT-ADA-GCN5-Acetyltransferase (SAGA) [156]. Interestingly, SAGA components SPT3, 7, and 20 have been shown to interact with TBP [157]. Further, TAF subunits are also members of the SAGA complex, implying that TFIID and SAGA may have somewhat redundant functional roles. Genome-wide binding studies of both SAGA and TFIID in yeast show that although each complex appears to regulate a subset of genes individually, both complexes bind to and regulate the majority of genes [158]. GAL4-VP16 transcription can be stimulated by SAGA and requires the HAT activity of the GCN5 subunit [159]. The recruitment of SAGA by activators such as GCN4 to promoters has been show to occur through interactions with the Tra1 subunit of yeast SAGA [160, 161]. Several studies have shown that SAGA is recruited to promoters early in PIC assembly, suggesting a possible role for SAGA in PIC formation [160, 162-164]. Once bound to promoters, SAGA can exert its HAT activity minimally on H3K9, K14, and K18 [156, 165, 166]. Other H3 tail modifications remain possible targets of SAGA; however, convincing reports of in vivo activity towards other residues still remains to be shown.

More recent studies in mammalian cells have provided evidence of GCN5 homologs in humans including GCN5L and P300/CBP associated factor (PCAF), the latter only being identified in vertebrates to date

[167-169]. These homologs have been shown to be present in four SAGA-like complexes: the PCAF complex, the TBP-Free TAFII-containing Complex (TFTC), the Ada2a-containing (ATAC) and the SPT3-TAFII31-GCN5L-acetyltransferase (STAGA) complex [167, 170-172]. It is important to note that two of these homologs, STAGA and TFTC, are capable of recruiting TBP to promoters in higher organisms, buttressing a model where SAGA and TFIID act redundantly in PIC formation [172-174].

These mammalian complexes, homologous to SAGA, have similar activity and specificity to the yeast SAGA complex in vitro and may be recruited to promoters via interactions with the yeast Tra1 homolog, TRRAP [175, 176]. Interestingly, an outlier is the ATAC complex, which appears to have preferential activity toward the histone H4 tail both in vitro and in vivo, suggesting a distinct functional difference compared to other GCN5-containing complexes [171, 177-179]. Further, genome-wide analysis of both STAGA and ATAC in human cells shows an overall lack of overlapping enrichment [180]. ATAC is enriched at both promoters and enhancers while SAGA is enriched at promoters only. Intriguingly, these two complexes show enrichment at a specific subset of active promoters, and ATAC binds to a significant portion of promoters that are P300 independent [180]. The various functional differences conferred by the accessory proteins of GCN5/PCAF present in each distinct complex suggest myriad roles for these HATs in transcriptional control.

Transcription in Development

The orchestration of specific profiles in gene expression of various cell types and tissues is critical for development of all but the simplest of multicellular organisms. During embryonic development, gene expression profiles change as cells differentiate into more specialized types. This can also be true for

cells of a specific type as development progresses through time. These complex gene regulatory events

are initiated through the action of transcription activators and repressors [181]. Once the cells within an

organism have executed their development programs, the maintenance of core gene expression

programs in the differentiated cells is also critical for the survival of the organism. An example of a

developmental program is body segmentation and identification, which is regulated by the expression of

the homeotic (Hox) genes [182]. The original and most extensive studies of Hox gene regulation have

been performed in *Drosophila*. Shortly after fertilization, the *Drosophila* embryo develops into distinct

body segments, which will continue to differentiate along specific paths to form the various tissues and

body parts of the fly. During early embryonic development in *Drosophila*, a set of transcription factors

including *engrailed* and other segment polarity genes stimulates the expression of specific Hox genes in

each segment [183]. Although the transcription factors controlling this initial expression rapidly

degrade, the segment-specific expression of the Hox genes is maintained to adulthood. Hox genes are

transcription factors that act as upstream regulators of differentiation pathways, acting on various

signaling networks to define a specific developmental fate.

Early studies in *Drosophila* used forward genetic screens to identify the regulators of Hox gene

expression, which include the Polycomb (PC) group proteins [184]. The general trend observed in the PC

group mutants was the shift of body segments toward the posterior of the developing embryo [185].

The first evidence that the PC group proteins acted as repressors came from the observation that the PC

group mutants ectopically expressed the Hox genes. Further, combinations of PC group mutations

displayed more severe phenotypes than each mutant individually, indicating that the genes may work

cooperatively [186, 187].   In further studies, the trithorax (Trx) group, antagonists of the PC group

proteins were identified in screens for suppressors of the PC phenotypes [188, 189]. It is not surprising

to find that the Trx mutants displayed a similar, yet opposing developmental phenotypes as the PC group mutants, but with a shift in body segments toward the anterior of the embryo. Based on evidence of homology to heterochromatin protein 1 (HP1), Paro was the first to hypothesize that Polycomb acted at the chromatin level to regulate and maintain the expression of key developmental regulators [190]. Studies using Chromatin Immunoprecipitation (ChIP) in support of this theory were published shortly after the hypothesis was proposed [191, 192]. Orthologs to both PC and Trx group proteins have been identified in numerous organisms, including plants, *C. elegans*, mice and humans [181, 193-195]. Mouse PC group mutants display a broad range of phenotypes including defects in hematopoiesis, gastrulation, and neurological development, as well as misplacement of axio-skeletal segments supporting previous findings that PC is essential for proper development of an organism [196-199].

Polycomb Repressive Complex 1

A number of studies elucidated the composition of the PC group of proteins, which can be subdivided into two main complexes, Polycomb Repressive Complex 1 and 2 (PRC1 and 2) [200, 201]. The initial isolation of PRC1 from Drosophila revealed that four subunits, polycomb (Pc), polyhomeotic (Ph), posterior sex combs (PSC), and dRING (also known as sex combs extra) comprise PRC1 [200]. In mice and humans, numerous homologs for each subunit exist, resulting in a variety of complex formations [202, 203]. The human chromobox (CBX) proteins 2, 4, 6, 7 and 8 are the major Pc homologs, each containing a chromodomain capable of binding H3K27me3 [204]. Further, the Ph homologs in humans include PH1, PH2, and PH3, and the dRING homologs are RING1a and RING1b. Finally, six different PSC homologs are present in humans, the most prominent being BMI-1 and Mel-18 [203]. The large number of homologs in humans favors the hypothesis that diverse PRC1 complexes exist. A recent study

confirms this hypothesis: using MuDPIT of PRC1 complexes isolated from cell lines tagged with differing subunits, the Reinberg group confirmed that distinct PRC1 complexes with specific subunits exist (Figure 2) [203]. Indeed, the authors observed mutual exclusion of RYBP- and CBX-containing complexes, supporting the existence of diverse complexes. Importantly, since RYBP-containing complexes lack a Pc homolog, these complexes are not traditional PRC1 complexes. As a hallmark of playing a critical role in development, genetic knockout models in mice reveal that PRC1 knockouts have various developmental phenotypes [205-208]. An exception is Ring1b null mice, which have an early lethal phenotype [199].

Once docked to chromatin, either by recognizing H3K27me3 or by an unknown mechanism, PRC1 complexes containing RING1b can monoubiquitinate histone H2A at lysine 119 (H2AK119Ub) [209]. The E3 ligase activity of RING1b has been mapped to the N-terminal Ring finger domain, which interacts with the UbcH5c E2 enzyme [210].   This ubiquitin mark is associated with repression and may lead to compaction of chromatin, although previous studies have suggested the binding of RING1b and subsequent compaction of chromatin is independent of H2AK119Ub in mice [95]. This result was recently refined to a model where although dispensable for compaction, the E3 ligase activity is required for repression of transcription [211]. Although the data is rather ambiguous in this area, point mutations in Ring1b that abrogate E3 ligase activity may affect chromatin compaction, or the stalling of RNA Pol II [95, 211, 212].

Polycomb Complexes

PRC1   PRC2
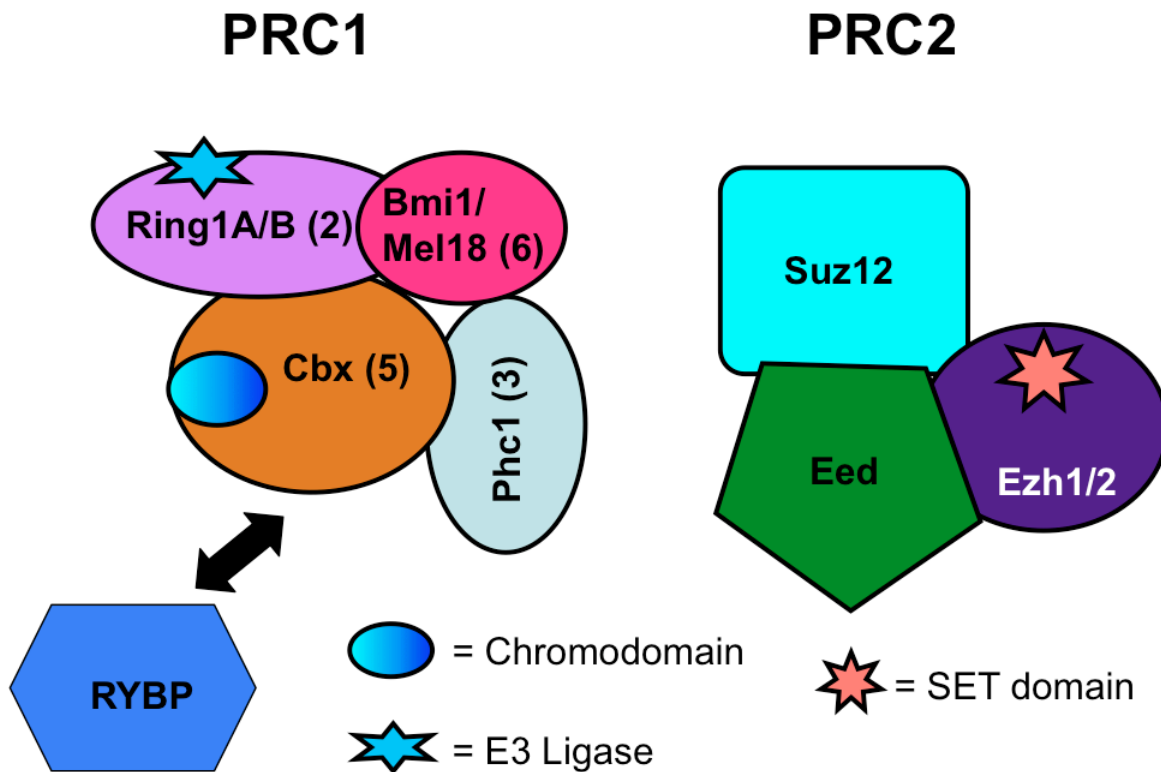
Figure 2. Diagram of the major polycomb complexes and subunits within each complex. Numbers in parentheses following subunit names indicates number of homologs in humans. Note, although PRC1 components can interact with RYBP in a mutually exclusive manner with Cbx, these complexes do not belong to the polycomb family.

Polycomb Repressive Complex 2

Originally isolated in *Drosophila*, the PRC2 complex is comprised of Enhancer of Zeste (Ez), Suppressor of Zeste 12 (Su(z)12), Nurf 55 and Extra Sec Combs (Esc) [213]. Ezh1 and Ezh2 encode the mammalian homologs of Ez, the SET-domain containing subunit responsible for H3K27me3 [214-217]. Despite a high degree of homology, Ezh1 and Ezh2 are expressed at different stages of the cell cycle, suggesting the

presence of two distinct complexes [218]. The mammalian homolog of Esc, embryonic ectoderm development (EED) is alternatively spliced into 4 different isoforms, supporting the existence of diverse PRC2 complexes [219]. The outstanding PRC2 homologs of Su(z)12 and Nurf 55 are SUZ12 and retinoblastoma-associated protein 46 and 48 (RbAp46/48). Interestingly, the activity of the SET domain containing Ezh subunit is stimulated when in complex with the other PRC2 members [219]. Genetic knockouts of PRC2 components in mice have embryonic lethal phenotypes consistent with the critical role of PRC2 activity for proper development [198, 220]. PRC2 has been shown to play a role in maintaining stem cell identity and proliferation, development, and stem cell plasticity, highlighting the critical role of PC in development [213].

Mechanism of Polycomb silencing

Since the discovery of the PC mutant in 1979, many groups have studied the mechanisms of PC-mediated gene silencing. Overall, a model where the HMT activity of PRC2 results in a docking site for the PRC1 complex has been accepted, although recent reports have started to call into question the need of H3K27me3 for silencing [215, 216, 221]. The original mutation described in PC was later determined to be the H3K27me3 binding member of PRC1, suggesting that the methylation mark itself was insufficient to convey silencing, and that PRC1 must have a role in silencing transcription in vivo [222]. An early in vitro study highlighted the possible role of PRC1 in gene silencing; cryo-electron microscopy studies by Woodcock and Kingston showed that a single PRC1 molecule could bind to and compact approximately three nucleosomes in vitro [96]. This raises the possibility that compaction of chromatin by PRC1, which in turn blocks the accessibility of activators and other PIC components, is at least partially responsible for gene silencing (Figure 3). Work from the Kingston group further supported this hypothesis with the finding that SWI/SNF remodeling activity was inhibited by PRC1 [223]. The

authors also provided evidence that native *Drosophila* and recombinant mouse PRC1 was able to silence the activator-induced transcription of an in vitro chromatin template, similar to that used in the Carey laboratory [223]. Intriguingly, in this study PRC1 was only able to silence transcription when pre-bound to the promoter. The role of ubiquitination in PRC1 silencing is yet to be fully understood. The majority of information about the role of PRC1 in gene silencing has come from biochemical assays that were either performed in the absence of ubiquitin or used subunits lacking E3 ligase activity. The Bickmore lab was the first to address the role of the Ring1b E3 ligase activity in the chromatin compaction in mouse embryonic stem cells [95]. Fluorescence in situ hybridization (FISH) was used on adjacent Hox loci to show that Ring1b was necessary for transcriptional silencing and normal spatial regulation of the loci. In agreement with earlier studies, the results revealed that juxtaposition and silencing of the Hox loci was independent of the E3 ubiquitin ligase activity of Ring1b [96].

In flies, at least three studies have utilized ChIP to investigate how PC affects the ability of the transcriptional machinery to bind gene promoters. Elgin and Pirrotta studied the effect of PRC1 silencing on the Hsp26 heat shock promoter [224]. ChIP assays revealed that PRC1 recruitment via a polycomb response element (PRE) did not prevent the recruitment of TBP, RNA Pol II and the heat shock factor to the promoter, but did silence transcription. Interestingly, this result suggests that PRC1 may function to alter the function or inhibit the recruitment of PIC factors critical for activated transcription. Work by the Orlando group focused on the Hox gene promoters and investigated whether TBP, TFIIB, TFIIF, and HDAC1 could be detected at natural PRC1 target genes using ChIP [225].
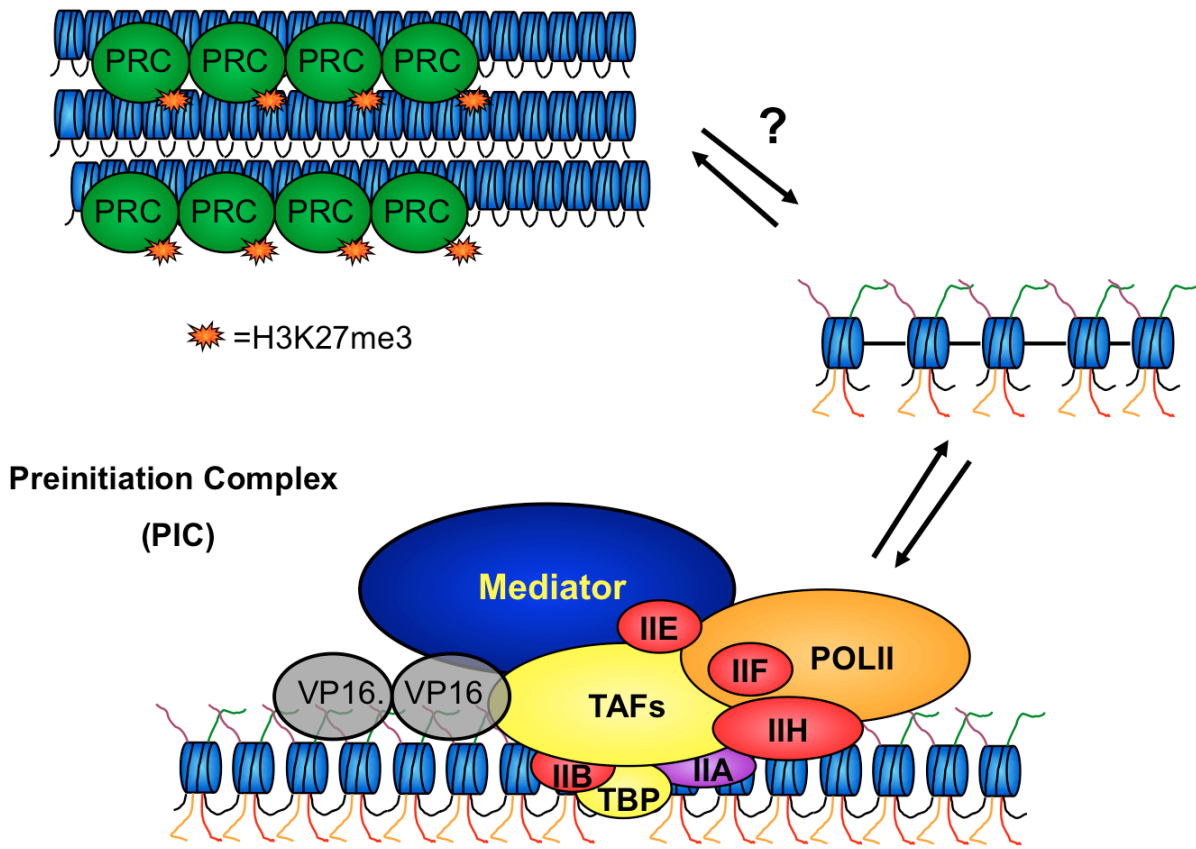
Figure 3. A model for the mechanism for transcriptional silencing by PRC1 via chromatin compaction is shown.

Their studies indicated that PRC1 binding to *Drosophila* gene promoters did not greatly affect the recruitment of the GTFs or HDAC [225]. Again, this indicated that PRC1 might be functioning to silence transcription by affecting the function of any PICs present at the promoters. The Fuller lab investigated this in *Drosophila* testes, where Polycomb silencing is reversed. Here, PC promotes tissue specific gene expression of the testes-specific TAF (tTAF) proteins, which are required for terminal differentiation of cells [226]. ChIP experiments of tTAF and PRC1 member Pc displayed that tTAFs bind to their target promoters, reduce the enrichment of PRC1, and promote the local accumulation of active H3K4me3 histone mark [226]. Interestingly, TFIID components were found to co-purify with PRC1 in Drosophila suggesting a direct connection between the two complexes [227]. The results of these studies revealed that PICs may form on PRC1-regulated promoters, that TAFs may be able to counteract PRC1 binding, and that PRC1 can interact with TFIID in flies. However, population effects, resulting from lower than optimal enrichments levels may make it challenging to gain mechanistic insight from ChIP studies. Prior to my studies investigating the mechanism of PRC1 silencing in mammals, there was a lack of insight as to how PRC1 precisely acted on PIC formation to silence transcription. My work in this area, using in vitro transcription and immobilized template experiments, revealed that PRC1 can functionally block PIC formation by inhibiting the recruitment of a number of PIC components and may allow TFIID binding to promoters (see chapter 4).

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Chapter 2

Mediator and SAGA have distinct roles in Pol II preinitiation

complex assembly and function

Cell Reports

Report

Mediator and SAGA Have Distinct Roles in Pol II Preinitiation Complex Assembly and Function

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SUMMARY

A key feature of RNA polymerase II (Pol II) preinitiation complexes (PICs) is their ability to coordinate transcription initiation with chromatin modification and remodeling. To understand how this coordination is achieved, we employed extensive proteomic and mechanistic analyses to study the composition and assembly of PICs in HeLa cell and mouse embryonic stem cell (ESC) nuclear extracts. Strikingly, most of the machinery that is necessary for transcription initiation on chromatin is part of the PIC. The PIC is nearly identical between ESCs and HeLa cells and contains two major coactivator complexes: Mediator and SAGA. Genome-wide analysis of Mediator reveals that it has a close correlation with Pol II, TATA-binding protein, and messenger RNA levels and thus may play a major role in PIC assembly. Moreover, Mediator coordinates assembly of the Pol II initiation factors and chromatin machinery into a PIC in vitro, whereas SAGA acts after PIC assembly to allow transcription on chromatin.

INTRODUCTION

Genes that are transcribed by RNA polymerase II (Pol II) contain chromatin modifications that facilitate initiation and early elongation (Wang et al., 2008, 2009; Agalioti et al., 2002; Li et al., 2007, 2008). These modifications often bind to ATP-dependent remodeling proteins such as the bromodomain-containing SWI/SNF and the chromodomain-containing CHD1, which mobilize nucleosomes to allow binding of the Pol II machinery (Hargreaves and Crabtree, 2011). Our groups are interested in determining how the assembly and function of the Pol II preinitiation complex (PIC) coordinate with the chromatin-modifying and -remodeling events at a promoter. To address this issue and study the mechanisms involved, we previously recreated transcription on naked DNA and chromatin templates, and captured the resulting PICs

using templates immobilized on magnetic beads (Lin et al., 2011; Black et al., 2006; Johnson et al., 2002). This approach permits a detailed examination of PIC composition and function.

In our initial studies on immobilized, naked DNA templates, we found that the activator GAL4-VP16 formed a complex with the TFIID and Mediator coactivators that was necessary for efficient recruitment of Pol II and the general transcription factors (GTFs; Johnson et al., 2002), in broad agreement with the view of these coactivators as bridging factors (D'Alessio et al., 2009; Näär et al., 2001; Kornberg, 2005). On chromatin, we found that the histone acetyltransferase p300 acts in concert with the Mediator coactivator very early in PIC assembly and prior to the recruitment of the GTFs (Black et al., 2006, 2008). The p300-mediated acetylation of itself and chromatin led to p300 dissociation and allowed binding of the TFIID complex to Mediator, which facilitated assembly of the PIC.

In our early studies we employed immunoblotting to identify factors thought to be involved in PIC assembly and function. To obtain a more detailed understanding, we employed multidimensional protein identification technology (MuDPIT) to detect factors captured by the immobilized template (IT) on unmodified and H3K4-trimethylated (H3K4me3) chromatin templates (Lin et al., 2011). Our analysis revealed that a wide range of protein complexes involved in chromatin modification and remodeling were recruited to the PIC along with Mediator, TFIID, and Pol II. Importantly, SAGA, a well-studied H3 histone acetyltransferase in yeast and mammals, and a major coactivator in yeast (Baker and Grant, 2007; Nagy and Tora, 2007), was typically among the highest abundance factors. Moreover, numerous Pol II elongation complexes, such as PAF and the CDK9-containing super-elongation complex (SEC), were detected (Smith et al., 2011).

After Pol II is initiated, it pauses 30–50 bp downstream of the transcription start site (TSS) through the action of DSIF and NELF. The SEC, which is recruited by Mediator (Takahashi et al., 2011), plays a postinitiation role by phosphorylating DSIF and NELF, thereby releasing the paused Pol II. Because many genes in vivo contain paused Pol II (Nechaev and Adelman, 2011), our work suggests that in these cases, Mediator's initial role may be in releasing the pause, after which it establishes a PIC to allow multiple rounds of transcription.

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A IN VITRO TRANSCRIPTION

B MuDPIT ANALYSIS OF IMMOBILIZED TEMPLATES

C IMMOBILIZED TEMPLATE
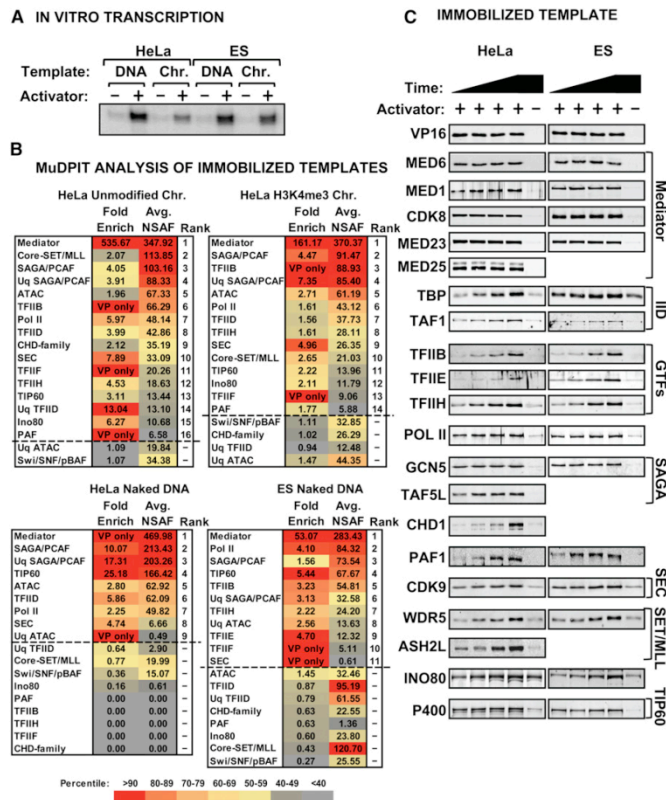
Figure 1. PIC Analysis in HeLa Cell and Mouse ESC Nuclear Extracts

(A) IVT from HeLa extract and mouse ESC nuclear extract on naked DNA and chromatin templates.

(B) MuDPIT analysis of PICs formed on naked DNA and chromatin templates in HeLa cell and ESC extracts in the presence and absence of GAL4-VP16. The primary data were analyzed by MS Sort (see Table S1) and the resulting activator-enriched (≥1.5-fold; above dotted line) protein complexes were scored by average NSAF abundance. Average unique NSAFs (Uq-) for some complexes are also shown as described in the text (see Extended Experimental Procedures for a detailed description of the analysis). The fold enrichment and average NSAF values are color-coded according to the percentile of proteins shown within each experimental condition (e.g., both fold enrichment and NSAF values for Mediator are above the 90th percentile of proteins shown in the chart for each experimental condition and therefore are colored red (see key).

(C) Immunoblots representing known PIC components assembled during a 54 min time course on naked DNA templates using either HeLa cell (left) or mouse ESC (right) nuclear extract. See also Figure S1.

The results of our initial proteomic study raised several important questions. First, are the chromatin modification and remodeling and Pol II elongation factors detected in our chromatin study components of PICs in vivo and in vitro? This bears on whether chromatin factors are general features of the PIC, whose recruitment is controlled by the activator or a major coactivator. Second, are the PICs that are formed in HeLa cells representative of PICs in other cell types, such as embryonic stem cells (ESCs), which are emerging as an exciting area of biological interest? Finally, given the abundance of SAGA, what is its role in PIC assembly and function? Previous findings in *Saccharomyces cerevisiae* showed that SAGA replaces TFIID at TATA-box-containing promoters (Bhaumik and Green, 2002; Lee et al., 2000; Huisinga and Pugh, 2004), whereas TFIID is employed at TATA-less promoters (Basehoar et al., 2004). These findings were surprising because SAGA was thought to be simply a histone acetyltransferase and deubiquitinase.

To address these questions, we employed MuDPIT to determine the composition of GAL4-VP16-stimulated PICs in vitro on DNA templates using transcriptionally active extracts from HeLa cells and mouse ESCs. We then compared the composition of the DNA PICs with that of our chromatin PICs. We found them to be highly similar, indicating that the chromatin modification and remodeling machinery is an inherent component of PICs. We then compared the in vitro PICs with Mediator-associated factors from HeLa and ESC nuclei isolated at a low salt concentration, where PIC components remain associated with chromatin (Dignam et al., 1983). The composition of the native PICs was remarkably similar to that of the in vitro PICs. Finally, we delineated the roles of SAGA and Mediator. Our data suggest that the coordinated binding of most chromatin and Pol II elongation factors, which act near the start site, is largely due to the Mediator coactivator. Importantly, we found that for the GAL4-VP16 activator, SAGA is not a coactivator in the traditional sense but functions independently of PIC assembly to allow transcription on chromatin templates.

RESULTS AND DISCUSSION

Mediator and SAGA Coactivators Are Abundant in PICs from HeLa Cells and ESCs

To study PIC assembly in HeLa versus mouse ESCs, we prepared nuclear extracts and compared their transcriptional activities using the model activator GAL4-VP16 along with a GAL4-responsive template. Figure 1A shows that ESC nuclear extracts are transcriptionally active and responsive to the activator (Lin et al., 2011). We then captured the transcriptionally

competent PICs using templates immobilized on magnetic beads (Lin and Carey, 2012). We compared unmodified chromatin, H3K4me3 chromatin, and naked DNA for HeLa nuclear extract, and naked DNA for the E14 ESC nuclear extract. The associated factors were analyzed by MuDPIT and expressed in units of normalized spectral abundance factor (NSAF; Washburn et al., 2001). Individual transcription-related proteins were sorted into complexes by an R-based program termed MS Sort, which was developed in house (see Extended Experimental Procedures). The complexes were then ranked according to activator inducibility and overall abundance. Because TFIID and SAGA share certain core subunits, we employed a "unique" average NSAF to identify whether each complex was definitively present. For cases in which unique subunit coverage of closely related complexes was too low, we relied on shared subunits or related family members to derive a score for the family of complexes (i.e., the SET1a/b and MLL1-4 complexes, and the CHD family of proteins; Nagy and Tora, 2007; Eissenberg and Shilatifard, 2010; Marfella and Imbalzano, 2007). The activator inducibility and rank abundance of the captured PICs are listed in Figure 1B. Proteins that were enriched >1.5-fold after addition of GAL4-VP16 were designated as activator stimulated. Proteins that were recruited from dialyzed nuclear extracts accurately reflect the preinitiation state because no nucleotides were added and similar recruitment data were obtained in the presence of α-amanitin and Apyrase (Figure S1).

Mediator was the most abundant activator-stimulated factor on both DNA and chromatin templates in both ES and HeLa cell extracts (Figure 1B; Table S1). Mediator is nearly identical between mice and humans (Conaway and Conaway, 2011), and 94% of the subunits were detected. SAGA was typically the second most abundant coactivator in both HeLa and ESC PICs and, like Mediator, was found on both DNA and chromatin templates (Figure 1B). SAGA contains the GCN5 HAT and a histone H2B monoubiquitination-specific protease submodule (Nagy and Tora, 2007). The abundance of SAGA on naked DNA templates was surprising. Most of our work and that of others in mammalian systems (Johnson and Carey, 2003; Johnson et al., 2002; Thomas and Chiang, 2006) suggested that Mediator and TFIID play the central roles in assembly of transcriptionally active PICs by recruiting the GTFs. Although TFIID was detected in both ESC and HeLa cell PICs, it was less abundant than SAGA and was less activator responsive in ESC extracts. Collectively, these data pointed to the possibility of a role for SAGA in PIC function.

Pol II and GTFs were also detected by MuDPIT in HeLa cell and in ESC PICs on naked DNA and chromatin. TFIIB recruits Pol II (Kettenberger et al., 2004; Chen and Hahn, 2003; Liu et al., 2010), and TFIIE and TFIIH represent the final steps in PIC assembly (Carey, 2012; Grünberg et al., 2012). Factors known to facilitate Pol II elongation, such as the CDK9-containing SEC, PAF, and TFIIF, were also found in PICs on DNA and chromatin. CDK9 promotes the release of Pol II paused by DSIF and NELF downstream of the TSS (Smith et al., 2011). PAF serves as a platform for binding of Pol II (Jaehning, 2010) but also associates with chromatin factors such as the SET1 complex (Smith et al., 2011) and CHD1 (Warner et al., 2007). Indeed, the core WDR5-RBBP5-ASH2L-DPY30 (WRAD) submodule from the SET1 trimethylase complexes was also de-

tected (Table S1). The SWI/SNF complex was abundant on chromatin and DNA templates but not activator stimulated, whereas the Ino80 chromatin-remodeling complex was modestly activator stimulated (Figure 1B).

Due to the crude nature of HeLa cell and ESC extracts, low-abundance proteins typically fall outside the dynamic range of detection. To compensate for this, we performed immunoblotting of select unique subunits of a protein identified by MuDPIT to validate the relative amounts in a DNA versus a chromatin PIC. An analysis of DNA PICs revealed that subunits from each of the complexes were present in both HeLa cell and ESC PICs, and their activator-inducibility and binding profiles in a time course were nearly identical between the two extracts (Figure 1C). Finally, immunoblotting of PICs on chromatin showed that most factors detected by MuDPIT were present and activator inducible on chromatin as on DNA (Figure S1).

Taken together, our data suggest a model of the mammalian PIC in which an activator stimulates recruitment of GTFs and coactivators, including Mediator and TFIID, along with numerous chromatin-remodeling and -modifying enzymes. The observation that numerous chromatin and early Pol II elongation factors are detected in activator-stimulated PICs alongside Pol II and the GTFs in HeLa cells and ESCs suggests that they are important components of all mammalian PICs. This conclusion suggests that the major coactivators that control assembly of the GTFs also control chromatin events associated with initiation. To address this issue further, we examined whether Mediator and SAGA associate with PIC components on native chromatin isolated from HeLa cells and ESCs to determine the roles they play in PIC assembly and transcription in vitro.

Roles of Mediator and SAGA in PIC Assembly

Figure 2A shows a heat map of Mediator distribution in ESCs generated from published genome-wide data sets sorted by Pol II abundance (Kagey et al., 2010; Shen et al., 2012). The data reveal a remarkable similarity among the relative amounts of Mediator, Pol II, and TATA-binding protein (TBP) bound to a given gene and a close correlation with messenger RNA (mRNA) levels. This observation, along with Mediator's abundance in PICs formed in vitro, suggests that Mediator is common to many Pol II PICs in vivo, where it likely acts both in PIC assembly and in release of paused Pol II (Figure 2A). If so, then isolation of Mediator from native chromatin, as opposed to soluble nuclear extracts, should be representative of PIC composition in vivo. We isolated Med29-tagged Mediator under different stringencies from sonicated HeLa cell and ESC nuclei isolated at low salt under conditions in which the transcriptional machinery is not solubilized (Dignam et al., 1983). MuDPIT revealed that many of the proteins found in an in-vitro-assembled PIC copurified with Mediator from HeLa cell and ESC chromatin (Figure 2B). However, in contrast to the in-vitro-assembled PIC, the most abundant Mediator-associated proteins from cellular chromatin were TFIIF, TFIIB, and Pol II in HeLa cells and TFIIF, and Pol II in ESCs (Figure 2B; Table S2). The association of TFIIF and Pol II with the Mediator was previously noted (Liu et al., 2008), and TFIIB is known to associate with Pol II when it is engaged at the TSS (Kostrewa et al., 2009). TFIID is the next most abundant complex in HeLa cells. TFIIH was also detected

Cell PRESS

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A GENOMEWIDE EXPRESSION AND BINDING

mRNA | TBP | MED1 | POL II

RPKM
≥25
0

-logP
8
0

-5.0 kb TSS +5.0 kb (for TBP, MED1, POL II)

B MuDPIT ANALYSIS

	HeLa				ES
[Salt]					
Mediator	480.0	602.4	1138.0	1061.9	508.5
TFIIF	0.0	0.0	322.7	528.8	123.7
TFIIB	0.0	0.0	428.5	486.6	0.0
Pol II	26.1	17.8	461.1	451.8	162.1
TFIID	12.5	8.2	45.3	52.7	4.1
SAGA/PCAF	0.6	0.0	27.0	32.3	9.5
Uq TFIID	15.6	10.2	32.2	36.8	0.7
SEC	0.0	0.0	25.9	33.4	4.6
Uq SAGA/PCAF	1.3	0.0	24.1	27.3	11.4
PAF	2.7	0.0	16.0	13.8	8.1
CHD-family	3.5	1.7	10.1	11.8	11.7
Core-SET/MLL	0.0	0.0	12.3	11.5	37.3
ATAC	0.0	0.0	6.3	9.3	4.7
TIP60	0.0	0.0	2.1	3.3	2.5
Swi/SNF/pBAF	0.0	0.0	2.5	2.4	18.9
TFIIH	0.0	0.0	0.8	2.2	14.9
Ino80	0.0	0.0	0.0	0.0	0.7
Uq ATAC	0.0	0.0	0.0	0.0	0.0
TFIIE	0.0	0.0	0.0	0.0	26.3

Percentile: >90 | 80-89 | 70-79 | 60-69 | 50-59 | 40-49 | <40

C PEARSON CORRELATIONS

	HeLa Med	Naïve PIC	ES Med
HeLa Med	1.00	0.75	0.90
Naïve PIC	0.75	1.00	0.85
ES Med	0.90	0.85	1.00

Pearson: 0.75 — 1.00

D IMMOBILIZED TEMPLATE

	Mock	Δ Med
Mediator:	− −	− − +
Activator:	− +	− + +

VP16
MED23 — Mediator
MED6
TFIID
TFIIH — GTFs
TFIIB
POL II
PAF1
CDK9 — SEC
GCN5
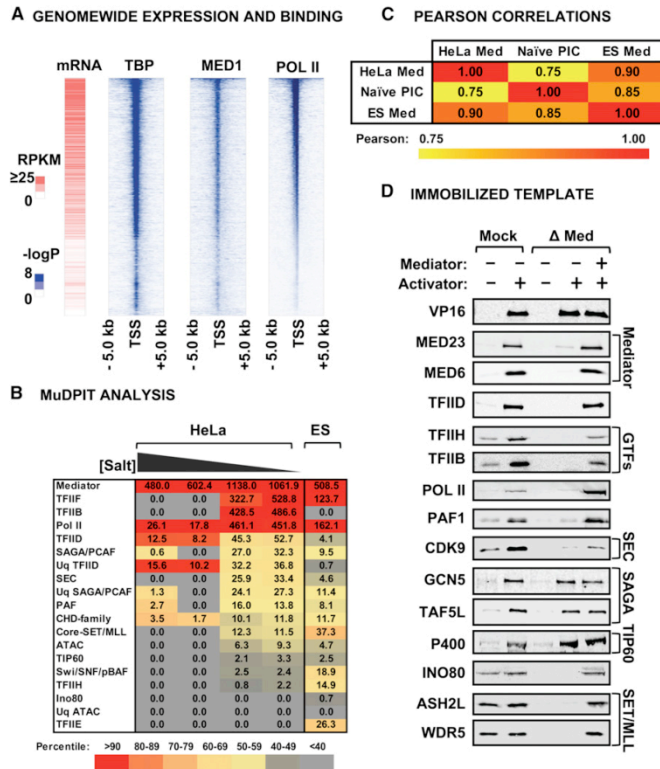TAF5L — SAGA
P400 — TIP60
INO80
ASH2L — SET1/MLL
WDR5

Figure 2. Mediator Composition and Function in PIC Assembly

(A) Genome-wide analysis of Mediator, TBP, and Pol II distribution in ESCs. Genes were ranked by average binding of Pol II (-log [p value]) within a 10 kb window surrounding the TSS of mouse promoters with a significant enrichment for any of the three factors ($p < 10^{-5}$). Expression data (reads per kilobase per million [RPKM]) were plotted consequently for the same ranking order.

(B) Chart depicting average NSAFs of complexes detected in MuDPIT analysis of purified FLAG-Med29 from sonicated HeLa and ESC nuclei at 100, 200, 300, and 500 mM KCl (indicated as a gradient). ESC Mediator was isolated from nuclei at 420 mM KCl. After MuDPIT and MS Sort, average NSAFs for proteins were ranked by abundance relative to the HeLa 100 mM data set. Values are color-coded according to percentile rank within all detected complexes.

(C) Pearson correlation comparison of Mediator-associated factors in unmodified chromatin and PICs assembled in vitro.

(D) Immunoblots of PIC assembly comparing the recruitment of Mediator-associated protein complexes identified by MuDPIT in Mock- or Mediator-depleted HeLa nuclear extracts. Pure Mediator was added back to depleted extracts to rescue binding. See also Figure S2.

SET1/MLL, and Ino80 complexes. The addition of pure Mediator to the depleted extract restored binding of the affected factors, which were recruited from the depleted extract because they are not found in highly purified Mediator (Figure 2D; Figure S2). Interestingly, despite previous work suggesting a TRRAP-subunit-independent interaction between Mediator and SAGA, Mediator was not necessary for recruitment of SAGA or TIP60 (Figure 2D; Liu et al., 2008). If SAGA were a bona fide coactivator for GAL4-VP16, it should associate with GTFs or Mediator, or be important for some other aspect of PIC assembly.

Interestingly, unlike Mediator, Spt3-tagged SAGA (Martinez et al., 1998) isolated from chromatin did not contain any other factors in abundance except for the chromatin remodeler NuRD (Figure 3A; Table S3). Indeed, many of the factors detected by MS Sort were not unique; for example, the TFIID subunits detected were shared by SAGA. To our knowledge, SAGA has not been examined by chromatin immunoprecipitation (ChIP) sequencing in ESCs; however, studies in human cell lines suggest that it binds only a subset of genes, in a somewhat exclusive manner, with another GCN5-containing complex called ATAC (Krebs et al., 2011).

To further analyze SAGA's role in the PIC, we depleted it from HeLa extracts using GCN5 and TAF5L antibodies (Figure S2). Surprisingly, we found that SAGA depletion did not significantly affect GAL4-VP16-mediated PIC assembly on chromatin (Figure 3B) or DNA templates (data not shown). This result coupled

in the HeLa cell and ESC preparations, and TFIIE was found in the ESCs, albeit at lower abundance. These observations are consistent with current models in which TFIID, Mediator, and Pol II cooperatively bind TFIIB and nucleate the assembly of other GTFs at the promoter. Other factors, such as PAF, SEC, and DSIF, were identified (reviewed in Nechaev and Adelman, 2011) along with the chromatin-modifying enzymes SAGA, SET1, and TIP60 (Figure 2B; Table S2).

A Pearson correlation comparison of the factors bound to Mediator revealed a strong similarity among HeLa cell and ESC chromatin and GAL4-VP16-stimulated PICs (Figure 2C). We conclude that the factors recruited by GAL4-VP16 in vitro are similar to those recruited by cellular activators in Mediator-associated PICs in vivo. Importantly, an IT MuDPIT analysis of ATF6α, on the *HSPA5* promoter, revealed a set of factors similar to those found in our PIC study (Sela et al., 2012).

Mediator plays a central role in recruitment of many new PIC factors. IT assays in Mediator-depleted extracts revealed that Mediator is necessary for efficient recruitment of TFIID, the GTFs, and the CDK9 subunit of PTEFb/SEC, in agreement with a previous study (Takahashi et al., 2011), and the PAF1,

A   MuDPIT ANALYSIS

[Salt]

	NE	Chromatin	
Uq SAGA/PCAF	2963.87	709.84	408.57
SAGA/PCAF	2372.39	439.02	245.68
TFIID	563.98	118.38	72.48
Core-SET/MLL	4.75	0.00	35.80
TIP60	149.04	37.47	17.43
CHD-family	2.42	0.80	0.82
ATAC	789.26	27.39	0.51
Swi/SNF/pBAF	0.60	0.00	0.26
Mediator	0.46	0.00	0.00
TFIIF	0.00	0.00	0.00
TFIIB	0.00	0.00	0.00
Pol II	0.00	0.00	0.00
Uq TFIID	0.00	0.93	0.00
SEC	0.62	0.00	0.00
PAF	0.00	0.00	0.00
TFIIH	0.00	0.00	0.00
Ino80	0.00	0.00	0.00
Uq ATAC	2.27	0.00	0.00
TFIIE	0.00	0.00	0.00

Percentile: >90   80-89   70-79   60-69   50-59   40-49   <40

B   IMMOBILIZED TEMPLATE

C   IN VITRO TRANSCRIPTION

E
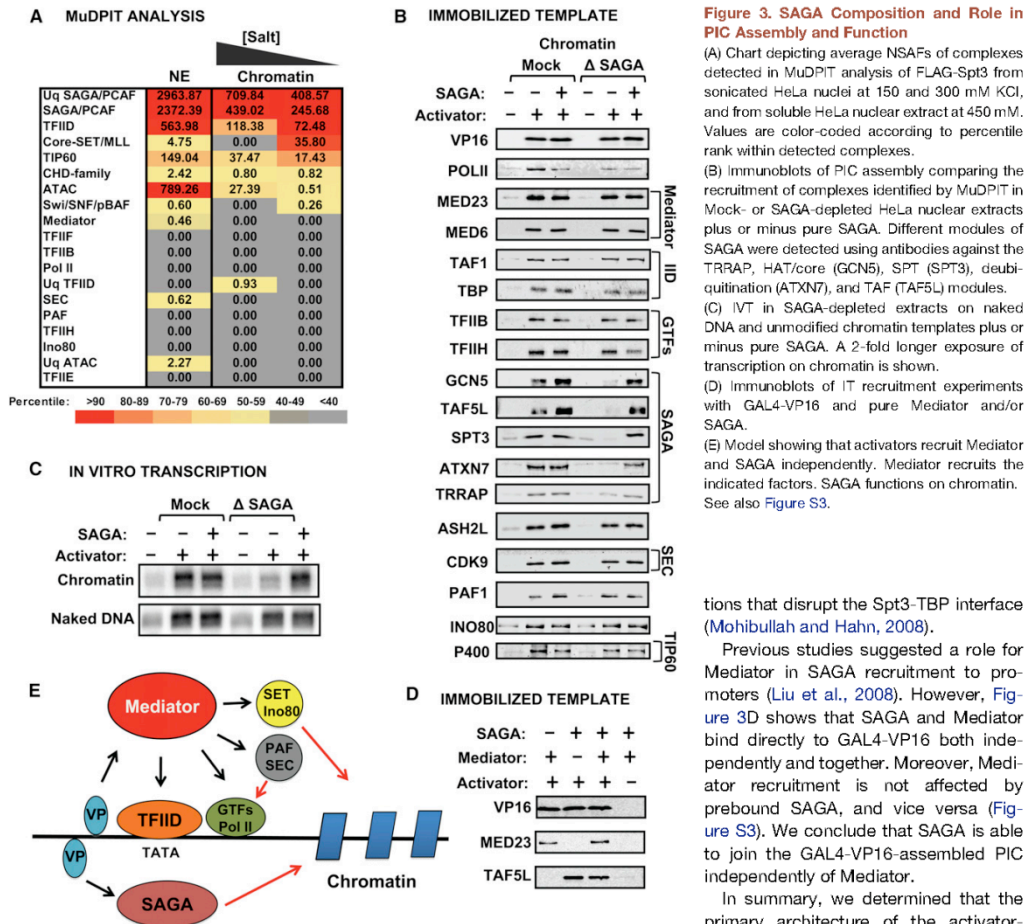

D   IMMOBILIZED TEMPLATE

Figure 3. SAGA Composition and Role in PIC Assembly and Function

(A) Chart depicting average NSAFs of complexes detected in MuDPIT analysis of FLAG-Spt3 from sonicated HeLa nuclei at 150 and 300 mM KCl, and from soluble HeLa nuclear extract at 450 mM. Values are color-coded according to percentile rank within detected complexes.

(B) Immunoblots of PIC assembly comparing the recruitment of complexes identified by MuDPIT in Mock- or SAGA-depleted HeLa nuclear extracts plus or minus pure SAGA. Different modules of SAGA were detected using antibodies against the TRRAP, HAT/core (GCN5), SPT (SPT3), deubiquitination (ATXN7), and TAF (TAF5L) modules.

(C) IVT in SAGA-depleted extracts on naked DNA and unmodified chromatin templates plus or minus pure SAGA. A 2-fold longer exposure of transcription on chromatin is shown.

(D) Immunoblots of IT recruitment experiments with GAL4-VP16 and pure Mediator and/or SAGA.

(E) Model showing that activators recruit Mediator and SAGA independently. Mediator recruits the indicated factors. SAGA functions on chromatin. See also Figure S3.

tions that disrupt the Spt3-TBP interface (Mohibullah and Hahn, 2008).

Previous studies suggested a role for Mediator in SAGA recruitment to promoters (Liu et al., 2008). However, Figure 3D shows that SAGA and Mediator bind directly to GAL4-VP16 both independently and together. Moreover, Mediator recruitment is not affected by prebound SAGA, and vice versa (Figure S3). We conclude that SAGA is able to join the GAL4-VP16-assembled PIC independently of Mediator.

In summary, we determined that the primary architecture of the activator-stimulated PIC consists of high-affinity interactions between Pol II and the GTFs, driven by Mediator. Mediator also plays an essential role in recruiting several key chromatin-modifying and -remodeling complexes, such as SET1, CHD1, and Ino80, along with two major Pol II elongation complexes, PAF1 and SEC (Figure 3E). Therefore, the activator-stimulated PIC contains most of the key factors that are thought to control promoter accessibility within chromatin and early Pol II elongation. Importantly, most of the interactions are found in PICs formed on naked DNA, suggesting that promoter-bound chromatin factors are targeted to the PIC not by chromatin but by interactions with Mediator, by direct interactions with activator, or through complex networks of interactions linked to both activator and Mediator. Despite SAGA's relative abundance, its binding is directed by GAL4-VP16, and it functions after PICs are assembled (Figure 3E).

with our proteomics data, argues that SAGA is not linked directly to PIC assembly and is therefore not a coactivator in the traditional sense, i.e., a bridging factor. Moreover, the depletion of SAGA had virtually no effect on naked DNA transcription (Figure 3C). Remarkably, chromatin transcription was strongly and reproducibly diminished by depletion and restored with pure SAGA. We conclude that SAGA does not affect PIC assembly but is required for efficient transcription of chromatin templates. It is unclear whether SAGA directly influences PIC assembly in yeast. Spt3 depletion does not affect TBP or TFIIA binding in PICs from yeast extracts (Warfield et al., 2004), but SAGA depletion abolishes activated transcription on naked DNA templates (Fishburn et al., 2005), which differs from our results in HeLa. Also, Tra1 mutants display some defects in recruitment of Pol II in vivo by ChIP assays (Knutson and Hahn, 2011), as do muta-

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EXPERIMENTAL PROCEDURES

Recruitment and In Vitro Transcription Assays

G5E4T (Johnson and Carey, 2003) was assembled into chromatin by salt dilution and immobilized as described previously (Steger et al., 1997). IT and in vitro transcription (IVT) assays, and corresponding scaled-up reactions for MuDPIT were performed as described previously (Lin and Carey, 2012; Lin et al., 2011).

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The data sets used for genome-wide analysis were TBP-GSE22303, MED1-GSE22557, and RNA Pol II-GSM723019 from the GEO database (http://www.ncbi.nlm.nih.gov/geo/). The expression levels for all annotated genes were determined using the GSM881355 GEO data set. All data analysis was performed as previously described (Ferrari et al., 2012)

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Supplemental Information

EXTENDED EXPERIMENTAL PROCEDURES

IT Recruitment Assay Antibodies

Antibodies used in immunoblotting included MED23 (BD PharMingen, 550429), MED25 (Carey Lab Stock), Pol II CTD 8WG16 (QED Bioscience), TFIIB (Tantin et al., 1996), TAF5L (Sigma, SAB4501194), CHD1 (Bethyl, A301-217A), PAF1 (Bethyl, A300-173A), WDR5 (Bethyl, A302-429A), INOC1 (Bethyl, A303-370A), EP400 (Bethyl, A300-541A), GAL4-VP16 (Carey Lab Stock), TBP (Santa Cruz, SC-204), TAF1 (Santa Cruz, SC-735), MED1 (Santa Cruz, SC-8998), MED6 (Santa Cruz, SC-9434), CDK8 (Santa Cruz, SC-1521), TFIIE-α (Santa Cruz, SC-237), CDK9 (Santa Cruz, SC-484), TFIIH-p89 (Santa Cruz, SC-293), GCN5 (Santa Cruz, SC- 20698X).

Immunodepletions

Immunodepletion of Mediator was performed as described (Lin et al., 2011). Briefly, antibodies were cross-linked to protein A and G paramagnetic beads (Invitrogen) using 20 mM dimethylpimelimidate in 0.1 M Sodium Borate buffer, pH 9, and washed extensively with 50 mM Glycine pH 2.5. The cross-linked beads were equilibrated in buffer D (20 mM HEPES, pH 7.9, 0.1 mM EDTA, 20% glycerol, 0.1 M KCl) and incubated with HeLa nuclear extract in buffer D, for 4 hr at 4°C. The supernatant was isolated and used for IT analysis as described above. The conditions used for purification are cited in the legends.

Genome-wide Analysis

Reads were mapped to the mouse (mm19) genome using Bowtie software. Only reads that aligned to a unique position in the genome with no more than two sequence mismatches were retained for further analysis. Duplicate reads that mapped to the same exact location in the genome were counted only once to reduce clonal amplification effects. The genome was tiled into 50-bp windows. Each read was extended by 150 bases (we refer to tags as the extended read counts within a bin) and was counted as one read to each window to which it partially or fully matched. The total counts of the input and ChIP samples were normalized to each other. The input sample was used to estimate the expected counts in a window; the average value for all windows was assigned to windows with zero counts. Finally, we used the Poisson distribution to estimate the probability of observing the ChIP counts within a window given the expected counts in the input sample window. We considered all windows with P-values less than 10^{-5} to have significant peaks. A p-value $< 10^{-5}$ was chosen to give a False Discovery Rate (FDR) of $< 1.5\%$. The FDR was calculated by applying the same statistic described above to the two halves of the same input library. Heat maps for promoter regions spanning a 10 kb window surrounding the TSS were generate by plotting the –log poisson p-val (-logP) obtained for each 50 bp intervals. Only –logP $\geq$ 5 were considered significant and thus genes considered bound. Genes were ranked based on the average –logP for the binding at the promoter regions.

MuDPIT Analysis of ITs and Purified Proteins

For MuDPIT analysis, the equivalent of 150 to 300 IT reactions, on 50 ng naked DNA template per reaction, were performed, washed, pooled together, and eluted as described previously for MuDPIT analysis of PICs on chromatin templates (Lin et al., 2011). MuDPIT of Mediator employed the equivalent of protein obtained from approximately 5×10^9 cells. Note: In Figure 1B, "CHD family" as a single entry represents the average of all CHD-family proteins due to the high degree of conservation between family members. Also, in some instances, we eliminated specific subunits from our calculations when we felt that its NSAF values were significantly over-represented relative to other detected subunits, occasionally due to their multimeric composition. For example, RuvB, a component of both TIP60 and INO80, is known to be a double hexamer, and inclusion in those complexes dramatically increases and almost certainly over-represents the amount of those complexes using our quantitation method. Also inclusion of actin related subunits would dramatically over-represent the same complexes. We do present these data in Table S1.

MS Sort Analysis of MuDPIT Data

Analysis of the MuDPIT data was performed using a custom built R function termed MS Sort (http://www.r-project.org/). MS Sort queries the original data, identifies and sums the NSAFs for all detected subunits of a given complex within the input file and divides by the total number of known subunits to calculate an average NSAF per complex. Briefly, identified proteins were matched to an input list of proteins involved in the regulation of transcription grouped by complex. The subunits and UniProt ID numbers used for each complex are presented in Table S1. The input files and program are available on request.

Comparison of Protein Complex and Protein Detection Reproducibility

To assess the reproducibility of protein complex and individual subunit detection across the PIC samples, Pearson correlation coefficients were calculated and the *r* value for each pair-wise comparison reported (Mosley et al., 2011).

SUPPLEMENTAL REFERENCE

Mosley, A.L., Sardiu, M.E., Pattenden, S.G., Workman, J.L., Florens, L., and Washburn M.P. (2011). Highly reproducible label free quantitative proteomic analysis of RNA polymerase complexes. Mol. Cell. Proteomics *10*, M110000687.
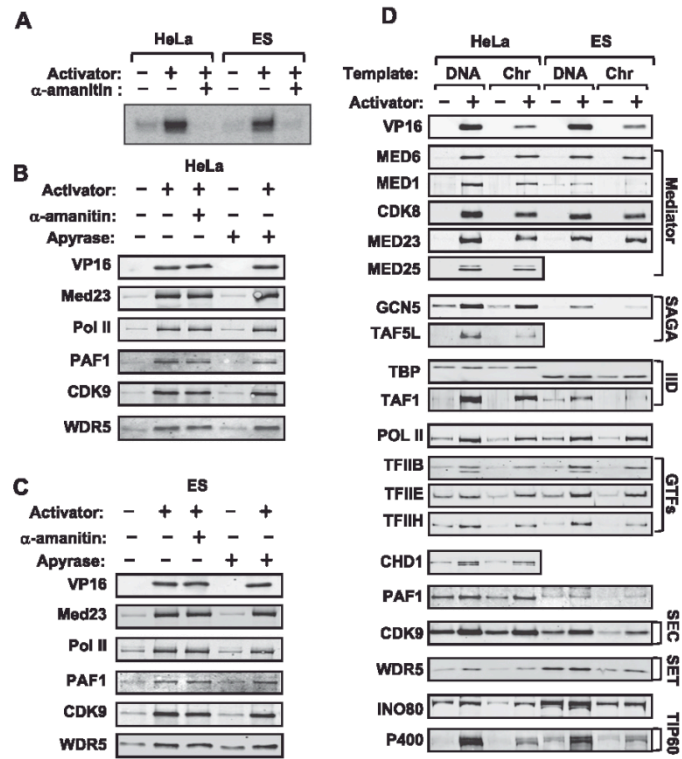
Figure S1. Verification of Activity and Recruitment Using Nuclear Extracts, Related to Figure 1

(A) IVT assay showing that α-amanitin inhibits transcription in our system.

(B and C) IT assays performed in the presence of α-amanitin and apyrase show that SEC/PTEFb (CDK9), PAF1(PAF1), and SET/MLL(WDR5) subunits are recruited to the PIC in both HeLa and mouse ES cell extracts under conditions that block initiation or elongation.

(D) Western blot analyzing PIC assembly at the 60 min time point from HeLa and mouse ES cell nuclear extracts on either naked DNA templates or chromatin (Chr) templates.

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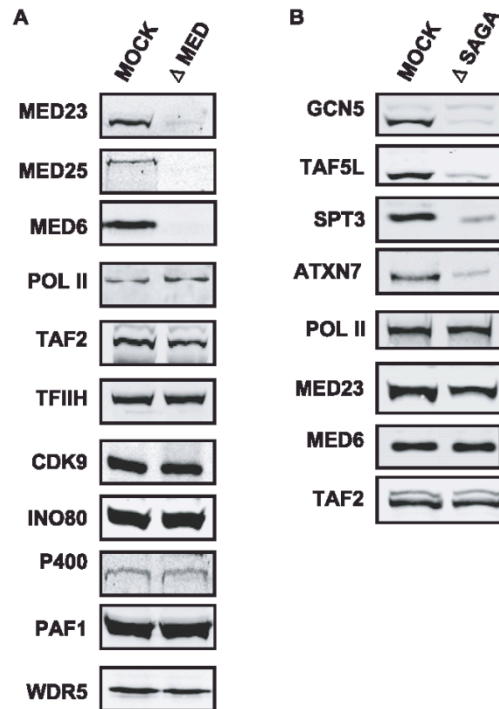
Figure S2. Western Blotting of Depleted Extracts, Related to Figure 2

(A and B) Depleted Mediator (A) or SAGA (B) and mock nuclear extracts were immunoblotted to identify immunodepleted proteins.
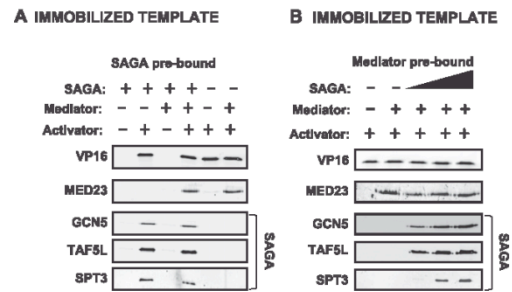
Figure S3. SAGA and Mediator Are Recruited Independently, Related to Figure 3
IT assays demonstrate that SAGA and Mediator are recruited independently by the activator and do not compete.
(A) SAGA was prebound to GAL4-VP16, and Mediator was added.
(B) Mediator was prebound, and increasing amounts of SAGA were added. Note that SAGA saturates at the middle point.

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Chapter 3

Mediator coordinates PIC assembly with

recruitment of CHD1

Mediator coordinates PIC assembly with recruitment of CHD1

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Murine Chd1 (chromodomain helicase DNA-binding protein 1), a chromodomain-containing chromatin remodeling protein, is necessary for embryonic stem (ES) cell pluripotency. Chd1 binds to nucleosomes trimethylated at histone 3 Lys 4 (H3K4me3) near the beginning of active genes but not to bivalent domains also containing H3K27me3. To address the mechanism of this specificity, we reproduced H3K4me3- and CHD1-stimulated gene activation in HeLa extracts. Multidimensional protein identification technology (MuDPIT) and immunoblot analyses of purified preinitiation complexes (PICs) revealed the recruitment of CHD1 to naive chromatin but enhancement on H3K4me3 chromatin. Studies in depleted extracts showed that the Mediator coactivator complex, which controls PIC assembly, is also necessary for CHD1 recruitment. MuDPIT analyses of CHD1-associated proteins support the recruitment data and reveal numerous components of the PIC, including Mediator. In vivo, CHD1 and Mediator are recruited to an inducible gene, and genome-wide binding of the two proteins correlates well with active gene transcription in mouse ES cells. Finally, coimmunoprecipitation of CHD1 and Mediator from cell extracts can be ablated by shRNA knockdown of a specific Mediator subunit. Our data support a model in which the Mediator coordinates PIC assembly along with the recruitment of CHD1. The combined action of the PIC and H3K4me3 provides specificity in targeting CHD1 to active genes.

[*Keywords*: Mediator; preinitiation complex; CHD1; H3K4me3]

Histone H3 Lys 4 trimethylation (H3K4me3) near the beginning of genes correlates with active transcription. However, although most active genes contain this modification, not all H3K4me3-bearing genes are actively transcribed (Kim et al. 2005; Ruthenburg et al. 2007). H3K4me3 is also found in bivalent domains alongside H3K27me3, which correlates with gene silencing. Bivalent domains are found on promoters of important developmental regulator genes that are hypothesized to be "primed" but not yet active or fully silenced (Bernstein et al. 2006). The mechanisms by which the context-specific effects of H3K4me3 are achieved have not been fully explored.

The coordination of transcription with histone modifications allows RNA polymerase II (Pol II) to overcome nucleosomal barriers presented by chromatin (Li et al. 2007). Specific histone modifications recruit distinct effector proteins that alter the chromatin landscape of active genes, making them permissive for transcription. It is largely unknown how the binding and function of this chromatin machinery are coordinated with assembly of

the preinitiation complex (PIC). The PIC comprises coactivators like the 30-subunit Mediator complex and the 14-subunit TFIID complex, along with Pol II and the general transcription factors (GTFs) TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH; TFIID is both a coactivator and a GTF (Kornberg 2005).

Mechanistic studies have established that activators contact and recruit TFIID and Mediator, while the GTFs and Pol II associate with these coactivators to complete PIC assembly (Roeder 1998; Johnson and Carey 2003). The GTFs bound at the promoter position Pol II at the start site, melt the DNA, and facilitate the catalytic steps of transcription initiation (Kornberg 2007). Cross-linking studies have shown that many of the GTFs contact promoter DNA (Lagrange et al. 1996). Moreover, Pol II, TFIIH, and the TBP-associated factor (TAF$_{II}$) subunits of TFIID all bind both upstream of and downstream from the start site (Martinez 2002). Additionally, proteins such as the PAF1 complex and P-TEFb are recruited at or near the start site to facilitate Pol II elongation. Indeed, most genes contain a well-characterized NELF- and DSIF-mediated Pol II pause site located 30 base pairs (bp) downstream from the start site that must be overcome by the action of P-TEFb, possibly in the context of the super elongation complex (SEC) (Peterlin and Price 2006; Smith

et al. 2011). Most human genes also contain nucleosomes positioned near the start site (Schones et al. 2008). The close proximity of the PIC and Pol II elongation machinery with nucleosomes emphasizes the need to understand how their functions are coordinated. Some chromatin factors—like the p300 histone acetyltransferase, which stimulates Pol II elongation, and the PAF1 complex, which controls H3K4me3—are known to be recruited to a functional PIC (Black et al. 2006; Wu et al. 2008; Kim et al. 2009). However, it is unknown whether the PIC or its associated factors control the recruitment of effector proteins that bind to H3K4me3.

The SET1 complex is responsible for the majority of H3K4me3 in yeast and mammalian cells (Ruthenburg et al. 2007; Wu et al. 2008). In budding yeast, recruitment of the SET1 complex (termed COMPASS) by PAF1, in combination with ubiquitinylation of H2B by Rad6-Bre1, leads to H3K4me3 (Wood et al. 2003; Kim et al. 2009). However, in mammals and other organisms, H3K4me3 is found at some gene promoters that have not yet generated productive transcript (Bernstein et al. 2006). Studies have shown that several DNA sequence-specific transcription factors associate directly with ASH2L, a core subunit of SET1 family complexes (Tan et al. 2008; Stoller et al. 2010). It is conceivable that SET1 might be recruited to a gene by such factors under conditions in which the gene is not actively transcribed.

The majority of H3K4me3-binding domains can be found in effector proteins associated with chromatin modification and remodeling (Ruthenburg et al. 2007). Chd1 (chromodomain helicase DNA-binding protein 1) is an H3K4me3-associated chromatin remodeler that is required for the expression of developmentally essential genes in mouse embryonic stem (ES) cells (Gaspar-Maia et al. 2009). However, although H3K4me3 is present across a large subset of genes, occupancy of mouse Chd1 correlates primarily with active gene promoters that display enrichment of Pol II. Human CHD1 has been reported to deposit the histone variant H3.3 in vivo (Konev et al. 2007). The CHD1 complex has been shown to associate with the PAF1 elongation complex in *Saccharomyces cerevisiae* (Warner et al. 2007). However, in *S. cerevisiae*, while CHD1 is capable of remodeling promoter nucleosomes, it does not exhibit preferential binding to H3K4me3 chromatin (Ehrensberger and Kornberg 2011). Thus, in higher eukaryotes, CHD1 is a model H3K4me3 effector used in developmental decisions and elsewhere; its enhancement of gene expression is linked to both transcription and H3K4me3. Since in vitro studies have demonstrated that the CHD1 double chromodomains bind H3K4me3 nucleosomes and tail peptides with seemingly high affinity (Sims et al. 2005; Bartke et al. 2010; Vermeulen et al. 2010), an important question is why Chd1 is found only at actively transcribed H3K4me3 genes and not at bivalent domains.

To investigate the biochemical mechanisms by which H3K4me3 effectors function in a transcription- and chromatin-specific context, we used multidimensional protein identification technology (MuDPIT) proteomics in conjunction with immunoblotting to identify factors found to be enriched in the context of a PIC assembled on chromatin in vitro. Proteomic techniques have been previously used to successfully analyze PICs on nonchromatin templates in yeast (Ranish et al. 2003). Surprisingly, we found that binding of CHD1 was stimulated by the activator on unmodified chromatin templates but was enhanced on methylated templates. CHD1 was observed to also participate in higher-order protein complex interactions with components of the transcription machinery located at or downstream from the start site and was a prime candidate for further mechanistic investigation. Using the immobilized chromatin template assay, we combined PIC-stimulated recruitment with functional transcription assays. Our studies show that the specific recruitment of CHD1 to an active gene is achieved by linking it to assembly of an active transcription complex via the action of Mediator.

Results

H3K4me3 stimulates transcription in vitro

To study the mechanism by which H3K4me3 contributes to active gene transcription, we used the methods developed by Shokat and colleagues (Simon et al. 2007) to synthetically methylate chromatin templates for biochemical analysis. In this method, Lys 4 of histone H3 was mutated to a cysteine to generate H3K4C (Fig. 1A). Recombinant H3K4C was reacted with an ethylamine substrate [(2-bromoethyl) trimethylammonium bromide] to generate the methyl-lysine analog (MLA) of H3K4me3. The MLA was validated by immunoblotting with an antibody to H3K4me3 (Fig. 1A) and quantitated by electrospray ionization mass spectrometry, which revealed that 66% of H3 was modified (data not shown). As shown in the Figure 1B schematic, unmodified naive or synthetically methylated H3 was assembled into octamers and then into chromatin on biotinylated DNA templates containing a GAL4-responsive promoter (Black et al. 2006). The amounts of chromatin template were first normalized based on the extent of chromatin assembly as shown in Figure 1B. Subsequently, in vitro transcription was performed in HeLa nuclear extracts on equivalent amounts of chromatin. The data revealed stimulation by the activator GAL4-VP16 on the H3K4me3 versus the naive unmodified templates (Fig. 1C). Multiple repeats were quantitated, graphed, and subjected to statistical analysis to reveal that the stimulation was indeed reproducible and significant (Fig. 1D). This result demonstrated an ability to recreate, in a cell-free system, the transcription stimulation imparted by H3K4me3 and offered the possibility of generating mechanistic insights into its function. We addressed the molecular basis for this stimulation by first analyzing the composition of the PICs formed via immobilized template assays.

Activator-mediated recruitment of CHD1

The immobilized template assay is a powerful method for understanding the mechanism of gene activation in vitro because it permits a comparison of transcriptional activity
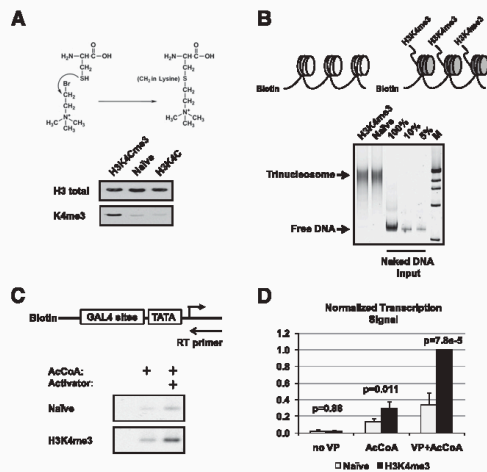
66

Figure 1. H3K4me3 stimulates in vitro transcription from nuclear extract. {A} Schematic of the trimethyl-lysine analog synthesis at histone 3 Cys 4. *Below* the schematic is a Western blot showing the specific detection of H3K4me3 MLA by antibody (Abcam anti-K4me3). {B} Schematic of the immobilized chromatin template and chromatin normalization. The extent of chromatin assembly was monitored by EMSA; equivalent amounts of chromatin were used in all experiments. {C} Schematic and autoradiograph of in vitro transcription as detected by primer extension on unmodified (Naive) and synthetic H3K4me3 chromatin templates in the presence and absence of the activator GAL4-VP16 and acetyl-CoA {AcCoA} using HeLa nuclear extract. (AcCoA was necessary for optimal levels of transcription.) {D} Signal quantitation and statistical analysis of the stimulation by H3K4me3. Three repeats of the transcription experiment were quantitated using Imagequant TL software, graphed, and subjected to a Student's *t*-test as a measure of statistical significance between naive unmodified and H3K4me3 chromatin.

with the composition of the PICs. In a typical experiment, biotinylated chromatin templates are attached to paramagnetic streptavidin-coated beads and incubated either in nuclear extract or with pure proteins. After washing the beads, the proteins captured by the template are eluted and analyzed by immunoblotting or MuDPIT.

Supplemental Figure 1A shows a typical time-course experiment on naive chromatin in which we determined the optimal conditions for chromatin binding of various GTFs, coactivators (Mediator and TAFs), and complexes associated with H3K4me3, including PAF1 and SET1. Immunoblotting for each subunit of every transcription factor is nearly impossible given the large number of polypeptides constituting the PIC. We therefore chose specific subunits to represent various factors within the PIC. The activator recruited the Mediator with the fastest kinetics as previously reported (Black et al. 2006). Importantly, we observed activator-stimulated recruitment of the PAF1 and SET1 complexes, although they bound more slowly than Mediator. The coordinated recruit-

ment of PAF1, SET1, and the PIC was expected based on Shilatifard's previous studies in yeast (Wu et al. 2008; Kim et al. 2009), with the caveat that PAF1 and SET1 bound in the absence of transcription, whereas cotranscriptional phosphorylation of Ser 5 in the Pol II C-terminal domain (CTD) is necessary for PAF1 binding in yeast. Immunoblotting revealed that no detectable Ser 5 phosphorylated Pol II was observed bound to the immobilized template in the absence of ATP (Supplemental Fig. 1B; data not shown). This result was consistent with numerous studies showing that phosphorylated Pol II does not join the PIC (Drapkin et al. 1994).

To more thoroughly interrogate and compare the components of PICs formed on naive and H3K4me3 chromatin, we scaled up the immobilized template reactions and performed MuDPIT analysis. We compared two primary criteria in our analysis—the proteins whose binding appeared to be stimulated by activator, and those that were further enriched by H3K4 methylation. Our goal was to identify H3K4me3-enriched factors that interact with the PIC and participate in transcription initiation on chromatin. PIC-related complexes detected in the MudPIT data are summarized as a table in Figure 2A. (The annotated data set with accompanying graphs representing peptide spectra focused on transcription/chromatin proteins are shown in Supplemental Table 1 and Supplemental Fig. 2A–D.) Relative enrichment of specific PIC components was calculated by average NSAF (normalized spectral abundance factor) for all subunits of a complex and ranked by color. A gradient of red to yellow to light blue represents proteins ranked in the 90th to 20th percentiles, while values shown in solid blue represent proteins ranking below the 20th percentile. The most enriched factor was Mediator. The Mediator is a direct target of VP16 (Uhlmann et al. 2007), and its recruitment by activator represented a validation of the overall approach (Fig. 2A). Indeed, for most of the Mediator subunits, the MuDPIT data indicated that no binding occurred in the absence of activator. Among other transcription factors, the number of peptides decreased substantially, but Pol II, TFIID, and TFIIH were readily detected. Surprisingly, among the known or suspected H3K4me3 effector proteins, CHD1 binding was strongly stimulated by activator on naive templates. CHD1 was further enriched on methylated templates in the absence of activator. In addition, we observed activator-stimulated enrichment of the PAF complex. The PAF1 complex is necessary for recruitment of the SET1 complex COMPASS in yeast (Krogan et al. 2003; Wood et al. 2003). CHD1 has been reported to associate with PAF1 in HeLa extracts (Sims et al. 2007; Warner et al. 2007).

Despite the fact that our approach detected activator-stimulated recruitment of proteins like Mediator, the spectral counting-based quantitation in MuDPIT is not ideal for detecting small differences in abundance as compared with other mass spectrometry techniques like SILAC (Bartke et al. 2010). We therefore used immunoblotting to validate candidates chosen for further mechanistic analysis. Figure 2B shows a recruitment time course of CHD1 versus the Mediator and TFIID on both naive and H3K4me3 templates. Quantitation of the blots from
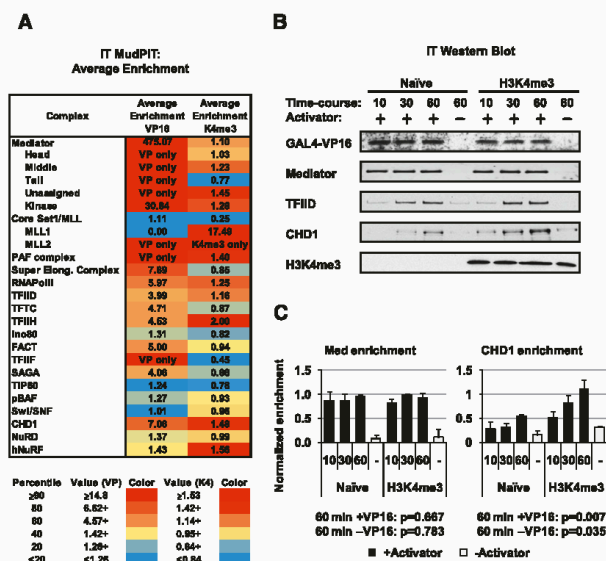
A

IT MudPIT:
Average Enrichment

Complex	Average Enrichment VP16	Average Enrichment K4me3
Mediator	475.07	1.10
Head	VP only	1.03
Middle	VP only	1.23
Tail	VP only	0.77
Unassigned	VP only	1.45
Kinase	30.84	1.28
Core Set1/MLL	1.11	0.35
MLL1	0.00	17.40
MLL2	VP only	K4me3 only
PAF complex	VP only	1.40
Super Elong. Complex	7.89	0.86
RNAPolII	5.97	1.25
TFIID	3.99	1.16
TFTC	4.71	0.87
TFIIH	4.63	2.00
Ino80	1.31	0.82
FACT	5.00	0.94
TFIIF	VP only	0.45
SAGA	4.06	0.66
TIP60	1.24	0.78
pBAF	1.27	0.93
SwI/SNF	1.01	0.96
CHD1	7.06	1.48
NuRD	1.37	0.99
hNuRF	1.43	1.56

Percentile	Value (VP)	Color	Value (K4)	Color
≥90	≥14.8		≥1.63	
80	6.62+		1.42+	
60	4.57+		1.14+	
40	1.42+		0.95+	
20	1.26+		0.64+	
<20	<1.26		<0.64	

B

IT Western Blot

Time-course:	Naïve				H3K4me3			
	10	30	60	60	10	30	60	60
Activator:	+	+	+	−	+	+	+	−

GAL4-VP16

Mediator

TFIID

CHD1

H3K4me3

C

Med enrichment

CHD1 enrichment

Normalized enrichment

| 10 | 30 | 60 | − | 10 | 30 | 60 | − |
| Naïve | | | | H3K4me3 | | | |

60 min +VP16: p=0.667
60 min −VP16: p=0.783

60 min +VP16: p=0.007
60 min −VP16: p=0.035

■ +Activator   □ −Activator

Figure 2. H3K4me3 effectors are recruited to the PIC. (A) MuDPIT analyses were performed on PICs assembled on chromatin arrays: a chart of PIC components, H3K4me3 effector proteins, and other factors recruited. Average enrichment in the presence of GAL4-VP16 and further enrichment on H3K4me3 chromatin are shown as a heat map from average NSAFe5 values for the factors in the complex. Average NSAFe5 values for each complex are ranked by percentile in a three-color gradient from high (red) to medium (yellow) to low (blue) as shown. (B) Time courses of PIC recruitment in HeLa extracts were compared between Naive and H3K4me3 templates by immunoblotting to validate enrichment of select factors as determined by the MuDPIT screen. (C) Quantitation and statistical analysis of Western blot signals for CHD1 and Mediator enriched on H3K4me3 versus Naive chromatin. Signals were normalized to VP16. Student's *t*-test was used to calculate the statistical significance of the differences at the 60-min time point. Assays were performed in triplicate.

multiple experiments revealed that the amount of Mediator remained roughly constant between naive versus H3K4me3 chromatin (Fig. 2C). In contrast, the activator stimulated a significant twofold to threefold increase with CHD1 on the H3K4me3 chromatin, with the effect being most evident at the 60-min time point. Moreover, CHD1 bound with a slightly higher affinity to the H3K4me3 versus naive templates in the absence of activator, although not to the extent observed in the MuDPIT analysis (Fig. 2B,C). The data suggest that the activator and H3K4me3 both contribute to the recruitment of CHD1. To distinguish between the effect of chromatin and the effect of activator on the recruitment of CHD1, we compared its binding on chromatin and naked DNA templates. The data in Supplemental 2E show a significant increase in CHD1 binding in response to activator even on naked DNA templates. These data imply that the recruitment of CHD1 might be linked directly to PIC assembly.

The role of Mediator in recruitment of CHD1

Biochemical analysis of PICs assembled in vitro revealed a dual requirement for the coactivators TFIID and Mediator to achieve efficient binding of the GTFs and Pol II (Johnson and Carey 2003). To assess the role of the coactivators in recruitment of CHD1, we prepared nuclear extracts depleted of either TFIID or Mediator. Our immunodepletion protocol removed ~90% or more of the Mediator and TFIID but did not significantly affect the levels of CHD1, other H3K4me3-related proteins, or Pol II (Fig. 3A; Supplemental Fig. 3A). We found that activator-dependent CHD1 recruitment was significantly reduced in immobilized template assays performed in the Mediator-

depleted extracts on both chromatin and naked DNA (Fig. 3B,C). Unlike Mediator, immunodepletion of TFIID had little effect on recruitment of CHD1 (Supplemental Fig. 3B). Our data suggest that the Mediator is required for recruitment of CHD1. Importantly, the Mediator dependence of CHD1 recruitment observed on naked DNA reinforces the idea that chromatin need not be present for CHD1 recruitment to PICs. The results suggest that Mediator coordinates the recruitment of a key chromatin remodeling enzyme with PIC assembly. These data reinforce the findings in Figure 2 and provide a basis for how Chd1 is preferentially localized to active genes in mouse ES cells.

Mediator stimulates CHD1 function

We took two approaches to determine the role of Mediator in recruiting CHD1. First, Mediator was immunoaffinity-purified from HeLa cell lines expressing Flag-tagged Med 29 and used to supplement our Mediator-depleted extract in add-back experiments to rescue CHD1 recruitment. Second, we expressed and purified Flag-CHD1 from the baculovirus system and used MuDPIT analysis to identify other factors that were consistently associated with CHD1, albeit in low abundance, in solution in HeLa extracts. This is a standard proteomic approach for identifying candidate proteins that might interact in the context of a PIC. The caveats in such analyses are that associated factors are usually present in substoichiometric amounts in solution, and the technology does not distinguish between direct and indirect associations.

We found that adding back Mediator, purified under high-stringency conditions, to Mediator-depleted extract
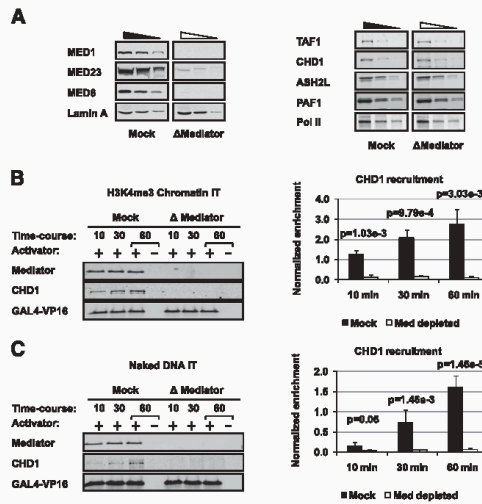
Figure 3. Mediator plays a key role in CHD1 recruitment to the PIC. (*A*) Immunoblot of Mediator subunits and control proteins in Mediator-depleted (ΔMediator) and mock-depleted (Mock) nuclear extract loaded in threefold steps. (*B*) Immunoblot (*left* panel) and quantitation (*right* panel) comparing CHD1 recruitment time courses in an immobilized template assay (IT) on H3K4me3 chromatin in Mock versus ΔMediator extracts. Western blot signal was quantified using LiCOR imaging software, and the statistical significance between Mock- and Mediator-depleted extracts was calculated using Student's *t*-test. Signals were normalized to VP16. (*C*) CHD1 recruitment in Mock-versus Mediator-depleted extracts repeated using naked DNA templates.

was able to rescue CHD1 recruitment to the immobilized template on naked DNA (Fig. 4A). Quantitation of triplicate repeats, normalized to VP16 activator binding, revealed that restoration of CHD1 recruitment in Mediator-depleted extract is reproducible and significant. One prediction of the hypothesis that CHD1 somehow attaches to the PIC is that CHD1 should interact with PIC components. To test this hypothesis, recombinant CHD1 was incubated with HeLa nuclear extracts in 100 mM KCl using lower and higher DNase/heparin conditions (see the Materials and Methods) and then repurified at high salt (300 mM NaCl) to examine interacting proteins. MuDPIT analysis of proteins bound to CHD1 revealed subunits from the Mediator and other PIC components present in the lower heparin conditions (Fig. 4B; Supplemental Table 2A). These components included the SEC, TFIID, TFIIH, INO80, and the SET1 and PAF1 complexes. TFIIH, PAF1, and FACT complexes were still detected at the higher heparin conditions (Fig. 4B; Supplemental Table 2B) and may possibly help bridge the CHD1–Mediator interaction. Nonetheless, the observation that CHD1 pull-down experiments mainly detect components of the PIC and associated chromatin remodeling factors

adds additional strength to the idea that CHD1 is indeed a component of the PIC bound at a promoter.

CHD1 is necessary for efficient transcription in vitro

We next focused on the function of CHD1 at a promoter. To establish that recombinant CHD1 from the baculovirus system is functional, we performed a chromatin remodeling assay. In the presence of ATP, CHD1 is active in the in vitro remodeling assay, as illustrated by the shift in mobility of the nucleosome on a gel upon CHD1 treatment (Fig. 5A). Additionally, the remodeling is significantly more pronounced on H3K4me3 chromatin. This result agrees with the generally accepted view that one role of H3K4me3 is to enhance CHD1 remodeling of nucleosomes (Petesch and Lis 2008).

To determine whether CHD1 contributes to transcriptional activation on H3K4me3 chromatin, we prepared extracts depleted of the protein using a native CHD1 antibody. Using this method, we depleted CHD1 from the HeLa extracts by >90% (Fig. 5B). The in vitro transcription experiment in Figure 5C shows that depletion of CHD1 decreases transcriptional activation, whereas addition of recombinant CHD1 restores the normal level of activated transcription. The effect is specific to the CHD1-depleted extracts, as addition of CHD1 to the mock-depleted extracts has no additional effect on transcription and even inhibits at high concentrations (Supplemental Fig. 4). Note that CHD1 depletion reduces but does not abolish transcription activation on the H3K4me3 chromatin template. This result could indicate that CHD1 is not absolutely required for, but does strongly contribute to, H3K4me3 chromatin transcription in our system. Collectively, the data in Figure 5, A–C, establish that CHD1 is active in remodeling and necessary for efficient activated transcription.

One possibility is that CHD1 recruitment to the PIC through the Mediator facilitates Pol II initiation on chromatin. If so, this could be a rate-limiting step that would be overcome by prebinding the Mediator and CHD1 to the template in the presence of ATP prior to adding HeLa extract and nucleotides. First, we tested binding of purified CHD1 and Mediator to chromatin. Figure 5D quantitates an immobilized template assay showing that purified CHD1 binds better in the presence of Mediator. Experiments on both naive unmodified and H3K4me3 chromatin revealed that the overall recruitment of CHD1 is further enriched on the H3K4me3 chromatin (Fig. 5D). We cannot say that this is a direct interaction, as neither protein is completely pure. Next, we tested the effect of prebinding Mediator and CHD1 on transcription of H3K4me3 templates. The data in Figure 5E establish that preincubation of Mediator and CHD1 with the chromatin template stimulates transcription in an ATP-dependent manner. Preincubation of Mediator and CHD1 in the absence of ATP failed to elicit a stimulatory effect within the short 10-min time frame of the in vitro transcription experiment. This result implies that the ATP-dependent activity shown in Figure 5A is necessary for transcription. We conclude that CHD1 enhances
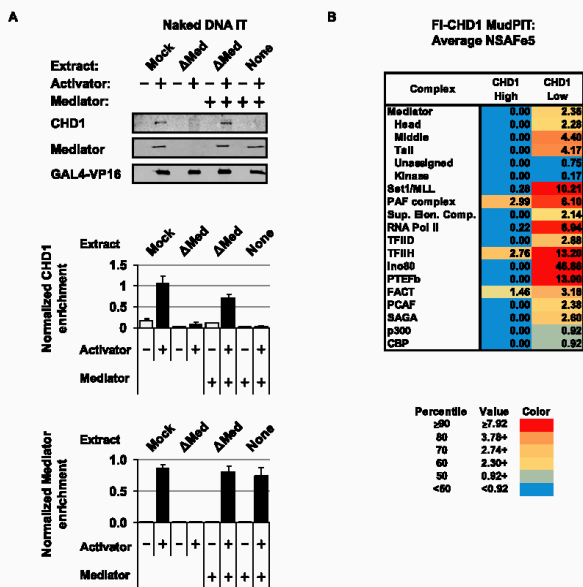
Figure 4. Characterization of CHD1 recruitment using purified complexes. (*A*) Immunoblot analysis of CHD1 recruitment to naked DNA templates in a Mediator-depleted extract supplemented with highly purified Mediator ± activator. Mediator was normalized to levels found in the HeLa nuclear extract used in Figures 2 and 3. The *top* panel is a representative immunoblot of the Mediator complementation experiment, while the *bottom* panels are graphs analyzing triplicate experiments. Quantitation was performed using LiCOR imaging software. Signals were normalized to VP16. (*B*) Chart of PIC-related complexes found associated with recombinant Flag-tagged human CHD1 incubated with HeLa nuclear extract at higher and lower heparin conditions. Average NSAFe5 values for each complex are ranked by percentile in a three-color gradient from high (red) to mid (yellow) to low (blue) as shown. Note that the cutoff values differ from the chart in Figure 2.

transcription in a Mediator- and ATP-dependent manner (Esnault et al. 2008; Boeing et al. 2010).

Genomic Chd1 binding overlaps with Mediator in vivo

Our hypothesis is that CHD1 binding to the PIC is controlled by the Mediator, possibly through bridging factors. CHD1 can then act on the H3K4me3 nucleosomes that are found at gene promoters. This hypothesis predicts that CHD1 occupancy would correlate with Mediator predominantly at active genes. To address this issue, we first examined the recruitment of CHD1 along with Mediator in a U2OS cell line bearing a doxycycline-inducible TetR-VP16-activated reporter gene (Fig. 6A). CHD1 recruitment closely follows Mediator recruitment in a time course of doxycycline induction at the promoter, but is not observed at the 3′ end of the reporter gene at the time points tested (Supplemental Fig. 5A). These data suggest that Mediator and CHD1 join the VP16-stimulated PIC as it is assembled in vivo.

We next examined two previously published genome-wide data sets in mouse ES cells (Gaspar-Maia et al. 2009; Sridharan et al. 2009; Kagey et al. 2010). We analyzed binding events for the Med1 Mediator subunit on the same promoter regions that were analyzed on promoter arrays for Chd1 and H3K4me3. Previous studies have reported that Chd1, which is required for ES cell self-renewal, localizes to only 12% of the promoter regions of bivalent genes and is highly enriched at active H3K4me3 genes. We found a significant genome-wide overlap for Chd1, Med1, and H3K4me3 (Fig. 6B; Supplemental Fig. 5B,C). As shown previously, Chd1 localizes predominantly to promoters occupied by H3K4me3. This correlation is even stronger when bivalent promoters are excluded from the pool of H3K4me3-positive promoters (Supplemental Fig. 5B). Similarly, Med1 occupancy is significantly reduced across bivalent promoters (Supplemental Fig. 5C). These data imply that the mechanism to "poise/prime" bivalent genes for future activation is not PIC-dependent. Although not all Med1-bound, H3K4me3-occupied genes are Chd1 targets, both Med1 and H3K4me3 are found at the vast majority of Chd1-bound promoters. Binding of Med1 and Chd1, along with H3K4me3, is indicative of highly active transcription as shown in Figure 6C (see also Supplemental Fig. 5D). We suggest, based on the data above, that the Mediator coordinates H3K4me3-stimulated initiation of transcription by Chd1.

Med1 is required for coimmunoprecipitation of Mediator and CHD1

One problem with studying cross-talk between Mediator and CHD1 in cells is that indiscriminate disruption of the Mediator is likely to have widespread indirect consequences. It was therefore necessary to identify specific Mediator subunits that might be involved in the recruitment. Using in vitro transcription and translation, we synthesized individual Mediator subunits and studied their affinity for Flag-CHD1 bound to the Flag antibody resin (Supplemental Fig. 6). In this type of analysis, binding does not necessarily indicate a direct interaction because transcription factors are known to be present in reticulocyte lysates and the baculovirus-synthesized CHD1 may contain contaminants from insect cells. Nevertheless, this
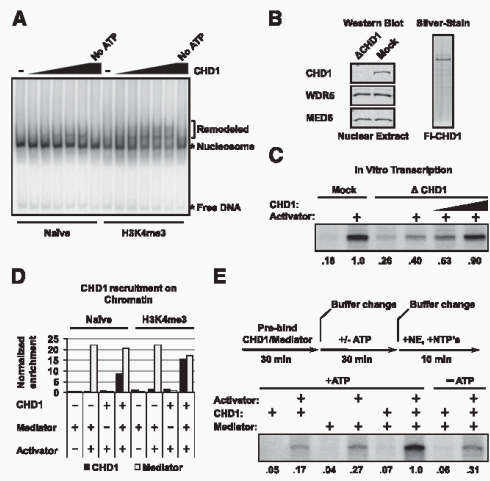
Figure 5. Characterization of CHD1 during transcription initiation. (*A*) Autoradiograph of nucleosome remodeling assay demonstrating that recombinant CHD1 is enzymatically active on Naive and H3K4me3 ^{32}P-labeled 601 mononucleosomal templates. (*B*) Western blot of CHD1-depleted nuclear extract and a silver-stained gel of purified recombinant Flag-CHD1 expressed in baculovirus. (*C*) Autoradiograph of in vitro transcription primer extension assays using CHD1-depleted nuclear extracts as shown. Transcription was compared between CHD1-depleted (ΔCHD1) and mock-depleted nuclear extracts and rescued with the addition of purified CHD1 as shown. Signals were quantitated using Imagequant TL and normalized to Mock-treated + activator. (*D*) Graph showing CHD1 recruitment to H3K4me3 versus Naive immobilized chromatin templates with purified Mediator and activator. Trace levels of CHD1 were detected by immunoblotting in the purified Mediator alone. CHD1 recruitment in each lane was normalized to recruited Mediator. (*E*) Levels of transcription were measured in a rate-limiting assay performed as illustrated by the schematic. H3K4me3 chromatin templates were preincubated with activator, Mediator, and CHD1 as indicated. After 30 min, unbound protein was removed, and buffer with or without ATP was added. After a further 30-min incubation, the buffer was removed and beads were suspended in transcription buffer containing HeLa nuclear extract and nucleotides. After 10 min, the transcription reaction was subjected to primer extension analysis to measure mRNA. Signals were quantitated using Imagequant TL and normalized to the signal in lane 6.

approach represents the most logical way to determine which Mediator subunit is involved in recruitment of CHD1. Our assay identified Med1 as the subunit with the highest affinity among the 29 subunits tested. This result, however, does not preclude the possibility that other Mediator subunits may also be involved.

Med1 was then knocked down in 293T cells along with a control subunit, Med23, using lentiviruses encoding shRNAs targeting the two subunits. Both Med1 and Med23 are known to be targeted by specific activators in vivo (Yuan et al. 1998; Boyer et al. 1999). Mediators

lacking Med1 and Med23 have been isolated (Ito et al. 2000; Stevens et al. 2002). Thus, disruption of Med1 or Med23 is unlikely to have a strong impact on Mediator structure. We then performed coimmunoprecipitation experiments with CHD1 as bait. We chose Med6, Med7, and Med14 as subunits indicative of the three known structural modules of Mediator (Conaway et al. 2005). The data in Figure 7 show that CHD1 coimmunoprecipitated with Mediator subunits in extracts from cells treated with the virus alone and with the Med23 shRNA knockdown virus but not in the Med1 shRNA knockdown. The data suggest that select disruption of the Mediator can abolish its ability to coimmunoprecipitate with CHD1.

Discussion

Our data support the concept that activator-stimulated PIC assembly is physically and mechanistically linked with the subsequent effects of H3K4me3. The use of MuDPIT and immunoblotting to interrogate the PICs formed on chromatin, coupled with the purification and analysis of CHD1, allowed us to determine that the Mediator
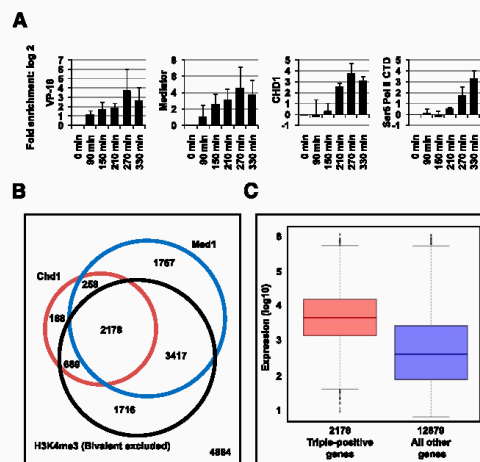
Figure 6. Chd1 and Mediator binding correlate in vivo. (*A*) ChIP analysis of doxycycline-induced enrichment of VP16, Med1-containing Mediator, CHD1, and Pol II on a stably integrated doxycycline-inducible Luciferase reporter in U2OS cells during a time course. (*B*) Venn diagram showing distribution and overlap of genes for Chd1 and Med1 binding and H3K4me3 (excluding H3K27me3) occupancy across the mouse ES cell genome. *P*-values are as follows: Chd1-Med1, *P*-value = 1.42×10^{-216}; Chd1-H3K4me3 (excluding H3K27me3), *P*-value < 9.34×10^{-322}; Med1-H3K4me3 (excluding H3K27me3), *P*-value < 1.05×10^{-321}. (*C*) Box plot of gene expression levels of Chd1-positive, Med1-positive, and H3K4me3 (excluding H3K27me3)-positive genes and all other genes, including singly and doubly bound as well as unbound genes. Chd1-, Med1-, and H3K4me3-cobound genes show an overall higher level of transcription. For triple-positive versus all other genes: $D = 0.4276$, *P*-value < 2.2×10^{-16}.
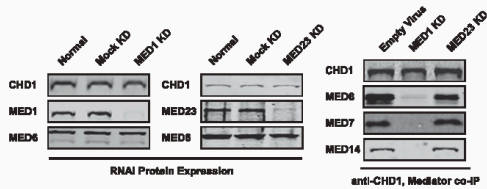
Figure 7. CHD1 associates with Med1-containing Mediator in vivo. Immunoblots showing specific shRNA knockdown of Med1 and Med23 subunits of Mediator in 293T cells (*left* panels) and Med1-dependent coimmunoprecipitation of Mediator by targeted immunoprecipitation of CHD1 (*right* panel). Subunits representing the head (Med6), middle (Med7), and tail (Med14) modules of Mediator are blotted.

coordinates assembly of the PIC and recruitment of CHD1. Our data provide a basis for the specificity of CHD1 for active genes.

Our major finding was that CHD1 binding was largely coupled to activator-mediated PIC assembly but was also weakly stimulated by H3K4me3 alone. Indeed, the PIC and H3K4me3 together caused the most efficient recruitment of CHD1. Previous studies have shown that CHD1 binds to H3K4me3, an observation highlighted by recent proteomic analyses (Sims et al. 2005; Bartke et al. 2010; Vermeulen et al. 2010). However, this event alone seems to contribute only a small amount of CHD1 binding to an active gene. Indeed, recent studies have demonstrated that in *Drosophila*, the CHD1 chromodomains, which recognize H3K4me3, are not required for its colocalization to active genes (Morettini et al. 2011). Moreover, Kornberg and colleagues (Ehrensberger and Kornberg 2011) have recently found that *S. cerevisiae* CHD1, whose chromodomains do not recognize H3K4me3, selectively removes nucleosomes at the promoter in vitro and in vivo in an activator-dependent manner. In mammalian cells, the close correlation between localization of Mediator, Chd1, and H3K4me3 and active transcription in mouse ES cells reinforces the notion that the major mechanism for Chd1 localization is PIC assembly. Dual interactions between the transcriptional machinery and the SET1-catalyzed H3K4me3 modification reinforce the specificity of CHD1.

Among our findings was that the SET1 complex is also recruited to the PIC (as shown in Supplemental Fig. 1A). It has been reported that some activators interact with subunits of the SET1 complex and that some promoters of inactive genes are bound by activators. CHD1, however, is found only at genes that are actively transcribed. Inactive genes are typically di- or trimethylated at H3K9 or H3K27. Hence, bivalent domains might be a natural byproduct of PIC absence. In such cases, where full PIC assembly might be limited by an incomplete complement of activators, CHD1 occupancy may be prevented by both the absence of Mediator and the presence of repressive complexes like PRC1 or HP1 that bind the modifications that correlate with inactive genes.

MuDPIT analysis of proteins bound to CHD1 led to the discovery that it associates with several proteins normally found near or immediately downstream from the start site, including the PAF1 complex, INO80, P-TEFb, NELF, TAFₙS, TFIIH, and Mediator, among others. P-TEFb releases the stalled Pol II found 30–50 bp downstream from many genes by phosphorylating DSIF and NELF (Peterlin and Price 2006). Similarly, numerous TAFₙ subunits and the TFIIH XPB subunit cross-link immediately downstream from the start site in vitro (Dvir et al. 2001). These data, along with the observation that many genes contain a nucleosome positioned near the start site (Schones et al. 2008), reinforce the notion that CHD1 action is functionally coupled to regulatory events associated with the promoter, including initiation and early elongation. The role of INO80 is unclear, but studies have implicated this ATP remodeling complex in promoter-dependent transcription (Cai et al. 2007). We also found small amounts of PAF1 associated with CHD1 as previously observed by others, suggesting the possibility that the two factors interact directly (Sims et al. 2007; Warner et al. 2007).

Analysis of higher-stringency CHD1 pull-downs suggested that among the interacting proteins, TFIIH has a high affinity (Fig. 4B). TFIIH has previously been shown to bind Mediator in work done by others (Esnault et al. 2008; Boeing et al. 2010). This suggests that TFIIH may contribute to the docking of CHD1 with Mediator. Indeed, our unpublished immobilized template data support a Mediator–TFIIH–CHD1 interaction. However, it remains a possibility that this may be enabled by factors copurifying with Mediator or with CHD1 from the insect cell expression system. These issues notwithstanding, our data suggest that Mediator controls CHD1 recruitment.

The idea that the Mediator serves as a platform for other proteins and conveys signals to the general transcription machinery was originally proposed by Young (Chao et al. 1996). The concept that Mediator coordinates events at chromatin is emerging and makes sense given that the machineries coevolved in almost all eukaryotes. In addition to Shilatifard's work on COMPASS (Krogan et al. 2003; Wu et al. 2008), a previous study in our laboratory (Black et al. 2006) linked p300-mediated acetylation with PIC assembly. We also observed in our MuDPIT analysis of PICs the enrichment of other chromatin factors—such as GCN5, NuA4, and other CHD family members—in an activator-stimulated fashion. Most importantly, Mediator has been linked directly to recruitment of Pol II SEC via the Med26 subunit (Takahashi et al. 2011). It would be interesting if this event was linked to the action of CHD1 at the start site. Boyer and colleagues (Ding et al. 2008) have shown that the connectivity also extends to silencing factors, as G9a links directly with the Cdk8 module of the Mediator.

Our current view of the protein–protein interactions identified by proteomic and mechanistic studies is that CHD1 is recruited only to active genes by its interaction with the PIC via the Mediator. Activators are known to interact directly with Mediator and TFIID. The VP16 activation domain docks with Mediator via the MED25 subunit (Yang et al. 2004; Uhlmann et al. 2007) and with TFIID via TAF9 (Goodrich et al. 1993). VP16 is known to recruit both complexes to DNA in vitro and in vivo (Berk

et al. 1998). TFIID and Mediator also interact to form a coactivator complex (Johnson and Carey 2003), which is necessary in vitro for binding of the GTFs and Pol II. Pol II binds tightly to the active form of Mediator in a somewhat mutually exclusive manner with the Cdk8 module (Paoletti et al. 2006).

In conclusion, our data suggest that the Mediator not only serves as a docking platform for activator-stimulated PIC assembly of the GTFs, but coordinates the recruitment of CHD1 during active transcription as well. As the Mediator signifies active genes, the specificity of CHD1 recruitment can best be described as cooperative protein–protein interactions between the PIC, CHD1, and H3K4me3.

Materials and methods

Methyl-lysine histone octamer preparation

Lys 4 of histone H3 was mutated to a cysteine by site-directed mutagenesis of *Xenopus* H3.1 bearing a C110A mutation, expressed and purified from *Escherichia coli* inclusion bodies, and subjected to chemical alkylation by (2-bromoethyl) trimethylammonium bromide (Simon et al. 2007) before assembly into histone octamers (Luger et al. 1997).

Chromatin preparation

A 602-bp biotinylated PCR fragment that directly encompasses G5E4T (Johnson and Carey 2003) was assembled into chromatin by salt dilution as described previously (Steger et al. 1997) and was validated by EMSA in native PAGE. Chromatin was immobilized on M280 streptavidin beads (Dynal) in chromatin-binding buffer (20 mM HEPES at pH 8.0, 150 mM KCl, 10% glycerol, 4 mM MgCl$_2$, 1 mM DTT, 200 μg/mL BSA).

Immobilized template recruitment assay

The 40-μL immobilized template recruitment assays contained 80 μg of HeLa nuclear extract, and 50 ng of chromatin or naked DNA template in immobilized template binding buffer (120 mM KCl, 10 mM HEPES at pH 8.0, 5% glycerol, 0.2 μg/mL BSA, 0.05% NP-40). After the indicated time periods, the beads were washed three times in immobilized template buffer. Captured PICs were incubated with 500 μM ATP in immobilized template binding buffer where indicated in Supplemental Figure 1B for Pol II phosphorylation assays. Bound protein was eluted in 10 μL of 2× Laemmli buffer, fractionated by SDS-PAGE, and immunoblotted. For proteomic analysis, the 1-h time point was scaled up and subjected to MuDPIT. Antibodies used in immunoblotting included MED23 (BD Pharmingen), Pol II CTD 8WG16 (QED Bioscience), TFIIB (Tantin et al. 1996), WDR5 (Upstate Biotechnology), ASH2L, RBBP5, PAF1, and CHD1 (Bethyl Laboratories). All other antibodies were purchased from Santa Cruz Biotechnologies.

Extract and protein preparation

HeLa nuclear extract (Dignam et al. 1983) and GAL4-VP16 were prepared as previously described. Immunodepletion of TFIID from 1 mL of HeLa nuclear extract was performed using 200 μg each of antibodies against TAF4, TAF1, and TAF3 (Santa Cruz Biotechnologies). Mediator was depleted from 1 mL of extract with antibodies against MED1, MED6, MED7, MED25, and CDK8.

CHD1 was depleted with an antibody against CHD1 (Bethyl). Antibodies were cross-linked to protein A and G paramagnetic beads (Invitrogen) using 20 mM dimethylpimelimidate in 0.1 M sodium borate buffer (pH 9) and washed extensively with 50 mM glycine (pH 2.5). The cross-linked beads were equilibrated in buffer D (20 mM HEPES at pH 7.9, 0.1 mM EDTA, 20% glycerol, 0.1 M KCl) and incubated with HeLa nuclear extract in buffer D for 4 h at 4°C. The supernatant was isolated and used for immobilized template analysis as described above.

Recombinant Flag-tagged human CHD1 was purified from SF9 cells using a baculovirus overexpression system (Invitrogen). Briefly, cells were resuspended in 0.3 M buffer F (0.3 M NaCl, 20% glycerol, 20 mM HEPES at pH 7.9, 4 mM MgCl$_2$, 0.2% Triton X-100, 0.1% NP-40) and sonicated. Lysates were then treated with DNase I (1 U/mL) and heparin (12.5 μg/mL) and cleared by centrifugation at 30,000g. The resulting lysate was bound to M2 anti-Flag resin (Sigma), washed extensively in 0.5 M buffer F, and eluted using 3× Flag peptide (0.25 mg/mL; Sigma). Mediator was purified from HeLa cells expressing Flag-tagged human Intersex (Med29) as described previously (Sato et al. 2003). The HeLa Intersex cell line was a gift from Joan and Ron Conaway.

MuDPIT analysis of immobilized templates and purified proteins

For MuDPIT analysis, the equivalent of 300 immobilized template reactions were pooled together. Samples were eluted in 50 mM Tris (pH 8.0) and 6 M urea. For CHD1, ~250 μg of Flag-CHD1 was immobilized on Flag antibody beads and incubated with 0.45 mL of HeLa nuclear extract in buffer containing 100 mM KCl in the presence of either 1 U/mL DNase and 12.5 μg/mL heparin (low stringency) or 75 μg/mL heparin and 10 U/mL DNase I in the presence of 2 mM CaCl$_2$ (high stringency). Bound proteins were washed in 0.3 M buffer F, and F-CHD1 and interacting proteins were then eluted using a 3× Flag peptide (0.25 mg/mL; Sigma). Protein samples were precipitated in 20% TCA, washed with cold acetone, and digested with trypsin. The digested peptide samples were then fractionated with sequential cation exchange and reverse-phase chromatography and eluted directly into a LTQ-Orbitrap mass spectrometer (Thermo Fisher). MS/MS spectra were collected as described (Law et al. 2010). Data analysis was performed with the SEQUEST and DTASelect2 algorithms and filtered with at least two peptides per protein and a peptide-level false-positive rate of <5% as estimated by a decoy database strategy (Law et al. 2010). NSAF values were calculated as described in Law et al. (2010). Proteins were assigned into complexes using the online resource CORUM (Ruepp et al. 2008) and Mediator submodules as published previously (Bourbon 2008).

In vitro transcription assays

The 40-μL standard reactions in Figure 1 contained 50 ng of linear chromatin template, nuclear extract, and GAL4-VP16 as described previously (Black et al. 2006). After 60 min, RNA was harvested and analyzed by primer extension as described previously. Transcription of immobilized chromatin templates in Figure 5 was carried out on 50 ng of linear H3K4me3 chromatin templates, which were bound with saturating levels of GAL4-VP16, followed by removal of excess activator. Templates were then bound with saturating amounts of Mediator and CHD1, as determined by immobilized template assays, and incubated for 30 min at 30°C in the presence or absence of ATP, after which unbound protein was removed. Eighty micrograms of HeLa extract and NTPs were added, and transcription was allowed to proceed for

10 min under standard conditions as described previously (Black et al. 2006).

Cross-correlation of genomic chromatin immunoprecipitation (ChIP) data sets

ChIP–chip data sets from experiments performed on mouse ES cells were used to determine whether gene promoters were enriched for Chd1 (Gaspar-Maia et al. 2009) and H3K4me3 or H3K27me3 (Sridharan et al. 2009). ChIP-seq data for Med1-occupied genes were from Supplemental Table 5 of the study by Young and colleagues (Kagey et al. 2010). The ChIP–chip experiments were performed on Agilent promoter arrays with probes covering the region −5.5 kb to +2.5 kb relative to the transcription start site. Hence, only those Med1-enriched regions that had significant peaks within this 8-kb region, based on the location of enriched regions from Supplemental Table 4 in Kagey et al. (2010), were used for further overlap analysis. The overlap of genes whose promoters were enriched for each feature (Med1, Chd1, and H3K4me3 without H3K27me3) was determined pairwise, and the significance of the overlap was evaluated using the hypergeometric test. Box plots were used to visualize the distribution of expression levels in mouse ES cells for the set of genes exhibiting co-occupancy by all three features as compared with those for gene sets with different combinations of the three features in their promoter regions or their complete absence. Gene expression levels were obtained from Supplemental Table 2 in Gaspar-Maia et al. (2009). A two-sample Kolmogorov-Smirnov test was used to compare the distributions of expression values between the two sets of genes.

shRNA knockdown of Mediator subunits and coimmunoprecipitation

293T cells were grown to 30% confluency in 10-cm dishes and infected with lentiviruses expressing shRNAs targeting MED23 (TTGTGAGTGTCATCAGCAGCC) and MED1 (GTCATGGA GAAGAGGGTTGTG). After 96 h, cell lysates were prepared and immunoblotted to determine the extent of MED1 and MED23 knockdown using the MED6 antibody as a control. The lysates were subjected to immunoprecipitation with a CHD1 antibody (Bethyl Laboratories). The immunoprecipitates were blotted with antibodies to CHD1, MED6, MED7, and MED14 (Santa Cruz Biotechnologies) to determine the amount of coimmunoprecipitation.

ChIP

ChIP from the U2OS cells, various times after doxycyline treatment, was performed using antibodies to CHD1, MED1, VP16, and Ser 5 Pol II (Abcam) as previously described (Black et al. 2006).

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Mediator Coordinates PIC Assembly with Recruitment of CHD1

by

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SUPPLEMENTAL FIGURES

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- Supplemental 1
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    – Immobilized Template MudPIT data set
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    – Immobilized Template MudPIT peptide spectra graphs
    – CHD1 recruitment to the PIC compared on naked DNA vs. chromatin
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    – Depletion controls and immobilized template immunodepletion assays for TFIID depletion experiment
- Supplemental Table 2
    – CHD1-interacting proteins MudPIT
- Supplemental 4
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- Supplemental 6
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A

Naïve Chromatin

Time-course: 10 30 60 60
Activator: + + + −

GAL4-VP16 — Activator

Mediator

TFIID

TFIIE

TFIIB

PAF1

Select Co-activators, GTF's and PIC components

JMJD3

SET1a

ASH2L

RBBP5

WDR5

Core SET1 complex and associated components

B

+ATP -treated PIC   Pre-ATP input

Time-course: 10 30 60 10 30 60
Activator: + + + − + + + −

RNA Pol II *

RNA Pol II *
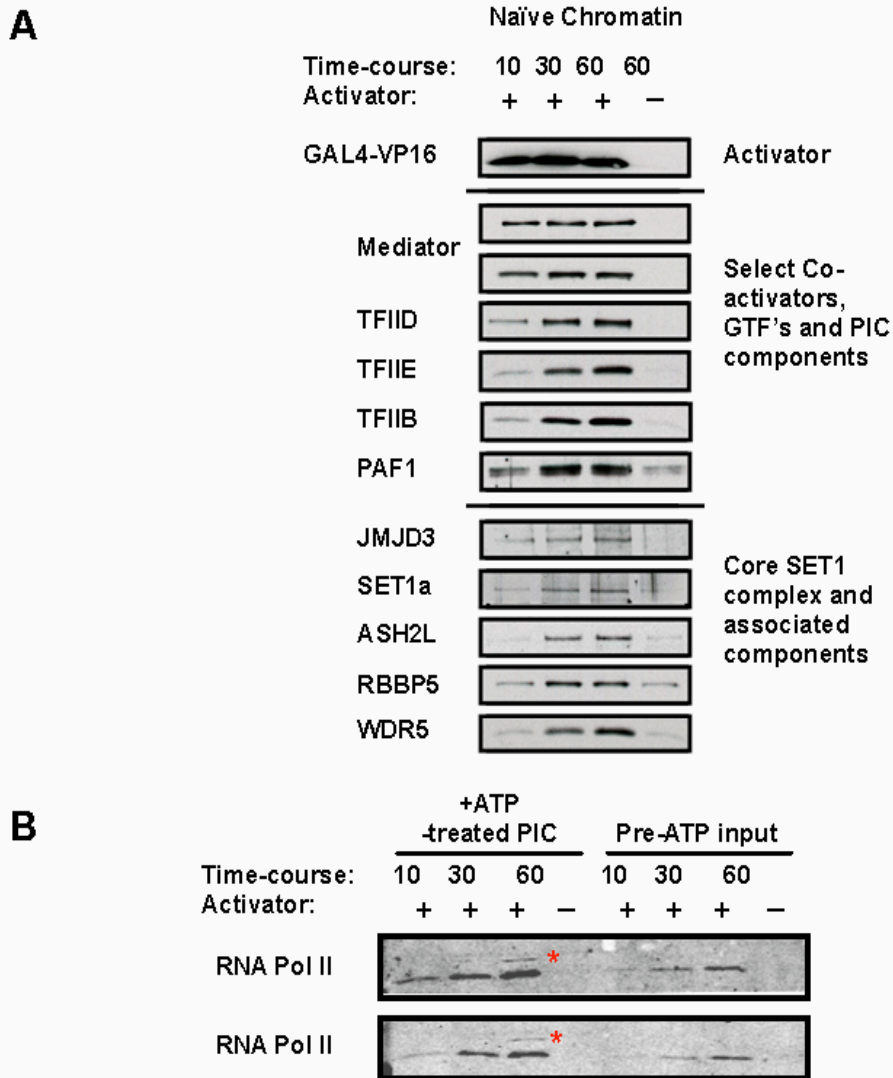
Preinitiation complex (PIC) capture assay: Chromatin assembled on a biotinylated GAL4-responsive promoter was immobilized on streptavidin-coated paramagnetic beads and incubated with HeLa nuclear extract for the times indicated (A). Representative general transcription factors, co-activators and subunits of the SET1 complex were immunoblotted. The + and − indicate the presence or absence of the activator, GAL4-VP16.

Immunoblotting of PICs captured from dialyzed nuclear extract on immobilized templates, treated with exogenous ATP: RNAP II is recruited in an unphosphorylated form, and does not become phosphorylated (red asterisk) in the absence of ATP in the reaction conditions (B).

Lin,U-20958_Supplemental Table 1

Naïve		VP Naïve		H3K4me3		VP		Protein
RNA Pol II								
P24928								
4.895		37.219		10.75		33.286		RPB1
MED Subunits								
Q9NWA0	0		71.743		0		128.322	MED9
Q96HR3	0		470.761		0		947.279	MED30
O75448	0		211.819		0		350.454	MED24
Q9P086	0		1074.3		0		1761.418	MED11
P24863	0		37.012		0		165.504	Cyclin C
Q9NPJ6	0		892.266		0		1387.784	MED4
Q9NVC6-1	0		434.423		0		633.136	MED17
Q15648-1	0		245.132		0		331.804	MED1
P49336-1	20.784		248.316		0		262.453	CDK8
Q9Y2X0-4	0		203.039		0		234.99	MED16
O95402-1	0		366.605		0		390.314	MED26
Q13503	0		290.956		73.536		390.314	MED21
Q9UHV7	0		168.632		0		198.209	MED13
Q93074	0		149.154		0		202.239	MED12
Q9Y3C7	0		319.83		0		357.54	MED31
Q9ULK4	0		153.135		0		198.581	MED23
Q9H204	0		176.535		0		105.253	MED28
Q96RN5	0		292.433		0		297.194	MED15
Q6P2C8	0		168.399		0		120.483	MED27
Q15528	0		628.466		0		421.539	MED22
Q9H944	0		592.892		0		486.052	MED20
Q9BUE0	0		201.431		0		180.145	MED18
O60244	0		216.116		0		167.508	MED14
O75586	0		298.053		0		152.318	MED6
Q71F56	0		37.916		0		16.955	MED13L
Q96G25	0		703.506		0		419.442	MED8
Q9BTT4	0		543.118		0		624.503	MED10
Q9NX70	0		1518.792		0		749.403	MED29
O43513	0		703.506		0		419.442	MED8
TAFs and TBP								
P21675	0		22.381		22.627		40.032	TAF1
P49848	0		30.944	(1/100%)	31.283	(1/100%)	55.347	(2/100%)	TAF6
Q15542	12.055	(1/100%)	26.186	(1/100%)	26.473	(1/100%)	11.709	(1/100%)	TAF5
Q16594	149.055		485.677		204.583		416.255	TAF9
Q15545	0		30.013		0		0	TAF7
Q15544	0		0		50.186		0	TAF11
Q5TBP5	0		28.962		9.76		25.901	TAF4
PCAF Subunits								
Q9Y6J9	62.018	(3/100%)	151.559	(4/100%)	34.049	(2/100%)	135.543	(5/100%)	TAF6L
O75529-1	32.747	(2/100%)	160.051	(5/100%)	0		95.425	(5/100%)	TAF5L
General Transcription Factors								
P19447	36.997	(3/100%)	66.972	(3/100%)	54.165	(2/100%)	131.768	(3/100%)	XPB (IIH)
Q6ZYL4	0		295.054		0		131.937	GTF2H5 (IIH)
P18074	0		13.782		0		0	p80 (IIH)
Q00403	0		66.294		0		88.932	TFIIB
PAF1 Subunits								
Q8N7H5	0		39.452		19.942		35.283	PAF1
Elongation								
P50750-2	0		168.942	(6/100%)	0		125.908	(4/100%)	CDK9 (pTEFb)
CHD Members								
O14646	11.279	(2/100%)	79.63	(9/92%)	74.31	(5/100%)	60.259	(5/91%)	CHD1
FACT Subunits								
Q9Y5B9	27.633		370.156		101.139		420.51	SPT16
Q08945	0.806		206.829		29.871		184.973	SSRP1
Histone Acetyltransferases								
Q9NPF5	0		44.858		45.35		80.236	DNMAP1 (NuA4)
Q92830	0		25.028		0		11.192	GCN5
NURF Subunits								
Q16576	68.074		98.583		124.579		132.248	RBBP7
Trithorax Subunits								
Q8IZD2	0		0		5.699		10.083	MLL5
Q15291	17.925		38.938		0		0	RBBP5
Q9UBL3	0		16.679		0		0	ASH2L
P61964	202.117	(5/100%)	188.163	(4/100%)	31.704	(1/100%)	84.14	(1/100%)	WDR5
Q9P2D1-1	17.301	(2/67%)	8.723	(2/67%)	18.4	(1/30%)	19.014	(3/75%)	CHD7*
Q14839	10.088	(1/50%)	60.261	(7/72%)	11.077	(1/50%)	24.497	(2/60%)	CHD4*
Q9HCK8	22.419	(2/50%)	40.583	(6/100%)	20.514	(1/40%)	43.553	(4/67%)	CHD8*

The NSAFs from transcriptionally relevant proteins identified on Naïve, GAL4-VP16-bound Naïve, H3K4me3, and GAL4-VP16-bound H3K4me3 templates are shown. For proteins containing common motifs (e.g., WD repeats, Chromodomains, etc.), the number and percent contribution of unique peptides to overall spectral abundances are reported. Proteins with fewer than 70% unique peptide contribution in their lowest abundances are listed below with an asterisk. Primary data are available upon request.
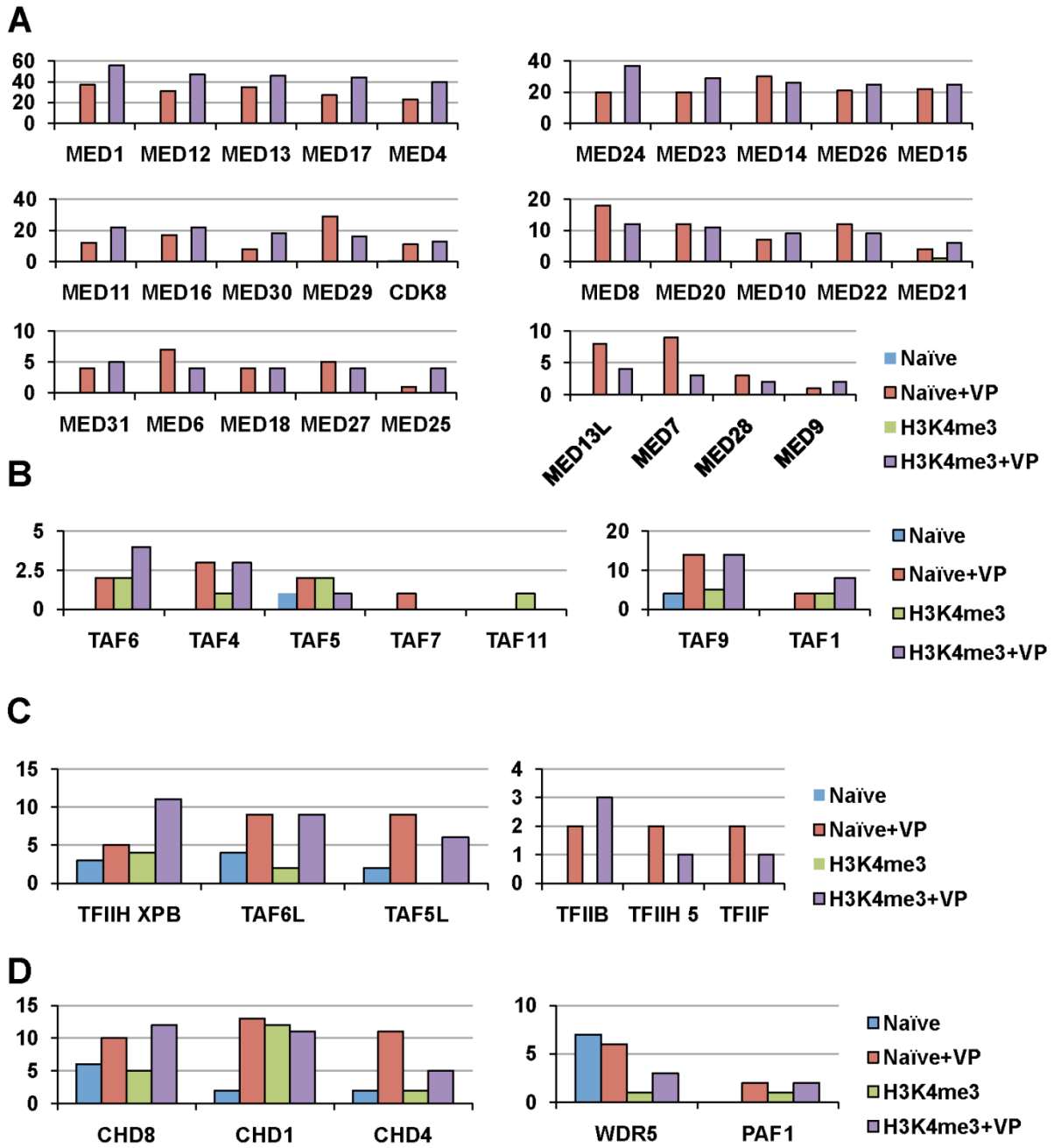
E
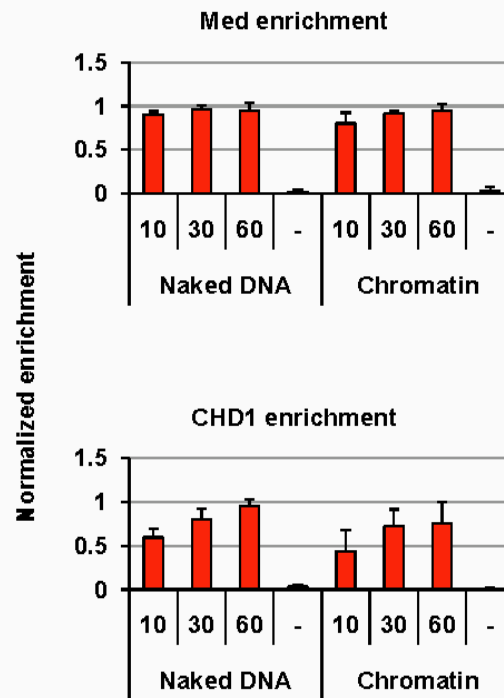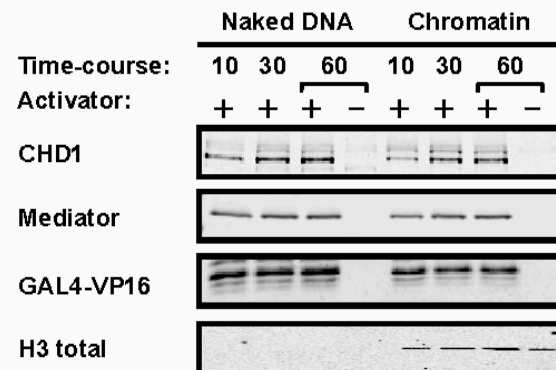
Graphical representations for Mediator subunits (A), TFIID subunits (B), GTF's and other transcription-related factors (C and D) are based on absolute spectral count detected.

Western blot analysis comparing CHD1 recruitment time courses on naked DNA templates vs chromatinized templates (E). Signals were quantified using LiCOR imaging software.

A

TAF1

TAF4

TBP

CHD1

ASH2L

PAF1

Δ TFIID   Mock

B

H3K4me3 Chromatin

Δ TFIID   Mock

30   60   30   60
+   +   −   +   +   −

TFIID
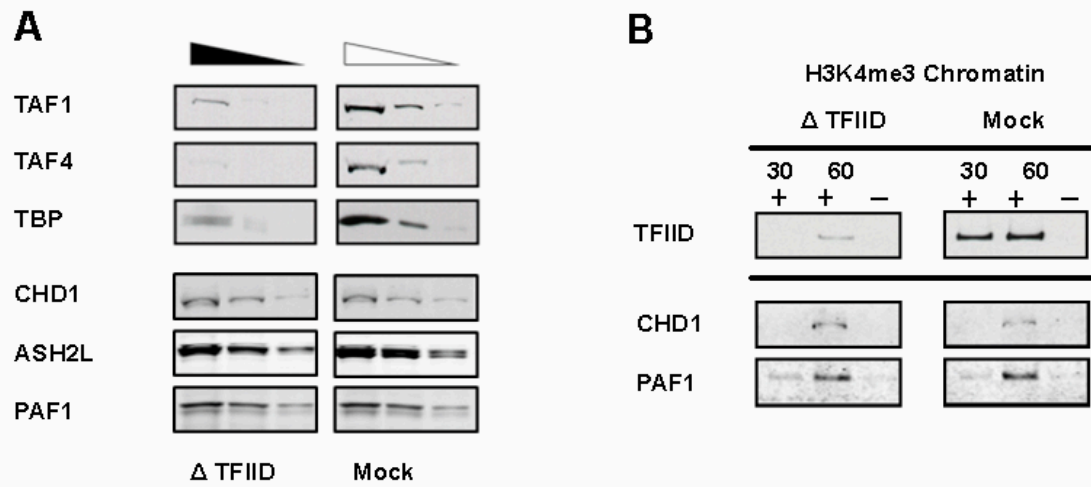
CHD1

PAF1

Immunoblots showing immunodepletion of TFIID from nuclear extract: TFIID was depleted using antibodies against four TFIID subunits (A). Sample titrations were loaded in 3-fold steps. PAF1 complex and CHD1 recruitment (B) were compared by western blotting of PIC-capture time courses in Mock vs. TFIID-depleted extracts.

A

	CHD1+NE (Low) NSAFe5	Protein
CHD		
O14646	10168.365	CHD1
INO 80 Subunits		
O96019	219.485	ACTL6A*
Q8NBZ0	176.870	INO80E
Q9C086	143.266	INO80B
Q9H981	138.321	ACTR8
Q9H9F9	122.805	ACTR5
Q9ULG1	65.556	INO80
Q6PI98	61.301	INO80C
Q53TQ3	8.937	INO80D
Elongation		
Q14241	162.241	SIII/Elongin
TFIIH Subunits		
Q13889	114.642	IIH3
Q6ZYL4	110.515	IIH 5/TFB5
Q92759	59.444	IIH4
P32780	57.274	IIH1
P51948	50.787	MAT1
P19447	50.170	XPB
Q6P1K8	49.662	IIH2 like
P50613	34.017	CDK7
P18074	25.811	XPD
P51946	24.293	Cyclin-H
TAFs and TBP		
Q16594	74.305	TAF9
P49848	23.180	TAF6
P20226	23.146	TBP
Q15572	18.059	TAF1c
Q15573	17.437	TAF1a
Q6P1X5	13.089	TAF2
O14776	10.719	TAF2s
O14981	4.244	BTAF1
P-TEFb		
Q7L2J0	68.330	MEPCE
P50750	31.639	CDK9
O60563	16.212	Cyclin-T1
SET1 Subunits		
Q9UBL3	62.473	ASH2L
Q15291	58.339	RBBP5
Q03164	16.804	MLL1
MED Subunits		
Q13503	54.490	MED21
Q6P2C8	37.845	MED27
O43513	33.676	MED7
A0JLT2	32.158	MED19
O75586	31.897	MED6
Q96RN5	24.894	MED15
Q15648	19.852	MED1
Q9ULK4	14.340	MED23
Q9Y2X0	13.421	MED16
O75448	11.901	MED24
Q71SY5	10.504	MED25
Q9UHV7	3.609	MED13
SWI/SNF Related		
Q12824	50.952	SNF5/BAF47
Q96GM5	45.708	BAF60A
Q969G3	38.183	BAF57
JMJds		
Q6NYC1	38.941	JMJD6
Q8NB78	19.068	LSD2/KDM1B
Q9BZ95	10.921	NSD3
Q9Y2K7	10.129	KDM2A

	CHD1+NE (Low) NSAFe5	Protein
PAF1 Subunits		
Q8N7H5	29.554	PAF1
Q6P1J9	22.165	CDC73
Q6PD62	13.379	CTR9
Q92541	11.711	RTF1
CAF1		
Q13112	28.073	CAF1-b
HIRA		
P54198	23.146	HIRA
FACT Subunits		
Q9Y5B9	11.241	SPT16
Q08945	11.067	SSRP-1
CHD Fam Members		
O14647	1023.740	CHD2 (8/3.35%)
Q9P2D1	155.779	CHD7 (20/24.37%)
Q8TD26	132.943	CHD6 (3/4.35%)
Q3L8U1	121.883	CHD9 (1/1.11%)
Q12873	35.309	CHD3 (9/61.11%)
Q14839	32.831	CHD4 (9/56.25%)

B

|	CHD1+NE (Hi) NSAFe5	Protein
CHD		
O14646	15133.000	CHD1
TFIIH Subunits		
Q13889	129.621	IIH3
Elongation		
P23193	82.171	TFIIS
Q14241	52.000	SIII/Elongin
O00267	45.508	SPT5
PAF1 Subunits		
Q92541	59.587	RTF1
Q6PD62	35.143	CTR9
NELF		
Q8WX92	56.859	NELF-B
P18615	43.392	NELF-E
Q9H3P2	31.229	NELF-A
TAFs and TBP		
O14776	22.526	TAF2s
FACT		
Q9Y5B9	15.749	SPT16

Shown here are charts listing NSF values for proteins associated with CHD1 purified under different conditions. Two charts are shown for CHD1: a longer list in which lower concentrations of heparin (12.5 micrograms/ml) were added (A) and a shorter list from a subsequent effort using higher heparin (75 micrograms/ml) and more active DNase I (B) to hone the list towards proteins interacting directly with CHD1. Due to the high degree of conservation between common domains in the CHD family, other CHD members were detected at high spectral abundance. These are grouped below with the absolute number of unique peptides detected and their percent contribution to total spectral abundance.

H3K4me3 Chromatin
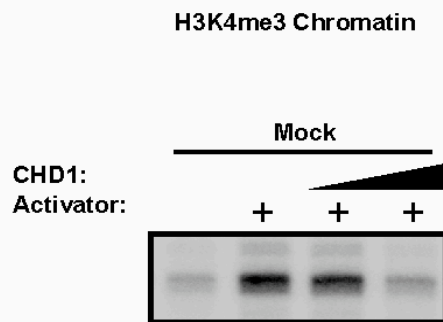
In vitro transcription in Mock depleted extract in the presence of activator and recombinant CHD1. The same points of the recombinant CHD1 titration seen in Figure 5C are used in this experiment. The combined amount of CHD1 from the extract and high point of recombinant CHD1 titration appear to inhibit transcription.

A

ChIP Timecourse at 3' Luciferase reporter

B

C

D

Box plot — Expression (log10) for groups a, b, c, d.

a) **2178 triple-positive genes**
b) **12879 all other genes**
 • vs (a): D = 0.4276, p-value < 2.2e-16
c) **7995 double/single-positive genes**
 • vs (a): D = 0.2598, p-value < 2.2e-16
d) **4884 unbound genes**
 • vs (a): D = 0.7438, p-value < 2.2e-16
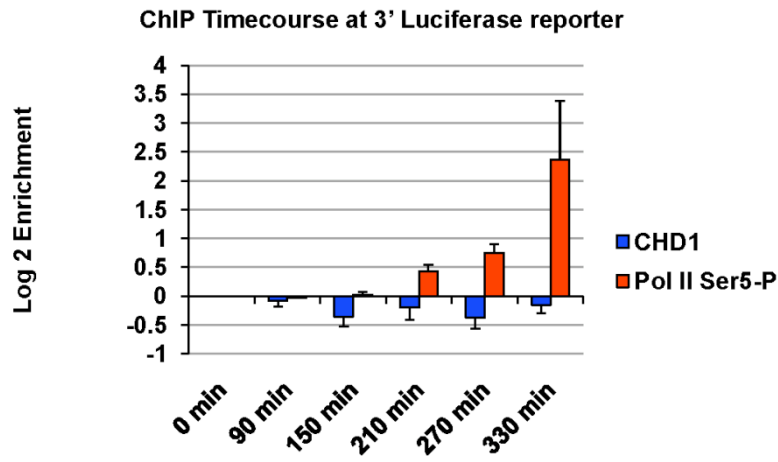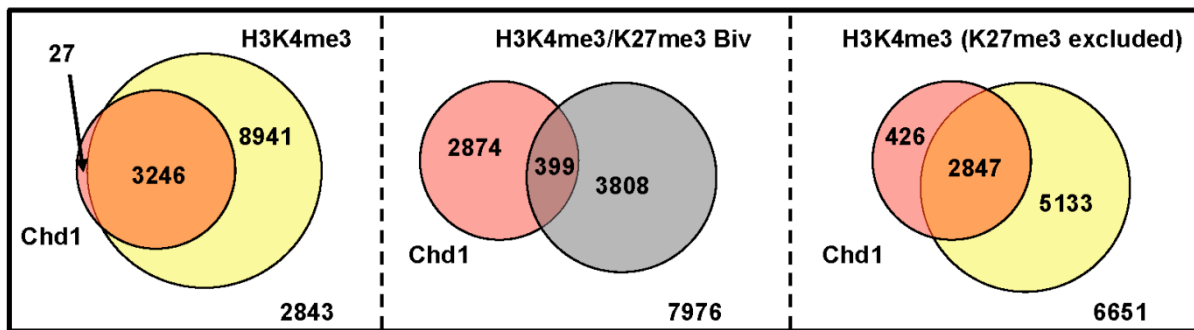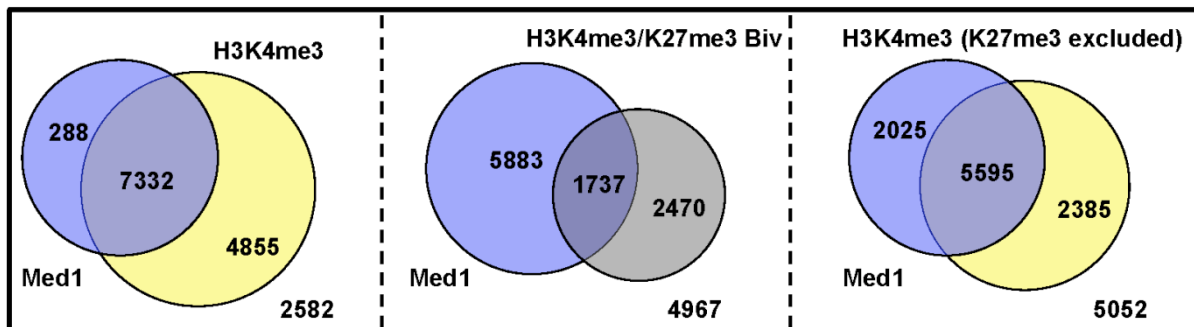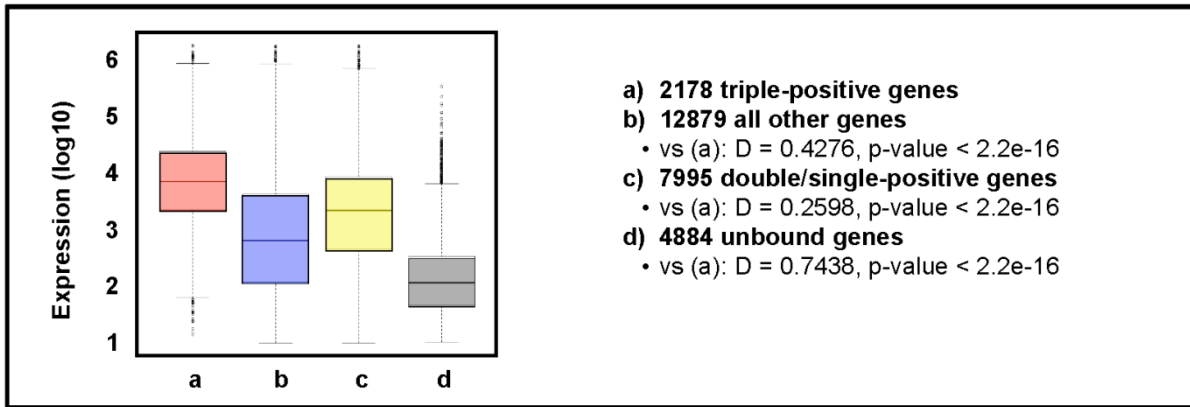
ChIP time course of Doxycycline induction against CHD1 and Pol II at the 3' end of the luciferase reporter gene in Tet-inducible U2OS cells (A).

Venn diagrams representing the genomic overlap between genes enriched for H3K4me3, non-bivalent H3K4me3, and bivalent H3K4/K27me3, and genes bound by Chd1 (B), with p-values of 4.8e-293, 9.3e-322, and 1, respectively. Similar Venn diagrams were constructed for the overlap of H3K4me3, non-bivalent H3K4me3, and bivalent H3K4/K27me3, with Mediator subunit Med1 (C), with p-values of 7.2e-322, 1.1e-321, and 1, respectively. Expression box plot for the indicated gene sets (D). Distribution and p-values were calculated by the KS test.

Mediator screen:

Module:	Subunit:	Enriched/ Background
Head	Med 6	=
Head	Med 8	=
Head	Med 11	=
Head	Med 17	=
Head	Med 18	=
Head	Med 19	=
Head	Med 20	+
Head	Med 22	+
Middle	Med 1	+++
Middle	Med 4	+
Middle	Med 7	=
Middle	Med 10	=
Middle	Med 21	=
Middle	Med 31	=
Tail	Med 29	=
Tail	Med 27	=
Tail	Med 24	=
Tail	Med 14	=
Tail	Med 15	=
Tail	Med 16	=
CDK	CDK8	+
CDK	CycC	=
CDK	Med 12	+
CDK	Med 13	=
Unassigned	Med 23	+
Unassigned	Med 25	=
Unassigned	Med 26	=
Unassigned	Med 28	=
Unassigned	Med 30	+
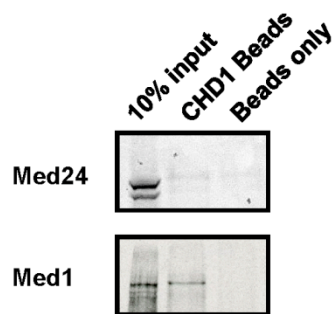
Table showing co-IP signals of Mediator subunits pulled down using CHD1-conjugated magnetic beads. Mediator subunits were labeled with ^{35}S using a rabbit reticulocyte lysate in vitro transcription-translation system. Enrichment of subunit signal was compared to background signal using beads alone. Autoradiograms of examples of no enrichment (Med24) and strong enrichment (Med1) are shown to the right of the table.

10 min under standard conditions as described previously (Black et al. 2006).

Cross-correlation of genomic chromatin immunoprecipitation (ChIP) data sets

ChIP–chip data sets from experiments performed on mouse ES cells were used to determine whether gene promoters were enriched for Chd1 (Gaspar-Maia et al. 2009) and H3K4me3 or H3K27me3 (Sridharan et al. 2009). ChIP-seq data for Med1-occupied genes were from Supplemental Table 5 of the study by Young and colleagues (Kagey et al. 2010). The ChIP–chip experiments were performed on Agilent promoter arrays with probes covering the region −5.5 kb to +2.5 kb relative to the transcription start site. Hence, only those Med1-enriched regions that had significant peaks within this 8-kb region, based on the location of enriched regions from Supplemental Table 4 in Kagey et al. (2010), were used for further overlap analysis. The overlap of genes whose promoters were enriched for each feature (Med1, Chd1, and H3K4me3 without H3K27me3) was determined pairwise, and the significance of the overlap was evaluated using the hypergeometric test. Box plots were used to visualize the distribution of expression levels in mouse ES cells for the set of genes exhibiting co-occupancy by all three features as compared with those for gene sets with different combinations of the three features in their promoter regions or their complete absence. Gene expression levels were obtained from Supplemental Table 2 in Gaspar-Maia et al. (2009). A two-sample Kolmogorov-Smirnov test was used to compare the distributions of expression values between the two sets of genes.

shRNA knockdown of Mediator subunits and coimmunoprecipitation

293T cells were grown to 30% confluency in 10-cm dishes and infected with lentiviruses expressing shRNAs targeting MED23 (TTGTGAGTGTCATCAGCAGCC) and MED1 (GTCATGGA GAAGAGGGTTGTG). After 96 h, cell lysates were prepared and immunoblotted to determine the extent of MED1 and MED23 knockdown using the MED6 antibody as a control. The lysates were subjected to immunoprecipitation with a CHD1 antibody (Bethyl Laboratories). The immunoprecipitates were blotted with antibodies to CHD1, MED6, MED7, and MED14 (Santa Cruz Biotechnologies) to determine the amount of coimmunoprecipitation.

ChIP

ChIP from the U2OS cells, various times after doxycyline treatment, was performed using antibodies to CHD1, MED1, VP16, and Ser 5 Pol II (Abcam) as previously described (Black et al. 2006).

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Chapter 4

Polycomb Repressive Complex 1 (PRC1) Disassembles RNA

Polymerase II Preinitiation Complexes

Polycomb Repressive Complex 1 (PRC1) Disassembles RNA Polymerase II Preinitiation Complexes*[s][◆]

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Background: PRC1 silences transcription by an unknown mechanism.
Results: PRC1 can both block and dissociate PICs in general with the exception of TFIID.
Conclusion: PRC1 gene silencing may involve the ability of TFIID to remain bound to gene promoters, leaving them in a poised state.
Significance: Understanding how PRC1 regulates transcription deepens our basic understanding of key developmental processes.

Despite the important role of Polycomb in genome-wide silencing, little is known of the specific biochemical mechanism by which it inactivates transcription. Here we address how recombinant Polycomb repressive complex 1 (PRC1) inhibits activated RNA polymerase II preinitiation complex (PIC) assembly using immobilized H3K27-methylated chromatin templates *in vitro*. Recombinant PRC1 inhibited transcription, but had little effect on binding of the activator as reported previously. In contrast, Mediator and the general transcription factors were blocked during assembly or dissociated from preassembled PICs. Importantly, among the PIC components, Tata Binding Protein (TBP) was the most resistant to eviction by PRC1. Immobilized template experiments using purified PRC1, transcription factor II D (TFIID), and Mediator indicate that PRC1 blocks the recruitment of Mediator, but not TFIID. We conclude that PRC1 functions to block or dissociate PICs by interfering with Mediator, but leaves TBP and perhaps TFIID intact, highlighting a specific mechanism for PRC1 transcriptional silencing. Analysis of published genome-wide datasets from mouse embryonic stem cells revealed that the Ring1b subunit of PRC1 and TBP co-enrich at developmental genes. Further, genes enriched for Ring1b and TBP are expressed at significantly lower levels than those enriched for Mediator, TBP, and Ring1b. Collectively, the data are consistent with a model in which PRC1 and TFIID could co-occupy genes poised for activation during development.

The Polycomb group of proteins plays a major role in transcriptional regulation during development. The first member of this group, *polycomb*, was identified in screens for *Drosophila* mutants defective in body patterning during development

(1). Subsequently, other genes that fell into this group were identified that encoded proteins assembled into two distinct Polycomb Repressive Complexes (PRC)[3] termed PRC1 and PRC2 (2, 3). PRC1 and PRC2 silence expression of the Hox gene network involved in development as well as the inactive X chromosome (4). The current models suggest that PRC2 methylates histone H3 at lysine 27 (H3K27) via its EZH2 subunit (5–7). This modification, in turn, provides a binding site for the chromodomain-containing Pc subunit of PRC1. Once bound, PRC1 can ubiquitinate H2AK119 via its Ring1a or Ring1b subunit (8). PRC1 binding is believed to be the major determinant in silencing, yet the precise mechanism remains unknown.

Kingston and colleagues (2) were the first to isolate and study the mechanism of PRC1 from *Drosophila*. They found that PRC1 comprises four subunits termed polycomb (Pc), polyhomeotic (PH), posterior sex combs (PSC), and dRING. Subsequent studies showed that mammalian counterparts of these proteins were highly conserved with chromodomain-containing Pc homologs termed CBXs, three PH homologs (PHC1–3), two dRING homologs Ring1a and Ring1b, and six human PSC homologs, the most prominent being BMI1 (9, 10).

Both native *Drosophila* and recombinant mouse PRC1 inhibited activated transcription on chromatin templates in mammalian *in vitro* systems (11). Importantly, PRC1 could only block transcription when prebound to the template. Analysis of individual subunits demonstrated that the PSC and PH subunits functioned most effectively at transcription inhibition on chromatin (11). Remarkably, PRC1 action *in vitro* was not dependent upon H3K27 methylation or ubiquitination. Indeed, the greatest effect was observed with PRC1 subunits lacking either the targeting or the ubiquitination functions (11). Further, a novel complex of RYBP, RING, and BMI1/MEL18 has been shown to be recruited to Polycomb-regulated genes independent of H3K27me3 (12).

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[3] The abbreviations used are: PRC, Polycomb repressive complex; PSC, posterior sex combs; PIC, preinitiation complex; TBP, Tata Binding Protein; TFII, transcription factor II; GTF, general transcription factor; TAF, TBP-associated factor; Pol II, RNA polymerase II; MLA, methyl-lysine analog; CBX, Chromobox protein homolog; GO, gene ontology.

The inhibition of transcription has been largely attributed to the ability of PRC1 to bind and compact chromatin. Cryo-EM analysis showed that a single molecule of PRC1 binds to three nucleosomes (13). This compaction is believed to limit access to factors necessary for transcription on chromatin. Indeed, PRC1 effectively blocked chromatin remodeling by SWI/SNF *in vitro*. Moreover, this inhibition occurred in the absence of ubiquitination (2).

A recent study by the Bickmore group (14) addressed the role of PRC1 and ubiquitination in gene expression and chromatin compaction *in vivo*. The authors found that Ring1b is necessary for silencing and chromatin compaction using fluorescence *in situ* hybridization (FISH) experiments on adjacent Hox genes in embryonic stem (ES) cells. Importantly, the compaction and silencing are dependent on the expression of Ring1b but independent of its ubiquitin ligase activity in agreement with the earlier *in vitro* work of Kingston and colleagues (13).

We were interested in understanding more specifically how PRC1 affects the assembly and function of the RNA polymerase II (Pol II) preinitiation complex. Chromatin immunoprecipitation studies performed in *Drosophila* showed that PRC1 co-bound to the Hox loci with the general transcription factors TBP, TFIIB, and TFIIF (15). Further, analysis of the composition of PRC1 in *Drosophila* identified TBP-associated factors (TAFs) as interacting proteins (16). Collectively, these studies raise the question of how PRC1 affects PIC assembly.

PICs contain Pol II, the 30-subunit Mediator co-activator complex, TFIID, the general transcription factors (GTFs) TFIIA, TFIIB, TFIIE, TFIIF, and TFIIH, and numerous chromatin-modifying and -remodeling complexes (17, 18). PIC formation in response to activator binding is a well studied and temporally regulated process. Initially, Mediator and p300 are recruited to the template directly by the activator. After p300 acetylates chromatin and itself, it dissociates from the PIC, and TFIID binds along with the GTFs (19). Mediator and TFIID binding is cooperative due to a direct interaction among the two co-activator complexes (20). The formation of the PIC also results in the recruitment of chromatin-remodeling and -modifying complexes, including CHD1, and Spt-Ada-Gcn5-acetyl-transferase (SAGA) (18, 19, 21). Importantly, HeLa nuclear extracts depleted of Mediator fail to recruit most GTFs, showing that Mediator is essential for PIC formation (20).

Here we examine the biochemical effect of PRC1 on PIC assembly and function *in vitro*. We employed the immobilized template assay, which allows us to correlate the functional effects of PRC1 on transcription with its effects on the composition of PICs formed on H3K27me3 chromatin templates. Next, we used previously published genome-wide datasets from mouse ES cells to address the hypothesis derived from our *in vitro* findings.

We found that PRC1 can block assembly of the Mediator and a variety of other PIC constituents. Remarkably, the activator and TFIID were most resistant to the action of PRC1. Further, we show that purified TFIID but not Mediator is recruited to templates in the presence of PRC1. Our analysis of genome-wide binding data for TBP, Ring1b, and Mediator shows that in mouse ES cells, most Ring1b-regulated genes are also bound by TBP. Further, genes bound by Ring1b and TBP are strongly

enriched for critical developmental genes. Collectively, these results suggest a more precise mechanism for PRC1-mediated transcriptional silencing and highlight the possibility that TFIID marks PRC1-regulated genes as poised for expression later in development.

EXPERIMENTAL PROCEDURES

Methyl-lysine Histone Octamer Preparation—Lysine 27 of histone H3 was mutated to a cysteine by site-directed mutagenesis of *Xenopus* H3.1 bearing a C110A mutation, expressed and purified from *Escherichia coli* inclusion bodies, and subjected to chemical alkylation by (2-bromoethyl)trimethylammonium bromide before assembly into histone octamers (22, 23). Modification of the histone tail was verified by using nano-spray mass spectrometry. Western blots were performed using standard protocols and a commercially available H3 and H3K27me3 antibody (Abcam catalog numbers ab1791 and ab6002).

Chromatin Preparation—A 602-bp biotinylated PCR fragment, which directly encompasses our GAL4-responsive promoter, was assembled into chromatin by salt dilution as described previously and validated by EMSA in native PAGE (24). Chromatin was immobilized on M280 streptavidin paramagnetic beads (Invitrogen) as described previously (25).

Immobilized Template Recruitment Assay—The 40-μl immobilized template recruitment assays contained HeLa nuclear extract and 125 fmol of a GAL4-responsive template assembled into chromatin (3 nM) as described previously (26). The template is termed G5E4T because it contains five GAL4 sites positioned upstream of the adenovirus E4 TATA box. Reactions were typically performed in the presence and absence of 250 nM PRC1. Bound protein was eluted from the immobilized templates in 10 μl of 2$\times$ Laemmli buffer, fractionated by SDS-PAGE, and immunoblotted. Antibodies used in immunoblotting included MED23 (BD Pharmingen), Pol II C-terminal domain 8WG16 (QED Bioscience), TFIIB (27), and CHD1 (Bethyl Laboratories). All other antibodies were purchased from Santa Cruz Biotechnology.

Extract and Protein Preparation—HeLa nuclear extract and GAL4-VP16 were prepared as described previously (28). Recombinant FLAG-tagged mouse BMI1 was purified from SF9 cells using a baculovirus overexpression system (Invitrogen). For the purification of the PRC1 complex, baculoviruses encoding mouse CBX2, Ring1b, and FLAG-BMI1 were co-infected into insect cells and harvested 44 h after infection. Briefly, cells were resuspended in 0.3 M Buffer F (0.3 M NaCl, 20% glycerol, 20 mM HEPES, pH 7.9, 4 mM MgCl$_2$, 0.2% Triton X-100, and 0.1% Nonidet P-40) and sonicated. Lysates were then treated with DNase I (1 unit/ml) and heparin (12.5 μg/ml) and cleared by centrifugation at 30,000 $\times$ g. The resulting lysate was bound to M2 anti-FLAG resin (Sigma), washed extensively in 0.5 M Buffer F, and eluted using 3$\times$ FLAG peptide (0.25 mg/ml Sigma). For the analysis of proteins that interact with the recombinant PRC1 complex from HeLa nuclear extracts, MudPIT was performed as described previously (18). Mediator was purified from HeLa cells expressing FLAG-tagged human Intersex (MED29) as described previously (29). The HeLa Intersex cell line was a gift from Joan and Ron Conaway (29).

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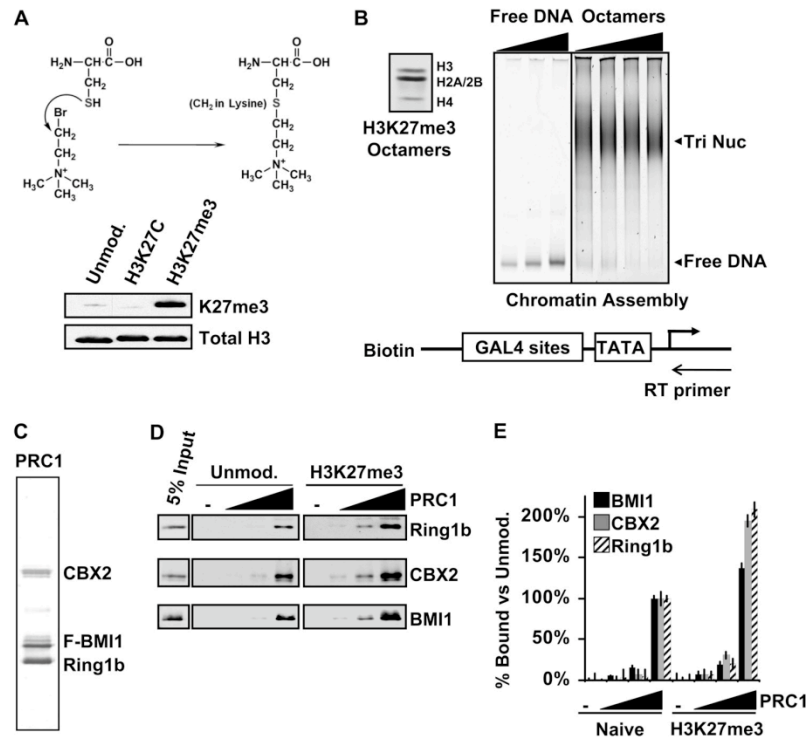
FIGURE 1. **Binding of PRC1 to H3K27me3 chromatin *in vitro*.** *A*, schematic of the MLA synthesis at histone H3 lysine 27. Below the schematic is a Western blot showing the specific detection of H3K27me3 MLA by antibody against HK27me3. *Unmod.*, unmodified. *B*, schematic of the immobilized chromatin template and chromatin assembly. A Coomassie Blue-stained gel of the purified recombinant *Xenopus laevis* histones used is shown on the *left*. The extent of chromatin assembly upon the addition of increasing amounts of histone octamers was monitored by EMSA; equivalent amounts of chromatin were utilized in all experiments. The positions of the free DNA and tri-nucleosome (*Tri Nuc*) are indicated with *arrows*. RT primer was used in primer extension to measure RNA during *in vitro* transcription. *C*, Coomassie Blue-stained gel of purified PRC1 showing the CBX2, BMI1, and Ring1b subunits. 2 μg of PRC1 expressed in and purified from the Bac-to-Bac baculovirus system (Invitrogen) is shown. *D*, immobilized template analysis of PRC1 binding to naive (unmodified) and H3K27me3 chromatin. 125 fmol of immobilized chromatin was incubated with increasing amounts of PRC1 from 62.5 to 250 nM. After washing, the bound proteins were eluted and subjected to immunoblotting. *E*, statistical analysis of data. Three replicates were quantitated using the LI-COR imaging package and graphed. The values were normalized to the highest point of naive chromatin used, which was set at 100%. *Error bars* indicate S.D.

TFIID was purified as described previously from a cell line expressing HA-TBP provided by Arnie Berk (30).

Genome-wide Analysis—Previous datasets for genome-wide binding of TBP, MED1, and Ring1b were obtained from the Gene Expression Omnibus (GEO) database (31, 32). The GEO accession codes for the data used are as follows: TBP, GSE22303; MED1, GSE22557; Ring1b, GSE13084; and CBX7, GSM820726. Reads were mapped to the mouse (mm9) genome using the Bowtie software (33). The mouse genome was tiled into 50-bp windows. The total counts of the input and chromatin immunoprecipitation (ChIP) samples were normalized to each other. The input sample was used to estimate the expected counts in a window. Poisson distribution analysis was used to estimate the probability of observing the ChIP counts within a window given the expected counts in the input sample window (34). In Figs. 6 and 7, all *p* values of enrichment are plotted to remove any bias. The expression levels for all annotated genes were determined using the GSM881355 GEO dataset.

Statistical Analysis—In Figs. 2 and 3, a two-tailed Student's *t* test was performed comparing the quantified signals of activator-stimulated PIC formation with PIC incubated with PRC1

from Western blots using the Odyssey imaging software from LI-COR. In Figs. 6 and 7, a Kolmogorov-Smirnov test was performed to compare datasets of unequal size (35).

RESULTS

Generation of H3K27me3 Chromatin Templates—Our first goal was to recreate PRC1 silencing *in vitro*. To generate the chromatin docking site for PRC1, we used the methyl-lysine analog (MLA) method developed by Shokat and colleagues (22) to synthetically methylate H3K27 for biochemical analysis. The H3K27me3 MLA was validated by immunoblotting with an antibody to H3K27me3 (Fig. 1*A*) and quantitated by electrospray ionization mass spectrometry, which revealed that 88% of H3 was modified (data not shown). Previous studies have shown that PRC1 binding genome-wide broadly spreads across promoters; however, the high amount of modification used in our experiments may also aid in the initial recruitment of PRC1 (32). As shown in the Fig. 1*B* schematic, the synthetically methylated H3 was assembled into octamers and then into chromatin on biotinylated DNA templates containing a GAL4-respon-

FIGURE 2. **PRC1 blocks transcription and PIC assembly.** *A*, PRC1 blocks *in vitro* transcription. The flowchart indicates the order of protein addition. GAL4-VP16 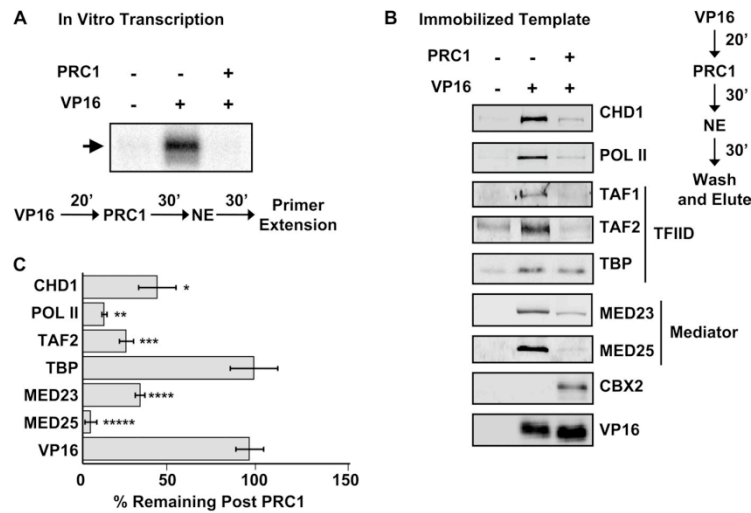was prebound to the template for 20 min, and 250 nM PRC1 was added. After 30 min, HeLa nuclear extract was added in the presence of nucleotides. After 30 min, the mRNA products were isolated and measured by primer extension. *B*, PRC1 blocks PIC assembly. An immobilized template assay using HeLa nuclear extract, in the presence and absence of activator and PRC1, was performed. An immunoblot of the isolated complexes comparing binding of select components of the PIC is shown. *C*, immunoblot signal was quantified using the LI-COR imaging software and graphed, and the statistical significance between mock- and PRC1-treated templates was calculated using a two-tailed Student's *t* test (*n* = 3). *p* values were determined to be *, 0.025, **, 0.03, ***, 0.015, ****, 0.022, and *****, 0.001 for the indicated signals quantified. Signals were normalized to that of activator-stimulated recruitment in *lane 2* (100%). *Error bars* indicate S.D.

sive promoter for immobilized template and *in vitro* transcription analysis (19).

Verification of in Vitro System to Study PRC1 Silencing—To generate a recombinant PRC1 complex, we co-expressed mouse CBX2 (Pc), Ring1b (dRING), and FLAG-BMI1 in a baculovirus expression system and isolated the complexes using FLAG antibody beads. Fig. 1C shows a Coomassie Blue-stained gel of the minimal core complex. To determine whether PRC1 displays a higher affinity for our H3K27me3 templates, we performed immobilized template analysis. Increasing concentrations of PRC1 were incubated with either naive or H3K27me3 templates immobilized on streptavidin-coated magnetic beads, and the bound proteins were captured using a magnetic particle concentrator. The immunoblot of Fig. 1D shows that recombinant PRC1 binds with higher affinity for the H3K27me3 MLA *versus* the naive chromatin. Approximately 5–10% of the PRC1 used in the assay is recruited to the template. Therefore, 12.5–25 nM PRC1 is binding to 3 nM H3K27me3 template in our assay. A bar graph of triplicates in Fig. 1E revealed a consistent 2-fold increase in affinity under the conditions of our binding assay, which is compatible with *in vitro* transcription conditions. Although previous studies with methylated histone tail peptides showed that PRC1 binds with a 4–5-fold higher affinity for H3K27me3, the use of chromatin may partially negate the effect of methylation because subunits of PRC1 other than Pc (CBX2), including BMI1, contribute strongly to its affinity (36).

As another measure of the function of the complex, we sought to confirm its ability to associate with subunits usually associated with PRC1 *in vivo* (9). We utilized MudPIT analysis to identify proteins from HeLa nuclear extracts able to interact with recombinant PRC1. MudPIT analysis of FLAG-PRC1

incubated with HeLa nuclear extract revealed an enrichment of numerous other PRC1 complex members as compared with FLAG beads alone (supplemental Fig. S1). Members of the PH, Pc, PSC, and dRING families associated with recombinant PRC1, showing that the complex is dynamic and can exchange subunits with those present in the nuclear extract. Using the recombinant complex in the presence of nuclear extract therefore allows for the recruitment of endogenous PRC1 members and may aid in PRC1 silencing *in vitro*.

Functional Consequences of PRC1 Binding—To validate the functional consequence(s) of PRC1 binding to a promoter, we carried out *in vitro* transcription of chromatin in HeLa nuclear extracts. We performed the assay two different ways. In one, the blocking assay, we prebound the activator GAL4-VP16 and then added PRC1 and measured transcription. In the other, the dissociation assay, we prebound GAL4-VP16, added nuclear extract, and allowed the PIC to assemble, after which we added PRC1.

PRC1 Blocks Functional PIC Formation—Our initial experiments focused on the role of PRC1 in blocking PIC formation, similar to the role that PRC1 plays in maintaining the silent state of developmental genes. The results of the *in vitro* transcription assay in Fig. 2A reveal stimulation by the activator GAL4-VP16 on the H3K27me3 templates. However, the addition of PRC1 completely blocked stimulation by the activator. This result demonstrated an ability to recreate, in a cell-free system, the silencing of a model reporter gene, as first reported by Kingston and colleagues (11).

We addressed the molecular basis for this inhibition by first analyzing the composition of the PICs formed in the presence of PRC1 via immobilized template assays. In this assay, the binding of our activator GAL4-VP16 to the template stimulates

FIGURE 3. **PRC1 dissociates preassembled PICs.** *A*, PRC1 silences *in vitro* transcription from preassembled PICs. The flowchart indicates the order of binding. GAL4-VP16 was prebound to the template for 20 min, after which HeLa nuclear extract was added to allow PIC assembly. After 30 min, PICs were isolated, and PRC1 was added in the presence of nucleotides. After 30 min, the mRNA products were isolated and measured by primer extension. *B*, PRC1 disassembles a PIC in an immobilized template assay. The flowchart indicates the order of protein addition. An immunoblot comparing binding of select components of the PIC in the presence of increasing amounts of PRC1 is shown. *C*, immunoblot signal was quantified using the LI-COR imaging software and graphed, and the statistical significance between recruitment of proteins to mock- and PRC1-treated templates was calculated using a two tailed Student's *t* test (*n* = 3). The *p* values determined were *, 0.001, **, 0.002, ***, 0.020, ****, 0.003, and *****, 0.001 for the indicated signals measured. Signals were normalized to that of activator-stimulated recruitment in *lane 2* (100%). *Error bars* indicate S.D.

PIC formation. Complexes bound to the template are captured using a magnet and immunoblotted to assay the extent of PIC formation. We assayed for previously identified PIC components and chose representative proteins from each complex including Pol II, Mediator, TFIID, and the chromatin remodeler CHD1. Analyzing which PIC components are blocked will define the step in PIC formation that is blocked by PRC1. Fig. 2*B* is a blocking experiment showing that PRC1 does not significantly alter the amount of GAL4-VP16 bound to the template but does block the recruitment of most other PIC proteins. TBP binding was the highest for all proteins tested. Similar results were obtained using a four-member PRC1 complex that included the PH subunit (supplemental Fig. S2). Importantly, Mediator recruitment, the initial step in PIC formation, is strongly blocked. The GTFs are recruited after and dependent on Mediator during PIC formation, and we found that these were also blocked (supplemental Fig. S3). The experiment was performed in triplicate, and the percentage of protein remaining after blocking by PRC1, as measured using the LI-COR detection system, is graphed in Fig. 2*C*. These results reveal that PRC1 silences transcription by inhibiting the binding of the Mediator, which is integral to the formation of an activator-stimulated PIC. Further, these results suggest that TBP recruitment to a PIC is only slightly affected by PRC1 binding.

PRC1 Functionally Dissociates PICs—PRC1 regulation is dynamic during development. It silences developmental genes before differentiation and pluripotency factors such as Sox2 at the onset of differentiation. With this in mind, we asked whether PRC1 has an effect on preassembled PICs. The results of the *in vitro* transcription assay in Fig. 3*A* show that PRC1 can functionally silence a pre-existing PIC. This result was surpris-

ing because Kingston and colleagues (11) had been unable to observe this effect in previous studies. Our result may stem from the use of the immobilized template approach as it allows us to isolate PICs from crude nuclear extracts and assay the affects of PRC1 in a more purified system.

We then performed immobilized template experiments, where PICs were preformed on the template prior to PRC1 addition to assess which PIC components were dissociated by PRC1 (Fig. 3*B*). Preformed PICs were relatively stable up to 1 h following formation (supplemental Fig. S4), yet the addition of PRC1 to the immobilized template at 20 min following PIC formation shows that many components, including the Mediator, Pol II, and GTFs, were dissociated from the template (Fig. 3, *B* and *C*). Importantly, TBP was consistently retained on the template. In summary, these results suggest that when PRC1 silences an active gene, the mechanism is similar to PIC blocking.

PRC1 Blocks the Recruitment of Purified Mediator, but Not TFIID, in Vitro—Our results using crude nuclear extracts revealed that TBP is somewhat resistant to PRC1-mediated blocking and dissociation relative to the GTFs. Moreover, PRC1 and TFIID can interact in *Drosophila*, and testes-specific TAFs counteract Polycomb during development (16, 37). These findings support models where PRC1 and TFIID might either co-occupy or compete for binding to a promoter during development in higher organisms. We investigated these possibilities *in vitro* using purified TFIID and PRC1 in an immobilized template assay. The results in Fig. 4, *A* and *B*, show that TFIID binding is resistant to PRC1 in both blocking and dissociation assays. Further, the quantification of triplicate experiments shown in Fig. 4, *C* and *D*, highlights the lack of effect by PRC1
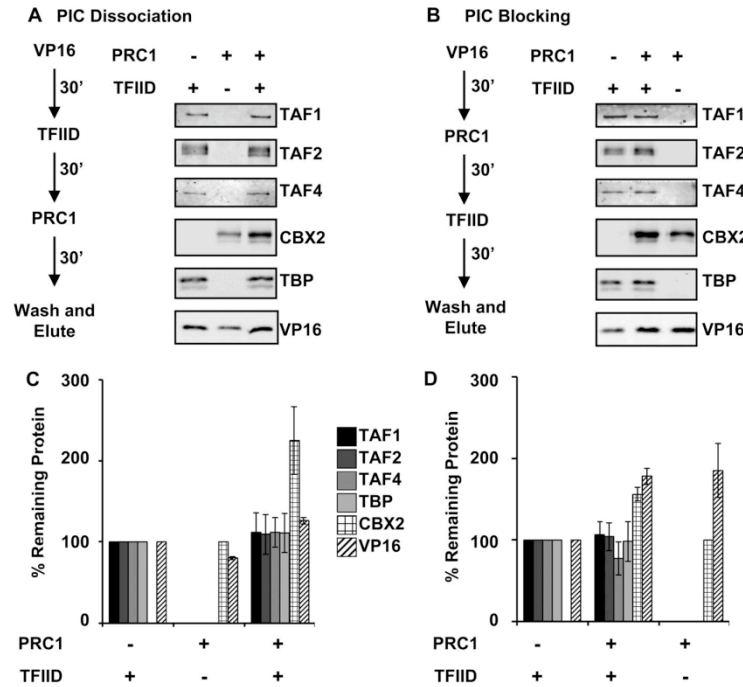
FIGURE 4. **TFIID binds the immobilized template in the presence of PRC1.** *A* and *B*, purified TFIID, PRC1, and GAL4-VP16 were incubated with 125 fmol of G5E4T template in immobilized template assays. Assays were performed to investigate both dissociation (*A*) and blocking (*B*) of TFIID binding. *C* and *D*, a quantitation of three replicate experiments for both dissociation (*C*) and blocking (*D*) assays is shown. The immunoblot signals were quantified using the LI-COR system, and values were normalized to the amount of protein bound when added alone. *Error bars* indicate S.D.

yet shows that CBX2 recruitment is modestly enhanced by the presence of TFIID. This result led us to question whether the recruitment of purified Mediator was affected by PRC1. We therefore performed immobilized template experiments using purified Mediator, TFIID, and PRC1. The data in Fig. 5 show that PRC1 blocks the recruitment of Mediator to the immobilized template when added either alone or with TFIID. This experiment was performed in triplicate, and amounts of binding for each protein were measured using the LI-COR detection system. The changes in Mediator binding were determined to be significant using a Student's *t* test comparing activator-stimulated recruitment of Mediator with PRC1-bound templates ($p < 0.001$). TFIID subunits were reproducibly unaffected by PRC1, similar to the results in Fig. 4. We noticed that TAFs in the extract were sensitive to PRC1, but TAFs in purified TFIID were not. To test whether an activity in extracts contributed to the effect, we also performed an immobilized template experiment where TFIID was prebound to the immobilized template followed by the addition of PRC1 and nuclear extract (supplemental Fig. S5). The results show that PRC1 has no effect on dissociating TFIID when PRC1 is recruited in the presence of nuclear extract. This result suggests that the extract does not contain an activity that dissociates the TAFs. We conclude that PRC1 blocks a critical step in PIC formation, binding of the Mediator, which is required for gene activation (38, 39).
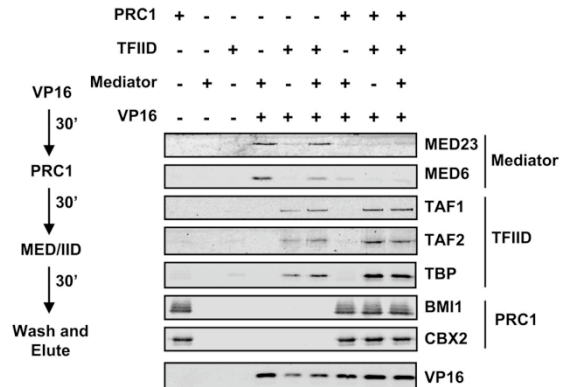

FIGURE 5. **TFIID but not Mediator binding is resistant to PRC1.** Purified TFIID, Mediator, PRC1, and GAL4-VP16 were incubated with 125 fmol of template in an immobilized template assay. Following prebinding of the activator, PRC1 was bound to the template. TFIID or Mediator was then incubated with the template either alone or in combination. The Western blot signals for representative subunits of TFIID (TAF1, TAF2, TAF4, TBP), Mediator (MED23, MED6), and PRC1 (BMI1, CBX2) are shown.

In Vivo Association of PRC1 and TBP Correlates with Gene Repression in Mouse ES Cells—The question of whether PRC1 and TBP can co-occupy a promoter *in vivo* is an interesting and logical next question. We therefore sought to address this hypothesis derived from our *in vitro* studies *in vivo*. We utilized previous genome-wide data from mouse embryonic stem cells,
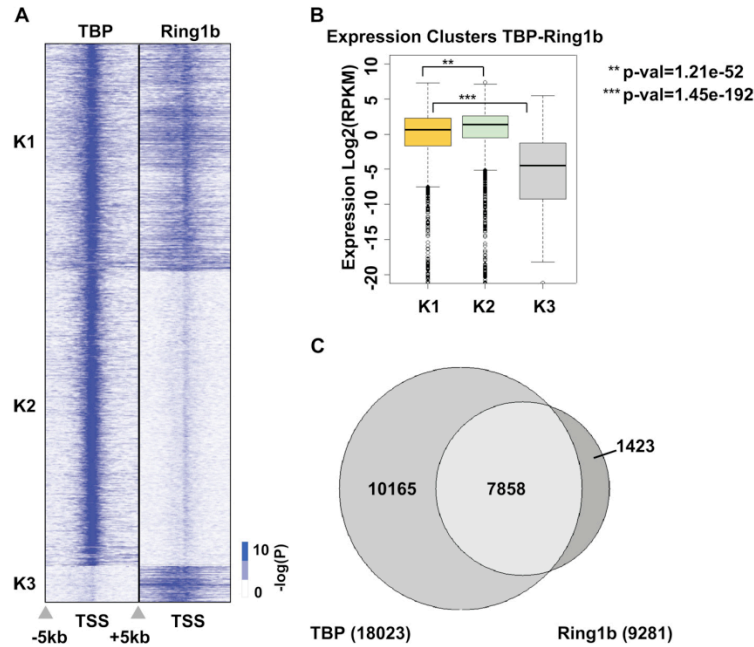
FIGURE 6. **PRC1 and TBP enrichment overlaps *in vivo.*** *A*, the distributions of TBP and Ring1b from −5 kb to +5 kb, centered at the transcriptional start site (*TSS*) for all annotated genes in mouse ES cells, are shown as heat maps of −log (*p* value (*p-val*)). Binding peaks were sorted into differential binding clusters (K1–3) comparing TBP and Ring1b across all genes in the genome. *B*, RNA-Seq expression analysis was used to determine the mean expression for genes in clusters K1–3. A box and whisker plot of the expression for each cluster is shown for reads per kilobase of exon per million (*RPKM*) fragments mapped. *Asterisks* indicate that the change in expression is significant between the indicated clusters as measured using a Kolmogorov-Smirnov test. *Error bars* indicate S.D. *C*, a Venn diagram comparing the enrichment of TBP alone, TBP and Ring1b, or Ring1b alone to all genes in mouse ES cells is shown.

where PRC1 is critical and required for the pluripotent state (40, 41).

ChIP-Seq datasets, deposited by investigators who previously reported the genome-wide binding of Ring1b, TBP, and MED1 in mouse embryonic stem cells, were obtained from the GEO database (31, 32). Peaks of enrichment were determined by segregating the mouse genome into 50-bp bins and comparing the ChIP and input signals in each bin. We then used Poisson distribution to calculate *p* values for the enrichment of ChIP signal reads in each bin. All values are plotted as −log (*p* value) (Figs. 6*A* and 7*A*). The mRNA expression levels for all annotated genes were assessed using a previously reported GEO dataset (GSM88135).

TBP Binding Is Retained at Ring1b-bound Promoters in Mouse ES Cells—The results of our *in vitro* studies implied that TFIID could bind to gene promoters regulated by PRC1. We analyzed the genome-wide binding of TBP and Ring1b in mouse ES cells from prior studies to investigate this possibility (31, 32). Fig. 6*A* shows a heat map of Ring1b and TBP binding distributions ±5 kb from the transcription start site for all annotated genes with each row representing one gene promoter. Clusters K1–3 were grouped based on combinatorial binding of TBP and Ring1b. For each cluster identified, the average gene expression level derived from an mRNA-Seq dataset is shown as a box plot in Fig. 6*B*. The Venn diagram in Fig. 6*C* shows the amount of overlap between TBP and Ring1b enrichments on promoters genome-wide.

We found a significant number of genes with ChIP enrichment for both Ring1b and TBP (Fig. 6, *A* and *C*). Interestingly, the mean expression of genes with ChIP enrichments for PRC1 and TBP (cluster K1) was significantly lower ($p = 1.21 \times 10^{-52}$) than genes with enrichment for TBP alone (cluster K2) (Fig. 6*B*). Also, gene ontology (GO) analysis revealed that genes in clusters K1 and K3 are highly enriched for processes associated with development. This result is not surprising, but it does imply that a large portion of gene promoters bound by PRC1 in ES cells may also be bound by TBP.

To further test our hypothesis and correlate it with our biochemical analysis, we analyzed previous genome-wide binding studies for the Mediator complex and compared its binding to PRC1 and TBP (31). We generated clusters of combinatorial binding to identify TBP-bound genes based on Ring1b and MED1 binding. The results in Fig. 7*A* show the different gene clusters identified, L1−4, visualized as heat maps of the enrichment score. A box plot of the expression for genes in clusters L1−4 is shown in Fig. 7*B*. We also utilized the DAVID software to determine the top six GO terms represented for each cluster. A histogram of the −log (*p* value) for each GO term enriched in clusters L1−4 is shown in Fig. 7, *C–F*. A representative gene from each cluster is shown, respectively, in Fig. 7, *G–J*, with levels of binding plotted as the enrichment *p* value. To validate the presence of the entire PRC1 complex in cluster L1, we also analyzed the genome-wide binding of CBX7 recently reported by the Brockdorff group (12). A comparison of the normalized
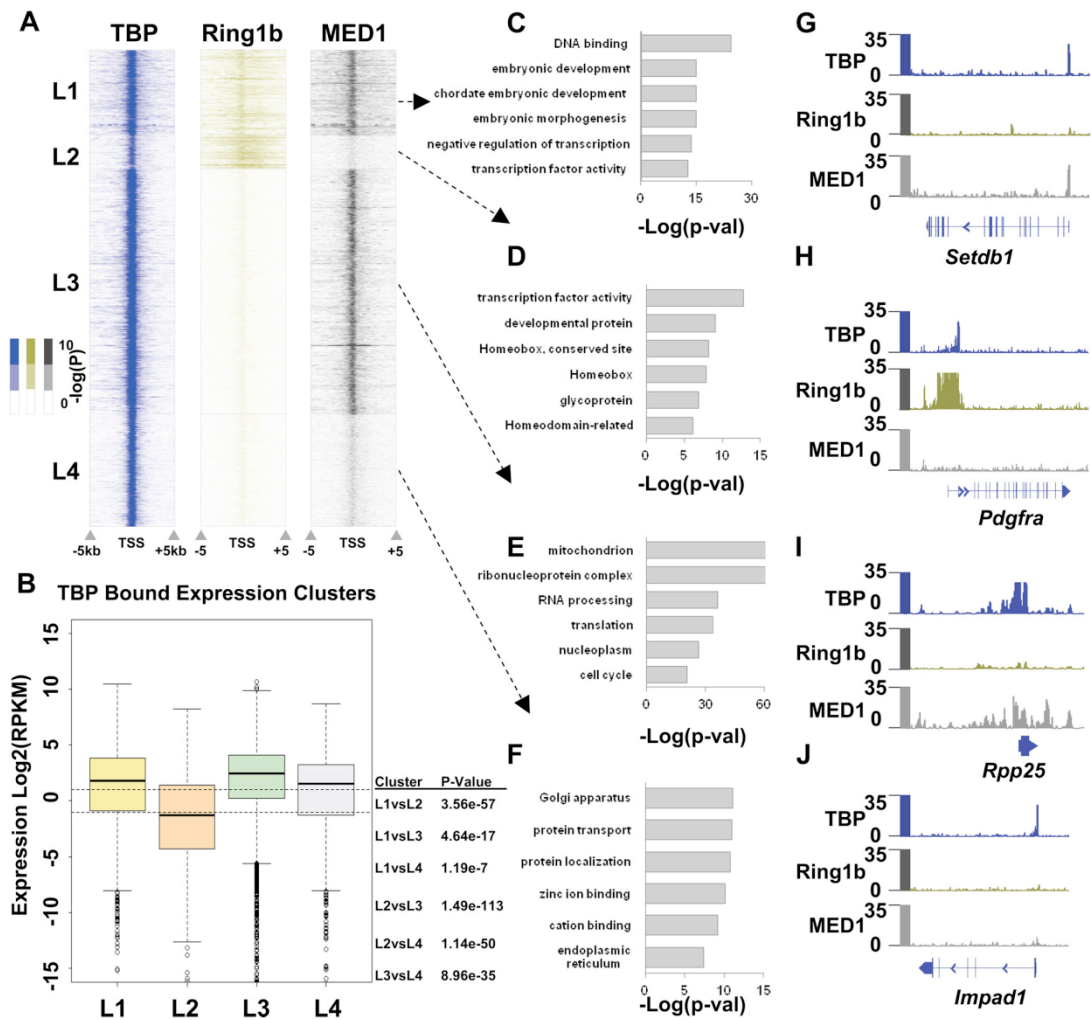
99

FIGURE 7. **Genome-wide analysis of Ring1b, TBP, and Mediator in mouse ES cells.** *A*, the distributions of TBP, Ring1b, and MED1 enrichment −5 kb to +5 kb, centered at the transcriptional start site (*TSS*) for all annotated genes in mouse ES cells, are shown as heat maps of −log (*p* value). Peaks were called and sorted into differential clusters (L1–4) based on the enrichments of TBP, Ring1b, and MED1 across all genes in the genome. Only genomic regions enriched for TBP are shown. *B*, mean expression analysis of genes in clusters L1–4 is shown. A box and whisker plot of the expression for each cluster indicates the mean expression for each cluster in reads per kilobase of exon per million (*RPKM*). The *dashed line* represents a 2-fold change in expression from the overall mean. The *p* values resultant from Kolmogorov-Smirnov test comparing clusters L1–4 are shown. *Error bars* indicate S.D. *C–F*, GO term analysis of clusters L1–4. The top six GO terms for each expression cluster are shown, and the −log of the *p* value of confidence for each term is graphed. *G–J*, the binding profile for a representative gene from each cluster is shown. Values plotted are −log (*p* value).

average enrichment for gene clusters L1–4 is shown in supplemental Fig. S6.

Our clustering reveals a number of intriguing observations. The first cluster, L1, defines a group of genes that are enriched for all proteins tested. The top six GO categories identified for this group include DNA binding and early embryonic developmental genes (Fig. 7, *C* and *G*). The L2 cluster contains genes enriched for Ring1b and TBP. Importantly, this gene cluster has a significantly lower mean expression as compared with L1, and the top GO terms include developmental proteins and homeobox genes (Fig. 7, *B*, *D*, and *H*). It is not a surprise to find that binding of Mediator correlates with a decreased level of Ring1b

binding (Fig. 7*A*, cluster L1 *versus* L2). Cluster L3 represents highly expressed genes as there is no Ring1b bound, and this group has a significantly higher mean expression as compared with L1 and L2 (Fig. 7, *A* and *B*). Also, this cluster is enriched for constitutively active mitochondrial and RNA processing genes based on GO analysis (Fig. 7, *E* and *I*). The last cluster, L4, is enriched for TBP only and represents genes involved in protein processing and cation binding proteins by GO analysis (Fig. 7, *A*, *F*, and *J*).

These results collectively support the hypothesis that the Mediator is the key PIC component inhibited from recruitment to promoters by PRC1. The analysis of genome-wide binding

correlated to expression levels reveals that PRC1 regulation of developmental genes in mouse ES cells may involve co-binding by TBP.

DISCUSSION

PRC1 Mechanism of Silencing—Young and colleagues (38, 39) originally postulated that the multimeric Mediator functions at the beginning of PIC formation to convey signals and efficiently recruit other machineries such as the GTFs and Pol II. The idea that Mediator coordinates these events has been verified by numerous groups including ours and is supported by the finding that Mediator recruitment is the earliest step in PIC formation *in vitro* using the immobilized template assay (19, 38, 39).

By utilizing a combination of the immobilized template assay and *in vitro* transcription, our study provides a more detailed mechanism for how PRC1 silences transcription. Our findings support a model where PRC1 blocks most PIC components with the exception of TFIID. The binding of TBP to PRC1-silenced genes in *Drosophila* was previously observed, and our results are consistent with that finding (42). However, our data indicate that PRC1 acts mainly by blocking Mediator, an early and integral event in activator-stimulated PIC formation (19). Curiously, we observe PRC1-mediated blocking of Pol II *in vitro*, whereas previous *in vivo* studies reported that Pol II remains on the gene (42). It is plausible that preinitiated Pol II is more sensitive to PRC1 than the paused Pol II frequently observed at promoters *in vivo*. We attempted but were unable to trap the paused Pol II *in vitro* and could not test this idea biochemically.

The finding that TFIID can dock to a promoter in the presence of PRC1 leads us to question whether genes co-bound by these two complexes may be in the poised transcriptional state. The poised state is a characteristic of bivalent promoters bearing both H3K27me3 and H3K4me3. Indeed, the TAF3 subunit of TFIID has been shown to bind to H3K4me3, and PRC1 binds to H3K27me3 (36, 43, 44). The presence of TBP, and possibly TFIID, at PRC1-regulated genes may facilitate rapid transcriptional activation when the gene is needed during development. Alternately, the binding of PRC1 to TBP may be a means of targeting PRC1 to recently inactivated genes where activators and thus Mediator have been lost. In this case, TBP or TFIID and its binding site may be acting analogous to the traditional *Drosophila* Polycomb repressive element. It was intriguing that in several of our experiments, PRC1 bound better when TFIID alone was present (*i.e.* Fig. 4) and stabilized TFIID binding even after dissociating Mediator (Fig. 5). Also, note in Fig. 6 that Ring1B binding appears centered near the transcription start site.

PIC Blocking and Dissociation by PRC1—Our *in vitro* transcription results verify previous studies by Kingston and colleagues (2, 11, 45) and others (42), but our immobilized template experiments provide further insight into the mechanism of PRC1 silencing, specifically, which activator-dependent PIC components are blocked from recruitment to the promoter. Specifically, we found that activator recruitment of Mediator, Pol II, and GTFs was significantly reduced, whereas TBP and GAL4-VP16 were more resistant. Using purified PRC1, TFIID,

and Mediator in an immobilized template assay, we then showed that PRC1 blocks the recruitment of Mediator, whereas TFIID binding is unaffected. The ability of holo-TFIID, not just TBP, to bind in the presence of PRC1 using the purified system is likely due to a change in the binding properties of TFIID when purified as compared with nuclear extract (supplemental Fig. S5). Our results argue against an activity in the nuclear extract that affects the stability of TFIID.

Activators are known to interact directly with Mediator and TFIID. In the case of VP16, Mediator interacts via MED25 (46, 47) and TFIID via TAF9 (48). VP16 is known to recruit both complexes to DNA *in vitro* and *in vivo* (49). TFIID and Mediator also interact to form a co-activator complex, which is necessary *in vitro* for binding of the general transcription factors and Pol II (20). Our findings suggest a model where the Mediator interaction with VP16 is blocked, either by chromatin compaction or by inhibiting the accessibility to the template.

It was rather interesting to find that PRC1 can dissociate functional PICs. Previous studies implicate a role for PRC1 in silencing genes active in stem cells, such as Sox2, following differentiation (50). In our immobilized template assays, PRC1 binding to a template post-PIC formation resulted in the demolition of PICs. The majority of proteins assayed for were significantly dissociated from the PICs, except for TBP and activator, similar to results obtained for PIC blocking. Our results reveal the ability of PRC1 to silence a gene by disrupting pre-existing PIC components *in vitro*.

Genome-wide Analysis of PRC1 Regulated Genes—We tested our hypothesis that TFIID is the most resistant to PIC inhibition by PRC1 by analyzing the genome-wide binding of TBP and Ring1b in mouse ES cells. Our analysis indicated that the majority of Ring1b-bound genes are also bound by TBP (Fig. 6, *A* and *C*). Further, the genes bound by both Ring1b and TBP have a significantly lower expression level as compared with those bound by TBP alone, suggesting that they are indeed targets of PRC1 silencing (Fig. 6*B*, clusters K1–3).

Our further analysis of genes bound by MED1, TBP, and Ring1b revealed a number of interesting binding clusters (Fig. 7*A*). Clusters L1 and L2 are bound by both PRC1 and TBP, whereas Mediator binds in cluster L1 only. It was interesting that MED1 is bound to genes that are also bound by Ring1b. Based on GO analysis, the genes in L1 are early differentiation genes that may be lowly expressed due to heterogeneity in the ES cultures (Fig. 7*C*). It is also possible that the Ring1b present at genes in the L1 cluster may not be sufficient for silencing or may be acting in a function not related to PRC1. The genes located in cluster L2 highlight an area where TBP and PRC1 cross-talk may occur. Importantly, the enrichment of Ring1b is increased in this cluster. Additionally, cluster L2 has the highest average enrichment for CBX7 (supplemental Fig. S6). Note that Ring1b enrichment appears to spread more broadly across the genes in this category and that MED1 binding is greatly reduced at these genes, possibly due to the higher levels of PRC1 present (Fig. 7*A*, supplemental Fig. S6). The GO terms associated with this category are homeobox genes and developmental proteins, potentially implicating TBP in helping to poise critical developmental genes for expression upon differentiation.

The ability of PRC1 to block Mediator *in vitro* is reminiscent of our previous study with a different silencing protein HP1. In that study, HP1 also blocked most of Mediator and the TAF subunits of TBP, but unlike PRC1, some components of Mediator, namely MED6, MED23, and MED25, were unaffected (51). This suggests that the two complexes have similar yet distinct mechanisms of silencing. This study, along with previous studies from our laboratory, supports the idea that transcriptional silencers work by targeting critical steps in PIC assembly (51).

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Supplemental Figure 1.

| PRC1-High	PRC1-Low	
5229.26	21790.008	Ring1b
84.686	371.475	CBX3
1461.806	7997.03	BMI 1
131.088	1105.313	CBX2
23.857	1783.218	Ring1a
122.607	107.563	Ubc9
0	121.393	CBX4
0	95.077	PHC2
0	253.434	RYBP
0	96.116	CBX8
0	62.24	PHC3
0	19.423	SCML2

Supplemental Figure S1. Recombinant PRC1 associates with endogenous PRC1. Purified Flag-tagged PRC1 was bound to Flag resin and then incubated with HeLa nuclear extract. Bound complexes were washed with 0.1M Buffer F (PRC1-Low) or 0.3M Buffer F (PRC1-High). The proteins were eluted with 0.2mg/ml Flag peptide (Sigma) and subjected to MuDPIT. The MuDPIT analysis was performed as previously described (18). A chart of PRC1 proteins found associated with recombinant FLAG-tagged mouse PRC1 is shown. Normalized spectral abundance factor values (NSAF) e5 values for each protein detected are shown.
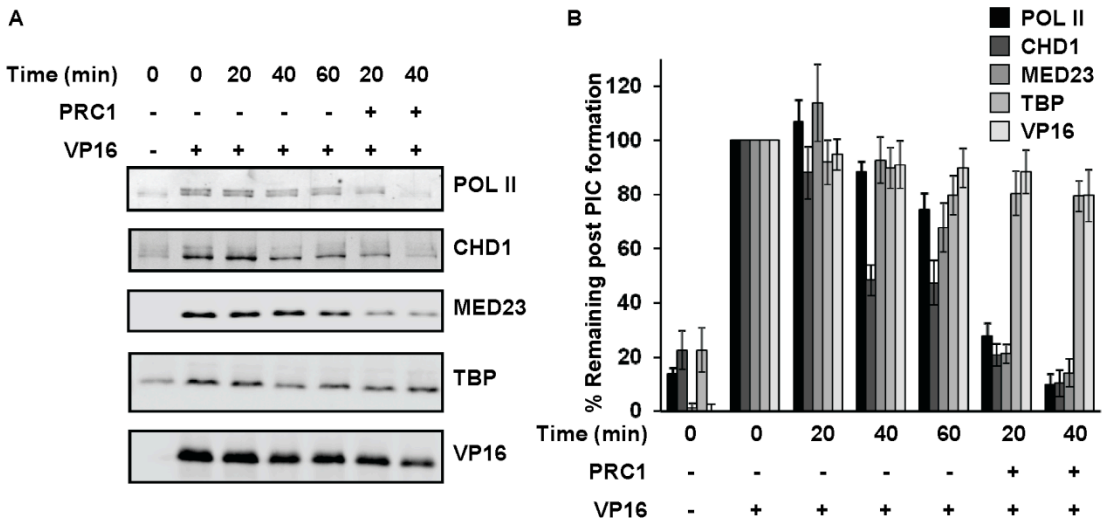
Supplemental Figure 2.
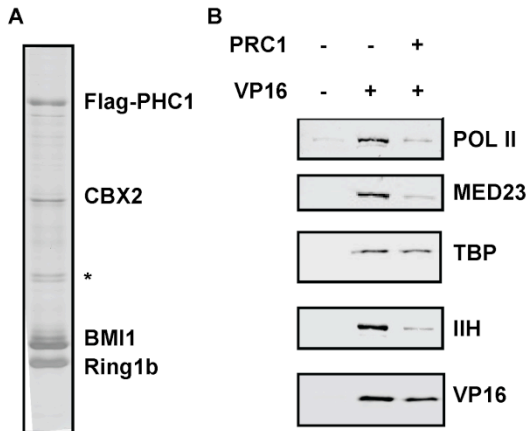
Supplemental Figure S2. The four member PRC1 complex has similar PIC blocking activity as the 3 member complex. A. The PRC1 complex containing Flag-PHC1, CBX2, BMI1 and Ring1B was overexpressed in insect cells and purified using Flag resin affinity. A silver stained gel of the eluted complex is shown. The asterisk indicates an unidentified contaminant.   B. PIC blocking immobilized template assay using the four member PRC1 complex. PRC1 was bound to templates after pre-binding of the activator, nuclear extract was then added, bound complexes were washed, and PIC formation was assayed by immunoblotting.
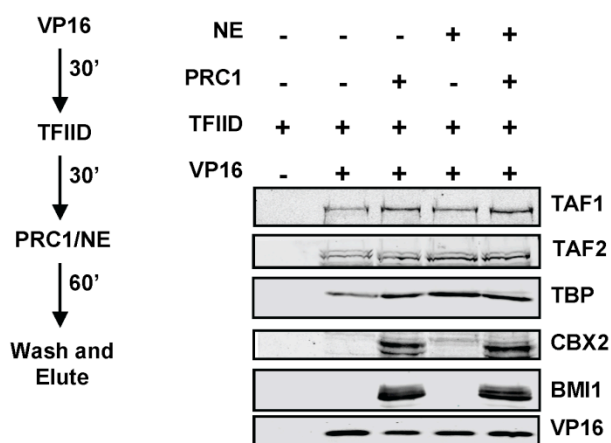
Supplemental Figure 3.

PRC1 - - +

VP16 - + +

TFIIE

TFIIB

TFIIH

BMI1
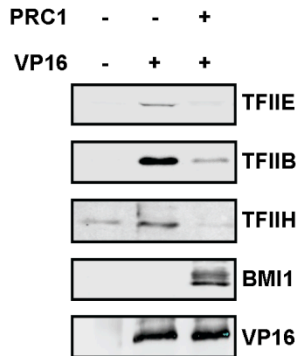
VP16

Supplemental Figure S3. PRC1 blocks the recruitment of the GTFs from the immobilized template. An immobilized template comparing PIC formation in the presence or absence of VP16 and PRC1 was performed. VP16 was pre-bound to the template, then PRC1 was added. After 30 minutes bound complexes were isolated, and HeLa nuclear extract was added for one hour. Bound complexes were again isolated, washed, and eluted using SDS. An immunoblot for GTFs, activator, and PRC1 proteins bound to the template is shown.

Supplemental Figure 4.

A

Time (min)	0	0	20	40	60	20	40
PRC1	-	-	-	-	-	+	+
VP16	-	+	+	+	+	+	+

POL II

CHD1

MED23

TBP

VP16

B

POL II
CHD1
MED23
TBP
VP16

Supplemental Figure S4. PIC dissociation timecourse of PRC1. A. An immobilized template assay was performed to test the effect of PRC1 on PIC stability over time. PICs were allowed to form on the template by the addition of activator for 30 min. Nuclear extract was then added to allow PIC formation for one hour. Bound complexes were isolated, then PRC1 (62.5nM) or buffer control was then added. PICs were incubated with PRC1 or immobilized template buffer alone for the times indicated. A western blot for PIC components bound to the template is shown. B. Quantification of three separate experiments as performed in (A) is shown. Western blot signals were quantified using LICOR sofware, and amounts of PIC components were graphed as compared to the initial formation of the PIC (lane 2), which was set at 100%. Error bars shown are standard deviation for the three replicate experiments.

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Supplemental Figure 5.

NE	-	-	-	+	+	
PRC1	-	-	+	-	+	
TFIID	+	+	+	+	+	
VP16	-	+	+	+	+	

TAF1
TAF2
TBP
CBX2
BMI1
VP16

VP16 → 30' → TFIID → 30' → PRC1/NE → 60' → Wash and Elute

Supplemental Figure S5. Nuclear extract has no effect on the stability of TFIID in the presence of PRC1. An immobilized template assay was performed using H3K27me3 chromatin. VP16 activator was pre-bound to the template, followed by TFIID binding for 30 minutes. PRC1 and nuclear extract were then incubated with the template either alone or in combination for 60 min.

Supplemental Figure 6.

CBX7 Average Profile Near TSS

Relative Distance (bp)
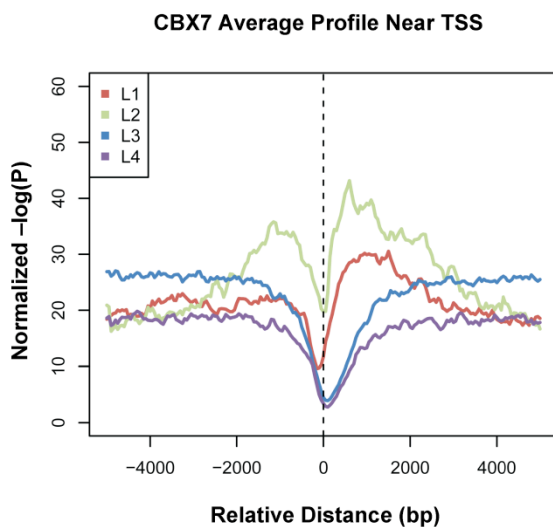
Supplemental Figure S6. Comparison of CBX7 enrichment for clusters L1-L4. Average normalized -log(P) of CBX7 binding across a 10 kb region spanning the TSS of genes within clusters of figure 7 was plotted. p-values levels were normalized to the size of the cluster.

Addendum to Chapter 4

My work investigating the mechanism of PRC1-mediated gene repression left open two major questions:

Does PRC1 silencing also affect the recruitment of SAGA and elongation factors that we found to be

components of PICs? Is the binding of PRC1 and TFIID to gene promoters a cooperative event?

I addressed these questions by performing immobilized template experiments using H3K27me3

chromatin templates and purified PRC1 in vitro. My published results showed that most PIC

components assayed were inhibited from binding to promoters in the presence of PRC1. The

experiment in Figure 4-1 is a dissociation experiment, in which purified PRC1 was incubated with the

immobilized template after PIC assembly. The assay demonstrated that the binding of the SAGA

complex (GCN5 and TAF5L) and elongation factors (CDK9, PAF1) to PICs is dissolved by PRC1 while TBP

remains refractory to this action. This result supports my hypothesis that PRC1 inhibits PIC formation by

blocking the binding of Mediator resulting in a lack of PIC assembly with the exception of TFIID.

While investigating the recruitment of purified TFIID and PRC1 to immobilized templates, I found that

the complexes could co-occupy promoters, suggesting that the two complexes may bind cooperatively

to promoters. To test this possibility, I performed an H3K27me3-based immobilized template

experiment, whereby increasing amounts of purified TFIID were pre-bound to the promoter, followed by

incubation with PRC1 (Figure 4-2). This experiment showed that the level of PRC1 recruitment to the

template does not change in the presence of TFIID. This result suggests that PRC1 and TFIID binding

events are independent, but not mutually exclusive. It is also possible that the order of addition plays a

role; perhaps PRC1 presence on a promoter allows for increased levels of TFIID binding.

The results of presented in this chapter and addendum of my thesis suggest that PRC1 acts to silence transcription by inhibiting the recruitment of the Mediator, GTFs, SAGA, transcription elongation factors, and RNA Pol II, but not activator or TFIID, the latter being an independent binding event.
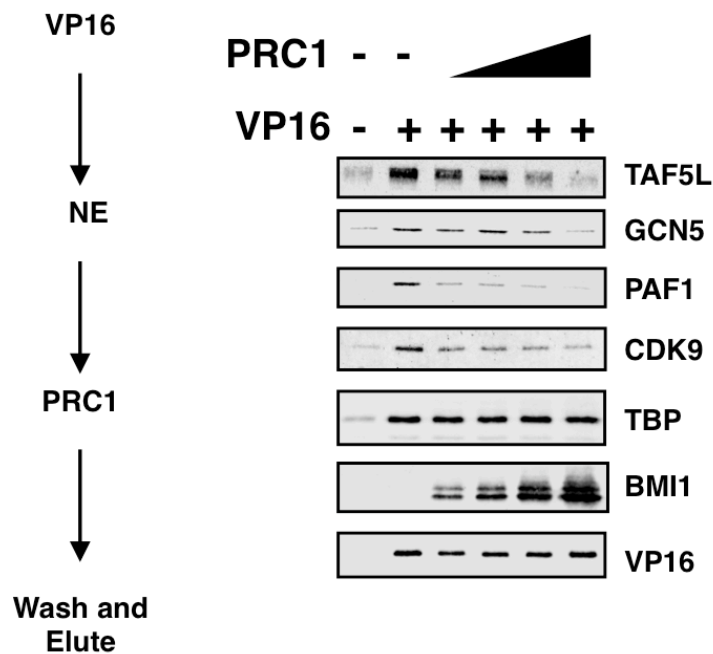

Figure 4-1. PRC1 causes the dissociation of SAGA and transcription elongation factors from the PIC. An immobilized template using HeLa nuclear extracts and purified PRC1 is shown. PICs were formed by incubating the template with GAL4-VP16, followed by the addition of nuclear extract. Assembled PICs were isolated using a magnetic particle concentrator, and incubated with increasing amounts of PRC1. Complexes associated with the template were again isolated, washed, and eluted. A western blot of the proteins bound to the template is shown, detecting subunits of the SAGA (TAF5L, GCN5), pTEFb (CDK9), PAF, TFIID, and PRC1 complexes.
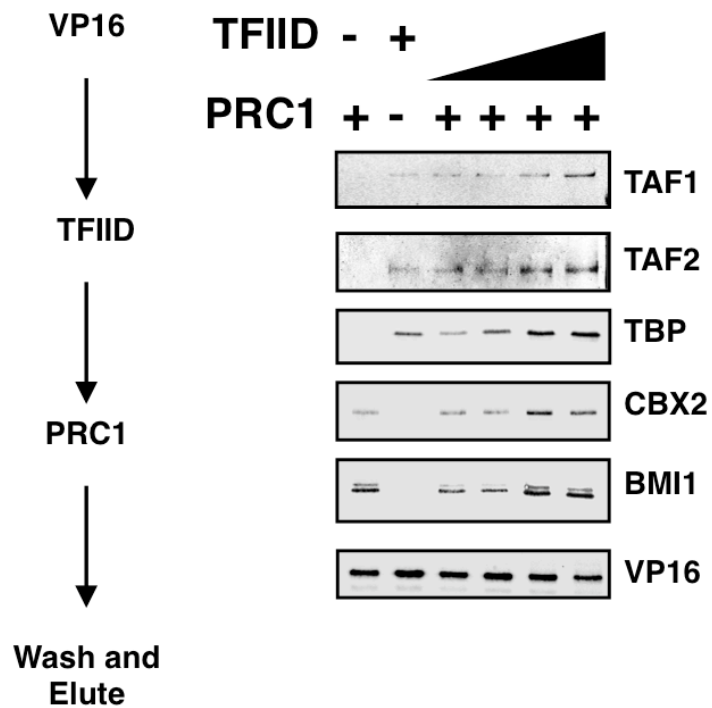
Figure 4-2. Pre-binding of TFIID effects on the recruitment of PRC1. An immobilized template using H3K27me3 chromatin templates and purified TFIID and PRC1 is shown. Activator bound templates were incubated with increasing amounts of TFIID. Complexes bound to the template were captured, and PRC1 was incubated with the template. Proteins interacting with the template were then isolated, washed, and eluted. A western blot detecting representative subunits of TFIID and PRC1 associated with the template is shown.

Conclusions

The conclusions of this dissertation are described below, with the major findings from each chapter highlighted with italic text. I present data supporting the hypothesis that the Mediator is the central scaffold protein that orchestrates PIC assembly. In addition, Mediator serves to recruit many more proteins to the PIC than originally postulated, including transcription elongation factors (SEC, pTEF-b), and chromatin remodelers (CHD1, Ino80). Finally, PRC1 functions to silence PIC formation by blocking or dissociating Mediator interactions with the activator protein. Surprisingly, TFIID recruitment is unaffected by PRC1, suggesting a role for the complex in defining a poised state capable of responding to differentiation cues.

Chapter two of this thesis is a reprint of work recently published in *Cell Reports*, "Mediator and SAGA Have Distinct Roles in Pol II Preinitiation Complex Assembly and Function." *MuDPIT of immobilized template experiments consistently identified Mediator and SAGA as the most activator-induced enriched complexes in PICs.* I optimized in vitro immobilized chromatin capture assays to accurately measure PIC formation. By immuno-depleting Mediator and SAGA, I was able to delineate the role each played in PIC assembly and function. *Nuclear extracts depleted of Mediator failed to support PIC formation on both naked and chromatin templates, supporting the hypothesis that Mediator is the central bridging factor critical for functional PIC formation.* In contrast, extracts depleted of SAGA supported PIC formation and transcription on naked DNA templates. *Moreover, extracts lacking SAGA failed to stimulate transcription on chromatin in response to activator, indicating that SAGA does not act as a traditional co-activator, but is essential for PIC function on chromatin.*

Chapter three is a reprint of a study published in *Genes and Development* describing how Mediator coordinates PIC assembly with the recruitment of CHD1. Importantly, the H3K4me3 modification stimulated transcription approximately three fold compared to unmodified chromatin templates in vitro. In this work, we performed MuDPIT of activator-bound chromatinized immobilized templates bearing unmodified H3K4 or H3K4me3. Our results revealed that H3K4me3-bearing templates enriched CHD1 binding three fold. I performed the cloning, expression in baculovirus, and purification of CHD1. I also performed the preparation of the MuDPIT samples used to determine the interaction partners by incubating the bead-bound purified CHD1 protein with HeLa nuclear extracts. Using the purified protein to study both the recruitment mechanisms to PICs and the effect of CHD1 on PIC function was a vital part of the study. *Immobilized template experiments showed that CHD1 recruitment and stimulation of transcription was enhanced by activator and further by Mediator. Functional studies using purified CHD1 and Mediator revealed that CHD1 stimulated transcription in the presence of ATP and Mediator.* Accordingly, genome-wide binding profiles of Mediator subunits and CHD1 in mouse ES cells show a significant overlap. *This chapter culminates with a model where CHD1 recruitment to promoters occurs through interactions with Mediator and H3K4me3.*

Chapter four is a reprint or a report published in the *Journal of Biological Chemistry*, which investigated the mechanism of PRC1 transcriptional silencing. In this study, I successfully recapitulated PRC1-based silencing of genes in vitro using the immobilized template approach. I purified the three-member "mini" PRC1 complex shown to have indistinguishable function from the four-member complex that includes the Ph subunit (See chapter for further elaboration). *Using this complex and crude HeLa nuclear extracts, I showed that PRC1 could both block and dissociate PIC formation functionally using in vitro*

transcription assays on immobilized chromatin templates bearing H3K27me3. Using immobilized template assays on HeK27me3 templates, I established that TFIID can be recruited to promoters and is resistant to the dissociation action of PRC1. Finally, I showed that the ability of TFIID to cooperate with Mediator is blocked by the binding of PRC1. We used previously published genome-wide datasets of Mediator, TBP, Cbx7, and Ring1b in mouse ES cells to show that the in vitro findings were further supported by in vivo studies. Overall, my thesis work defines the composition of the PIC, and the molecular basis of how H3K4me3 and H3K27me3 chromatin modifications recruit effector proteins to subsequently alter gene expression.

The ability of PRC1 to block Mediator *in vitro* is reminiscent of our previous study with a different silencing protein HP1. In that study, HP1 also blocked most of Mediator and the TAF subunits of TBP, but unlike PRC1, some components of Mediator, namely MED6, MED23, and MED25, were unaffected (51). This suggests that the two complexes have similar yet distinct mechanisms of silencing. This study, along with previous studies from our laboratory, supports the idea that transcriptional silencers work by targeting critical steps in PIC assembly (51).

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