Mediator and Cohesin Connect Gene Expression and Chromatin Architecture

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Summary

Transcription factors control cell specific gene expression programs through interactions with diverse coactivators and the transcription apparatus. Gene activation may involve DNA loop formation between enhancer-bound transcription factors and the transcription apparatus at the core promoter, but this process is not well understood. We report here that Mediator and Cohesin physically and functionally connect the enhancers and core promoters of active genes in embryonic stem cells. Mediator, a transcriptional coactivator, forms a complex with Cohesin, which can form rings that connect two DNA segments. The Cohesin loading factor Nipbl is associated with Mediator/Cohesin complexes, providing a means to load Cohesin at promoters. DNA looping is observed between the enhancers and promoters occupied by Mediator and Cohesin. Mediator and Cohesin occupy different promoters in different cells, thus generating cell-type specific DNA loops linked to the gene expression program of each cell.

Transcription factors control the gene expression programs that establish and maintain cell state 1,2. These factors bind to enhancer elements that can be located some distance from the core promoter elements where the transcription initiation apparatus is bound 3,4. The

ChIP-Seq and Microarray Data

ChIP-Seq and Microarray data have been deposited in Gene Expression Omnibus: GSE22557

Competing Financial Interests

The authors declare no competing financial interests.
enhancer-bound transcription factors bind coactivators such as Mediator and p300, which in turn bind the transcription initiation apparatus \(^5\)\(^-\)\(^9\). This set of interactions, well established in vitro, implies that activation of gene expression is accompanied by DNA loop formation. Indeed, Chromosome Conformation Capture (3C) experiments have confirmed that some enhancers are brought into proximity of the promoter during active transcription \(^10\)\(^-\)\(^12\). If DNA looping does occur between the enhancers and core promoters of active genes, it would be valuable to identify the proteins that play key roles in the formation and stability of such loops.

### Mediator and Cohesin contribute to ES cell state

We used a small hairpin RNA (shRNA) library to screen for regulators of transcription and chromatin necessary for the maintenance of ES cell state (Supplementary Fig. 1a, b). The screen was designed to detect changes in the level of the ES cell transcription factor Oct4, a master regulator of the pluripotent state, in cells that remain viable during the course of the experiment. Most known regulators of ES cell state were identified in this screen, including Oct4, Sox2, Nanog, Esrrb, Sal4, and Stat3 (Fig. 1a and Supplementary Tables 1, 2), suggesting that other components identified in this screen may also be important for maintenance of ES cell state. It was particularly striking that many of the subunits of the Mediator complex (Med6, Med7, Med10, Med12, Med14, Med15, Med17, Med21, Med24, Med27, Med28 and Med30), the Cohesin complex (Smc1a, Smc3 and Stag2) and the Cohesin loading factor Nipbl emerged from the screen. Mediator, Cohesin and Nipbl are thought to play essential roles in gene expression and chromosome segregation \(^5\)\(^-\)\(^9\), \(^13\)\(^-\)\(^15\), so their identification in this screen suggests that ES cell state may be highly sensitive to a reduction in the levels of these protein complexes.

The loss of ES cell state is characterized by reduced levels of Oct4 protein, a loss of ES cell colony morphology, reduced levels of mRNAs specifying transcription factors associated with ES cell pluripotency (e.g., Oct4, Sox2 and Nanog) and increased expression of mRNAs encoding developmentally important transcription factors \(^16\), \(^17\). We confirmed that shRNAs targeting Mediator, Cohesin and Nipbl produced all these effects (Fig. 1b, c, Supplementary Table 3 and Supplementary Figures 1c-f and 2). Thus, reduced levels of Mediator, Cohesin and Nipbl have the same effect on these key characteristics of ES cell state as loss of Oct4 itself.

### Mediator occupies enhancers and promoters

Transcription factors bound to enhancers bind coactivators such as the Mediator complex, which in turn can recruit RNA polymerase II to the core promoter \(^5\)\(^-\)\(^9\). It is not clear, however, how often Mediator is employed as a coactivator at active genes in vivo. We used chromatin immunoprecipitation coupled with massively parallel DNA sequencing (ChIP-Seq) to identify sites occupied by Mediator subunits Med1 and Med12 in the ES cell genome (Fig. 2., Supplementary Fig. 3 and Supplementary Tables 4–6). Med1 and Med12 were studied because they occupy different functional domains within the Mediator complex \(^18\). Analysis of the results revealed that Mediator occupied the promoter regions of at least 60% of actively transcribed genes (Supplementary Fig. 4).

More detailed examination of the ChIP-Seq data for Mediator with that of key transcription factors (Oct4, Nanog and Sox2) and components of the transcription initiation apparatus (Pol2 and TBP) revealed that Mediator is found at both the enhancers and core promoters of actively transcribed genes (Fig. 2a). For example, Mediator was detected at the well-characterized enhancers of the Oct4 (Pou5f1) and Nanog genes \(^19\)\(^-\)\(^21\), which are bound by the ES cell master transcription factors Oct4, Sox2 and Nanog \(^22\), \(^23\). Mediator was also detected at the Oct4 (Pou5f1) and Nanog core promoters together with RNA polymerase II.
and TATA-binding protein (TBP). These observations provide in vivo support for the model that Mediator bridges interactions between transcription factors at enhancers and the transcription initiation apparatus at core promoters.

**Mediator and Cohesin co-occupy active genes**

Cohesin has been shown to occupy sites bound by CTCF and to contribute to DNA loop formation associated with gene repression or activation. Cohesin has also been demonstrated to occupy sites independently of CTCF, but the role of Cohesin at these sites is not known. We used ChIP-Seq to determine the genome-wide occupancy of the two Cohesin core complex proteins, Smc1a and Smc3, whose knockdown resulted in a loss of Oct4 (Fig. 2, Supplementary Fig. 3 and Supplementary Tables 4–6). The results show that Cohesin occupies sites bound by CTCF, as expected, but also occupies the enhancer and core promoter sites bound by Mediator (Fig. 2a, b and Supplementary Fig. 5). The regions co-occupied by Cohesin and Mediator were associated with RNA polymerase II whereas those co-occupied by Cohesin and CTCF were not (Fig. 2c). These results demonstrate that there is a population of Cohesin that is associated with the enhancer and core promoter sites occupied by Mediator in many active promoters of ES cells.

The Cohesin loading factor Nipbl, which was also identified in the shRNA screen, has been implicated in transcriptional regulation and is mutated in the majority of individuals afflicted with Cornelia de Lange Syndrome (CdLS), a developmental disorder. To our surprise, ChIP-Seq data revealed that Nipbl generally occupies the enhancer and core promoter regions bound by Mediator and Cohesin, but is rarely found at CTCF and Cohesin co-occupied sites (Fig. 2a-c and Supplementary Fig. 5). The association between Nipbl and Mediator/Cohesin sites was highly significant (P-val <10^{-300}) whereas the association of Nipbl with CTCF/Cohesin sites was no greater than expected by chance (P-val =1). Thus, the Cohesin loading factor Nipbl is associated with Cohesin/Mediator sites but not with Cohesin/CTCF sites in ES cells. These results link Nipbl and CdLS to a form of Cohesin associated with Mediator at actively transcribed genes.

The co-occupancy of Mediator, Cohesin and Nipbl at the promoter regions of Oct4 (Pou5f1) and other active ES cell genes (Fig. 2a, c) suggests that these complexes may all contribute to control of transcription. If Mediator, Cohesin and Nipbl function together to regulate the genes they occupy, then we would expect that knockdown of Nipbl or key components of the Mediator or Cohesin complexes would have similar effects on expression of these genes. Analysis of changes in mRNA levels in knockdown cells revealed that this is the case (Fig. 2d). Of the approximately 2700 genes that are co-occupied by Mediator, Cohesin, Nipbl and Pol2 at high confidence, approximately 700 showed significant expression changes (P-val <0.01) in each of the Mediator, Cohesin and Nipbl knockdown datasets (Fig. 2d and Supplementary Table 3). The three knockdowns had strikingly similar effects at this set of genes, which may explain why Mediator, Cohesin and Nipbl knockdowns cause very similar ES cell phenotypes (Supplementary Fig. 6). These results indicate that actively transcribed genes occupied by Mediator, Cohesin and Nipbl typically depend on each of these factors for normal expression.

**Mediator and Cohesin interact**

The ChIP-Seq results show that Mediator, Cohesin and Nipbl co-occupy thousands of sites in the ES cell genome and thus suggest that these complexes may physically interact. To investigate this possibility, we crosslinked ES cells using the ChIP protocol, immunoprecipitated complexes using antibodies against Mediator (Med1 and Med12) and Cohesin (Smc1a, Smc3) and determined whether the Mediator subunit Med23 could be detected in the immunoprecipitate. (Fig. 3a). The results showed that Mediator and Cohesin
components can coprecipitate with one another. Furthermore, an antibody against Nipbl co-
precipitated both Cohesin and Mediator subunits (Fig. 3b). These results suggest that
Mediator, Cohesin and Nipbl interact.

If Mediator and Cohesin do indeed interact, then they should copurify. Mediator was affinity
purified from ES cell nuclei using a multi-step approach (Fig. 3c). First, the activation
domain of SREBP-1a, which is known to bind Mediator, was used for an initial affinity
purification step. Following a series of high-salt washes, bound proteins were eluted and
subjected to a second orthogonal immunoprecipitation step, with an anti-CDK8 antibody resin. CDK8 is a Mediator specific subunit, which ensured that Mediator and
Mediator-associated factors would be specifically retained on this antibody column. After
binding, the CDK8 antibody resin was subjected to a series of high-salt washes, and bound
proteins were then eluted and examined by silver stain and western blot. The results show
that Cohesin and Nipbl co-purified with Mediator throughout this protocol (Fig. 3c).

Additional evidence for a Mediator-Cohesin interaction came from an unbiased,
Multidimensional Protein Identification Technology (MudPIT) based screen for Mediator
associated factors in HeLa cells. Collectively, these results indicate that Mediator, Cohesin
and Nipbl physically interact and suggest that this interaction accounts for their co-
occupancy at active promoters in vivo.

Mediator and Cohesin predict DNA looping

Our evidence shows that Mediator, Cohesin and Nipbl interact and co-occupy the enhancer
and core promoter regions of a set of active genes in ES cells, suggesting that they
contribute to DNA looping between the enhancer and core promoter of these genes. We
selected four different loci, Nanog, Phc1, Oct4 (Pou5f1) and Lefty1, to test enhancer-
promoter interaction frequencies in ES cells and in Murine Embryonic Fibroblasts (MEFs).
These genes were selected because Mediator and Cohesin occupy their enhancer and core
promoter regions in ES cells, where they play a positive role in their transcription, whereas
Mediator and Cohesin are not present at these genes in MEFs, where these genes are
transcriptionally silent.

We utilized Chromosome Conformation Capture (3C) technology to determine whether a
looping event could be detected between the enhancer and promoter of Nanog, Phc1, Oct4
(Pou5f1) and Lefty1 loci in both ES cells and MEFs (Fig. 4 and Supplementary Fig. 7). For
all loci tested we observed an increased interaction frequency between the core promoter
and the enhancer in ES cells, indicating the presence of a DNA loop. Importantly, this
interaction was not observed in MEFs where Nanog, Phc1, Oct4 (Pou5f1) and Lefty1 are
silent and not occupied by Mediator and Cohesin. Furthermore, a reduction in SMC1a or
Med12 expression levels resulted in a decreased interaction frequency between the core
promoter and enhancer of Nanog (Supplementary Fig. 8). These 3C results are consistent
with a model where the Mediator/Cohesin/Nipbl complex promotes cell-type specific gene
activation through enhancer/promoter DNA looping.

Cell-type specificity

The observation that Mediator, Cohesin and Nipbl occupied the promoters of ES cell
specific genes such as those encoding the pluripotency regulators Oct4 and Nanog (Fig. 2a),
led us to ask whether Mediator and Cohesin tend to occupy cell-type specific genes. Indeed,
Mediator and Cohesin were found to co-occupy very different sets of promoters in ES cells
and MEFs (Fig. 5a and Supplementary Tables 4–6). In contrast, many of the sites occupied
by Cohesin and CTCF in ES cells were also co-occupied by these proteins in MEFs (Fig. 5b
and Supplementary Tables 4–6). The levels of Mediator were found to be considerably
higher in ES cells than in MEFs (Fig. 5c), accounting for the differences in the number of
sites co-occupied by Mediator and Cohesin in the two cell types. These observations suggest that Mediator and Cohesin play especially important roles in cell-type specific gene expression and thus, in cell-type specific chromosome structure.

Discussion

Evidence for specific DNA loop formation during transcription initiation was first described in bacteria and bacteriophage gene expression systems \(^34-39\). For example, bacterial DNA-binding factors can bind elements located upstream of sites occupied by sigma-54 RNA polymerases and cause looping of the intervening DNA when the transcription factors bind to polymerase. Proteins that act to stabilize these DNA loops and thus contribute to gene activity were also identified in these systems \(^40-42\). Our results suggest a similar model for the contributions of Mediator and Cohesin to gene regulation and DNA looping in vertebrate cells. In this model, DNA loop formation between enhancers and core promoters occurs as a consequence of the interaction between enhancer-bound transcription activators, Mediator and promoter-bound RNA polymerase II. When the transcription activators bind Mediator, the Mediator complex undergoes a conformational change \(^32,43\), and this activator-bound form of Mediator binds Cohesin and its loading factor Nipbl, which all contribute to gene activity.

Through their roles in DNA loop formation at a subset of active promoters, Mediator, Cohesin and Nipbl link gene expression with cell-type specific chromatin structure. In this context, it is interesting that mutations in the genes encoding Mediator and Cohesin components and Nipbl can cause an array of human developmental syndromes and diseases. Mediator mutations have been associated with Opitz-Kaveggia (FG) syndrome, Lujan syndrome and schizophrenia \(^44-47\). Mutations in Nipbl are responsible for most cases of Cornelia de Lange syndrome, which is characterized by developmental defects and mental retardation and appears to be the result of mis-regulation of gene expression rather than chromosome cohesion or mitotic abnormalities \(^28,29,48\). It is possible that these disorders and diseases are due to deficiencies in the chromatin structure generated by Mediator and Cohesin, which we have shown is essential for normal transcriptional programs in ES cells.

METHODS SUMMARY

A detailed description of all materials and methods can be found in Supplementary Information.

High Throughput shRNA Screening

High-throughput RNAi screening was performed at the Broad Institute RNAi Platform. Murine ES cells were seeded in 384-well plates, infected with an individual lentiviral shRNA construct, treated with puromycin, and crosslinked with 4% paraformaldehyde five days post infection. Cells were stained with Hoechst and for Oct4 and imaged with an ArrayScan HCS Reader (Cellomics). Cells were identified with Cellomics software, the average Oct4 pixel intensity was quantified and an average was calculated for all cells identified in the well.

ChIP-Seq

Chromatin immunoprecipitations (ChIPs) were performed and analyzed as previously described \(^49\).
**Microarray Analysis**

Expression analyses were carried out with Agilent DNA microarrays using labeled cDNA generated from shRNA GFP (control), Smc1a, Med12 and Nipbl infected mES cells.

**Mediator Complex Purification**

The Mediator complex was purified from mES cell nuclear extracts, essentially as described.

**Chromosome Conformation Capture (3C)**

Murine ES cells or MEFs were crosslinked, lysed and chromatin was digested with 1000 units HaeIII or 2000 units MspI. Crosslinked fragments were ligated with 50 units T4 DNA ligase for 4 hours at 16°C. 3C product detection was done in triplicate by qPCR and averaged for each primer pair. Each data point was first corrected for PCR bias by dividing the average of three PCR signals by the average signal in the BAC control template. Data from ES cells and MEFs were normalized to each other using the interaction frequencies between fragments in control regions.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

We thank Tony Lee for helpful discussions, David Root, Serena Silver, Thomas Neiland, and Hanh Le at the RNAi Screening Platform at the Broad Institute for screening advice and technical support, and Jeong-Ah Kwon, Jennifer Love, Sumeet Gupta and Thomas Volkert for assistance with ChIP-Seq. Immunofluorescence images were collected using the W.M. Keck Foundation Biological Imaging Facility at the Whitehead Institute and Whitehead-MIT Bioimaging Center. This work was supported by an NIH Fellowship (MK), a Canadian Institutes of Health Research Fellowship (DJT), a Keck Distinguished young scholar award (JD) and by NIH grants HG003143 (JD) and HG002668 (RY).

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Figure 1. Mediator and Cohesin Contribute to ES Cell State

**a,** Mediator and Cohesin components were highly represented in an shRNA screen for regulators of ES cell state. Complete results are listed in Supplementary Tables 1, 2. **b,** Knockdown of Mediator (Med12), Cohesin (Smc1a) or Nipbl caused reduced Oct4 protein levels and changes in ES cell colony morphology. Murine ES cells were infected with GFP control, Med12, Smc1a or Nipbl shRNAs, and stained for Oct4 and with Hoechst. Scale bar = 100 μM. **c,** Mediator, Cohesin and Nipbl knockdowns all cause reduced expression of ES cell regulators and increased expression of developmental regulators. ES cells were infected with the indicated shRNA and gene expression levels relative to a control GFP infection were determined with microarrays. Log2 fold expression changes were rank ordered from lowest to highest for all genes.
Figure 2. Genome-wide Occupancy of Mediator and Cohesin in ES cells

**a**, Binding profiles for ES cell transcription factors (Oct4, Nanog and Sox2), Mediator (Med1 and Med12), Cohesin (Smc1a, Smc3 and Nipbl), CTCF and components of the transcription apparatus (Pol2 and TBP) at the Oct4 (*Pou5f1*) and Nanog loci. ChIP-Seq data is shown in reads/million with the y-axis floor set to 0.5 reads/million. Oct4/Sox2, CTCF and TBP (TATA Box) sequence motifs are indicated.

**b**, Venn diagram showing the overlap of high confidence (P-val <10^{-9}) Cohesin (Smc1a) occupied sites with those bound by CTCF, Mediator (Med12) and Nipbl.

**c**, Region map showing that Smc1a, Nipbl and Med12 co-occupied sites generally occur in close proximity to Pol2 and in the absence of CTCF. For each Smc1a occupied region, the occupancy of Med12, Nipbl, Pol2 and CTCF is indicated within a 10kb window centered on the Smc1a region.

**d**, Heat map indicating that regions co-occupied by Smc1a, Med12 and Nipbl, which are associated with active genes, exhibit similar expression changes with knockdown of Smc1a, Med12 or Nipbl. Log_{2} expression data was ordered based on the Smc1a knockdown data and is shown for all Smc1a, Med12 and Nipbl co-occupied regions that could be mapped to a gene, as described in supplementary information.
**Figure 3. Mediator and Cohesin Interact**

**a**, Mediator (Med23) is detected by western blot when crosslinked, sheared chromatin is subjected to immunoprecipitation with antibodies against Mediator (Med1 or Med12) or Cohesin (Smc1a or Smc3). **b**, Cohesin (Smc1a, Smc3) and Mediator (Med23) are detected by western blot following immunoprecipitation of uncrosslinked ES cell nuclear extracts with a Nipbl antibody. **c**, Cohesin (Smc3) and Nipbl co-purify with Mediator. The Input fractions and IP Eluate were examined by western blot and silver staining.
Figure 4. Mediator and Cohesin Binding Profiles Predict Enhancer-Promoter Looping Events

a-d, A looping event was detected between the upstream enhancer and the core promoter of Nanog, Phc1, Oct4(Pou5f1) and Lefty1 by Chromosome Conformation Capture (3C) in ES cells, but not in MEFs. ES cell and MEF crosslinked chromatin was digested by MspI or HaeIII and religated under conditions that favor intramolecular ligation events. The interaction frequency between the anchoring point and distal fragments was determined by PCR and normalized to BAC templates and control regions. Error bars represent the standard error of the average of 3 independent PCR reactions. The ChIP-Seq binding profiles for Med12, Smc1a and Nipbl are shown as in Fig. 2a. Restriction enzyme sites are indicated above the 3C graph. Biological replicates of the 3C experiments and the full 3C profile are presented in Supplementary Fig. 7.
Figure 5. Cell Type Specific Occupancy of Mediator and Cohesin

a, Region map of a 10kb window around Mediator and Cohesin co-occupied sites for ES cells (Smc1a and Med12) and MEFs (Smc1a and Med1) indicates that co-occupied regions are different between the cell types. 
b, Region map of a 10kb window around Cohesin (Smc1a) and CTCF co-occupied sites indicates that many of these regions are co-occupied in ES cells and in MEFs. 
c, Western blot of ES and MEF cell extracts indicates that Cohesin protein levels are similar for both cell types, whereas Mediator protein levels are substantially lower in MEFs.