Dynamic PRC1-CBX8 stabilizes a porous structure of chromatin condensates

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Abstract

The compaction of chromatin is a prevalent paradigm in gene repression. Chromatin compaction is commonly thought to repress transcription by restricting chromatin accessibility. However, the spatial organisation and dynamics of chromatin compacted by gene-repressing factors are unknown. Using cryo-electron tomography, we solved the three-dimensional structure of chromatin condensed by the Polycomb Repressive Complex 1 (PRC1) in a complex with CBX8. PRC1-condensed chromatin is porous and stabilised through multivalent dynamic interactions of PRC1 with chromatin. Within condensates, PRC1 remains dynamic while maintaining a static chromatin structure. In differentiated mouse embryonic stem cells, CBX8-bound chromatin remains accessible. These findings challenge the idea of rigidly compacted polycomb domains and instead provides a mechanistic framework for dynamic and accessible PRC1-chromatin condensates.

Main

Chromatin structure is intricately linked to transcriptional activity. Compacted or “closed” chromatin is generally associated with inhibition of transcription while “open”, more accessible chromatin is more prone to being transcribed. Polycomb Repressive Complex 1 (PRC1) is a repressive chromatin modifier critical for organismal development. PRC1 has been proposed to inhibit gene expression by tightly compacting chromatin in a process that often is considered to restrict chromatin accessibility. However, direct evidence for...
PRC1-compacted chromatin being inaccessible is sparse and mechanistic explanations remain unsatisfactory (reviewed in11). Furthermore, recent studies show that changes in chromatin accessibility are more gradual than the simple binary classification into “open” and “closed” chromatin suggests12–15. A challenge in consolidating these seemingly contradictory findings is limited information into how PRC1 influences the three dimensional structure of chromatin.

PRC1 complexes can include one of five different chromobox proteins (CBX), all homologous to the fly Polycomb (Pc)16. The CBX protein CBX2 forms condensates through liquid-liquid phase separation, providing a potential mechanism for the compartmentalization of facultative heterochromatin9,10. Phase separation is emerging as a mechanism for chromatin organisation through the association of self-similar domains17. Chromatin can form condensates in the presence of divalent cations and histone tails17–20. Within these condensates, chromatin has variably been described as liquid-like, formed through liquid-liquid phase separation17,19, or as solid20. A recent structure of liquid-liquid phase-separated chromatin, condensed by magnesium cations without protein binding partners, revealed that nucleosomes organise into irregular assemblies19. The lack of apparent periodicity in chromatin geometry has also been noted in computational simulations and in first attempts to image chromatin in cells by cryo-electron tomography (cryo-ET)21,22. However, the structural arrangement of chromatin condensed by a repressive factor remained unknown.

Herein we describe the three dimensional cryo-ET structure of chromatin condensed by a polycomb-repressive complex. We focus on a PRC1 complex that includes CBX8 (PRC1C8), a chromobox protein that is upregulated during cell differentiation23 and has oncogenic potential24,25. We show that dynamic interactions between PRC1C8 and chromatin promote condensates through polymer-polymer phase separation. Contrary to expectations, PRC1-condensed chromatin is not tightly compacted but stabilises a porous chromatin structure that allows largely unhindered diffusion of PRC1C8.

**Results**

**PRC1-chromatin condensates are porous and accessible**

To determine the structure of polycomb-compacted chromatin and the mechanisms of polycomb-driven chromatin compaction, we reconstituted the system *in vitro*. The reconstitution included a chromatinized polycomb target gene (referred to as chromatin hereafter) and a purified recombinant PRC1 complex composed of RING1b, BMI1 and CBX8 (PRC1C8) (Fig. 1a). The PRC1C8 complex is pure (Fig. 1b), monodispersed (Fig. 1c) and retains H2A ubiquitylation activity comparable to the RING1b-BMI1 heterodimer (Fig. 1d).

When combined, chromatin and PRC1C8 were sufficient to form spherical phase-separated condensates, apparent in phase-contrast and fluorescence imaging (Fig. 1e). Using two different fluorescence labels, we confirmed the presence of both chromatin and PRC1C8
within the same condensates (Fig. 1e). Importantly, both PRC1C8 and chromatin are necessary for chromatin condensation, while the individual components do not phase-separate (Fig. 1e). PRC1C8-chromatin condensates are preserved on an EM grid after vitrification (Fig. 1f). This allowed us to study the internal molecular structure of PRC1C8-chromatin condensates by cryo-electron tomography (Fig. 1g). We collected tomograms near the condensate border to observe the boundary conditions. The reconstruction allows individual nucleosomes to be identified, revealing a dense network of hundreds of nucleosomes with a distinct condensate boundary (Fig. 1G and Movie S1). We could not unambiguously assign density to PRC1C8, possibly because it adapts various conformations while simultaneously using multiple surfaces to interact with chromatin (more below). The final structure reflects the arrangement of nucleosomes in PRC1C8-chromatin condensate (Fig. 1g, second panel). Unexpectedly, the structure shows that PRC1C8 does not compact nucleosomes into an impassable barrier. Instead, PRC1C8 rather stabilises chromatin in a porous mesh-like structure (Fig. 1g). Analysing the orientation of individual nucleosomes towards their neighbouring nucleosomes shows no obvious orientation bias (Extended Data Fig. 1a,b). We conclude that PRC1C8 does not induce a substantial inter-nucleosome orientation bias, but rather supports forming a porous chromatin structure.

We next wished to determine the size of macromolecules that could diffuse into PRC1-chromatin condensates. We used the condensate structure to calculate solvent-excluded volumes with variable probe radii ranging from 0.2 nm to 20 nm (Fig. 1h,i). Interestingly, the analysis shows that the condensate is accessible for macromolecules of a considerable size of up to 8 nm in radius (equivalent to approximately 600 kDa). Small macromolecules (<10 kDa), with radii below 2 nm, would have enough room to access every single nucleosome. Conversely, access is increasingly restricted for molecules with a radius above 8 nm (approximately 600 kDa). This suggests that PRC1-chromatin condensates are surprisingly accessible and that PRC1C8 itself would be able to move within these condensates largely unhindered. To test this we next ventured to analyse PRC1 dynamics within condensates experimentally.

**PRC1C8 is mobile and chromatin is static within PRC1-chromatin condensates**

Chromatin condensates form under close to physiological salt concentrations, at PRC1C8 concentrations as low as 500 nM and are dependent on PRC1C8 (Fig. 2a). We conclude that PRC1C8 is sufficient to drive the formation of the chromatin condensates under physiologically relevant conditions. We next asked if chromatin and PRC1 show different dynamics within the condensates. Fluorescence recovery after photobleaching (FRAP) of PRC1C8-chromatin condensates shows fast recovery kinetics for GFP-labelled PRC1 (Fig. 2b, in green). Conversely, we observed a very slow recovery for Cy5-labelled chromatin (Fig. 2b, in red). We conclude that, within condensates, PRC1 is mobile while chromatin itself is static (Fig. 2c). This confirms that PRC1C8 can diffuse largely unhindered within PRC1C8-chromatin condensates, in line with our structural analysis (Fig. 1h,i).
Multivalent interactions between PRC1C8 and chromatin induce phase separation

Previous studies have concluded that magnesium-driven chromatin condensates form in vitro via liquid-liquid phase separation\textsuperscript{17,19}. This view is challenged by the observation of solid-like chromatin condensates in living cells\textsuperscript{20}. Our observation that chromatin is static within PRC1-chromatin condensates does not agree with liquid-like behaviour, but rather suggests polymer-polymer phase separation (PPPS) as a mechanism for condensate formation\textsuperscript{27–29}. PPPS depends on one polymer acting as a scaffold that is condensed by the second molecule binding to it via multivalent interactions, effectively acting like a crosslinker\textsuperscript{27–29}.

To test for polymer-polymer phase separation of condensates, we next probed for multivalent interactions between PRC1 and chromatin. The whole PRC1\textsuperscript{C8} complex (RING1b, BM1 and CBX8) is necessary and sufficient to condense chromatin, while the PRC1 core or CBX8 alone do not condense chromatin (Fig. 3a). This suggests multivalent interactions between the PRC1\textsuperscript{C8} complex and chromatin, involving different chromatin interacting surfaces in both PRC1 and CBX8. To identify the different interaction sites, we first used crosslinking mass spectrometry (XL-MS) to probe for protein-protein interactions within PRC1\textsuperscript{C8}—chromatin condensates (Fig. 3b). The results show multiple crosslinks from the CBX8 chromodomain to the H3 histone tail (Fig. 3b), indicative of binding. Interactions between CBX-proteins and H3K27me3-modified H3-histone tails have been proposed to recruit PRC1 to chromatin modified by PRC2\textsuperscript{2,3}. However, CBX8 did crosslink to unmethylated H3 tails (Fig. 3b) and a trimethyl-lysine analogue (MLA) at H3K27 did not improve the chromatin-condensation activity of PRC1\textsuperscript{C8} (Fig. 3d). We conclude that H3K27me3 is not necessary for the chromatin condensation activity of PRC1\textsuperscript{C8} and that the H3 histone tail, even if unmodified, provides an interaction site for PRC1 on chromatin.

DNA has previously been shown to bind CBX8\textsuperscript{30} and could provide another interaction site for PRC1\textsuperscript{C8} on chromatin. We tested DNA binding in solution using a 24bp double stranded DNA probe and found that CBX8 is necessary for the DNA-binding activity of PRC1\textsuperscript{C8} (Fig. 3c). Hence, CBX8 binding to DNA provides a second interaction site of PRC1 with chromatin.

We conclude that PRC1 interacts with chromatin via at least three distinct sites: PRC1\textsuperscript{C8} binds to DNA and the H3 tail via CBX8, as shown herein (Fig. 3), and binds the nucleosome acidic patch via RING1b-BMI1 as shown elsewhere\textsuperscript{31}. These multivalent interactions would have to change dynamically while PRC1 maintains the condensed state of chromatin and diffuses through it at the same time (Fig. 2b). Collectively, we propose that PRC1 induces chromatin condensation via polymer-polymer phase separation, through dynamic multivalent interactions between PRC1 and chromatin.

CBX8 binding sites on chromatin in mouse embryonic stem cells are accessible
Given the porous structure of PRC1C8-condensed chromatin in vitro (Fig. 1) and the dynamic diffusion of PRC1C8 within condensates (Fig. 2b), we next wished to probe for the accessibility of PRC1C8-bound chromatin in cells. We carried out the Assay for Transposase Accessible Chromatin (ATAC-seq) in differentiated mESC, combined with ChIP-seq for CBX8 and H3K27me3. We used differentiated mESC, because CBX8 is expressed at very low levels in pluripotent mESC and is upregulated during retinoic acid-induced cell differentiation (23 and Fig. 4a). The DNA-loaded Tn5 used in ATAC-seq experiments forms a dimeric complex of approximately 130 kDa with an estimated hydrodynamic radius of 4.6 nm (based on PDB code 1MUH33). In agreement with the accessibility analysis in vitro (Fig. 1h), the majority of CBX8 ChIP-seq peaks in cells overlapped with ATAC-seq peaks (Figure 4B), indicating they are accessible to Tn5. This observation was persistent across the genome, where ATAC-seq peaks are co-localised with CBX8 and H3K27me3 peaks (Fig. 4c), indicating that CBX8-target genes are largely accessible. The insufficiency of CBX8 to restrict chromatin accessibility is further supported by the similar ATAC-seq profiles of wildtype and Cbx8 knockout mESCs (Fig. 4c,f, compare blue to orange). Hence, although the overall chromatin accessibility is reduced during mESC differentiation (Fig. 4d,e), in agreement with previous works, this process is not dependent on CBX8. Collectively, we show that CBX8-bound polycomb-repressed chromatin is largely accessible in differentiated mECSs (Fig. 4b-f).

**Discussion**

In conclusion, we have shown that PRC1-CBX8 binds chromatin via multivalent interactions and induces chromatin condensation through polymer-polymer phase separation. PRC1C8 is dynamic within condensates while keeping chromatin in a static, solid-like state. We established that PRC1C8 is sufficient to induce the solid condensed state of chromatin.

How can PRC1 condense chromatin but yet maintain a highly dynamic behaviour in the nucleus? PRC1 is characterised with a short residence time on chromatin. Solid-like chromatin that is condensed by a mobile chromatin binder has been shown in vitro for a truncation of the SAM-domain protein Polyhomeotic (Ph)36 and in cells for HP1a at chromocenters. Yet, the mechanism allowing PRC1 and other gene-repressing factors to condense chromatin while constantly diffusing in it remained largely unknown. Our cryo-EM structure of PRC1-condensed chromatin explains how PRC1 can move in condensed chromatin (Fig. 1), owing to the large pores that are formed between condensed nucleosomes. The multivalent interactions between PRC1-CBX8 to chromatin provide PRC1 with multiple docking sites on chromatin: unmodified and modified H3 tail (Fig 3b-d), DNA (Fig 3c) and the acidic patch of the nucleosome. Hence, it is possible that PRC1-CBX8 can constantly change its interactions with chromatin to maintain its condensed structure while utilising its different chromatin-interacting surfaces to dynamically move around. Our data indicate that chromatin condensation together with dynamic behaviour within chromatin condensates is an intrinsic biophysical property of PRC1-CBX8. It is plausible that the dynamic behaviour of PRC1 within chromatin condensates is required in order to allow PRC1
to modify nucleosomes by the H2AK119ub mark while holding them together. This phenomenon might represent a broad paradigm of repressive chromatin. The internal structure of PRC1-chromatin condensates is a porous network of nucleosomes (Fig. 1). Such a structure could present a size-selective diffusion barrier, in agreement with its permeability to PRC1 diffusion in vitro (Fig. 2) and Tn5 accessibility in cells (Fig. 4). Such a size-selective diffusion barrier may contribute to gene repression by selectively excluding transcriptional coactivators, which are commonly large protein complexes (>1MDa\textsuperscript{12,37–39}). This is in agreement with the inverse correlation between the density of chromatin domains and the molecular weight of the chromatin modifiers present in them\textsuperscript{12}. Transcription factor size has also been suggested to determine access to different chromatin domains based on simulations\textsuperscript{40}. Combining our results with earlier findings\textsuperscript{12,19,20,40}, we propose that size-selective exclusion may be part of a broader mechanism by which chromatin-interacting proteins regulate the accessibility of repressive chromatin.

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**Authors contributions:** C.D. and M.U. conceptualised the project and acquired funding, M.U., V.L., C.T., X.H.N and M.L. carried out experiments and investigated, P.P.D. and V.P. generated cell lines, S.F. and Q.Z. cloned and purified histone mutants, M.L. and M.U. developed software, C.D., A.d.M. and P.P.D. supervised, M.U. and C.D. wrote the original draft and all authors reviewed and edited the manuscript.

**Declaration of interests:** The authors declare no conflict of interest.

**Data and materials availability:** Next generation sequencing data (ATAC-seq and ChIP-seq) are available under GEO accession number GSE220140. The map for tomogram #1 has been deposited to the EMD (EMD-29022). All cryo-ET raw data has been deposited to EMPIAR under accession number EMPIAR-11344. XL-MS data has been deposited to Pride (PXD039589).
Fig. 1. The molecular structure of PRC1-chromatin condensates is porous and accessible to macromolecules. **a**, Schematics introducing the workflow. **b**, SDS-PAGE of purified PRC1<sup>C8</sup> complex that includes RING1b, BMI1 and MBP-tagged CBX8. **c**, Size exclusion chromatography of the purified PRC1<sup>C8</sup> using a HiLoad Sephacryl 300 16/60 column. **d**, In vitro ubiquitylation assay comparing PRC1<sup>C8</sup> to a RING1b-BMI1 heterodimer. Samples in lane 2 and 3 included E1, E2, ubiquitin and ATP. Ubiquitylation is detected by western blot using an anti-H2A antibody. **e**, Chromatin condensates induced by the PRC1<sup>C8</sup> complex and the individual proteins, visualised by confocal (left and centre) and phase contrast (right, from independent experiments) microscopy. CBX8 is GFP-labelled and chromatin is Cy5 labelled. Protein and chromatin concentrations are 1 μM and 20 nM, respectively. **f**, Cryo-confocal microscopy of vitrified PRC1<sup>C8</sup>-chromatin condensates. **g**, Cryo-electron tomography of a PRC1<sup>C8</sup>-chromatin condensate. Shown is a central slice through the
reconstruction (left image). Nucleosome subtomogram averages (centre, bottom) are then placed in a volume the size of the tomographic slice, at the position and orientation determined by template matching and subtomogram averaging (right image). h, Surface representation of the volume of a subset of the PRC1C8-chromatin condensate structure that is inaccessible to probes of given radii. i, Inaccessible volumes for a given probe radii are plotted, with exemplary molecules indicated (in grey) and selected probes coloured as in h. For the indicated complexes, the hydrodynamic radius was estimated using resolved domains from published structures (see Methods section for PDB accessions) as a minimum size estimate. See also Extended Data Figure 1.
Fig. 2. PRC1C8 is mobile while chromatin is static within PRC1-chromatin condensates.

a, Chromatin condensation in response to salt and PRC1 concentration measured by confocal microscopy using Cy5-labelled chromatin at a constant concentration of 50 ng/μl DNA. b, Representative micrographs of FRAP recorded in PRC1C8-chromatin condensates. CBX8 is GFP labelled and chromatin is Cy5 labelled. Mean fluorescence intensity of the bleached area, normalised to pre-bleach mean signal, is plotted for every time point. Error bars show standard deviation from n=7 (GFP) and n=8 (Cy5) measurements recorded from two independent experiments. c, Schematic representation of the FRAP experiment.
Fig. 3. Multivalent interactions between PRC1C8 and chromatin.  

a, Chromatin condensation in response to the whole PRC1C8 complex (RING1b, BMI1 and CBX8) or the individual components CBX8 and the RING1b–BMI1 heterodimer. Representative images from two replicates.  
b, Intermolecular (purple lines) and intramolecular (green lines) protein-protein interactions mapped within PRC1-chromatin condensates using crosslinking mass spectrometry (XL-MS). Data is from three independent replicates.  
c, PRC1C8 (blue) or PRC1 core (red) binding to a fluorescein labelled 24bp DNA probe measured by fluorescence anisotropy. Error bars show standard deviation based on two independent replicates.  
d, Titration of PRC1C8 to unmodified chromatin (top) and H3K27me3-MLA chromatin (bottom) at an identical chromatin concentration (50 ng/μl DNA) and 150 mM KCl. Micrographs are representative of two independent replicates.
Fig. 4. CBX8 binding sites on chromatin in mouse embryonic stem cells are accessible.

a, Schematics of the experimental setup (left) and anti-CBX8 western blot (right) of wildtype and Cbx8 knockout mESC after 48 hours of retinoic acid (RA) treatment. b, Overlap of ATAC-seq peaks and CBX8 and H3K27me3 ChIP-seq peaks. ATAC-seq peaks are defined from two biological replicates. c, ChIP-seq traces for H3K27me3 and CBX8 in wildtype mESC and representative ATAC-seq traces at four genes in wildtype and CBX8 knockout mESC after 48 hours of RA treatment. d, Accessibility changes at all ATAC-seq peaks in wildtype (WT) mESC, in response to retinoic acid (RA) treatment. e, Accessibility changes at all CBX8-target sites in wildtype mESC, in response to RA treatment. f, Comparison of accessibility at CBX8-target sites in wildtype mESC, in response to RA treatment.
target sites between wildtype and Cbx8 knockout cells after RA treatment. Model: PRC1 forms multivalent interactions with chromatin, thereby stabilizing chromatin condensates. These interactions dynamically change as PRC1 diffuses through condensates.
Extended Data

Extended Data Fig. 1. Related to Fig. 1; Nucleosomes in PRC1<sup>18</sup>-chromatin condensates show no orientation bias towards neighbouring nucleosomes. 

**a**, Orientations of nucleosomes towards neighbouring nucleosomes within a cut-off of 20 nm. Individual points represent nucleosomes and lines between points are coloured according to the relative orientation of neighbouring nucleosomes as indicated in the colour key (left). 

**b**, Distribution of nucleosome-nucleosome orientation for the three nearest neighbours and the 150th neighbour of each nucleosome in tomogram #1. Colours correspond to the same respective orientations as in **a**.
Extended Data Movie S1. Related to Fig.1; Cryo-tomogram of a PRC1\textsuperscript{C8}-chromatin condensate. The movie shows a scan through the z-axis of denoised\textsuperscript{51} tomogram #1.
Materials and Methods

Plasmids and cloning

Human RING1b (Uniprot ID Q99496) and human BMI1 (Uniprot ID P35226) were cloned into a pFBOH-mhl vector (Addgene plasmid # 62304) cleaved with BseRI using Gibson Assembly® Master Mix (NEB #E2611L) using the primers indicated in Table S1.

Human CBX8 open reading frame (Uniprot ID Q9HC52-1 and NCBI Reference Sequence was NM_020649.2) was obtained from gene synthesis (Gen9) cloned into a modified pFastBac1 pFB1.HMBP.A3.PrS.ybbR vector digested by XhoI and Xmal sites to include a N-terminal 6xHis-MBP tag. Cloning of the polycomb target gene ATOH1 into the pUC18 vector was described previously41. Plasmids for expression of human histones (H2A, H2B, H3.1 and H4) in E.coli were a kind gift from David Tremethick, Australian National University. UbcH5c WT pET28a was a gift from Rachel Klevit (Addgene plasmid # 12643; http://n2t.net/addgene:12643; RRID:Addgene_12643)42. pET3a-hUBA1 was a gift from Titia Sixma (Addgene plasmid # 63571; http://n2t.net/addgene:63571; RRID:Addgene_63571)43.

To generate a baculovirus expression vector of a monomeric EGFP-CBX8 (mEGFP-CBX8) construct, first GFP was amplified from a pSpCas9(BB)-2A-GFP vector and CBX8 was amplified from a pFB1.HMBP.A3.PrS.ybbR vector containing CBX8 as an insert. Subsequently, EGFP-CBX8 was cloned into the pFBOH-mhl vector cleaved with BseRI via Gibson assembly, with a Serine-Glycine-Serine linker between EGFP and CBX8. Finally, to generate monomeric mEGFP-CBX8, alanine residue 207 in EGFP was mutated to Lysine using a site directed mutagenesis kit (Takeda) and the mutagenesis primers listed in Table 1.

<table>
<thead>
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<th>Table 1: Cloning and mutagenesis primers (5’-3’)</th>
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<tr>
<td>mEGFP mutagenesis A207K fw</td>
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<tr>
<td>mEGFP mutagenesis A207K rv</td>
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<tr>
<td>EGFP-fw_pFBOH_MHL</td>
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<tr>
<td>EGFP rv with SGS linker</td>
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<tr>
<td>CBX8 5’ with SGS linker</td>
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<tr>
<td>CBX8 rv pFBOH_MHL</td>
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<td>Protein expression and purification</td>
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PRC1<sup>C</sup>, PRC1 core and CBX8 were co-expressed in *Trichoplusia ni* insect cells using the Baculovirus system. CBX8 variably carried an N-terminal 6xHis-mEGFP or a N-terminal 6xHis-MBP tag. The purification protocols were identical regardless of the tag. Baculoviruses were generated as per manufacturers instructions (Thermo Fisher). The viral titre was determined using the MTT assay (Promega #G3580). *Trichoplusia ni* insect cells were infected at a density of 1.5 - 2 x 10<sup>6</sup> cells and incubated for 60 hours at 27 °C in a shaker. The cells were spun down at 1500 relative centrifugal force (RCF). The pellet was resuspended in 100 ml of lysis buffer per litre of cell culture (20 mM HEPES-KOH pH 7.5, 400 mM NaCl, 25 mM Imidazol, 10 % Glycerol, 0.2 mM TCEP, 1 mM PMSF and EDTA-free Complete protease inhibitor (Thermo Fisher)). Lysis proceeded for 30-45 minutes at 4 °C while rotating. The lysate was then centrifuged at 29000 RCF for 20 minutes at 4 °C. The supernatant was transferred to a fresh tube and 5 ml of Ni-NTA resin (Qiagen) was added.

| 24bp DNA duplex Fluorescein labeled | GGGCCCTGCCCCCGCCTCGCTCTG | Fluorescein labeled DNA duplex used for binding assays. Only the top strand is shown. The dye was attached to the 3’ end of the top strand. |

| 20bp DNA duplex Fluorescein labeled | GAGAAGATCAAGTTGCTGATGa | Fluorescein labeled DNA duplex used for binding assays. Only the top strand is shown. The dye was attached to the 3’ end of the top strand. |

### Table

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### ATOH1 fw
- GCAGAGCCCAACATTACACA
  - Used to amplify ATOH1 from pUC18 plasmid

### ATOH1 rv
- GCCGAABTTTACTAAAGACGCC
  - Used to amplify ATOH1 from pUC18 plasmid

### H2BT120C fw
- GTGACCTGCTATACCAGCAGCAAATAT
  - Used for mutagenesis of H2B

### H2BT120C rv
- GGTATAGCAGGTACGGCTTGGTGCC
  - Used for mutagenesis of H2B

### RING1b pFBOH-mhl fw
- ttgtatttccagggcTCTCAGGCTGTGCAGACAAAC
  - Used for Gibson assembly into pFBOH-mhl vector

### RING1b pFBOH-mhl rv
- caagcttcgtcatcaTTTGTGCTCCTTGTAGGTGC
  - Used for Gibson assembly into pFBOH-mhl vector

### BMI1 pFBOH-mhl fw
- ttgtatttccagggcCATCGAACAACGAGAATCAAG
  - Used for Gibson assembly into pFBOH-mhl vector

### BMI1 pFBOH-mhl rv
- caagcttcgtcatcaACCAGAAGAAGTTGCTGATGa
  - Used for Gibson assembly into pFBOH-mhl vector

### Protein expression and purification

**PRC1**<sup>C</sup>, PRC1 core and CBX8 were co-expressed in *Trichoplusia ni* insect cells using the Baculovirus system. CBX8 variably carried an N-terminal 6xHis-mEGFP or a N-terminal 6xHis-MBP tag. The purification protocols were identical regardless of the tag. Baculoviruses were generated as per manufacturers instructions (Thermo Fisher). The viral titre was determined using the MTT assay (Promega #G3580). *Trichoplusia ni* insect cells were infected at a density of 1.5 - 2 x 10<sup>6</sup> cells and incubated for 60 hours at 27 °C in a shaker. The cells were spun down at 1500 relative centrifugal force (RCF). The pellet was resuspended in 100 ml of lysis buffer per litre of cell culture (20 mM HEPES-KOH pH 7.5, 400 mM NaCl, 25 mM Imidazol, 10 % Glycerol, 0.2 mM TCEP, 1 mM PMSF and EDTA-free Complete protease inhibitor (Thermo Fisher)). Lysis proceeded for 30-45 minutes at 4 °C while rotating. The lysate was then centrifuged at 29000 RCF for 20 minutes at 4 °C. The supernatant was transferred to a fresh tube and 5 ml of Ni-NTA resin (Qiagen) was added.

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| 24bp DNA duplex Fluorescein labeled | GGGCCCTGCCCCCGCCTCGCTCTG | Fluorescein labeled DNA duplex used for binding assays. Only the top strand is shown. The dye was attached to the 3’ end of the top strand. |
The samples were then incubated for 60 minutes at 4 °C while rotating and subsequently centrifuged at 500 RCF for 5 minutes at 4 °C to settle the beads. About 90 % of the supernatant was removed. The beads were resuspended in the remaining 10 % of supernatant and transferred to 25 mm diameter gravity flow columns (Biorad). The beads were allowed to settle before the remaining buffer was drained and the beads were then washed with 12 CV of Buffer B (20 mM HEPES-KOH pH 7.5 at 20 °C, 500 mM NaCl, 25 mM Imidazole, 10 % Glycerol, 0.2mM TCEP), followed by 30 CV of Buffer A (20 mM HEPES pH 7.5 at 20 °C, 100 mM NaCl, 25 mM Imidazole, 10 % Glycerol, 0.5 mM DTT or 0.2 mM TCEP). The protein was then eluted in 6 CV Elution Buffer (20 mM HEPES-KOH pH 7.5 at 20 °C, 100 mM NaCl, 400 mM Imidazole, 10 % Glycerol, 0.2 mM TCEP). The eluted protein was loaded onto a Hitrap 5 ml Heparin column (Cytvia) equilibrated in IX Buffer A (20 mM HEPES pH 7.5 at 20 °C, 100 mM NaCl, 0.5 mM DTT or 0.2 mM TCEP) and the column was washed with 5 CV of IX Buffer A. The proteins were resolved over a 20 CV gradient ranging from 0 % to 100 % IX Buffer B (20 mM HEPES pH7.5 at 20 °C, 1000 mM NaCl, 0.5 mM DTT or 0.2mM TCEP). The fractions were analysed on SDS-PAGE and fractions containing the protein complex of interest with the expected subunits stoichiometry were pooled. The pooled fractions were concentrated using a Amicon ultra 30K centrifugal filter (Merck, cat UFC903024) and purified by size exclusion chromatography using a HiLoad Sephacryl 300 16/60 column (Cytiva) equilibrated in GF Buffer (20 mM HEPES-KOH pH 7.5 at 20 °C, 150 mM NaCl, 0.5 mM DTT). The collected fractions were analysed on SDS-PAGE and fractions containing the protein complex of interest at the expected stoichiometry were pooled and concentrated to a concentration of 1-2 mg/ml using an Amicon ultra 30K centrifugal filter (Merck, cat UFC903024). The purified protein was then aliquoted and frozen in liquid nitrogen. The purified proteins were stored at -80 °C until use.

Human UBA1, UBCH5C and Ubiquitin were purified as described previously44. Histone proteins H2A, H2B, H2BT120C, H3 and H4 were purified as described previously45, except that the gel filtration step was omitted.

### Production and purification of DNA for chromatin reconstitution

The ATOH1 polycomb target gene was amplified in a large scale 10 ml PCR reaction including 500 nM ATOH1 fwd and reverse primers (see Table 1), 4 μg of ATOH1-pUC18 template41, 200 μM dNTP mix (Invitrogen), 3 % DMSO, 50 mM KCl, 10 mM Tris-HCl, pH 8.8, 1.5 mM MgCl2, 0.01% sterile gelatin. The reaction mixture was divided into 96-well plates to include 50 μl per well and the following PCR program was run:

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
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</tr>
<tr>
<td>72</td>
<td>3:00</td>
<td></td>
</tr>
</tbody>
</table>

The PCR product was purified via ion exchange chromatography using a 5 ml HiTrap Q column (GE Healthcare). The column was equilibrated in Buffer A (25 mM HEPES 7.5, 250 mM NaCl) and the sample was resolved using a linear gradient ranging from 0 % to 100 % Buffer B (25 mM HEPES pH 7.5, 2 M NaCl). The fractions containing pure DNA were pooled, subjected to ethanol-precipitation and resuspended in 10 mM Tris-HCl pH 7.5 at 20 °C, 0.1 mM EDTA.

Chromatin reconstitution

Histone Octamers were refolded as described previously. All steps were done at 4 °C or on ice. Chromatin was reconstituted following the salt-gradient dialysis protocol described previously. For large scale reconstitution, DNA and octamer were combined at an optimal ratio that was determined at trial experiments for each batch of octamers and DNA. To determine the optimal ratio of histone octamer to DNA, titration was carried out using increasing amounts of octamer to a constant amount of DNA (molar ratios of 16:1, 18:1, 20:1, 22:1 of Octamer:DNA) following by salt gradient dialysis. For the salt gradient dialysis, samples were initially dialysed in a buffer containing 10 mM Tris-HCl pH 7.5 at 20 °C, 2 M KCl, 1 mM EDTA, 1 mM DTT. The initial buffer was then gradually exchanged for a low salt buffer containing 10 mM Tris-HCl pH 7.5 at 20 °C, 250 mM KCl, 1 mM EDTA, 1 mM DTT over the course of 18 hours, after which the salt exchange was complete. Samples were then centrifuged at 21000 RCF for one minute, separated on a 1% agarose gel in TAE buffer and stained with SYBR Safe (Sigma). The highest Octamer:DNA ratio at which chromatin remained soluble after finishing salt dialysis was finally used for large scale reconstitution.

For large scale reconstitutions, the salt gradient dialysis was performed as above while scaling up the reaction accordingly. Additionally, after conclusion of the salt gradient, the samples were transferred to 400 ml of low salt buffer (10 mM TrisRIS-HCl pH 7.5 at 20 °C, 250 mM KCl, 1 mM EDTA, 1 mM DTT) and dialysed for another 2 hours. The samples were finally dialysed against 1 litre of Chromatin Storage Buffer (10 mM TRIS-HCl pH 7.5, 10 mM KCl) overnight.
Reconstitution of Fluorescently labelled chromatin

To allow site-specific labelling of histone H2B, a cysteine was introduced via site-directed mutagenesis (H2BK120C, as previously described\textsuperscript{46}) using mutagenesis primers indicated in Table S1. H2BK120C was labelled with Cyanine5-maleimide (Lumiprobe cat #13080) as described previously\textsuperscript{46}. Labelled octamers were refolded as described above. Before chromatin reconstitution, the unlabelled and labelled octamers were combined at a molar ratio of 7:1 (unlabelled:labelled). Chromatin was then reconstituted as described above.

Chromatin condensation assays

Fluorescently labelled proteins and chromatin were protected from light whenever possible. Chromatin condensation assays were done as described previously\textsuperscript{17} with some modifications. Assays were done in 384-well plates with #1.5 glass bottoms (MatTek PBK384G-1.5-C). The wells were treated with 1 M NaOH for 1 hour at room temperature, NaOH was removed and wells were washed with copious amounts of MilliQ water (MQ). MQ was removed and 70 ul of 5k mPEG-silane (Sigma #JKA3037-1G, dissolved in 95% EtOH to a final concentration of 25 mg/ml) was added to each well. The plates were sealed and incubated overnight at room temperature. The mPEG-silane was removed, the wells were washed once with 95% EtOH, then rinsed with copious amounts of MQ and dried completely in the fume hood. The wells were then passivated by adding 40 ul of 20 mg/ml BSA (NEB) and incubated for at least one hour at room temperature. The BSA was removed and the wells were washed three times with Condensation Buffer (20 mM HEPES pH 7.5 at 20 °C, 0.2 mg/ml BSA, 10% Glycerol, 5 mM DTT and 150 mM KCl unless stated otherwise). The chromatin stock was adjusted to a DNA concentration of 100 ng/ul and 150 mM KCl. The PRC1 complex was diluted in Condensation buffer to a protein concentration equal to twice the final PRC1 assay concentration as stated. To induce condensation, 16 ul of the diluted PRC1 were combined with 16 ul of the salt-adjusted chromatin dilution in PCR tubes. The samples were incubated for 30 minutes at room temperature before being transferred to the 384-well plate and incubated for a further 60 minutes at room temperature, so that the first images were recorded 90 minutes after induction of condensation. Images were recorded with a Nikon C1 scanning confocal microscope. GFP was excited with a 488 nm laser, Cy5 was excited with a 561 nm laser. Linear contrast adjustments were made with ImageJ. Where several micrographs are compared to each other, the same contrast settings were used for all micrographs. Phase contrast images were recorded with a Leica DMI8 imaging system.

Chromatin ubiquitylation assay
The salt concentration of the chromatin stock was adjusted to 100 mM KCl. The nucleosome equivalent concentration of chromatin arrays was calculated by measuring the molar DNA concentration and assuming that one DNA molecule is populated by 18 nucleosomes. The reaction mixture included 750 nM of chromatin (nucleosome equivalent concentration), 500 nM PRC1C8 or RING1b-BMI1 dimer, 100 nM hUBA1, 500 nM UBCH5C and 50 μM ubiquitin in Ub-Buffer (25 mM HEPES-KOH pH 7.5 at 20 °C, 100 mM KCl, 3 mM MgCl and 2 mM DTT) and started by adding ATP to a final concentration of 3 mM. 15 ul reactions were incubated at 30 °C for 45 minutes and the reaction was stopped by adding 5 ul of 4X NuPage LDS-loading dye (Thermo Scientific cat #NP0008) supplemented with 5 % 2-mercaptoethanol. The samples were separated on a 4-12% NuPage gel (Thermo Scientific cat #NP0321BOX) using MES buffer (Thermo Scientific cat #NP0002) in the tank. The gels were then blotted onto a nitrocellulose membrane (Amersham, cat #GE10600002) in Tris-Glycine transfer buffer + 20 % Ethanol (v/v) for 90 minutes in the cold room at 310 mAmp in a blotting tank (BioRad). H2A was detected using anti-H2A primary antibodies (Merck Millipore Cat. # 07-146, 1:1000 titer) and HRP-conjugated secondary antibodies (Santa Cruz, cat #sc-2357, titler 1:5000).

Sample preparation for cryo-electron tomography

The PRC1C8 complex was combined with chromatinized ATOH1 at a final concentration of 1.6 μM PRC1C8 and 500 ng/ul DNA at a final salt concentration of 25 mM NaCl and 8.3 mM KCl. Samples were incubated at room temperature for 30 minutes. Just before freezing, 5 nm gold nanoparticles were added at a 1:6 ratio (v/v). 3.5 ul of sample was applied to a Quantifoil grid (R1.2/1.3 on 200 copper mesh, Quantifoil cat #N1-C14nCu20-01) and vitrified in liquid ethane using the Vitrobot plunge freezer (Thermo Scientific) with the following settings: Temperature 4°C, blot force -3, blot time 4 seconds, humidity 100%.

Cryo-electron tomography data collection and processing

The data was collected with a Titan Krios electron microscope (Thermo Fisher) at 300 keV acceleration voltage using a Gatan K2 Summit camera (Gatan). A tilt series was acquired ranging from -60 to 60 degrees with 3 degree increments and a nominal defocus of -2.5 μm. A dose symmetric collection scheme was followed as described previously. The pixel size was 1.32 Angstrom, the total dose per tomogram was 144.32 e/A². Four frames were collected per tilt (dose per frame 0.88 e/A²). The movies were motion corrected using motioncor2. The motion corrected images were combined into stacks and further processed with Imod version 4.9.9. Imaging artefacts were identified and removed with Imod’s Ccderaser function. Tilts were aligned using the gold fiducial markers and the final aligned stack was binned by a factor of 4. The defocus was estimated using emClarity and the estimated defocus was used in Imod for CTF correction. The final tomogram was calculated using Imod’s implementation of weighted back projection. For visualisation, the tomogram was denoised using Topaz. The denoised
tomogram was only used for visualisation (Figure 1 and Movie S1). The original non-
denoised tomograms were used for all further processing, including template matching and
subtomogram averaging.

Template matching, subtomogram averaging and modelling the chromatin structure of
condensates from cryo-electron tomograms

Two tomograms were selected for further processing (Tomogram #1 and Tomogram #2 in
the following). To avoid user bias, we used a template matching algorithm\textsuperscript{52} with the
structure of a single nucleosome as template (EMD-8140\textsuperscript{53}) to identify initial positions and
orientations for each nucleosome. The pixel size of the template was adjusted using
emClarity to match the unbinned pixel size of the tomogram\textsuperscript{50}. The template was then
subjected to a low-pass filter of 30 Angstrom and binned by a factor of four to match the
binned tomogram. Template matching was done with the Matlab implementation of
Dynamo\textsuperscript{52} using the “dynamo_match” function. The template was masked with a tight-
fitting mask with smooth edges. The scanning range was set to 360 degrees with 10 degree
steps. In-plane rotation was also scanned over 360 degrees with 10 degrees steps. Particles
that passed a cross-correlation cut-off of 0.17 (Tomogram #1) or 0.19 (Tomogram #2) were
selected for further analysis. Obvious false positives were removed manually.

The particles were cropped from the tomogram with a box size of 36 pixels. Nucleosome
position and orientation was refined over three rounds of subtomogram averaging\textsuperscript{52}. As an
initial template for subtomogram averaging, we used an average from all cropped particles
after template matching, masked with a tight-fitting mask with smooth edges generated in
the Dynamo mask editor. Specific settings for the different rounds of subtomogram
averaging are detailed in Table 2. The resulting average structure of a nucleosome from the
subtomogram averaging was then used to populate a volume the size of the tomogram with
nucleosomes at the determined positions and orientations. The graphic depiction of the
final model (Figure 1G) was generated using the Dynamo Matlab implementation\textsuperscript{52}.

<p>| Table 2: Settings for subtomogram averaging in Dynamo |
|---------------------------------|--|--|
| Iterations | Round 1 | Round 2 | Round 3 |
| References | 1 | 1 | 1 |
| Cone Aperture | 100 | 16 | 8 |
| Cone Sampling | 5 | 2 | 1 |</p>
<table>
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<tr>
<td>Threshold Modus</td>
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</tbody>
</table>

**Analysis of exclusion volume**

Exclusion volumes for spherical molecules of various radii were calculated using $3V^{26}$ as follows. First, the positional and rotational coordinates of the nucleosomes within Tomogram #1 were determined by template matching followed by subtomogram averaging, as described above. Next, the positional and rotational coordinates of the nucleosomes were tabulated from within a section of 300 pixels x 300 pixels x 150 pixels (corresponding to 158.4 nm x 158.4 nm x 87.0 nm at the x, y and z axes, respectively) centred on the pixel at location (650,650,80), which was large enough to include hundreds of nucleosomes but yet
sufficiently small to carry out the subsequent computational analysis. The dimensions of the resulting table were converted to Angstrom by multiplying with a tomogram pixel size of 5.28 Angstrom/pixel. Next, the table including nucleosome positions was used by the Dynamo function ‘dtchimera’ to generate a chimera cmd script that placed a pdb structure of a single nucleosome (PDB 1KX4) at the position and orientation defined for each nucleosome in the table. At the end of this process, each of the nucleosomes within the tomogram section is represented by the pdb coordinates of the nucleosome template. The resulting model was saved as a pdb file and used as input for 3V. Varying probe radii ranging from 2 to 20 nm were used.

**Hydrodynamic radius calculation using protein structures**

The hydrodynamic radii of various proteins (Figure 1I) were calculated using the HullRad web server (http://52.14.70.9/). The PDB codes for the structures used are: 6LTJ (BAF37), 1MUH (Tn533), 7O4J (PolII-PIC), 6C24 (PRC2.255), 8GXS (PolII-Mediator). 6LTJ, 6C24, 8GXS and 7O4J are only partial models as not all residues were assigned, therefore the calculated hydrodynamic radius represents an estimate of the minimal complex size, while the actual size of these complexes could be larger.

**Analysis of nucleosome-nucleosome orientation**

Nucleosome-nucleosome orientation was classified into face-face, face-side and side-side, as described in. The orientations were calculated from the output table of the template matching and subtomogram averaging process in Dynamo using the Matlab script ‘calculate_orientation.m’. The nucleosome-nucleosome orientation plot (Fig. S1A) was generated using the ‘plot_edges’ function from the python notebook NCP_orientation_analysis.ipyn. Both scripts are available on Github (https://github.com/MichaUckelmann/Chromatin-Structure-Analysis).

**Cryo-light microscopy**

The samples were prepared as described for cryo-electron tomography, without gold nanoparticles and in a buffer containing 100 mM KCl. The vitrified grids were imaged using a ZEISS LSM900 Airyscan2 with a Linkam CMS196V Cryo stage.

**Fluorescence recovery after photobleaching (FRAP)**

Samples and plates were prepared and images were recorded as described above for chromatin condensation assays. 1 μM PRC1 with an N-terminal GFP tag was used. The FRAP experiments were set up with the NIS-Elements software (Nikon). Regions of interest (ROIs) were defined and a single image was recorded before bleaching. Then the ROI was...
bleached using 488 nm (GFP) and 561 (Cy5) lasers. The bleaching time and laser power was
set so that approximately 80% of fluorescence signal within the ROI was quenched.
Recovery was measured over 424 seconds, recording a total of 13 images.
The data was analysed with ImageJ, the mean pixel intensity of the bleached ROI was
quantified for each timepoint.

DNA binding assays
DNA binding was assayed using a 24 bp DNA (sequence see Table 1) with Fluorescein
attached to the top strand. The probe was protected from light wherever possible. The
probes were synthesised and delivered as duplexes (IDT). Probes were dissolved at a
concentration of 5 mM DNA in milliQ water. The probes were then diluted to 4 μM in
annealing buffer (20 mM HEPES-KOH, 150 mM NaCl) and heated to 95 °C for 5 minutes. The
probe was then left at room temperature for at least 1 hour to anneal before being diluted
to 20 nM in 20 mM HEPES-KOH pH 7.5 at 20 °C, 150 mM NaCl, 1 mg/ml BSA (NEB cat
#B9000S), 0.1% Tween20, 1 mM DTT. Serial protein dilutions of the PRC1C8 and RING1b-
BMI1 complexes were prepared in Protein Dilution Buffer (20 mM HEPES-KOH, 150 mM
NaCl, 1 mM DTT) ranging from 10000 nM to 2.44 nM. 20 ul of probe were mixed with 20 ul
of the respective protein dilution and transferred to a 384-well plate. The samples were
incubated for 30 minutes in the dark at room temperature and then read using a Pherastar
plate reader (BMG Labtech). The fluorescence anisotropy signal was normalised and the
curves were fitted with a specific binding model with Hill slope (GraphPad Prism).

Crosslinking mass spectrometry (XL-MS)
Reconstituted chromatin was dialysed overnight against 1 litre of XL buffer (25 mM HEPES-
KOH pH 7.5 at 20 °C, 150 mM NaCl, 1 mM DTT). The chromatin was combined with PRC1C8 in
a 1:1.5 molar ratio (Nucleosomes:PRC1C8). Specifically, the nucleosome equivalent
concentration of the chromatin array was calculated from the measured DNA concentration
assuming 18 nucleosomes per DNA molecule. 1.5 μM PRC1 and 1 μM nucleosome
equivalent concentration of chromatin arrays were then combined in XL buffer and
incubated for 30 minutes at room temperature. Crosslinking was done as described
before57. The bis(sulfosuccinimidyl)suberate (BS3) crosslinker was added to a final
concentration of 500 μM and crosslinking proceeded for 20 minutes at room temperature at
a reaction volume of 15 μl. The reaction was stopped by the addition of Tris-HCl pH 8 at
20 °C to a final concentration of 125 mM. The samples were then diluted to a volume of
100 μL using a buffer containing 50 mM Tris pH 8 at 20 °C and 150 mM NaCl. TCEP was
added to a final concentration of 10 mM and the samples were incubated at 60 °C for
30 min. Chloroacetamide was added to a final concentration of 40 mM and the samples
were incubated in the dark for 20 min. The samples were then digested using trypsin
(Promega cat #V5280) at 37 °C overnight. The digest was stopped by adding formic acid to a
The digested samples were purified using 100 µl ZipTip pipette tips (Merck cat #ZTC185960) according to the manufacturer’s instructions. Samples were then concentrated to ~5 µL using a SpeedVac vacuum centrifuge and diluted with 20 µL Buffer A (0.1% v/v formic acid).

The peptides were analyzed by online nano-high-pressure liquid chromatography (UHPLC) electrospray ionization-tandem mass spectrometry (MS/MS) on an Q Exactive Plus Instrument connected to an Ultimate 3000 UHPLC (Thermo-Fisher Scientific). Peptides reconstituted in 0.1% formic acid were loaded onto a trap column (Acclaim C18 PepMap 100 nano Trap, 2 cm × 100 µm I.D., 5-µm particle size and 300-Å pore size; Thermo-Fisher Scientific) at 15 µL/min for 3 min before switching the precolumn in line with the analytical column (Acclaim C18 PepMap RSLC nanocolumn, 75 µm ID × 50 cm, 3-µm particle size, 100-Å pore size; Thermo-Fisher Scientific). The separation of peptides was performed at 250 nL/min using a non-linear acetonitrile (ACN) gradient of buffer A (0.1% formic acid) and buffer B (0.1% formic acid, 80% ACN), starting at 2.5% buffer B to 42.5% over 95 min. Data were collected in positive mode using a Data Dependent Acquisition m/z of 375–2000 as the scan range, and higher-energy collisional dissociation (HCD) for MS/MS of the 12 most intense ions with z 2–5. Other instrument parameters were: MS1 scan at 70,000 resolution, MS maximum injection time 118 ms, AGC target 3E6, ion intensity threshold of 4.2e4 and dynamic exclusion set to 15 s. MS/MS resolution of 35000 at Orbitrap with the maximum injection time of 118 ms, AGC of 5e5 and HCD with collision energy = 27%.

For the data analysis, Thermo raw files were analysed using the pLink 2.3.4 search engine to identify crosslinked peptides, searching against the sequences of RING1b, BMI1, CBX8, H2A, H2B, H3 and H4. The default settings for searches were used. N-terminal acetylation and methionine oxidation were used as variable modifications and carbamidomethyl on cysteines as a fixed modification. False discovery rates of 1% for peptide spectrum match level were applied by searching a reverse database. Crosslinked peptides were further analysed using the crisscrosslinkeR package. Specifically, peptides were retained by crisscrosslinkeR only if they passed a p-value cutoff of 0.05 or were present in at least two of three replicates. Subsequent visualisation of retained peptide was carried out with xiNET.

### Generation of Cbx8 KO mESC lines using CRISPR/Cas9

Paired sgRNAs were designed to delete exons 1–4 of the murine Cbx8 gene. The Golden Gate Cloning method was used to clone the sgRNAs (sequence below) into the lentiguide-mCherry-Cas9 plasmid. 2 million mESCs were transfected with 1 µg of each plasmid carrying sgRNAs-mCherry-Cas9, using electroporation (Neon™ Transfection System MPK5000). The following day, mCherry-positive mESCs were sorted by FACS and plated on a 10 cm dish at a very low density for single-cell clone picking. After 5-6 days, individual colonies (derived from single cells) were picked, expanded, and genotyped using genomic PCR to identify homozygous/biallelic deletions of Cbx8 KO mESC colonies. Selected Cbx8 KO
mESC lines were further confirmed by western blot for CBX8 (Cell Signalling, CBX8 (D2O8C), cat # 14696S, titer 1:50 (Fig. 4a)) and HRP-conjugated secondary antibodies (Santa Cruz, cat #sc-2357, titer 1:10000).

Cbx8 sgRNA sequences (5’ and 3’ sgRNAs)

CBX8-5’Fw: CACCTGCGAATGCGCCGCTTCAGG
CBX8-5’Rv: AAACCCTGAAGCGGCGCATTCGCA
CBX8-3’Fw: CACCCTCTATGGCCCCAAAAAGCG
CBX8-3’Rv: AAAACGCTTTTTTGGGGCCATAGAG

Cbx8 genotyping primers:

Deletion_Fw: GCCTTCTGGTGCAGCTAAGT
Deletion_Rv: GACGTCAGCGGGAGAGTATT
Internal_Fw: CACCAAATGAATGCTCCAAA
Internal_Rv (same as the Deletion_Rv): GACGTCAGCGGGAGAGTATT

Mouse embryonic stem cell culture

Wildtype and Cbx8 knockout mES cells were grown on gelatinized culture dishes in DMEM, 20 % FBS, 1x non-essential amino acids (Gibco #11140-050), 1x Glutamax (Gibco #35050-061), PenStrep 100 u/ml (Thermo Fischer), 0.5 x EmbryoMax 2-Mercaptoethanol (Merck Millipore #ES-007-E), 2.5 μg/ml Plasmocin (Invivogen), 1000 U/ml ESGRO Leukemia Inhibitory Factor (LIF) (Merck Millipore cat #ESG1107).

Mouse embryonic stem cell differentiation ahead of ChIP-seq and ATAC-seq

Differentiation was induced by seeding cells at a density of 0.3x10^6 cells per well in 6-well plates (for ATAC-seq) or at 1.5x10^6 cells per 10-cm culture dish (for ChIP-seq) in media containing 1 μM all-trans retinoic acid (RA, Sigma-Aldrich R2625-50MG) and no LIF. Cells were differentiated for 48 hours and the media was changed after 24 hours. Control cells were treated with a DMSO volume equivalent to the RA volume in the differentiating cells. After 48 hours, the cells were harvested, washed once with PBS, counted and used immediately in either ChIP-seq or ATAC-seq experiments.

ChIP-seq

ChIP-seq was done as described previously. 1.5 μg of CBX8 antibody (Cell Signalling, CBX8 (D2O8C), cat # 14696S) and 3 μg of H3K27me3 antibody (Tri-Methyl-Histone H3 (Lys27) (C36B11), cat #35861SF) were used. Libraries were prepared using NEBNext ultra II DNA library kit for Illumina (NEB Biolabs) according to the manufacturer instructions. The
resulting libraries were assessed for quality on a High Sensitivity D1000 Screen Tape (Agilent) and were sequenced using an Illumina Novaseq 6000 sequencer (Genewiz/Azenta).

**Data processing for Chip-seq**

The reads were quality-trimmed and adapters were removed using Trim Galore!, a wrapper script to cutadapt, in paired end mode and using default settings. The reads were then aligned to the mouse mm10 genome build using bowtie2 with the option “very-sensitive”. The data was reduced to only properly paired reads using “samtools view” with the flag “-f 3”. PCR duplicates were removed using the RemoveDuplicates function from Picard Tools. Read mates were fixed using samtools fixmate. BigWig files were calculated using BamCoverage with CPM normalisation.

Peaks were called with Macs2 callpeak function, using the input sample as control. For the CBX8 ChIP-seq data set, default settings with a q-value cut-off of 0.05 were used. For H3K27me3 ChIP-seq data set, broad mode was used with a q-value and broad cutoff of 0.001. Peaks overlapping with ENCODE blacklisted regions were removed.

**ATAC-seq**

ATAC-seq was done using a commercial kit (Diagenode Cat.# C01080002) according to the instructions of the manufacturer. The resulting libraries were assessed for quality control on a High Sensitivity D1000 Screen Tape (Agilent) and libraries were sequenced using an Illumina Novaseq 6000 sequencer (Genewiz/Azenta).

**Data processing ATAC-seq**

ATAC-seq data was processed as described previously. Specifically, reads were quality-trimmed and adapters were removed using Trim Galore!, a wrapper script to cutadapt, in paired end mode using default settings. The reads were then aligned to the mouse mm10 genome build using bowtie2 with the option “very-sensitive”. Reads were sorted and indexed using Samtools. Mitochondrial reads were removed using a python script from Harvard Bioinformatics (available at [https://github.com/harvardinformatics/ATAC-seq](https://github.com/harvardinformatics/ATAC-seq)). The data was reduced to only properly paired reads using “samtools view” with the flag “-f 3”. The library complexity was estimated and the data sets were subsampled to reach a similar complexity as described previously. PCR duplicates were removed using the RemoveDuplicates function from Picard Tools. Read mates were fixed using samtools fixmate. BigWig files were calculated using BamCoverage with CPM normalisation.

For peak calling the bam files were converted to BEDPE files and the Tn5 shift was corrected by running a bash script provided at [https://github.com/reskejak/ATAC-seq](https://github.com/reskejak/ATAC-seq) (bedpeTn5Shift.sh). Files were then converted to minimal bed format and peaks were called using Macs2 callpeak function in broad mode with broad-cutoff set to 0.05. Peaks...
overlapping with ENCODE blacklisted regions\textsuperscript{67} were removed. Consensus peaks for each condition were defined as the intersect of peaks from both biological replicates. Venn diagrams were generated using the ChIPPeakAnno package\textsuperscript{69}. 

\textsuperscript{67}See \textsuperscript{67}

\textsuperscript{69}See \textsuperscript{69}
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