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. Mechanistically, positively charged residues on the internally disordered regions (IDRs) of CBX8 mask negative charges

on the DNA to stabilize the condensed state of 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[1]. Compacted or "closed" chromatin is generally associated with inhibition of transcription while "open", more accessible chromatin is more prone to being transcribed[1]. Polycomb Repressive Complex 1 (PRC1) is a repressive chromatin modifier critical for organismal development[2,3]. PRC1 has been proposed to inhibit gene expression by tightly compacting chromatin in a process that often is considered to restrict chromatin accessibility[2–11]. However, direct evidence for PRC1-compacted chromatin being inaccessible is sparse and mechanistic explanations remain unsatisfactory (reviewed in[11]). Furthermore, recent studies show that changes in chromatin accessibility are more gradual than the simple binary classification into "open" and "closed" chromatin suggests[12–15]. 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 heterochromatin[9,10]. Phase separation is emerging as a mechanism for chromatin organisation through the association of self-similar domains[17]. Chromatin can form condensates in the presence of divalent cations and histone tails[17–20]. Within these condensates, chromatin has variably been described as liquid-like, formed through liquid-liquid phase separation[17,19], or as solid[20]. A recent structure of liquid-liquid phase-separated chromatin, condensed by magnesium cations without protein binding partners, revealed that nucleosomes organise into irregular assemblies[19]. 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 ($PRC1^{C8}$), a chromobox protein that is upregulated during cell differentiation[23] and has oncogenic potential[24,25]. We show that dynamic interactions between $PRC1^{C8}$ and chromatin promote condensates through phase separation. Mechanistically, positive charges on the internally disordered regions (IDRs) of CBX8 are required for DNA binding and chromatin condensation. Contrary to expectations, PRC1-condensed chromatin is not tightly

compacted but stabilises a porous chromatin structure that allows largely unhindered diffusion of PRC1^{C8}.

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 (3,631 bp DNA) with a sequence from the human ATOH1 locus, which can harbour roughly up to 20 nucleosomes, assuming 150-200 bp per nucleosome. This construct is referred to as chromatin hereafter. We used a native DNA sequence for chromatin reconstitution, as regular spacing using artificial nucleosome stabilising sequences were previously reported to drive the liquid-liquid phase separation of chromatin[17]. The nucleosomes on the chromatin that we reconstituted are not evenly phased (Extended Data Fig. 1a). The purified recombinant PRC1 complex is composed of RING1B, BMI1 and CBX8 (PRC1^{C8}) (Fig. 1a). The PRC1^{C8} complex is pure (Fig. 1b), monodispersed (Fig. 1c) and retains H2A ubiquitylation activity comparable to the RING1b-BMI1 heterodimer (Fig. 1d). This also applies to all other protein complexes used in this study (Extended Data Fig. 2 and 1b,c).

When combined, chromatin and PRC1^{C8} were sufficient to form spherical phase-separated condensates, apparent in differential interference contrast (DIC) and fluorescence imaging (Fig. 1e). Using two different fluorescence labels, we confirmed the presence of both chromatin and PRC1^{C8} within the same condensates (Fig. 1e). Importantly, both PRC1^{C8} and chromatin are necessary for chromatin condensation, while the individual components do not phase-separate (Fig. 1e). PRC1^{C8}-chromatin condensates are preserved on an EM grid after vitrification (Fig. 1f). To study these structures by cryo-electron tomography, we reduced the salt concentration and increased chromatin concentration (see Methods section). This was done to decrease condensate size and abundance, which was necessary for high-quality data collection (Fig. 1g). We used a PRC1 complex with MBP-tagged CBX8 for cryo-tomography. The MBP tag does not affect condensation, as condensates still form after tag cleavage (Extended Data Fig. 1d). We collected tomograms near the borders of condensates, to observe the boundary conditions (Extended Data Fig. 3a for an example of a condensate on the grid). Subtomogram averaging allowed us to identify the orientation and position of individual nucleosomes in the tomographic volume (Extended Data Fig. 3b-d). The reconstruction reveals a dense network of hundreds of nucleosomes with a distinct condensate boundary (Fig. 1g and Movie S1). We could not unambiguously assign density to PRC1^{C8}, 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 PRC1^{C8}-chromatin condensate (Fig. 1g, second panel). Unexpectedly, the structure shows that PRC1^{C8} does not compact nucleosomes into an

111 impassable barrier. Instead, PRC1^{C8} rather stabilises chromatin in a porous mesh-like
112 structure (Fig. 1g). Analysing the orientation of individual nucleosomes towards their
113 neighbouring nucleosomes shows no obvious orientation bias (Extended Data Fig. 3c,d). We
114 conclude that PRC1^{C8} does not induce a substantial inter-nucleosome orientation bias, but
115 rather supports forming a porous chromatin structure.

116 We next wished to determine the size of macromolecules that could diffuse into PRC1-
117 chromatin condensates. We used the condensate structure to calculate solvent-excluded
118 volumes[26] with variable probe radii ranging from 0.2 nm to 20 nm (Fig. 1h,i). Interestingly,
119 the analysis shows that the condensate is accessible for macromolecules of a considerable
120 size of up to 8 nm in radius (equivalent to approximately 600 kDa). Small macromolecules
121 (<10 kDa), with radii below 2 nm, would have enough room to access every single
122 nucleosome. Conversely, access is increasingly restricted for molecules with a radius above
123 8 nm (approximately 600 kDa). This suggests that PRC1-chromatin condensates are
124 surprisingly accessible and that PRC1^{C8} itself would be able to move within these
125 condensates largely unhindered.

126 To compare the structure of PRC1^{C8}-chromatin condensates to PRC1^{C8}-free chromatin, we
127 generated cryo-tomograms of chromatin without PRC1^{C8} (Fig. 1j, movie S2). Low
128 magnification cryo-EM images show that condensation happens only in the presence of
129 PRC1^{C8} (Extended Data Fig. 4a,b) and condensates are preserved on the EM grid. The
130 structure of chromatin in the absence of PRC1^{C8} is less dense than in the presence of PRC1^{C8},
131 with distances between neighbouring nucleosomes that are on average significantly longer
132 (Fig. 1j,k). These results confirm that PRC1^{C8} facilitates large-scale chromatin restructuring.

133 We observe some sporadic areas of high nucleosome density, even without PRC1 (Extended
134 Data Fig. 4c,d). The median distance to the next neighbouring nucleosomes in these
135 sporadic dense PRC1^{C8}-free condensates are very similar to distances measured in the
136 PRC1^{C8}-chromatin condensates (9.8 nm and 10.9 nm, respectively, Extended Data Fig. 4d).
137 This matches closely to distances reported for chromatin condensed by MgCl2, where the
138 radial distribution function of nucleosomes peaked at 10.6 nm[19]. At the nuclear periphery in
139 cells, the median distance between neighbouring nucleosomes is about 12 nm, which is
140 again remarkably similar[27]. Overall, this raises the possibility that PRC1 thermodynamically
141 stabilises a naturally-occurring condensed chromatin state, rather than actively compacts
142 chromatin. By doing so, PRC1 may cause multiple compacted arrays to cluster together
143 more often.

144 **PRC1^{C8} is mobile and chromatin is static within PRC1-chromatin condensates**

145 To gain further insights into PRC1^{C8}-chromatin condensation, we tested the dependency of
146 condensation on the concentration of PRC1 and chromatin. Condensates form under close
147 to physiological monovalent salt concentrations of 122.5 mM (90 mM KCl and 32.5 mM

148 NaCl), at PRC1^{C8} concentrations as low as 250 nM and are dependent on PRC1^{C8} (Fig. 2a). At
149 a high concentration of PRC1^{C8} (2,000 nM), most efficient condensation occurs at high
150 chromatin concentration (850 nM nucleosome concentration). Yet, at lower concentration
151 of PRC1^{C8} (250 nM), ideal condensation appears at lower chromatin concentration and the
152 condensation efficiency is then reduces when the chromatin concentration increases (Fig
153 2a). This is possibly because at high chromatin concentration the large amount of potential
154 binding sites for PRC1^{C8} reduces the average per-site-occupancy of PRC1^{C8}. This may lead to
155 less efficient phase separation. A similar observation was recently made for a PRC1 complex
156 with CBX2 and PHC2[28]. Altering the salt concentrations confirms that PRC1^{C8}-chromatin
157 condensates most readily form close to physiological salt concentration (Extended Data Fig.
158 5). We conclude that PRC1^{C8} is sufficient to drive the formation of the chromatin
159 condensates under physiologically relevant conditions. Furthermore, the efficiency of phase
160 separation depends on the ratio of PRC1 to chromatin. We next asked if chromatin and
161 PRC1 show different dynamics within the condensates.

162 Fluorescence recovery after photobleaching (FRAP) of PRC1^{C8}-chromatin condensates shows
163 fast recovery kinetics for GFP-labelled PRC1 (Fig. 2b, in green; $T_{1/2}$ = 71 ± 8 s). Conversely, we
164 observed a very slow recovery for Cy5-labelled chromatin (Fig. 2b, in red). The GFP signal
165 does not recover up to 100 % but rather plateaus at 72 ± 2%. This is possibly because a
166 substantial percentage of the condensate has been bleached, while redistribution of GFP-
167 labelled PRC1^{C8} within the same condensate likely drives fluorescence recovery during the
168 monitored timescale. However, we cannot exclude the possibility that incomplete recovery
169 is due to an immobile fraction of CBX8. We conclude that, within condensates, PRC1 is
170 mobile while chromatin itself is static (Fig. 2c). This confirms that PRC1^{C8} can diffuse largely
171 unhindered within PRC1^{C8}-chromatin condensates, in line with our structural analysis (Fig.
172 1h,i). Recent publications have been at odds as to the solid or liquid state of chromatin[17,20].
173 Our data suggests that chromatin behaves as a solid-like material when condensed by
174 PRC1^{C8}.

175 We next wished to gain insights into PRC1^{C8} -chromatin condensate formations at low
176 protein concentrations that better resemble physiological concentrations. To this end, we
177 employed a single-molecule confocal microscope that allows tracking of individual
178 condensates through a confocal volume[29] (Extended Data Fig. 6a,b). We used GFP-labelled
179 PRC1^{C8}, where GFP peaks indicate the formation of bright protein assemblies. Importantly,
180 this system allows the detection of protein assemblies smaller than what can be identified
181 by standard fluorescence microscopes[30]. In the presence of chromatin, assemblies are
182 observed at PRC1^{C8} concentrations as low as 62.5 nM (Extended data Fig. 6c). This indicates
183 that condensates can form at physiologically relevant PRC1 concentrations, previously
184 estimated as 130 nM in polycomb bodies in cells[31]. In the absence of chromatin, GFP peaks
185 are only detected sporadically, even at the highest PRC1^{C8} concentration (Extended Data Fig.
186 6 b,c). Overall, this data indicates that PRC1^{C8}-chromatin condensates form under
187 physiologically relevant PRC1^{C8} concentration, but do not form without chromatin.

188 **Multivalent interactions between PRC1^{C8} and chromatin induce phase separation**

189 A scaffold-client based phase separation model has recently been proposed for PRC1-CBX2
190 complexes[28,35], where CBX2[35] or chromatin[28] act as a scaffold that induces phase separation
191 of PRC1 proteins. Since PRC1^{C8} is insufficient to phase-separate without chromatin (Fig 1e),
192 we wished to test if chromatin might act as a scaffold that concentrates PRC1^{C8} and induces
193 phase separation. To test this model, we probed the different interaction sites between
194 PRC1 and chromatin. The whole PRC1^{C8} complex (RING1b, BMI1 and CBX8) is necessary and
195 sufficient to condense chromatin, while the PRC1 core or CBX8 alone do not condense
196 chromatin (Fig. 3a). This suggests multivalent interactions between the PRC1^{C8} complex and
197 chromatin, involving different chromatin interacting surfaces in both PRC1 and CBX8. To
198 identify the different interaction sites, we first used crosslinking mass spectrometry (XL-MS)
199 to probe for protein-protein interactions within PRC1^{C8}–chromatin condensates (Fig. 3b and
200 Supplementary Data 4; PRC1 with an MBP-tagged CBX8 was used). As expected, we
201 identified extensive crosslinks between the RING domains of RING1B and BMI1. RING1B and
202 BMI1 did not crosslink to histones. This is likely because these proteins bind to the acidic
203 patch of the nucleosome[36], which is unlikely to be crosslinked by the BS3 crosslinker that
204 reacts preferentially with lysine residues. The results show multiple crosslinks from the CBX8
205 chromodomain to the H3 histone tail (Fig. 3b), indicative of binding. Interactions between
206 CBX-proteins and H3K27me3-modified H3-histone tails have been proposed to recruit PRC1
207 to chromatin modified by PRC2[2,3]. However, CBX8 did crosslink to unmethylated H3 tails
208 (Fig. 3b) and a trimethyl-lysine analogue (MLA) at H3K27 did not improve the chromatin-
209 condensation activity of PRC1^{C8} (Fig. 3d). We conclude that H3K27me3 is not necessary for
210 the chromatin condensation activity of PRC1^{C8} and that the H3 histone tail, even if
211 unmodified, provides an interaction site for PRC1 on chromatin.

212 We also observe extensive self-crosslinks in the IDRs of CBX8 (Fig. 3b). This may indicate
213 inter- or intramolecular interaction within this flexible lysine-rich region. However, these
214 self-crosslinks within the IDRs appear even in the absence of chromatin, suggesting that
215 they are not related to condensation (Extended Data Fig. 7 and Supplementary Data 3).

216 DNA has previously been shown to bind CBX8[37] and could provide another interaction site
217 for PRC1^{C8} on chromatin. We tested DNA binding in solution using a 24bp double stranded
218 DNA probe and found that CBX8 is necessary for the DNA-binding activity of PRC1^{C8} (Fig. 3c).
219 Hence, CBX8 binding to DNA provides a second interaction site of PRC1 with chromatin.

220 We conclude that PRC1 interacts with chromatin via at least three distinct sites: PRC1^{C8}
221 binds to DNA and the H3 tail via CBX8, as shown herein (Fig. 3), and binds the nucleosome
222 acidic patch via RING1b-BMI1 as shown elsewhere[36]. These multivalent interactions would
223 have to change dynamically while PRC1 maintains the condensed state of chromatin and
224 diffuses through it at the same time (Fig. 2b). Collectively, we propose that PRC1 induces

225 chromatin condensation via phase separation, through dynamic multivalent interactions
226 between PRC1 and chromatin.

227 **DNA binding by the CBX8 IDRs is required for efficient phase separation**

228 To test how different $PRC1^{C8}$-chromatin interaction sites affect phase separation, we
229 generated several different chromatin and CBX8 mutants (Fig. 4a and Extended Data Fig. 8).
230 Removing the CBX8 chromodomain ($PRC1^{C8\Delta Chromo}$), which interacts with the H3 tail[37,38] (Fig.
231 3b), does not have a significant effect on phase separation (Fig. 4b,c). We then mutated 21
232 positively charged residues in the CBX8 IDRs to alanine ($PRC1^{C8KR21A}$). $PRC1^{C8KR21A}$ is
233 analogous to a phase separation-deficient CBX2 mutant that was previously studied[10].
234 Accordingly, $PRC1^{C8KR21A}$ shows a clear defect in phase separation activity (Fig. 4b,c).

235 To further dissect the mechanism, we tested the DNA binding activity of $PRC1^{C8KR21A}$ and
236 $PRC1^{C8\Delta Chromo}$ against a 24 bp double-strand DNA probe with a sequence from a polycomb-
237 target gene. Both mutants are defective in DNA binding (Fig 4d). Hence, while DNA binding
238 of the chromodomain was reported previously[37], our data now suggests that the IDRs of
239 CBX8 are also contributing to DNA binding. We hypothesise that electrostatic interactions
240 between the negatively charged DNA to positive charges in the IDR lead to charge screening,
241 which promotes phase separation of chromatin (Fig. 4e).

242 We next set to determine if the H2A acidic patch of the nucleosome affects $PRC1^{C8}$-
243 chromatin condensation. For that, we mutated residues in the H2A acidic patch that were
244 previously shown to interfere with the interaction between PRC1 and the nucleosome[36]
245 (Extended Data Fig. 8). We observed a change in the condensate morphology (Extended
246 Data Fig. 8a): while $PRC1^{C8}$-chromatin condensates appear spherical, the condensates with
247 mutated acidic patch chromatin adapt elongated and branched structures (Extended Data
248 Fig. 8a; most apparent at lower $PRC1^{C8}$ concentrations). The effects of the $PRC1^{C8KR21A}$
249 mutant and the acidic patch chromatin mutant are additive (Extended Data Fig. 8b),
250 suggesting that both interaction sites contribute to phase separation independently. The
251 $PRC1^{C8\Delta Chromo}$ mutant does not substantially affect phase separation, regardless of the
252 chromatin used (Extended Data Fig. 8c). Overall, our data supports a model where $PRC1^{C8}$
253 uses its chromodomain and IDRs to bind DNA. Then, $PRC1^{C8}$ condenses chromatin through
254 interactions between the IDRs and DNA and, independently, between PRC1 and the acidic
255 patch on the nucleosomes (Fig 4e).

256

257 **CBX8 binding sites on chromatin in mouse embryonic stem cells are accessible**

258 Given the porous structure of $PRC1^{C8}$-condensed chromatin in vitro (Fig. 1) and the dynamic
259 diffusion of $PRC1^{C8}$ within condensates (Fig. 2b), we next wished to probe for the
260 accessibility of $PRC1^{C8}$-bound chromatin in cells. We carried out the Assay for Transposase

261 Accessible Chromatin (ATAC-seq) in differentiated mESC, combined with ChIP-seq for CBX8
262 and H3K27me3. We used differentiated mESC, because CBX8 is expressed at very low levels
263 in pluripotent mESC and is upregulated during retinoic acid-induced cell differentiation ([23]
264 and Fig. 5a). The DNA-loaded Tn5 used in ATAC-seq experiments forms a dimeric complex of
265 approximately 130 kDa with an estimated hydrodynamic radius[39] of 4.6 nm (based on PDB
266 code 1MUH[40]). In agreement with the accessibility analysis *in vitro* (Fig. 1h), the majority of
267 CBX8 ChIP-seq peaks in cells overlapped with ATAC-seq peaks (Figure 5b), indicating they
268 are accessible to Tn5. This observation was persistent across the genome, where ATAC-seq
269 peaks are co-localised with CBX8 and H3K27me3 peaks (Fig. 5c), indicating that CBX8-target
270 genes are largely accessible. The insufficiency of CBX8 to restrict chromatin accessibility is
271 further supported by the similar ATAC-seq profiles of wildtype and *Cbx8* knockout mESCs
272 (Fig. 5c,f, compare blue to orange). Hence, although the overall chromatin accessibility is
273 reduced during mESC differentiation (Fig. 5d,e), in agreement with previous works,[41] this
274 process is not dependent on CBX8. Collectively, we show that CBX8-bound polycomb-
275 repressed chromatin is largely accessible in differentiated mESCs (Fig. 5b-f).

276 Data thus far suggest that the deletion of CBX8 does not change chromatin accessibility in
277 mESCs (Fig.5c,f). Yet, other CBX proteins could potentially compensate for the loss of CBX8
278 in endogenous polycomb target genes. Therefore, we wished to test CBX8 in a system
279 where its recruitment is sufficient to trigger gene repression. To this end, we used a mESC
280 line that expresses a TetR-CBX8 fusion and includes a GFP reporter cassette downstream of
281 a TetO DNA binding array, stably integrated at chromosome 15 (coordinates: mm10 chr15:
282 79,013,675; Fig. 5g) [42]. In the absence of doxycycline (Dox), the TetR-CBX8 fusion is recruited
283 to the TetO-GFP reporter together with RING1B and initiates transcriptional repression (Fig.
284 5g and Extended Data Fig. 9a). However, GFP repression is reversible upon Dox addition,
285 which causes release of both TetR-CBX8 and RING1B from the TetO DNA binding array (Fig.
286 5g and Extended Data Fig. 9a).

287 Remarkably, ATAC-seq after prolonged Dox treatment did not reveal a substantial increase
288 in chromatin accessibility over the promoter region (Fig 5h). Small changes in the ATAC-seq
289 signal can be measured across samples and replicates, but these follow the sample-to-
290 sample variations globally (see Extended Data Fig. 9b for ATAC-seq coverage over the HoxA
291 genes cluster) and are therefore unlikely to be indicative of increased local accessibility.

292 The only increment in accessibility upon Dox treatment occurred downstream of the GFP
293 cassette, at a considerable distance from the TetO recruitment site and its adjacent PGK
294 reporter (Fig. 5h). That distal site includes another PGK promoter that is placed over 5 kbp
295 downstream to the TetO array. That distal PGK promoter originated from the construct that
296 was used to generate the reporter ([42] and references therein) and does not express a
297 functional protein-coding mRNA. Hence, we cannot exclude the possibility that CBX8 affects
298 accessibility at sites distant from the main recruitment hub, possibly through indirect

299 effects. Nevertheless, the data indicate that CBX8 recruitment and subsequent gene
300 repression are insufficient to restrict chromatin accessibility at the recruitment site.

301 **Discussion**

302 In conclusion, we have shown that PRC1-CBX8 binds chromatin via multivalent interactions
303 and induces chromatin condensation using both the nucleosome interacting surface of PRC1
304 and the IDRs of CBX8. $PRC1^{C8}$ is dynamic within condensates while keeping chromatin in a
305 static, solid-like state (Fig 3). This is in contrast to the liquid-like state of chromatin
306 condensates that were observed in vitro, in the absence of PRC1[17] . We established that
307 $PRC1^{C8}$ is sufficient to induce the solid condensed state of chromatin.

308 How can PRC1 condense chromatin but yet maintain a highly dynamic behaviour in the
309 nucleus? PRC1 is characterised with a short residence time on chromatin[31]. Solid-like
310 chromatin that is condensed by a mobile chromatin binder has been shown *in vitro* for a
311 truncation of the SAM-domain protein Polyhomeotic (Ph)[43] and in cells for HP1a at
312 chromocenters[20]. Yet, the mechanism allowing PRC1 and other gene-repressing factors to
313 condense chromatin while constantly diffusing in it remained largely unknown.

314 Our cryo-EM structure of PRC1-condensed chromatin explains how PRC1 can move in
315 condensed chromatin (Fig. 1), owing to the large pores that are formed between condensed
316 nucleosomes. The multivalent interactions between PRC1-CBX8 to chromatin provide PRC1
317 with multiple docking sites on chromatin: unmodified and modified H3 tail (Fig 3b-d), DNA
318 (Fig 3c) and the acidic patch of the nucleosome[36]. Hence, it is possible that PRC1-CBX8 can
319 constantly change its interactions with chromatin to maintain its condensed structure while
320 utilising its different chromatin-interacting surfaces to dynamically move around. While
321 doing so, the positively charged IDRs of CBX8 mask the negative charge of the DNA to bring
322 together chromatin segments and stabilize the condensed state of chromatin (Fig. 5i).
323 Neither the DNA binding activity of the chromo domain (Fig. 4b) nor H3K27me3 (Fig. 3d)
324 seem to be necessary for efficient $PRC1^{C8}$-chromatin phase separation. Given that the
325 chromodomain is required for DNA binding (Fig. 4d) and implicated in binding to
326 H3K27me3[37,38], it may play a role predominantly in recruitment.

327 In cells, canonical PRC1 includes an additional PHC protein, which was previously implicated
328 in chromatin compaction and condensation [8,28,43]. We reasoned that an in vitro study of a
329 simplified three-subunit complex, devoid of a PHC subunit, would allow us to characterize
330 the chromatin condensation activity of the CBX subunit. This minimal complex also allowed
331 us to overcome difficulties in purifying a PHC-bound PRC1 complex in sufficient quantities
332 and purity for structural studies. It is plausible that the chromatin compaction activities of
333 CBX8 and the PHC subunit cooperate in vivo.

334 Our data indicate that chromatin condensation together with dynamic behaviour within
335 chromatin condensates is an intrinsic biophysical property of PRC1-CBX8. It is plausible that

336 the dynamic behaviour of PRC1 within chromatin condensates is required in order to allow
337 PRC1 to modify nucleosomes by the H2AK119ub mark while holding them together. This
338 phenomenon might represent a broad paradigm of repressive chromatin. The internal
339 structure of PRC1-chromatin condensates is a porous network of nucleosomes (Fig. 1). Such
340 a structure could present a size-selective diffusion barrier, in agreement with its
341 permeability to PRC1 diffusion in vitro (Fig. 2) and Tn5 accessibility in cells (Fig. 5). The
342 existence of such a size-selective diffusion barrier remains to be identified in vivo, where it
343 may contribute to gene repression by selectively excluding transcriptional coactivators,
344 which are commonly large protein complexes- (>1MDa[12,44–46]). The hypothesis that
345 polycomb-mediated repression antagonises Pol II transcription without blocking all proteins
346 has been made nearly three decades ago[47]. This idea was conceived based on the
347 observation that T7 polymerase (~100 kDa) can initiate transcription from a polycomb-
348 repressed locus but GAL4-dependent transcriptional activation does not take place there.
349 This is in agreement with the inverse correlation between the density of chromatin domains
350 and the molecular weight of the chromatin modifiers present in them[12]. Transcription factor
351 size has also been suggested to determine access to different chromatin domains based on
352 simulations[48]. Combining our results with earlier findings[12,19,20,48], we propose that size-
353 selective exclusion may be part of a broader mechanism by which chromatin-interacting
354 proteins regulate the accessibility of repressive chromatin.

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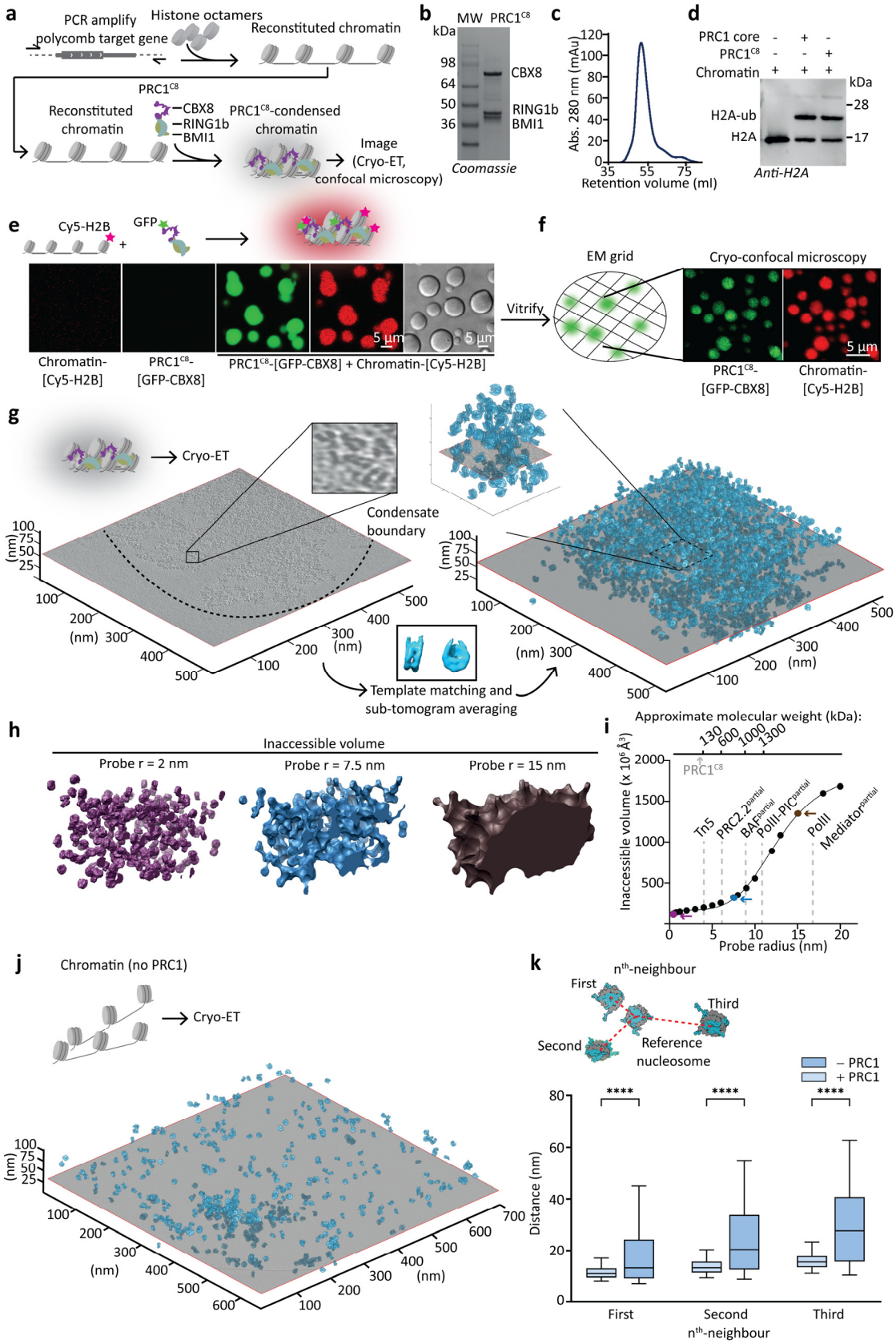
375

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 maps for tomograms in Fig. 1 g, and j have been deposited to the EMD (EMD-29022 and EMD-43554, respectively). All cryo-ET raw data has been deposited to EMPIAR under accession numbers EMPIAR-11344 and EMPIAR-11883, of chromatin in the presence and absence of PRC1-CBX8, respectively. XL-MS data has been deposited to Pride (PXD039589 and PXD049094).


a
PCR amplify polycomb target gene
Histone octamers
Reconstituted chromatin
PRC1C8
Reconstituted chromatin
CBX8
RING1b
BMI1
PRC1C8-condensed chromatin
Image (Cryo-ET, confocal microscopy)
b
MW PRC1C8
kDa
98
64
50
36
CBX8
RING1b
BMI1
Coomassie
c
Abs. 280 nm (mAu)
Retention volume (ml)
d
PRC1 core
PRC1C8
Chromatin
kDa
H2A-ub
H2A
28
17
Anti-H2A
e
Cy5-H2B
GFP
5 µm
Chromatin-[Cy5-H2B]
PRC1C8-[GFP-CBX8]
PRC1C8-[GFP-CBX8] + Chromatin-[Cy5-H2B]
f
EM grid
Cryo-confocal microscopy
Vitrify
5 µm
PRC1C8-[GFP-CBX8]
Chromatin-[Cy5-H2B]
g
Cryo-ET
Condensate boundary
(nm)
Template matching and sub-tomogram averaging
h
Inaccessible volume
Probe r = 2 nm
Probe r = 7.5 nm
Probe r = 15 nm
i
Approximate molecular weight (kDa):
130
600
1000
1300
PRC1C8
Tn5
PRC2.2partial
BAFfirtial
PolII-PICpartial
PolII
Mediatorpartial
Inaccessible volume (x 10⁶ Å³)
Probe radius (nm)
j
Chromatin (no PRC1)
Cryo-ET
(nm)
k
nth-neighbour
First
Second
Third
Reference nucleosome
– PRC1
+ PRC1
****
Distance (nm)
First
Second
Third
nth-neighbour

386 **Fig. 1. The molecular structure of PRC1-chromatin condensates is porous and accessible to**
387 **macromolecules. a,** Schematics introducing the workflow. **b,** SDS-PAGE of purified $PRC1^{C8}$
388 complex that includes RING1b, BMI1 and MBP-tagged CBX8. **c,** Size exclusion
389 chromatography of the purified $PRC1^{C8}$ using a HiLoad Sephacryl 300 16/60 column. **d,** In
390 vitro ubiquitylation assay comparing $PRC1^{C8}$ to a RING1b-BMI1 heterodimer. Samples in
391 lane 2 and 3 included E1, E2, ubiquitin and ATP. Ubiquitylation is detected by western blot
392 using an anti-H2A antibody. **e,** Chromatin condensates induced by the $PRC1^{C8}$ complex and
393 the individual proteins, visualised by confocal (left and centre) and phase contrast (right,
394 from independent experiments) microscopy. CBX8 is GFP-labelled and chromatin is Cy5
395 labelled. Protein and chromatin concentrations are 1 µM and 20 nM (estimated nucleosome
396 concentration), respectively. **f,** Cryo-confocal microscopy of vitrified $PRC1^{C8}$-chromatin
397 condensates. **g,** Cryo-electron tomography of a $PRC1^{C8}$-chromatin condensate. Shown is a
398 central slice through the reconstruction (left image). Nucleosome subtomogram averages
399 (centre, bottom) are then placed in a volume the size of the tomographic slice, at the
400 position and orientation determined by template matching and subtomogram averaging
401 (right image). 1330 nM $PRC1^{C8}$ and 3500 nM chromatin (estimated nucleosome
402 concentration) were assayed in 3.5 mM HEPES-KOH pH 7.5, 6.8 mM TRIS-HCl PH 7.5, 21 mM
403 NaCl, 7 mM KCl, 0.8 mM DTT. See Supplementary Data 1 for a list of cross correlation peaks.
404 **h,** Surface representation of the volume of a subset of the $PRC1^{C8}$-chromatin condensate
405 structure that is inaccessible to probes of given radii. **i,** Inaccessible volumes for a given
406 probe radii are plotted, with exemplary molecules indicated (in grey) and selected probes
407 coloured as in **h**. For the indicated complexes, the hydrodynamic radius was estimated[32]
408 using resolved domains from published structures (see Methods section for PDB accessions)
409 as a minimum size estimate. **j,** As in g but without $PRC1^{C8}$. See Supplementary Data 2 for a
410 list of cross correlation peaks. **k**, Pairwise distances of each individual nucleosome to its
411 nearest three neighbouring nucleosomes in 3D space in tomograms with and without
412 $PRC1^{C8}$. Only unique pairs are plotted from two tomograms with (+PRC1) and three
413 tomograms without (-PRC1). Whiskers extend from the 5th to the 95th percentiles.
414 Significance was tested using a Brown-Forsythe ANOVA with a Games-Howell post hoc test.
415 **** = p-value < 0.0001.

416

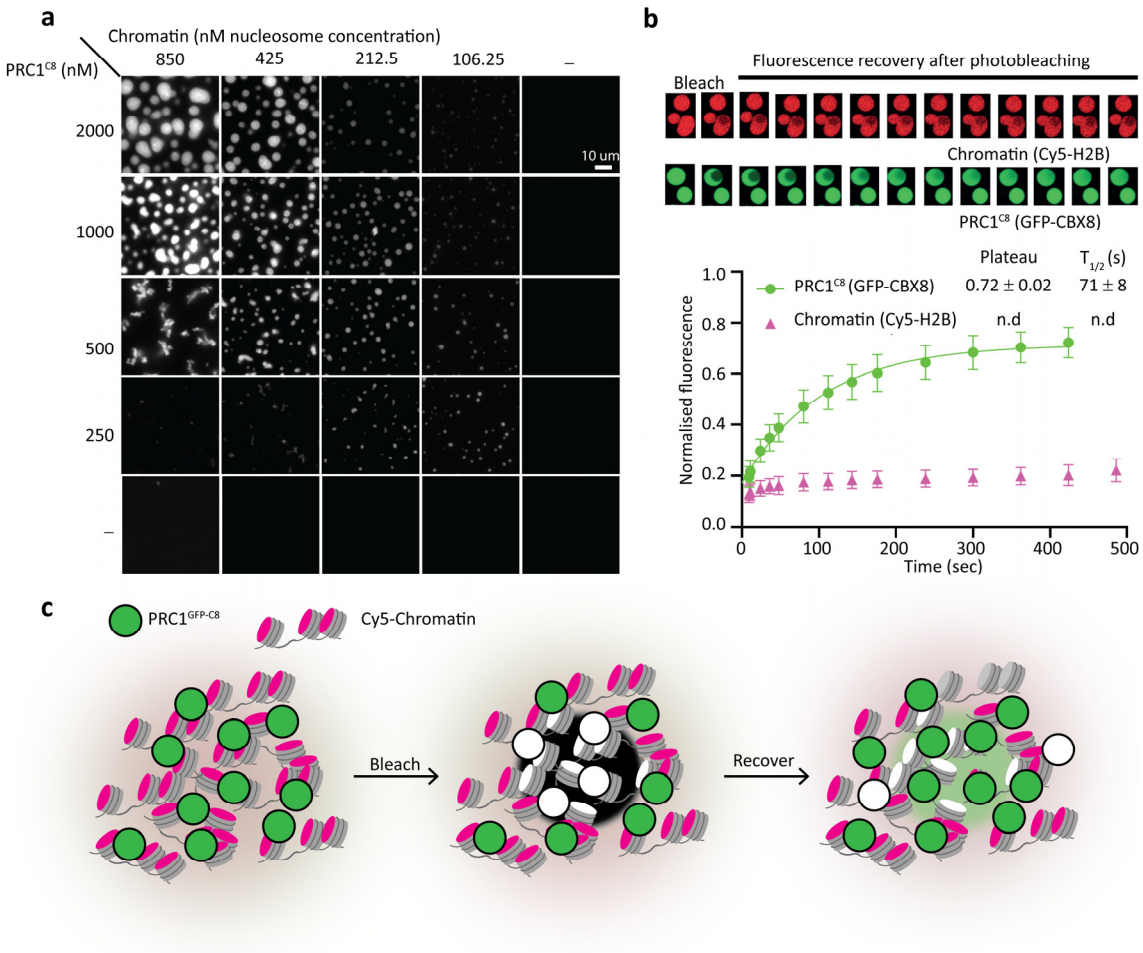


Fig. 2. PRC1^{C8} is mobile while chromatin is static within PRC1-chromatin condensates. **a,** Titration of Chromatin against PRC1^{C8}. Condensates were assessed with a fluorescence widefield microscope imaging a Cy5 label on the chromatin. Presented are representative micrographs of three replicates, including two with MBP-tagged PRC1^{C8} (presented) and one with GFP-tagged PRC1^{C8}. **b,** Representative micrographs of FRAP recorded in PRC1^{C8}-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. The GFP signal recovery was fit with an exponential association model, best fit values for Plateau and fluorescence recovery half time ($T_{1/2}$) are shown with standard error. **c,** Schematic representation of the FRAP experiment.

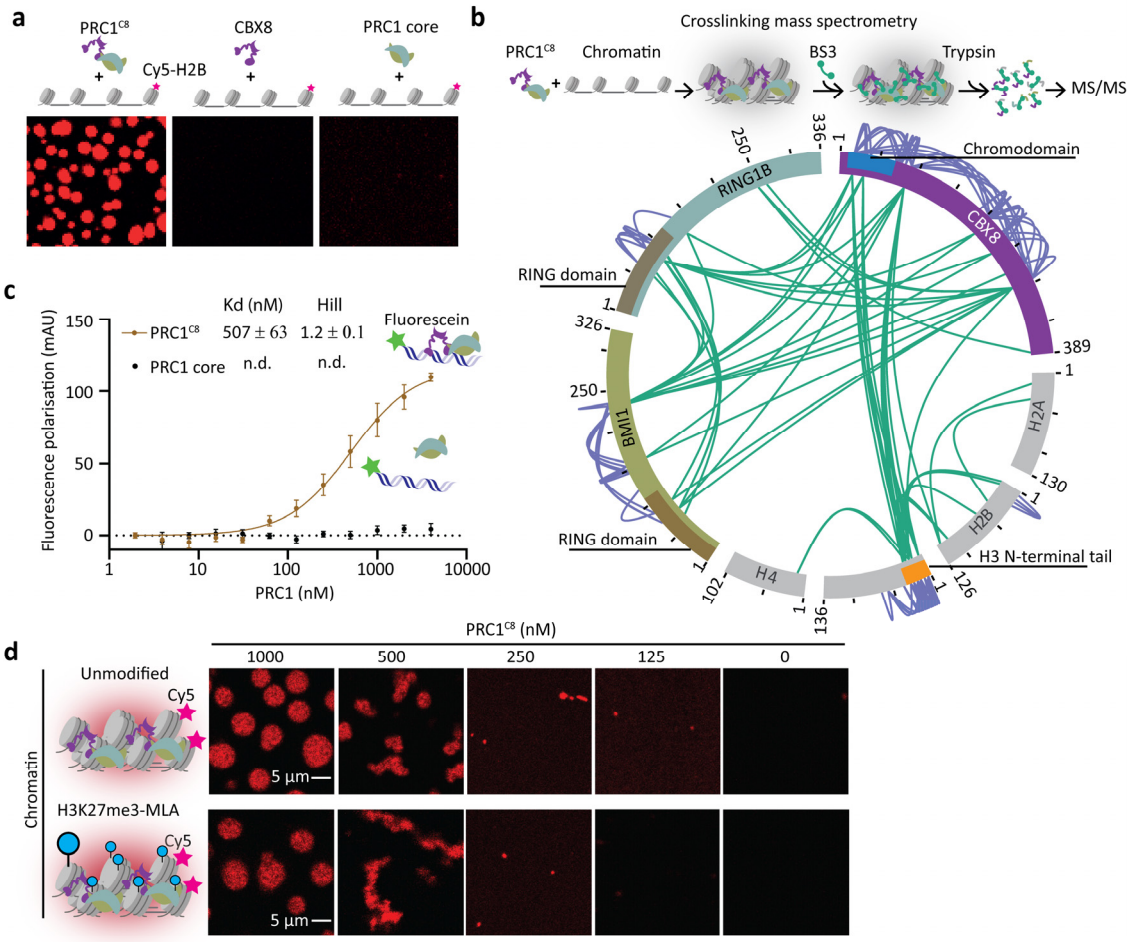


Fig. 3. Multivalent interactions between PRC1^{C8} and chromatin. a, Chromatin condensation in response to the whole PRC1^{C8} complex (RING1b, BMI1 and CBX8) or the individual components CBX8 and the RING1b–BMI1 heterodimer. Representative images from two replicates. **b,** Intermolecular (purple lines) 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,** PRC1^{C8} or PRC1 core binding to a fluorescein labelled 24bp DNA probe measured by fluorescence polarisation. Data points show the mean (baseline subtracted) of three independent replicates and the error bars indicate the standard error. The continuous line represents the fit to a Hill binding model, when applicable. **d,** Titration of PRC1^{C8} 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.

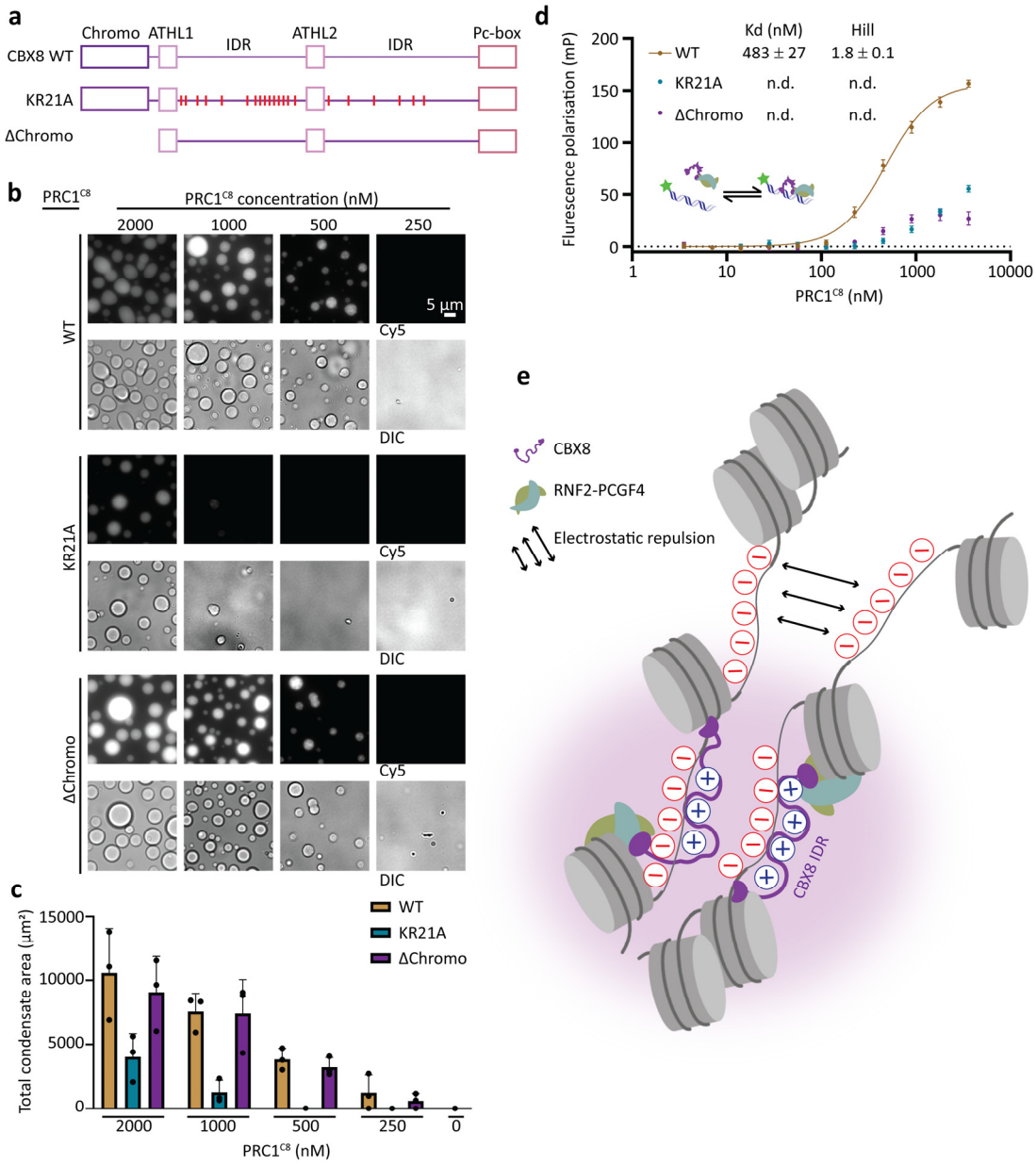
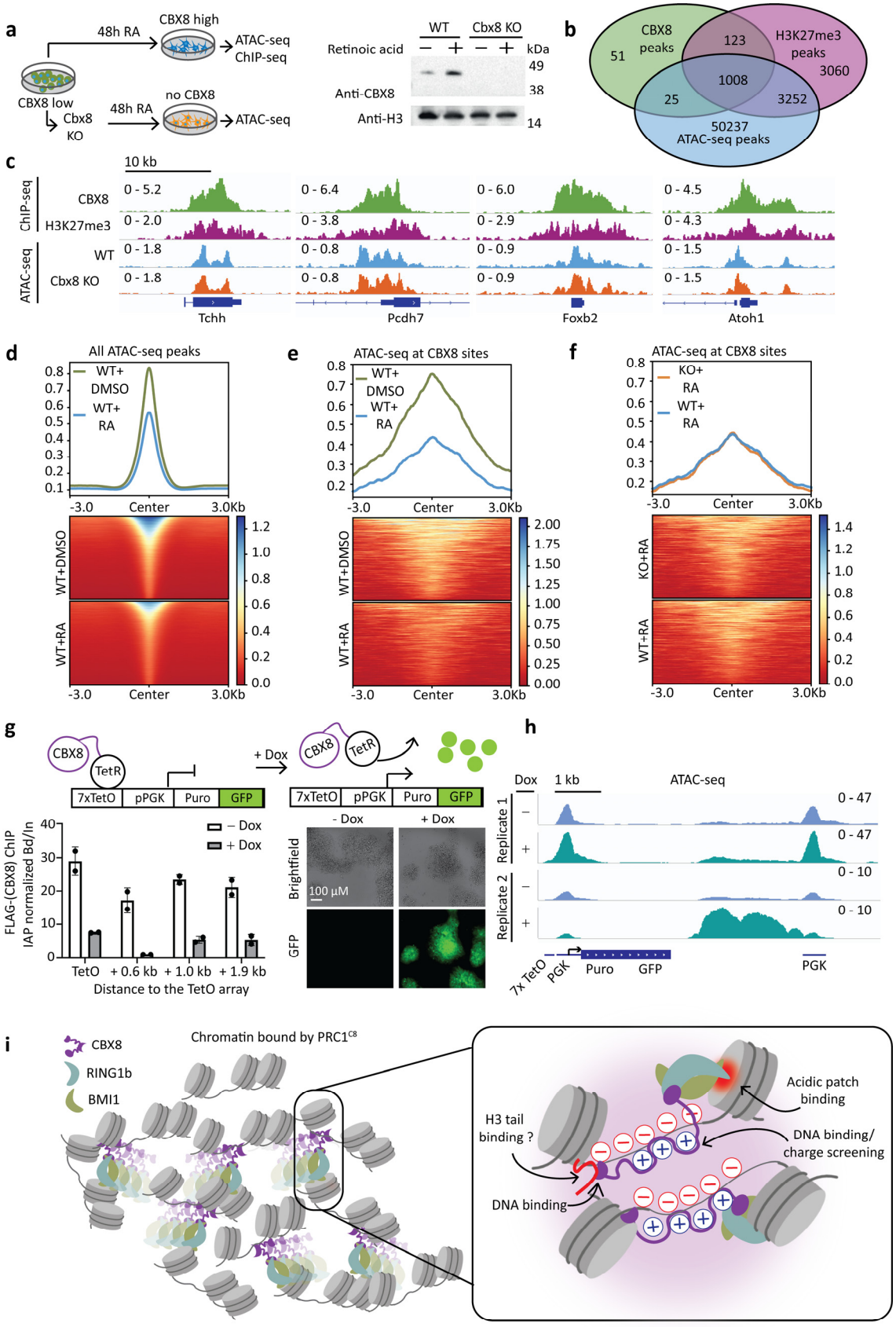


Fig. 4. Positive charges in the CBX8 IDRs are required for DNA binding and phase separation. a, Schematics depicting the different CBX8 mutants used, drawn to scale. **b,** PRC1^{C8}-chromatin condensates in the context of different CBX8 mutants. Varying concentrations of PRC1^{C8} were titrated to a constant concentration of C5-labelled reconstituted chromatin (50 ng/µl). Widefield fluorescence and differential interference contrast (DIC) micrographs are representative of three replicates. **c** Quantification of the total area covered by condensates per micrograph for different PRC1^{C8} mutants and concentrations. Bars represent the means from three independent replicates and error bars represent the standard deviation. **d,** Fluorescence polarisation assay measured the affinity of different PRC1^{C8} mutants for a Fluorescein-labelled 24 bp DNA probe. Data points are the mean (baseline subtracted) of three independent replicates and error bars indicate the

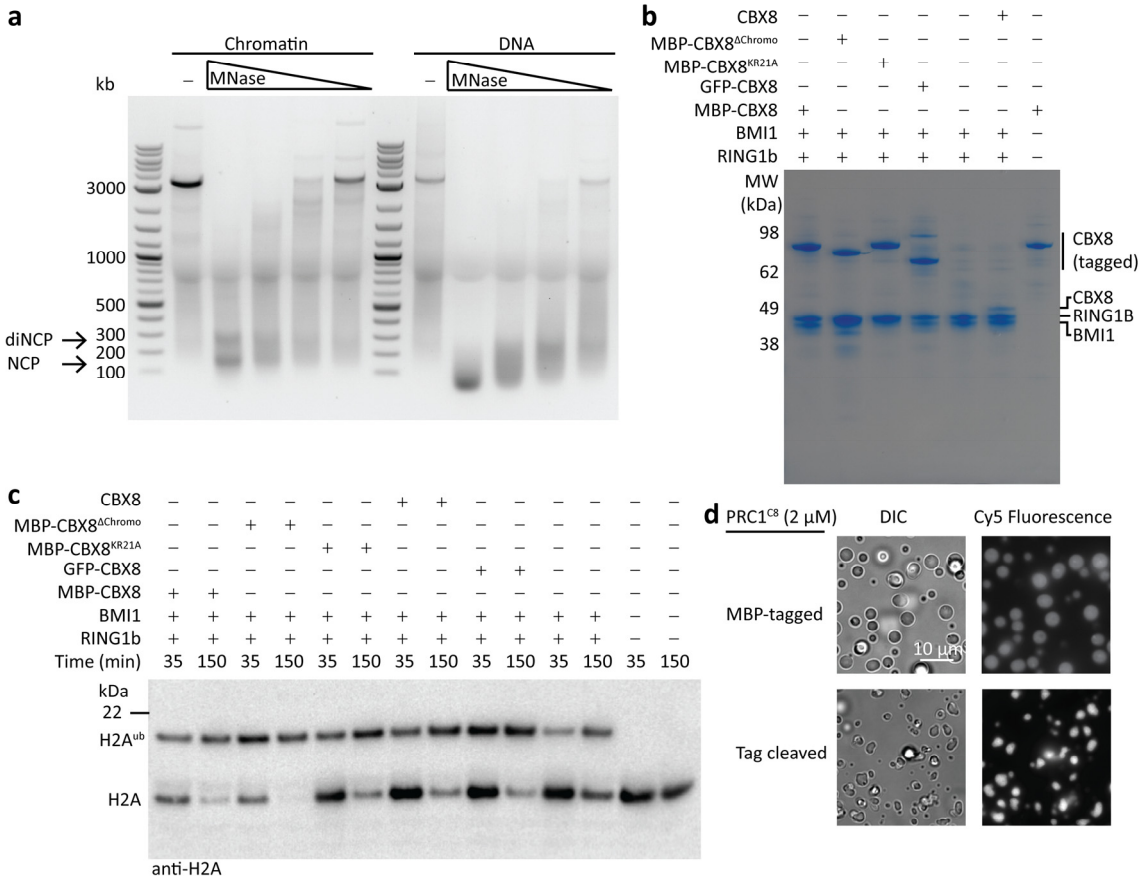
457 standard error. The continuous line represents the fit to a Hill binding model, when
458 applicable. **e,** Model for chromatin condensation by PRC1^{C8}: Electrostatic interaction
459 between the CBX8 IDR with DNA provides charge screening and promotes phase separation.


a
48h RA
CBX8 high
ATAC-seq
ChIP-seq
CBX8 low
Cbx8 KO
48h RA
no CBX8
ATAC-seq
WT
Cbx8 KO
Retinoic acid
kDa
Anti-CBX8
Anti-H3
49
38
14
b
CBX8 peaks
51
123
H3K27me3 peaks
3060
1008
25
3252
50237 ATAC-seq peaks
c
10 kb
ChIP-seq
CBX8
H3K27me3
ATAC-seq
WT
Cbx8 KO
0 - 5.2
0 - 6.4
0 - 6.0
0 - 4.5
0 - 2.0
0 - 3.8
0 - 2.9
0 - 4.3
0 - 1.8
0 - 0.8
0 - 0.9
0 - 1.5
Tchh
Pcdh7
Foxb2
Atoh1
d
All ATAC-seq peaks
WT+ DMSO
WT+ RA
-3.0
Center
3.0Kb
WT+DMSO
WT+RA
e
ATAC-seq at CBX8 sites
f
ATAC-seq at CBX8 sites
KO+ RA
KO+RA
g
CBX8
TetR
+ Dox
7xTetO
pPGK
Puro
GFP
FLAG-(CBX8) ChIP IAP normalized Bd/In
– Dox
+ Dox
TetO
+ 0.6 kb
+ 1.0 kb
+ 1.9 kb
Distance to the TetO array
Brightfield
100 µM
h
Dox
1 kb
ATAC-seq
Replicate 1
Replicate 2
0 - 47
0 - 10
7x TetO
PGK
Puro
GFP
i
CBX8
RING1b
BMI1
Chromatin bound by PRC1^C8
Acidic patch binding
H3 tail binding ?
DNA binding/ charge screening
DNA binding

460

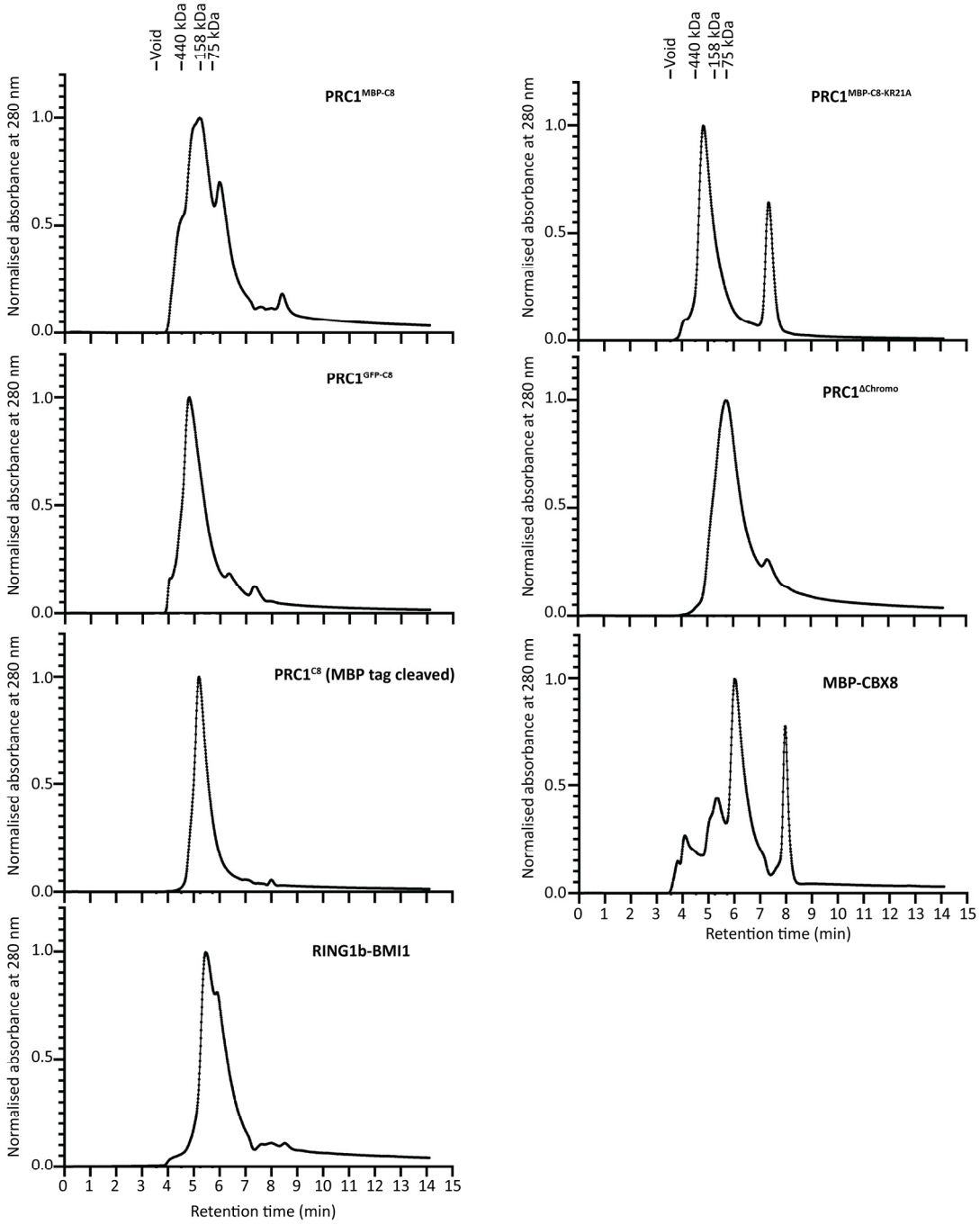
461 **Fig. 5. CBX8 binding sites on chromatin in mouse embryonic stem cells are accessible.**
462 **a,** Schematics of the experimental setup (left) and anti-CBX8 western blot (right) of wildtype
463 and *Cbx8* knockout mESC after 48 hours of retinoic acid (RA) treatment. **b,** Overlap of ATAC-
464 seq peaks and CBX8 and H3K27me3 ChIP-seq peaks. ATAC-seq peaks are defined from two
465 biological replicates. **c,** ChIP-seq traces for H3K27me3 and CBX8 in wildtype mESC and
466 representative ATAC-seq traces at four genes in wildtype and CBX8 knockout mESC after 48
467 hours of RA treatment. **d,** Accessibility changes at all ATAC-seq peaks in wildtype (WT)
468 mESC, in response to retinoic acid (RA) treatment. **e,** Accessibility changes at all CBX8-target
469 sites in wildtype mESC, in response to RA treatment. **f,** Comparison of accessibility at CBX8-
470 target sites between wildtype and *Cbx8* knockout cells after RA treatment. **g,** Top: schematic
471 representation of the chromosome-integrated reporter. Left panel: ChIP-qPCR using FLAG
472 antibody (CBX8 is FLAG tagged) at indicated distances from the TetO array, in the presence
473 and absence of doxycycline (Dox) treatment for six hours. Bars represent the mean bound
474 over input (Bd/In) normalised to the IAP gene and points represent two replicates. See
475 Extended Data Fig 9a for ChIP-qPCR using additional antibodies. Right panel: Brightfield and
476 GFP-fluorescence images of the mECS cells before and after Dox treatment. **h,** ATAC-seq
477 signal reporting accessibility of the integrated locus before and after Dox treatment for six
478 days. From left to right: annotated are the TetO array and its proximal PGK promoter that
479 controls the Puromycin-GFP reporter gene, and the distal PGK promoter. **i,** Model: PRC1
480 forms multivalent interactions with chromatin, thereby stabilizing chromatin condensates
481 potentially through charge screening of negatively charged DNA by positive charges in the
482 CBX8 IDR. These interactions dynamically change as PRC1 diffuses through condensates.
483

484 **Extended Data**

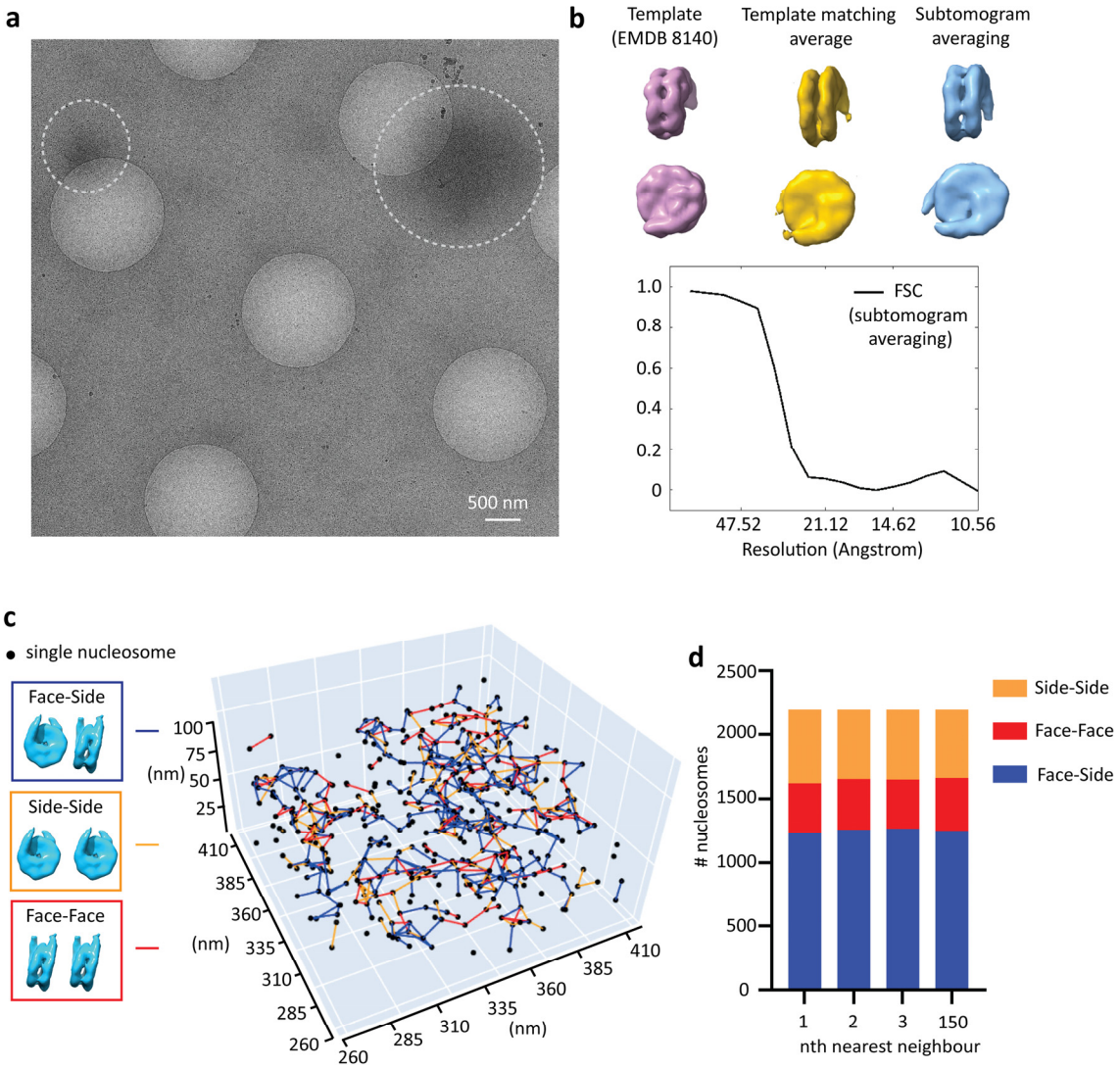


485
486

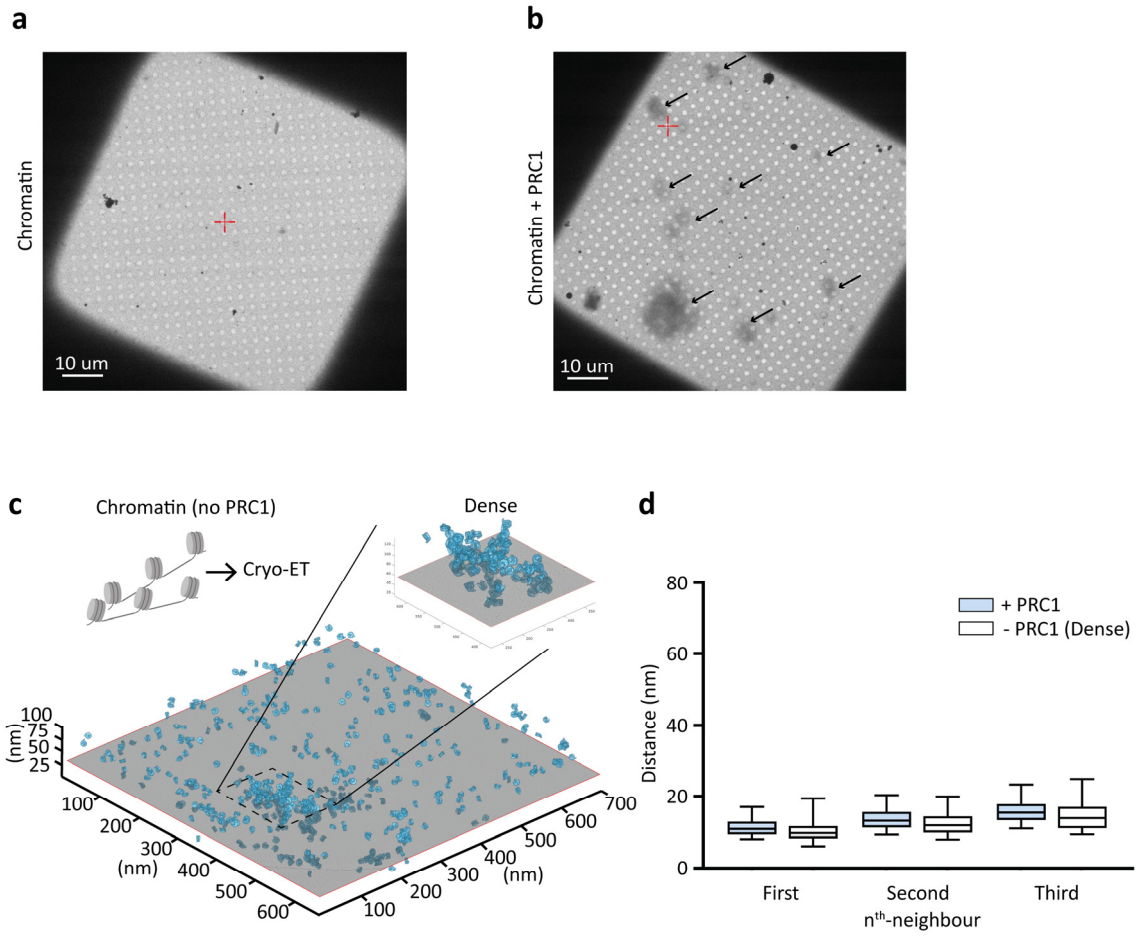
487 **Extended Data Fig 1. Quality control of chromatin and protein constructs. a,** MNase
488 digestion of reconstituted Chromatin and a naked DNA (same sequence as used for
489 chromatin reconstitution). DNA fragments post digestion are resolved on a 1.2 % Agarose
490 gel. Protected bands indicating mono- and di-nucleosome core particles (NCP and diNCP)
491 are indicated by the arrows. **b,** 3 ug of each protein complex used in this study resolved on a
492 4-12% SDS-PAGE gel stained with Coomassie. **c,** Ubiquitylation activity of each protein
493 complex used in this study visualized on a western blot. All samples include UBA1, UBCH5C,
494 Ubiquitin, ATP and 1 uM chromatin (nucleosome concentration). **d,** Phase separation
495 experiment comparing chromatin condensation activity of $PRC1^{C8}$ with MBP-tagged CBX8 to
496 $PRC1^{C8}$ with the tag cleaved.



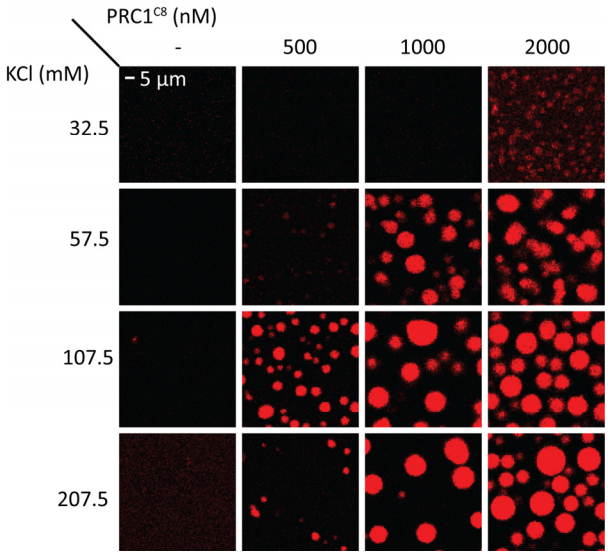
Extended Data Fig.2. PRC1 complexes used in this study are monodispersed. HPLC elution profiles from a Shim-pack Bio Diol 200 HPLC column for each of the purified protein complexes, as indicated.



Extended Data Fig. 3. Nucleosomes in PRC1^{C8}-chromatin condensates show no orientation bias towards neighbouring nucleosomes. a, Low-magnification micrograph showing examples of dense regions suspected to be condensates (dashed circles). Tomograms were collected at the borders of regions such as these. Micrographs are from the same gird from which tomograms were collected. **b,** Structures of the template used for template matching (left), the averaged structure after template matching (middle) and the final structure after the subtomogram averaging routine (right) with the related Fourier shell correlation curve (bottom). **c,** 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). **d,** 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 **c**.



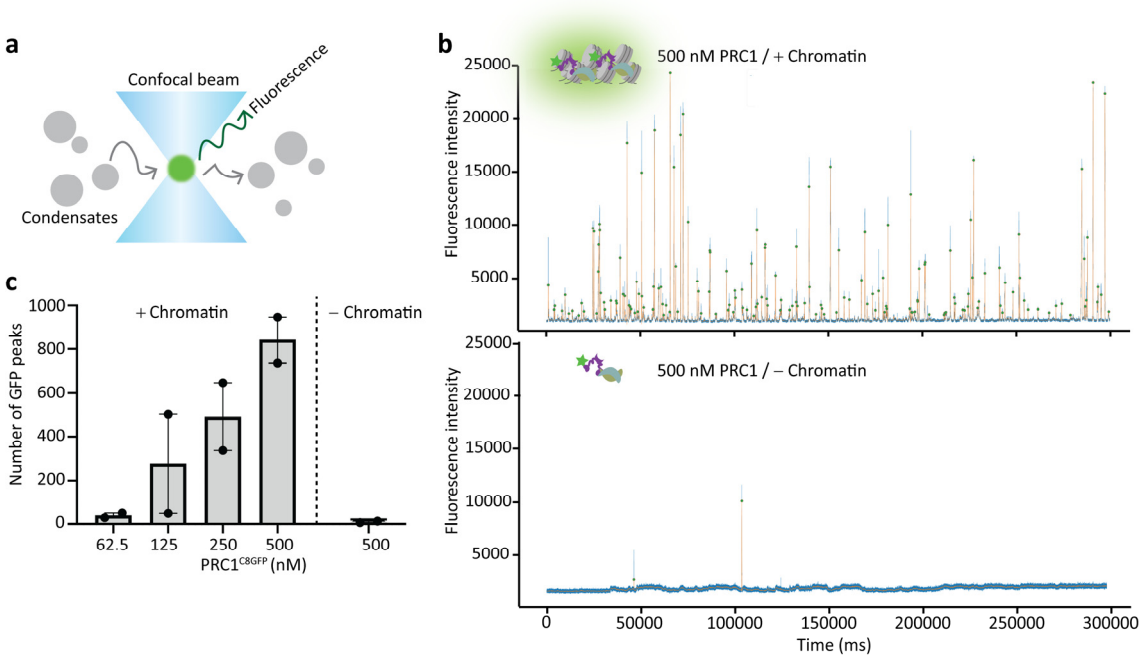
Extended Data Fig. 4. a, Representative low-magnification cryo-EM micrograph of a grid square with vitrified chromatin in absence of PRC1. **b,** Same as (**a**) but with PRC1. Black arrows indicate presumed condensates. Red crosses relate to stage movement and do not indicate features in the context of this figure. **c,** The same cryo-tomogram as in Fig. 1j, of chromatin in the absence of PRC1, with dense regions highlighted. **d,** Distances to the three nearest neighbouring nucleosomes, where +PRC1 as in Fig. 1k and -PRC1 shows distances for the highlighted dense region in **c**.



525

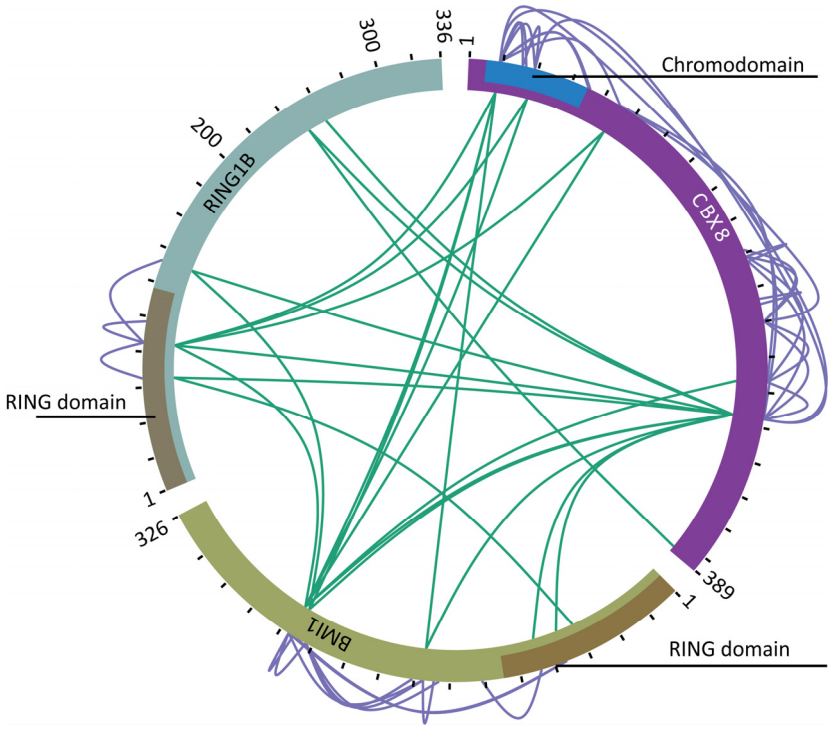
526 **Extended Data Fig. 5.** Chromatin condensation in response to variations in salt and PRC1
527 concentration measured by confocal microscopy using Cy5-labelled chromatin at a constant
528 concentration of 50 ng/µl DNA (approximately 400 nM nucleosomes).

529

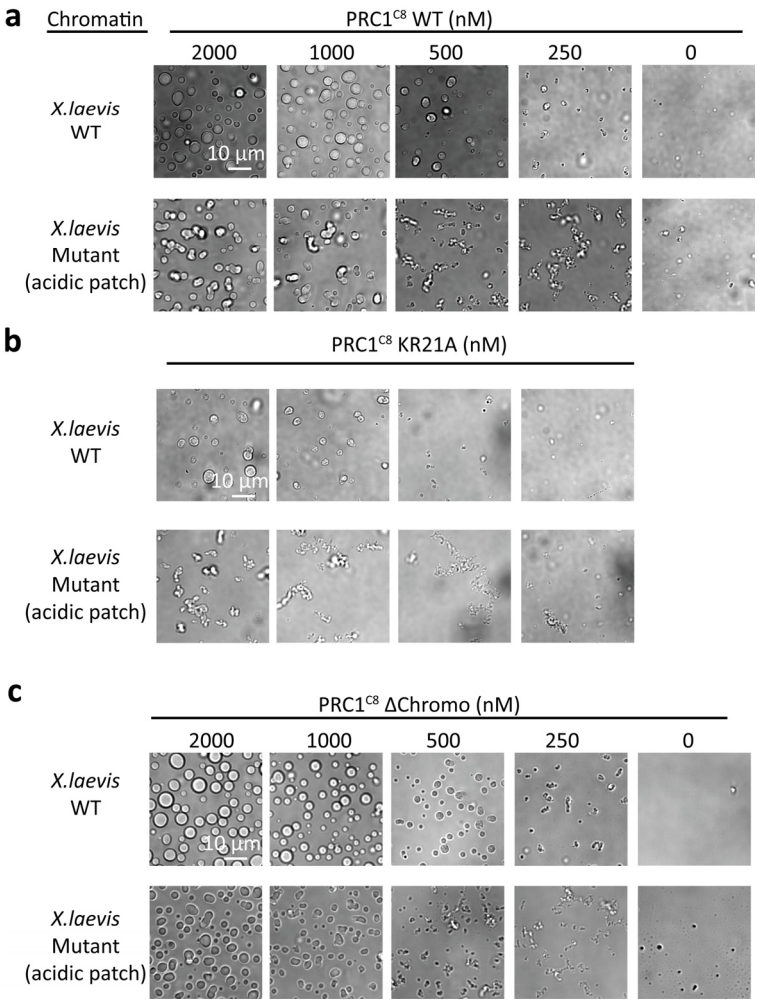


530

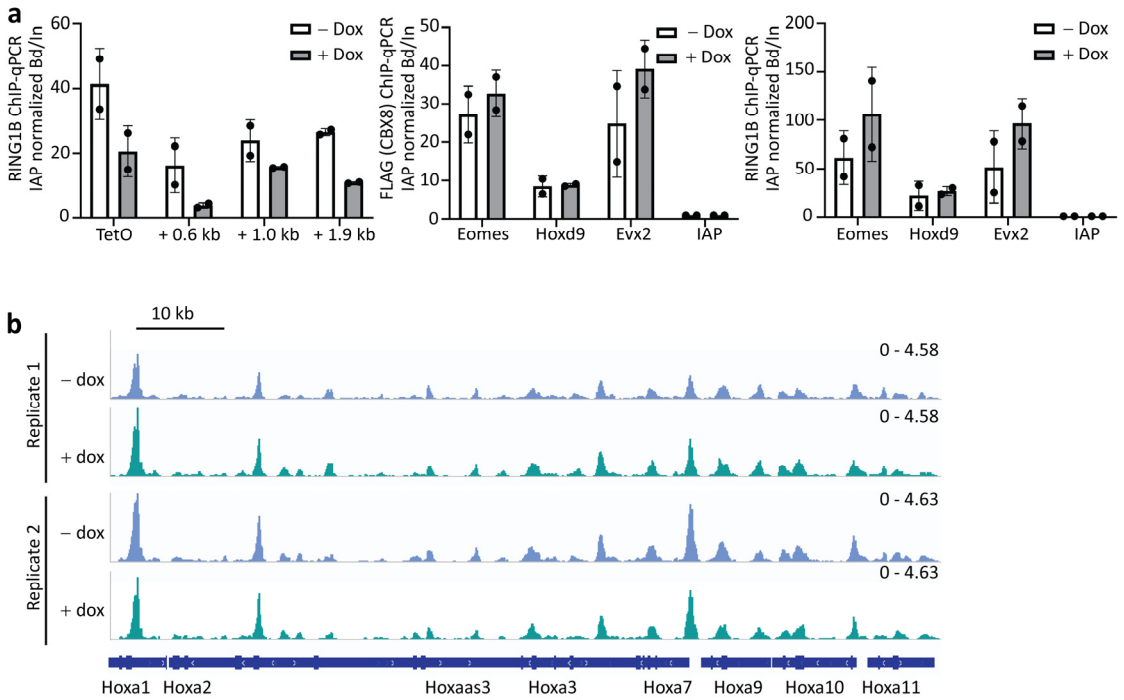
531 **Extended Data Fig. 6. Condensates can be detected at physiologically relevant PRC1^{C8}**
532 **concentrations. a,** An illustration of single molecule confocal microscopy. Individual
533 condensates (in grey) are detected when diffusing through the confocal volume (in blue)
534 and emitting GFP fluorescence (in green). **b,** Top: representative trace tracking GFP signal
535 over time for samples with 500 nM GFP-labelled PRC1^{C8} and chromatin (200 nM nucleosome
536 concentration). Traces show a 5 minute window from a 20 minute experiment. Blue lines
537 show the raw GFP signal and orange lines show the GFP signal after low-pass filtering using
538 a Butterworth filter. Green dots indicate the maxima of the detected peaks. Bottom: same
539 as the top plot, but in the absence of chromatin. **c,** GFP peak counts at different PRC1^{C8}
540 concentrations. Data from two replicates are shown. Bars indicate the mean of two
541 independent replicates that were carried out on different days, the error bars show the
542 standard error of the mean and individual data points are presented.
543



Extended Data Fig. 7. Crosslinking mass spectrometry (XL-MS) profile of PRC1^{C8} is similar in the absence of chromatin. Same experiment as in Fig. 3b, but without chromatin.



Extended Data Fig. 8. The nucleosome acidic patch is required for efficient PRC1-chromatin phase separation. a Phase separation of *X. laevis* wildtype and acidic patch mutant chromatin in response to increasing concentrations of $PRC1^{C8}$ wildtype. DIC micrographs are representative of two replicates. **b** Same as (**a),** but with the $PRC1^{C8KR21A}$ mutant. **c** Same as (**a)** but with the $PRC1^{C8\Delta Chromo}$ mutant.



Extended Data Fig. 9. a, ChIP-qPCR in the presence and absence of doxycycline (Dox) treatment using RING1B antibody at indicated distances from a chromosome-integrated TetO array (left), at control genes (right) and using FLAG (CBX8) antibodies at control genes (middle). Bars represented the bound over input (Bd/In) normalised to the IAP gene and the dots represent individual data points. **b,** ATAC-seq signal at the HoxA locus of the reporter-integrated mECS cell line before and after dox treatment. Two independent replicates are shown.

563 **Extended Data Movie S1. Related to Fig.1; Cryo-tomogram of a PRC1^{C8}-chromatin**
564 **condensate.** The movie shows a scan through the z-axis of denoised[49] tomogram of
565 chromatin in presence of PRC1.
566
567 **Extended Data Movie S2. Related to Fig.1; Cryo-tomogram of chromatin without PRC1.**
568 The movie shows a scan through the z-axis of denoised[49] tomogram of chromatin in absence
569 of PRC1.
570
571 **Supplemental Table 1. Template matching results for PRC1^{C8}-chromatin condensates.**
572 Results from the Dynamo template matching process before manually removing false
573 positive hits, in Excel format. Every entry describes a cross correlation peak above the cut-
574 off of 0.17. Each peak indicates the position of a nucleosome at x-y-z coordinates given in
575 columns 24-26 and at rotations applied to the template as defined by Euler angles in
576 columns 7-9. The table is also provided in Dynamo format (.tbl) for direct import into
577 Dynamo (Supplementary Data 1).
578
579 **Supplemental Table 2. Template matching results for chromatin without PRC1^{C8}.** Results
580 from the dynamo template matching process before manually removing false positive hits in
581 Excel format. Every entry describes a cross correlation peak above the cut-off of 0.23. Each
582 peak indicates the position of a nucleosome at x-y-z coordinates given in columns 24-26 and
583 at rotations applied to the template as defined by Euler angles in columns 7-9. The table is
584 also provided in Dynamo format (.tbl) for direct import into Dynamo.
585
586 **Supplementary Data 1. Template matching results for chromatin without PRC1^{C8}.** Same as
587 Supplemental Table 1, provided in Dynamo format (.tbl) for direct import into Dynamo.
588
589 **Supplementary Data 2. Template matching results for PRC1^{C8}-chromatin condensates.**
590 Same as Supplemental Table 3, provided in Dynamo format (.tbl) for direct import into
591 Dynamo.
592
593 **Supplementary Data 3. XL-MS-identified crosslinks within PRC1^{C8}.** Provided is a list of the
594 crosslinks that were detected using XL-MS within PRC1^{C8} in the absence of chromatin.

595 **Supplementary Data 4. XL-MS-identified crosslinks within PRC1^{C8}-chromatin.** Provided is a
596 list of the crosslinks that were detected using XL-MS within PRC1^{C8} in the presence of
597 chromatin.
598
599
600

601 **Materials and Methods**

602 **Plasmids and cloning**

603

604 Human RING1b (Uniprot ID Q99496) and human BMI1 (Uniprot ID P35226) were cloned into
605 a pFBOH-mhl vector (Addgene plasmid # 62304) cleaved with BseRI using Gibson Assembly®
606 Master Mix (NEB #E2611L) using the primers indicated in Table S1.
607 Human CBX8 wildtype open reading frame (Uniprot ID Q9HC52-1 and NCBI Reference
608 Sequence was NM_020649.2) was obtained from gene synthesis (Gen9). The CBX8^{KR21A}
609 mutant open reading frame was codon optimised for expression in *Trichoplusia ni* insect
610 cells and synthesised (Genscript). The CBX8$^{\Delta Chromo}$ truncation was amplified from the
611 wildtype ORF using primers indicated in Table 1 and then subcloned into a vector with the
612 same backbone as used to expresses the wild type protein. All three CBX8 constructs were
613 cloned into a modified pFastBac1 pFB1.HMBP.A3.PrS.ybbR vector digested by XhoI and XmaI
614 sites to include a N-terminal 6xHis-MBP tag Cloning of the polycomb target gene ATOH1
615 into the pUC18 vector was described previously[50]. Plasmids for expression of human
616 histones (H2A, H2B, H3.1 and H4) in *E.coli* were a kind gift from David Tremethick,
617 Australian National University. UbcH5c WT pET28a was a gift from Rachel Klevit (Addgene
618 plasmid # 12643; http://n2t.net/addgene:12643; RRID:Addgene_12643)[51].
619 pET3a-hUBA1 was a gift from Titia Sixma (Addgene plasmid # 63571;
620 http://n2t.net/addgene:63571; RRID:Addgene_63571)[52].
621 To generate a baculovirus expression vector of a monomeric EGFP-CBX8 (mEGFP-CBX8)
622 construct, first GFP was amplified from a pSpCas9(BB)-2A-GFP vector and CBX8 was
623 amplified from a pFB1.HMBP.A3.PrS.ybbR vector containing CBX8 as an insert.
624 Subsequently, EGFP-CBX8 was cloned into the pFBOH-mhl vector cleaved with BseRI via
625 Gibson assembly, with a Serine-Glycine-Serine linker between EGFP and CBX8. Finally, to
626 generate monomeric mEGFP-CBX8, alanine residue 207 in EGFP was mutated to Lysine using
627 a site directed mutagenesis kit (Takeda) and the mutagenesis primers listed in Table 1.
628

629 Table 1: Cloning and mutagenesis primers (5'-3') and sequences

mEGFP mutagenesis A207K fw	CTGAGCACCCAGTCCAAGCTGAG CAAAGACCC	Used for mutagenesis of CBX8-EGFP
mEGFP mutagenesis A207K rv	GGGGTCTTTGCTCAGCTTGGACT GGGTGCTCA	Used for mutagenesis of CBX8-EGFP
EGFP_fw_PFBOH_MHL	TTGTATTTCCAGGGCATGGTGAG CAAGGGCGAG	Used for Gibson assembly of mEGFP-CBX8 into pFBOH-mhl vector
EGFP rv with SGS linker	TGAAAGCTCACTTCCACTCTTGTA CAGCTCGTCCATGC	Used for Gibson assembly of mEGFP-CBX8 into pFBOH-mhl vector

CBX8 5' with SGS linker	TGTACAAGAGTGGAAGTGAGCTTTCAGCGGTGGGG	Used for Gibson assembly of mEGFP-CBX8 into pFBOH-mhl vector
CBX8 rv pFBOH_MHL	CAAGCTTCGTCATCATCATCTTTTCTCTTTAAAAAAGCCT	Used for Gibson assembly of mEGFP-CBX8 into pFBOH-mhl vector
ATOH1 fw	GCAGAGCCCAAACATTCACACA	Used to amplify ATOH1 from pUC18 plasmid
ATOH1 rv	GCGGAGTTTCCTAAAAGACGCC	Used to amplify ATOH1 from pUC18 plasmid
H2BT120C fw	GTGACCTGCTATACCAGCAGCAAATAA	Used for mutagenesis of H2B
H2BT120C rv	GGTATAGCAGGTCACGGCTTTGGTGCC	Used for mutagenesis of H2B
RING1b pFBOH-mhl fw	ttgtatttccagggcTCTCAGGCTGTGCAGACAAAC	Used for Gibson assembly into pFBOH-mhl vector
RING1b pFBOH-mhl rv	caagcttcgtcatcaTTTGTGCTCCTTTGTAGGTGC	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
24bp DNA duplex Fluorescein labeled	GGCGCCCTGCCCCGCCTCGCTCTG	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.
CBX8 KR21A	ATGGAATTATCAGCAGTAGGTGAACGCGTGTTTGCAGCGGAAGCGCTGCTCAAACGTAGGATTCGGAAGGGTCGCATGGAGTACCTAGTTAAGTGGAAAGGATGGTCACAGAAGTATAGCACATGGGAACCGGAGGAGAACATACTGGATGCTCGCTTGCTAGCAGCGTTTGAGGAGCGGGAACGAGAAATGGAGCTGTACGGACCTAAAAAACGTGGACCCAAGCCAAAGACCTTCCTTTTGAAGGCTCAAGCGGCTGCCAAGGCTGCCACGTATGAATTCAGATCGGATTCTGCCGCAGGTATAAGAATTCCCT	CBX8 KR21A mutant open reading frame (obtained by gene synthesis from GeneScript).

	ACCCAGGGGCCAGTCCACAGGA CTTGGCCTCCACGTCTAGGGCA GCAGAGGGCTTACGGAACATGG GCCTCTCTCCTCCGGCTTCATCTA CTAGTACGAGTAGTACATGTGCC GCGGAAGCGCCAAGGGACGCA GACCGAGACGCAGATCGGGATG CCGAGAGAGATGCGGAACGAGA GGCGGAAAGAGAAGCCGAGCGC GAGGCTGAGCGTGAAGCGGAAC GTGGCACAAGCGCCGTTGATGAC AAACCATCGAGCCCTGGTGATTC CAGCAAAAAACGGGGACCCAAG CCTAGGAAGGAGTTGCCGGACCC ATCCCAAGCCCCGCTTGGTGAAC CATCGGCGGGCCTCGGGGAATAT CTTAAAGGCCGAGCTTTGGACGA TACCCCTAGTGGTGCAGGAAAA TTTCCTGCGGGACATTCGGTTATC CAACTTGCTGCACGACAAGACTC AGATTTAGTACAGTGCGGGGTGA CATCCCCCAGCTCTGCAGAG GCGACCGGGGCCCTAGCTGTCGA CACCTTCCCAGCACGCGTGATAG CGCACCGAGCCGCATTTTTAGAA GCTGCTGGGCAGGGCGCGCTA GATCCCAACGGCACTAGGGTGCG GCACGGTTCAGGACCTCCCTCGT CCGGGGGGGGCCTATATAGAGA CATGGGAGCTCAGGGGGGTAGA CCGTCGCTTATCGCTCGTATCCCG GTAGCCCGTATTCTGGGTGACCC GGAGGAAGAATCCTGGTCTCCCA GTTTAACGAATTTAGAGAAA GTCGTAGTCACTGACGTTACGTC AAATTTTCTGACAGTCACCATCAA AGAGAGCAATACTGATCAAGGAT TCTTCAAGGAAAAGCGCTAA	
C8_PFB1_68_fw	TTCTGTTCCAGGGGCCCGGCCCC AAAAAGCGTGGA	Primer used to generate the CBX8$^{\Delta Chromo}$ truncation.
C8_PFB1_68_rv	TCGAGACTGCAGGCTTCATCTTTT CTCTTTAAAAAAGCCT	Primer used to generate the CBX8$^{\Delta Chromo}$ truncation.

Protein expression and purification

634
635 PRC1^{C8}, PRC1 core and CBX8 were co-expressed in *Trichoplusia ni* insect cells using the
636 Baculovirus system. CBX8 variably carried an N-terminal 6xHis-mEGFP or a N-terminal 6xHis-
637 MBP tag. The purification protocols were identical regardless of the tag. Baculoviruses were
638 generated as per manufacturers instructions (Thermo Fisher). The viral titre was determined
639 using the MTT assay (Promega #G3580). *Trichoplusia ni* insect cells were infected at a
640 density of 1.5 - 2 x 10^6 cells and incubated for 60 hours at 27 °C in a shaker.
641 The cells were spun down at 1500 relative centrifugal force (RCF). The pellet was
642 resuspended in 100 ml of lysis buffer per litre of cell culture (20 mM HEPES-KOH pH 7.5, 400
643 mM NaCl, 25 mM Imidazol, 10 % Glycerol, 0.2 mM TCEP, 1 mM PMSF and EDTA-free
644 Complete protease inhibitor (Thermo Fisher)). Lysis proceeded for 30-45 minutes at 4 °C
645 while rotating. The lysate was then centrifuged at 29000 RCF for 20 minutes at 4 °C. The
646 supernatant was transferred to a fresh tube and 5 ml of Ni-NTA resin (Qiagen) was added.
647 The samples were then incubated for 60 minutes at 4 °C while rotating and subsequently
648 centrifuged at 500 RCF for 5 minutes at 4 °C to settle the beads. About 90 % of the
649 supernatant was removed. The beads were resuspended in the remaining 10 % of
650 supernatant and transferred to 25 mm diameter gravity flow columns (Biorad). The beads
651 were allowed to settle before the remaining buffer was drained and the beads were then
652 washed with 12 CV of Buffer B (20 mM HEPES-KOH pH 7.5 at 20 °C, 500 mM NaCl, 25 mM
653 Imidazole, 10 % Glycerol, 0.2mM TCEP), followed by 30 CV of Buffer A (20 mM HEPES pH 7.5
654 at 20 °C, 100 mM NaCl, 25 mM Imidazole, 10 % Glycerol, 0.5 mM DTT or 0.2 mM TCEP). The
655 protein was then eluted in 6 CV Elution Buffer (20 mM HEPES-KOH pH 7.5 at 20 °C, 100 mM
656 NaCl, 400 mM Imidazole, 10 % Glycerol, 0.2 mM TCEP). The eluted protein was loaded onto
657 a Hitrap 5 ml Heparin column (Cytvia) equilibrated in IX Buffer A (20 mM HEPES pH 7.5 at
658 20 °C, 100 mM NaCl, 0.5 mM DDT or 0.2 mM TCEP) and the column was washed with 5 CV of
659 IX Buffer A. The proteins were resolved over a 20 CV gradient ranging from 0 % to 100 % IX
660 Buffer B (20 mM HEPES pH7.5 at 20 °C, 1000 mM NaCl, 0.5 mM DTT or 0.2mM TCEP). The
661 fractions were analysed on SDS-PAGE and fractions containing the protein complex of
662 interest with the expected subunits stoichiometry were pooled. The pooled fractions were
663 concentrated using a Amicon ultra 30K centrifugal filter (Merck, cat UFC903024) and
664 purified by size exclusion chromatography using a HiLoad Sephacryl 300 16/60 column
665 (Cytiva) equilibrated in GF Buffer (20 mM HEPES-KOH pH 7.5 at 20 °C, 150 mM NaCl, 0.5 mM
666 DTT). The collected fractions were analysed on SDS-PAGE and fractions containing the
667 protein complex of interest at the expected stoichiometry were pooled and concentrated to
668 a concentration of 1-2 mg/ml using an Amicon ultra 30K centrifugal filter (Merck, cat
669 UFC903024). The purified protein was then aliquoted and frozen in liquid nitrogen. The
670 purified proteins were stored at -80 °C until use.
671
672 Human UBA1, UBCH5C and Ubiquitin were purified as described previously[53]. Human
673 histone proteins H2A, H2B, H2BT120C, H3 and H4 were purified as described previously[54],
674 except that the gel filtration step was omitted. Purified *Xenopus laevis* histone proteins H2A,

675 H2B, H3, H4 and H2A acidic patch mutant (E56T, E61T, E64T, D90S, E91T, E92T) were bought
676 form from the Histone Source of the Colorado State University, Fort Collins.
677
678
679 **Production and purification of DNA for chromatin reconstitution**
680
681 The ATOH1 polycomb target gene was amplified in a large scale 10 ml PCR reaction including
682 500 nM ATOH1 fwd and reverse primers (see Table 1), 4 µg of ATOH1-pUC18 template[50],
683 200 µM dNTP mix (Invitrogen), 3 % DMSO, 50 mM KCl, 10 mM Tris-HCl, pH 8.8, 1.5 mM
684 MgCl2, 0.01% sterile gelatin. The reaction mixture was divided into 96-well plates to include
685 50 µl per well and the following PCR program was run:
686
687

Temperature (°C)	Time (minutes)	Number of Cycles
95	3:00	1
95	0:15	25
63	0:20	
72	8:00	
72	3:00	1

688
689
690 The PCR product was purified via ion exchange chromatography using a 5 ml HiTrap Q
691 column (GE Healthcare). The column was equilibrated in Buffer A (25 mM HEPES 7.5, 250
692 mM NaCl) and the sample was resolved using a linear gradient ranging from 0 % to 100 %
693 Buffer B (25 mM HEPES pH 7.5, 2 M NaCl). The fractions containing pure DNA were pooled,
694 subjected to ethanol-precipitation and resuspended in 10 mM Tris-HCl pH 7.5 at 20 °C, 0.1
695 mM EDTA.
696
697 **Chromatin reconstitution**
698
699 Histone Octamers were refolded as described previously[54]. All steps were done at 4 °C or on
700 ice. Chromatin was reconstituted following the salt-gradient dialysis protocol described
701 previously[54]. For large scale reconstitution, DNA and octamer were combined at an optimal
702 ratio that was determined at trial experiments for each batch of octamers and DNA. To
703 determine the optimal ratio of histone octamer to DNA, titration was carried out using
704 increasing amounts of octamer to a constant amount of DNA (molar ratios of 16:1, 18:1,

705 20:1, 22:1 of Octamer:DNA) following by salt gradient dialysis. For the salt gradient dialysis,
706 samples were initially dialysed in a buffer containing 10 mM Tris-HCl pH 7.5 at 20 °C, 2 M
707 KCl, 1 mM EDTA, 1 mM DTT. The initial buffer was then gradually exchanged for a low salt
708 buffer containing 10 mM Tris-HCl pH 7.5 at 20 °C, 250 mM KCl, 1 mM EDTA, 1 mM DTT over
709 the course of 18 hours, after which the salt exchange was complete. Samples were then
710 centrifuged at 21000 RCF for one minute, separated on a 1% agarose gel in TAE buffer and
711 stained with SYBR Safe (Sigma). The highest Octamer:DNA ratio at which chromatin
712 remained soluble after finishing salt dialysis was finally used for large scale reconstitution.
713 For large scale reconstitutions, the salt gradient dialysis was performed as above while
714 scaling up the reaction accordingly. Additionally, after conclusion of the salt gradient, the
715 samples were transferred to 400 ml of low salt buffer (10 mM TrisRIS-HCl pH 7.5 at 20 °C,
716 250 mM KCl, 1 mM EDTA, 1 mM DTT) and dialysed for another 2 hours. The samples were
717 finally dialysed against 1 litre of Chromatin Storage Buffer (10 mM TRIS-HCl pH 7.5, 10 mM
718 KCl) overnight.
719
720 **Reconstitution of Fluorescently labelled chromatin**
721
722 To allow site-specific labelling of histone H2B, a cysteine was introduced via site-directed
723 mutagenesis (H2BK120C, as previously described[55]) using mutagenesis primers indicated in
724 Table S1. H2BK120C was labelled with Cyanine5-maleimide (Lumiprobe cat #13080) as
725 described previously[55]. Labelled octamers were refolded as described above. Before
726 chromatin reconstitution, the unlabelled and labelled octamers were combined at a molar
727 ratio of 7:1 (unlabeled:labelled). Chromatin was then reconstituted as described above.
728
729 **Chromatin condensation assays**
730
731 Fluorescently labelled proteins and chromatin were protected from light whenever possible.
732 Chromatin condensation assays were done as described previously[17] with some
733 modifications. Assays were done in 384-well plates with #1.5 glass bottoms (MatTek
734 PBK384G-1.5-C). The wells were treated with 1 M NaOH for 1 hour at room temperature,
735 NaOH was removed and wells were washed with copious amounts of MilliQ water (MQ).
736 MQ was removed and 70 ul of 5k mPEG-silane (Sigma #JKA3037-1G, dissolved in 95% EtOH
737 to a final concentration of 25 mg/ml) was added to each well. The plates were sealed and
738 incubated overnight at room temperature. The mPEG-silane was removed, the wells were
739 washed once with 95 % EtOH, then rinsed with copious amounts of MQ and dried
740 completely in the fume hood.
741 The wells were then passivated by adding 40 ul of 20 mg/ml BSA (NEB) and incubated for at
742 least one hour at room temperature. The BSA was removed and the wells were washed
743 three times with Condensation Buffer (20 mM HEPES pH 7.5 at 20 °C, 0.2 mg/ml BSA, 10 %
744 Glycerol, 5 mM DTT and 150 mM KCl). The chromatin stock was adjusted to a DNA
745 concentration of 100 ng/ul (unless otherwise stated) and 150 mM KCl. The PRC1 complex

746 was diluted in Condensation buffer to a protein concentration equal to twice the final PRC1
747 assay concentration as stated. To induce condensation, 16 ul of the diluted PRC1 were
748 combined with 16 ul of the salt-adjusted chromatin dilution in PCR tubes. The samples were
749 incubated for 30 minutes at room temperature before being transferred to the 384-well
750 plate and incubated for a further 60 minutes at room temperature, so that the first images
751 were recorded 90 minutes after induction of condensation.
752 Images were recorded with a Nikon C1 scanning confocal microscope. GFP was excited with
753 a 488 nm laser, Cy5 was excited with a 561 nm laser. Linear contrast adjustments were
754 made with ImageJ. Where several micrographs are compared to each other, the same
755 contrast settings were used for all micrographs.
756 For condensation assays in Fig. 2a, Fig. 4, Extended Data Fig. 1d and Extended Data Fig. 8,
757 chromatin and PRC1 dilutions were prepared in Chromatin Buffer (10 mM TRIS-HCl pH 7.5 at
758 20 °C, 10 mM KCl, 1 mM DTT) and PRC1 buffer (20 mM HEPES-KOH pH 7.5 at 20 °C, 150 mM
759 NaCl, 1 mM DTT), respectively. After adjusting BSA and salt concentration and combining
760 PRC1 and chromatin, the final reaction contained the PRC1 and chromatin concentration
761 stated in the figures and the following components: 20 mM HEPES-KOH, pH 7.5, 5 mM TRIS-
762 HCl pH 7.5 at 20 °C, 90 mM KCl, 32.5 mM NaCl, 0.2 mg/ml BSA (NEB), 1mM DTT.
763 DIC images and fluorescence wide field images were recorded with a Leica DMI8 imaging
764 system equipped with a 4.2 MP (2k x 2k) sCMOS monochromatic K8 camera and a 63x oil
765 immersion objective with numerical aperture of 1.3. DIC images in Extended Data Fig. 8
766 were recorded with a Leica DMI8 imaging system equipped with a 4.2 MP (2k x 2k) sCMOS
767 monochromatic DFC 9000GT camera and a 40x dry objective with a numerical aperture of
768 0.8.
769 Condensate quantification was done using ImageJ. A threshold was set for each image to
770 segment condensates in the frame using the Cy5 signal. Condensates overlapping the frame
771 edges were excluded. Then the total area of all condensates in the frame was calculated.
772
773 **Chromatin ubiquitylation assay**
774
775 The salt concentration of the chromatin stock was adjusted to 100 mM KCl. The nucleosome
776 equivalent concentration of chromatin arrays was calculated by measuring the molar DNA
777 concentration and assuming that one DNA molecule is populated by 20 nucleosomes. The
778 reaction mixture included 750 nM (Fig. 1d) or 1000 nM (Extended Data Fig.1d) of chromatin
779 (nucleosome equivalent concentration), 500 nM $PRC1^{C8}$ or RING1b-BMI1 dimer, 100 nM
780 hUBA1, 500 nM UBCH5C and 50 µM ubiquitin in Ub-Buffer (25 mM HEPES-KOH pH 7.5 at
781 20 °C, 100 mM KCl, 3 mM MgCl and 2 mM DTT) and started by adding ATP to a final
782 concentration of 3 mM. 15 ul reactions were incubated at 30 °C for 45 minutes or the
783 indicated time points (Extended Data Fig 1d). The reaction was stopped by adding 5 ul of 4X
784 NuPage LDS-loading dye (Thermo Scientific cat #NP0008) supplemented with 5 % 2-
785 mercaptoethanol. The samples were separated on a 4-12% NuPage gel (Thermo Scientific
786 cat #NP0321BOX) using MES buffer (Thermo Scientific cat #NP0002) in the tank. The gels

787 were then blotted onto a nitrocellulose membrane (Amersham, cat #GE10600002) in Tris-
788 Glycine transfer buffer + 20 % Ethanol (v/v) for 90 minutes in the cold room at 310 mAmp in
789 a blotting tank (BioRad). H2A was detected using anti-H2A primary antibodies (Merck
790 Millipore Cat. # 07-146, 1:1000 titer) and HRP-conjugated secondary antibodies (Santa Cruz,
791 cat #sc-2357, titer 1:5000).
792
793 **Sample preparation for cryo-electron tomography**
794
795 The $PRC1^{C8}$ complex (MBP-tagged CBX8) was combined with chromatinized ATOH1 at a final
796 concentration of 1.6 µM $PRC1^{C8}$ and 500 ng/ul DNA at a final salt concentration of 25 mM
797 NaCl and 8.3 mM KCl. Samples were incubated at room temperature for 30 minutes. Just
798 before freezing, 5 nm gold nanoparticles were added at a 1:6 ratio (v/v). 3.5 ul of sample
799 was applied to a Quantifoil grid (R1.2/1.3 on 200 copper mesh, Quantifoil cat #N1-
800 C14nCu20-01) and vitrified in liquid ethane using the Vitrobot plunge freezer (Thermo
801 Scientific) with the following settings: Temperature 4°C, blot force -3, blot time 4 seconds,
802 humidity 100%. The final sample composition after addition of gold was 1330 nM $PRC1^{C8}$
803 and 3500 nM chromatin (estimated nucleosome concentration) in 3.5 mM HEPES-KOH pH
804 7.5, 6.8 mM TRIS-HCl PH 7.5, 21 mM NaCl, 7 mM KCl, 0.8 mM DTT. The sample of chromatin
805 in absence of PRC1 was prepared the same but instead of adding PRC1 an equivalent
806 volume of the PRC1 buffer was added.
807
808 **Low magnification cryo-EM image collection**
809
810 The images in Extended Data Fig. 3a were collected just before cryo-ET data collection using
811 the Titan Krios (Thermo Fisher) at an acceleration voltage of 300 keV and are of the same
812 grid from which tomograms were collected. The images in Extended Data Fig. 4a were
813 recorded using a Talos Arctica TEM (Thermo Fisher) at an acceleration Voltage of 200 keV.
814
815 **Cryo-electron tomography data collection and processing**
816
817 The data was collected with a Titan Krios electron microscope (Thermo Fisher) at 300 keV
818 acceleration voltage using a Gatan K2 (+PRC1 sample) or K3 (-PRC1 sample) Summit camera
819 (Gatan). A tilt series was acquired ranging from -60 to 60 degrees with 3 degree increments
820 and a nominal defocus of -2.5 µm. A dose symmetric collection scheme was followed as
821 described previously[56]. Chromatin in the presence of PRC1 was images at pixel size of 1.32
822 Angstrom. The chromatin samples in the absence of PRC1 were collected in super resolution
823 mode with a nominal pixel size of 1.632 Angstrom (0.815 Angstrom super resolution pixel
824 size). The total dose per tomogram was 144.32 e/A^2 (+PRC1) and 160.72 e/A^2 (-PRC1). Four
825 frames were collected per tilt (dose per frame 0.88 e/A^2 for the +PRC1 sample and 0.98 e/A^2
826 for the -PRC1 sample). The movies were motion corrected using motioncor2[57]. The motion
827 corrected images were combined into stacks and further processed with Imod[58] version

828 4.9.9. Imaging artefacts were identified and removed with Imod's Ccderaser function. Tilts
829 were aligned using the gold fiducial markers and the final aligned stack was binned by a
830 factor of 4. The defocus was estimated using emClarity[59] and the estimated defocus was
831 used in Imod for CTF correction. The final tomogram was calculated using Imod's
832 implementation of weighted back projection. For visualisation, the tomogram was denoised
833 using Topaz[49]. The denoised tomogram was only used for visualisation (Figure 1 and Movie
834 S1). The original non-denoised tomograms were used for all further processing, including
835 template matching and subtomogram averaging.
836
837 **Template matching, subtomogram averaging and modelling the chromatin structure of**
838 **condensates from cryo-electron tomograms**

839 Two tomograms were selected for further processing (Tomogram #1 and Tomogram #2 in
840 the following). To avoid user bias, we used a template matching algorithm[60] with the
841 structure of a single nucleosome as template (EMD-8140[61]) to identify initial positions and
842 orientations for each nucleosome. The pixel size of the template was adjusted using
843 emClarity (version 1.0.0) to match the unbinned pixel size of the tomogram[59]. The template
844 was then subjected to a low-pass filter of 30 Angstrom and binned by a factor of four to
845 match the binned tomogram. Template matching was done with the Matlab
846 implementation of Dynamo (version v-1.1.514)[60] using the "dynamo_match" function. The
847 template was masked with a tight-fitting mask with smooth edges. The scanning range was
848 set to 360 degrees with 10 degree steps. In-plane rotation was also scanned over 360
849 degrees with 10 degrees steps. Particles that passed a cross-correlation cut-off of 0.17
850 (Tomogram #1), 0.19 (Tomogram #2), 0.24 (Tomogram #27), 0.23 (Tomogram #41) and 0.25
851 (Tomogram #49) were selected for further analysis. Obvious false positives were removed
852 manually.

853 The particles were cropped from the tomogram with a box size of 36 pixels. Nucleosome
854 position and orientation was refined over three rounds of subtomogram averaging[60]. As an
855 initial template for subtomogram averaging, we used an average from all cropped particles
856 after template matching, masked with a tight-fitting mask with smooth edges generated in
857 the Dynamo mask editor. Specific settings for the different rounds of subtomogram
858 averaging are detailed in Table 2. The resulting average structure of a nucleosome from the
859 subtomogram averaging was then used to populate a volume the size of the tomogram with
860 nucleosomes at the determined positions and orientations. The graphic depiction of the
861 final model (Figure 1G) was generated using the Dynamo Matlab implementation[60].
862
863 Table 2: Settings for subtomogram averaging in Dynamo

	Round 1	Round 2	Round 3

Iterations	3	3	3
References	1	1	1
Cone Aperture	100	16	8
Cone Sampling	5	2	1
Azymuth Rotation Range	100	16	8
Azymuth Rotation Sampling	5	2	1
Refine	0	0	4
Refine Factor	0	0	2
High Pass	2	2	2
Low Pass	7	8	8
Symmetry	c1	c1	c1
Particle Dimension	36	36	36
Shift Limits	5	4	2
Shift Limiting Ways	1	1	1
Seperation in Tomogram	0	0	0
Basic MRA	0	0	0
Threshold Parameter	0.5	0.6	0.6
Threshold Modus	2	2	2

866 **Analysis of exclusion volume**
867
868 Exclusion volumes for spherical molecules of various radii were calculated using 3V[26] as
869 follows. First, the positional and rotational coordinates of the nucleosomes within
870 Tomogram #1 were determined by template matching followed by subtomogram averaging,
871 as described above. Next, the positional and rotational coordinates of the nucleosomes
872 were tabulated from within a section of 300 pixels x 300 pixels x 150 pixels (corresponding
873 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
874 location (650,650,80), which was large enough to include hundreds of nucleosomes but yet
875 sufficiently small to carry out the subsequent computational analysis. The dimensions of the
876 resulting table were converted to Angstrom by multiplying with a tomogram pixel size of
877 5.28 Angstrom/pixel. Next, the table including nucleosome positions was used by the
878 Dynamo function 'dtchimera' to generate a chimera cmd script that placed a pdb structure
879 of a single nucleosome (PDB 1KX4[62]) at the position and orientation defined for each
880 nucleosome in the table. At the end of this process, each of the nucleosomes within the
881 tomogram section is represented by the pdb coordinates of the nucleosome template. The
882 resulting model was saved as a pdb file and used as input for 3V[26]. Varying probe radii
883 ranging from 2 to 20 nm were used.
884
885 **Hydrodynamic radius calculation using protein structures**
886
887 The hydrodynamic radii of various proteins (Figure 1I) were calculated using the HullRad[39]
888 web server (http://52.14.70.9/). The PDB codes for the structures used are: 6LTJ (BAF[44]),
889 1MUH (Tn5[40]), 7O4J (PolII-PIC[46]), 6C24 (PRC2.2[63]), 8GXS (PolII-Mediator[64]). 6LTJ, 6C24, 8GXS
890 and 7O4J are only partial models as not all residues were assigned, therefore the calculated
891 hydrodynamic radius represents an estimate of the minimal complex size, while the actual
892 size of these complexes could be larger.
893
894 **Analysis of nucleosome-nucleosome orientation and distances**
895
896 Nucleosome-nucleosome orientation was classified into face-face, face-side and side-side,
897 as described in[22]. The orientations were calculated from the output table of the template
898 matching and subtomogram averaging process in Dynamo[60] using the Matlab script
899 'calculate_orientation.m'. The nucleosome-nucleosome orientation plot (Fig. S1A) was
900 generated using the 'plot_edges' function from the python notebook
901 NCP_orientation_analysis.ipyn. Both scripts are available on Github
902 (https://github.com/MichaUckelmann/Chromatin-Structure-Analysis).
903 Centre-centre distances between neighbouring nucleosomes were calculated using the
904 Matlab function 'knnsearch'. Double-counted distances were removed before further
905 analysis.
906

907 **Cryo-light microscopy**
908
909 The samples were prepared as described for cryo-electron tomography, without gold
910 nanoparticles and in a buffer containing 100 mM KCl. The vitrified grids were imaged using a
911 ZEISS LSM900 Airyscan2 with a Linkam CMS196V Cryo stage.
912
913 **Fluorescence recovery after photobleaching (FRAP)**
914
915 Samples and plates were prepared and images were recorded as described above for
916 chromatin condensation assays. 1 µM $PRC1^{C8}$ with an N-terminal GFP tag was used. The
917 FRAP experiments were set up with the NIS-Elements software (Nikon). Regions of interest
918 (ROIs) were defined and a single image was recorded before bleaching. Then the ROI was
919 bleached using 488 nm (GFP) and 561 (Cy5) lasers. The bleaching time and laser power was
920 set so that approximately 80 % of fluorescence signal within the ROI was quenched.
921 Recovery was measured over 424 seconds, recording a total of 13 images.
922 The data was analysed with ImageJ, the mean pixel intensity of the bleached ROI was
923 quantified for each timepoint.
924
925 **Single molecule confocal microscopy**
926
927 Cy5 labelled reconstituted chromatin (200 nM nucleosome concentration) and GFP labelled
928 $PRC1^{C8}$ at the concentration indicated in the figure were combined in assay buffer (25 mM
929 HEPES-KOH pH 7.5, 100 mM KCl, 15 mM NaCl, 1 mM DTT, 0.1 mg/ml BSA). Samples were
930 immediately loaded into a custom-made silicone well plate with a 70 x 80 mm glass
931 coverslip (ProSciTech, Kirwan, QLD, Australia). Plates were analysed at room temperature on
932 a custom setup based on a Zeiss Axio Observer microscope (Zeiss, Oberkochen, Germany).
933 Illumination is provided by a 488 nm and a 561 nm laser beams, cofocussed in the sample
934 volume using a ×40 magnification, 1.2 Numerical Aperture water immersion objective (Zeiss,
935 Oberkochen, Germany). This creates a very small observation volume in solution (~1 fL),
936 through which fluorescent proteins diffuse and emitting light in specific wavelengths as their
937 fluorescent tags are excited by the laser beams. Light emitted by the fluorophores is split
938 into GFP and mCherry channels by a 560 nm dichroic mirror. The fluorescence of GFP is
939 measured through a 525/50 nm band-pass filter and the fluorescence of mCherry is
940 measured through a 580 nm long-pass filter. Fluorescence is detected by two photon
941 counting detectors (Micro Photon Devices, Bolzano, Italy). Photons of the two channels are
942 recorded simultaneously in 1 ms time bins by a custom Lab-VIEW 2018 program (National
943 Instruments)[65]. The data were analysed using a custom python script (Spyder version 4.1.5)
944 that automatically detects peaks, as described in[66,67].
945
946 **DNA binding assays**
947

948 DNA binding was assayed using a 24 bp DNA (sequence see Table 1) with Fluorescein
949 attached to the top strand. The probe was protected from light wherever possible. The
950 probes were synthesised and delivered as duplexes (IDT). Probes were dissolved at a
951 concentration of 5 mM DNA in milliQ water. The probes were then diluted to 4 µM in
952 annealing buffer (20 mM HEPES-KOH, 150 mM NaCl) and heated to 95 °C for 5 minutes. The
953 probe was then left at room temperature for at least 1 hour to anneal before being diluted
954 to 20 nM in 20 mM HEPES-KOH pH 7.5 at 20 °C, 150 mM NaCl, 1 mg/ml BSA (NEB cat
955 #B9000S), 0.1% Tween20, 1 mM DTT. Serial protein dilutions of the $PRC1^{C8}$ and RING1b-
956 BMI1 complexes were prepared in Protein Dilution Buffer (20 mM HEPES-KOH, 150 mM
957 NaCl, 1 mM DTT) ranging from 8000 nM to 3.9 nM (Fig. 3) and from 7200 nM to 7 nM (Fig.
958 4). 20 ul of probe were mixed with 20 ul of the respective protein dilution and transferred to
959 a 384-well plate. The samples were incubated for 30 minutes in the dark at room
960 temperature and then read using a Pherastar plate reader (BMG Labtech). The fluorescence
961 anisotropy signal was normalised and the curves were fitted with a specific binding model
962 with Hill slope (GraphPad Prism).
963
964 **Crosslinking mass spectrometry (XL-MS)**
965
966 Sample preparation, processing and data analysis was identical for samples with and
967 without chromatin. Reconstituted chromatin (if used) was dialysed overnight against 1 litre
968 of XL buffer (25 mM HEPES-KOH pH 7.5 at 20 °C, 150 mM NaCl, 1 mM DTT). The chromatin
969 was combined with $PRC1^{C8}$ in a 1:1.5 molar ratio (Nucleosomes:$PRC1^{C8}$). Specifically, the
970 nucleosome equivalent concentration of the chromatin array was calculated from the
971 measured DNA concentration assuming 18 nucleosomes per DNA molecule. 1.5 µM PRC1
972 and 1 µM nucleosome equivalent concentration of chromatin arrays were then combined in
973 XL buffer and incubated for 30 minutes at room temperature. Crosslinking was done as
974 described before[68]. The bis(sulfosuccinimidyl)suberate (BS3) crosslinker was added to a final
975 concentration of 500 µM and crosslinking proceeded for 20 minutes at room temperature at
976 a reaction volume of 15 µl. The reaction was stopped by the addition of Tris-HCl pH 8 at
977 20 °C to a final concentration of 125 mM. The samples were then diluted to a volume of
978 100 µL using a buffer containing 50 mM Tris pH 8 at 20 °C and 150 mM NaCl. TCEP was
979 added to a final concentration of 10 mM and the samples were incubated at 60 °C for
980 30 min. Chloroacetamide was added to a final concentration of 40 mM and the samples
981 were incubated in the dark for 20 min. The samples were then digested using trypsin
982 (Promega cat #V5280) at 37 °C overnight. The digest was stopped by adding formic acid to a
983 final concentration of 1% v/v. The digested samples were purified using 100 µl ZipTip pipette
984 tips (Merck cat #ZTC18S960) according to the manufacturer's instructions. Samples were
985 then concentrated to ~5 µL using a SpeedVac vacuum centrifuge and diluted with 20 µL
986 Buffer A (0.1% v/v formic acid).
987

988 The peptides were analyzed by online nano-high-pressure liquid chromatography (UHPLC)
989 electrospray ionization-tandem mass spectrometry (MS/MS) on an Q Exactive Plus
990 Instrument connected to an Ultimate 3000 UHPLC (Thermo-Fisher Scientific). Peptides
991 reconstituted in 0.1% formic acid were loaded onto a trap column (Acclaim C18 PepMap 100
992 nano Trap, 2 cm × 100 µm I.D., 5-µm particle size and 300-Å pore size; Thermo-Fisher
993 Scientific) at 15 µL/min for 3 min before switching the precolumn in line with the analytical
994 column (Acclaim C18 PepMap RSLC nanocolumn, 75 µm ID × 50 cm, 3-µm particle size, 100-
995 Å pore size; Thermo-Fisher Scientific). The separation of peptides was performed at
996 250 nL/min using a non-linear acetonitrile (ACN) gradient of buffer A (0.1% formic acid) and
997 buffer B (0.1% formic acid, 80% ACN), starting at 2.5% buffer B to 42.5% over 95 min. Data
998 were collected in positive mode using a Data Dependent Acquisition m/z of 375–2000 as the
999 scan range, and higher-energy collisional dissociation (HCD) for MS/MS of the 12 most
1000 intense ions with z 2–5. Other instrument parameters were: MS1 scan at 70,000 resolution,
1001 MS maximum injection time 118 ms, AGC target 3E6, ion intensity threshold of 4.2e4 and
1002 dynamic exclusion set to 15 s. MS/MS resolution of 35000 at Orbitrap with the maximum
1003 injection time of 118 ms, AGC of 5e5 and HCD with collision energy = 27%.
1004 For the data analysis, Thermo raw files were analysed using the pLink 2.3.4 search engine[69]
1005 to identify crosslinked peptides, searching against the sequences of RING1b, BMI1, CBX8,
1006 H2A, H2B, H3 and H4. The default settings for searches were used. N-terminal acetylation
1007 and methionine oxidation were used as variable modifications and carbamidomethyl on
1008 cysteines as a fixed modification. False discovery rates of 1% for peptide spectrum match
1009 level were applied by searching a reverse database. Crosslinked peptides were further
1010 analysed using the crissrosslinkeR package[70]. Specifically, peptides were retained by
1011 crissrosslinkeR only if they passed a p-value cutoff of 0.05 or were present in at least two of
1012 three replicates. Subsequent visualisation of retained peptide was carried out with xiNET[71].
1013
1014 **Generation of Cbx8 KO mESC lines using CRISPR/Cas9**
1015
1016 Paired sgRNAs were designed to delete exons 1–4 of the murine *Cbx8* gene. The Golden
1017 Gate Cloning method was used to clone the sgRNAs (sequence below) into the lentiguide-
1018 mCherry-Cas9 plasmid[72,73]. 2 million mESCs were transfected with 1 µg of each plasmid
1019 carrying sgRNAs-mCherry-Cas9, using electroporation (Neon™ Transfection System
1020 MPK5000). The following day, mCherry-positive mESCs were sorted by FACS and plated on a
1021 10 cm dish at a very low density for single-cell clone picking. After 5-6 days, individual
1022 colonies (derived from single cells) were picked, expanded, and genotyped using genomic
1023 PCR to identify homozygous/biallelic deletions of Cbx8 KO mESC colonies. Selected Cbx8 KO
1024 mESC lines were further confirmed by western blot for CBX8 (Cell Signalling, CBX8 (D2O8C),
1025 cat # 14696S, titer 1:50 (Fig. 4a)) and HRP-conjugated secondary antibodies (Santa Cruz, cat
1026 #sc-2357, titer 1:10000).
1027
1028 **Cbx8 sgRNA sequences (5' and 3' sgRNAs)**

1029 CBX8-5'Fw: CACCTGCGAATGCGCCGCTTCAGG
1030 CBX8-5'Rv: AAACCCTGAAGCGGCGCATTCGCA
1031 CBX8-3'Fw: CACCCTCTATGGCCCCAAAAAGCG
1032 CBX8-3'Rv: AAACCGCTTTTTGGGGCCATAGAG
1033 **Cbx8 genotyping primers:**
1034 Deletion_Fw: GCCTTCTGGTGCAGCTAAGT
1035 Deletion_Rv: GACGTCAGCGGGAGAGTATT
1036 Internal_Fw: CACCAAATGAATGCTCCAAA
1037 Internal_Rv (same as the Deletion_Rv): GACGTCAGCGGGAGAGTATT
1038
1039 **Mouse embryonic stem cell culture**
1040
1041 Wildtype and *Cbx8* knockout mES cells were grown on gelatinized culture dishes in DMEM,
1042 20 % FBS, 1x non-essential amino acids (Gibco #11140-050), 1x Glutamax (Gibco #35050-
1043 061), PenStrep 100 u/ml (Thermo Fischer), 0.5 x EmbryoMax 2-Mercaptoethanol (Merck
1044 Millipore #ES-007-E), 2.5 µg/ml Plasmocin (Invivogen), 1000 U/ml ESGRO Leukemia
1045 Inhibitory Factor (LIF) (Merck Millipore cat #ESG1107). For ATAC-seq experiments with the
1046 reporter-integrated mECS line, the cells were treated for 6 days with 1 µg/ml Doxycycline
1047 (passaged every 48 h) before ATAC-seq was performed as described below.
1048
1049 **Mouse embryonic stem cell differentiation ahead of ChIP-seq and ATAC-seq**
1050
1051 Differentiation was induced by seeding cells at a density of $0.3x10^6$ cells per well in 6-well
1052 plates (for ATAC-seq) or at $1.5x10^6$ cells per 10-cm culture dish (for ChIP-seq) in media
1053 containing 1 µM all-trans retinoic acid (RA,Sigma-Aldrich R2625-50MG) and no LIF. Cells
1054 were differentiated for 48 hours and the media was changed after 24 hours. Control cells
1055 were treated with a DMSO volume equivalent to the RA volume in the differentiating cells.
1056 After 48 hours, the cells were harvested, washed once with PBS, counted and used
1057 immediately in either ChIP-seq or ATAC-seq experiments.
1058
1059 **Mouse embryonic stem cell culture for ChIP-qPCR**
1060
1061 TetR-CBX8 reporter-integrated mESCs were cultivated without feeders in high-glucose-
1062 DMEM (Corning 10-013-CV) supplemented with 13.5% fetal bovine serum (Corning 35-015-
1063 CV), 10 mM HEPES pH 7.4 (Corning, 25-060-Cl), 2 mM GlutaMAX (Gibco, 35050-061), 1 mM
1064 Sodium Pyruvate (Corning 25-000-Cl), 1% Penicillin/Streptomycin (Sigma, P0781), 1X non-
1065 essential amino acids (Gibco, 11140-050), 50 mM β-mercaptoethanol (Gibco, 21985-023)
1066 and recombinant LIF. Cells were incubated at 37 °C and 5 % CO2 and were passaged every
1067 48 h by trypsinization in 0.25 % 1x Trypsin-EDTA (Gibco, 25200-056). To reverse TetR-CBX8
1068 binding, 1 µg/ml Doxycycline (Sigma, D9891) was added to cell culture medium for 6 hours.

1069

1070 **ChIP qPCR**

1071

1072 25 x 10^6 reporter-integrated mESCs were collected, washed once in 1x PBS and cross-linked
1073 for 7 min in 1 % formaldehyde. The crosslinking was quenched by addition of 125 mM
1074 glycine and incubated on ice. The cross-linked ESCs were pelleted by centrifugation for 5 min
1075 at 1200 g at 4 °C. Nuclei were prepared by washes with NP-Rinse buffer 1 (10 mM Tris pH
1076 8.0, 10 mM EDTA pH 8.0, 0.5 mM EGTA, 0.25 % Triton X-100) followed by NP-Rinse buffer 2
1077 (10 mM Tris pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 200 mM NaCl). Afterwards, the nuclei were
1078 washed twice with shearing buffer (1 mM EDTA pH 8.0, 10 mM Tris-HCl pH 8.0, 0.1 % SDS)
1079 and subsequently resuspended in 900 µL shearing buffer including 1x protease inhibitors
1080 Complete Mini cocktail (Roche). Chromatin was sheared by sonication in 15 ml Bioruptor
1081 tubes (Diagenode, C01020031) with 437.5 mg sonication beads (Diagenode, C03070001) for
1082 6 cycles (1 min on and 1 min off) on a Bioruptor Pico sonicator (Diagenode). ChIP lysates
1083 equivalent to 50 ug DNA were incubated in 1x IP buffer (50 mM HEPES/KOH pH 7.5, 300 mM
1084 NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% DOC, 0.1% SDS), and 1.5 ul of FLAG M2 antibody
1085 (Sigma Aldrich Sigma F1804) overnight. Antibody-bound chromatin was captured using
1086 Dynabeads protein G beads (Thermofisher #10004D) for 4 h at 4 °C. ChIPs were washed 5x
1087 with 1x IP buffer (50 mM HEPES/KOH pH 7.5, 300 mM NaCl, 1 mM EDTA, 1% Triton-X100,
1088 0.1 % DOC, 0.1 % SDS), followed by 3x washes with DOC buffer (10 mM Tris pH 8, 0.25 mM
1089 LiCl, 1 mM EDTA, 0.5 % NP40, 0.5 % DOC) and 1x with TE/50 mM NaCl. ChIP DNA was eluted
1090 twice with elution buffer (1 % SDS, 0.1 M $NaHCO_3$) at 65 °C for 20 min, and subsequently
1091 treated with RNase A (60 ug final, Invitrogen) for 30 min at 37 °C, and Proteinase K (15ug,
1092 NEB) for 3 h at 55 °C and crosslinks were reversed overnight at 65 °C. The following day, ChIP
1093 samples and corresponding inputs were purified by AMPure XP beads (Beckman Coulter
1094 A63880).

1095

1096 ChIP-qPCR primers:

Name	Forward primer sequence	Reverse primer sequence
TetO	AAGATGGGCTGCAGGAATTC	ATACACGCCTACCTCGACATAC
+0.6 kb	GCAGGACGTGACAAATGGAAG	AAAGCGAAGGAGCAAAGCTG
+1.0 kb	GATCCGGACCGCCACATC	ACACCTTGCCGATGTCGAG
+1.9 kb	CAGTGCCACGTTGTGAGTTG	GCCCCTTGTTGAATACGCTTG
IAP	CTCCATGTGCTCTGCCTTCC	CCCCGTCCCTTTTTTAGGAGA

1097

1098 **ChIP-seq**

1099

1100 ChIP-seq was done as described previously[74]. 1.5 µg of CBX8 antibody (Cell Signalling, CBX8
1101 (D2O8C), cat # 14696S) and 3 µg of H3K27me3 antibody (Tri-Methyl-Histone H3 (Lys27)
1102 (C36B11), cat #35861SF) were used. Libraries were prepared using NEBNext ultra II DNA
1103 library kit for Illumina (NEB Biolabs) according to the manufacturer instructions. The

1104 resulting libraries were assessed for quality on a High Sensitivity D1000 Screen Tape
1105 (Agilent) and were sequenced using an Illumina Novaseq 6000 sequencer (Genewiz/Azenta).
1106
1107 **Data processing for Chip-seq**
1108
1109 The reads were quality-trimmed and adapters were removed using Trim Galore!, a wrapper
1110 script to cutadapt[75], in paired end mode and using default settings. The reads were then
1111 aligned to the mouse mm10 genome build using bowtie2 (version 2.3.5)[76] with the option
1112 "very-sensitive". The data was reduced to only properly paired reads using "samtools view"
1113 (Samtools version 1.9) with the flag "-f 3". PCR duplicates were removed using the
1114 RemoveDuplicates function from Picard Tools (version 2.19.0). Read mates were fixed using
1115 samtools fixmate. BigWig files were calculated using BamCoverage (Deeptools version 3.5.2)
1116 with CPM normalisation.
1117 Peaks were called with Macs2 (version 2.1.1)[77] callpeak function, using the input sample as
1118 control. For the CBX8 ChIP-seq data set, default settings with a q-value cut-off of 0.05 were
1119 used. For H3K27me3 ChIP-seq data set, broad mode was used with a q-value and broad
1120 cutoff of 0.001. Peaks overlapping with ENCODE blacklisted regions[78] were removed.
1121
1122 **ATAC-seq**
1123
1124 ATAC-seq was done using a commercial kit (Diagenode Cat.# C01080002) according to the
1125 instructions of the manufacturer. The resulting libraries were assessed for quality control on
1126 a High Sensitivity D1000 Screen Tape (Agilent) and libraries were sequenced using an
1127 Illumina Novaseq 6000 sequencer (Genewiz/Azenta).
1128
1129 **Data processing ATAC-seq**
1130
1131 ATAC-seq data was processed as described previously[79]. Specifically, reads were quality-
1132 trimmed and adapters were removed using Trim Galore!, a wrapper script to cutadapt[75], in
1133 paired end mode using default settings. The reads were then aligned to the mouse mm10
1134 genome build using bowtie2 (version 2.3.5)[76] with the option "very-sensitive". Reads were
1135 sorted and indexed using Samtools (version 1.9). Mitochondrial reads were removed using a
1136 python script from Harvard Bioinformatics (available at
1137 https://github.com/harvardinformatics/ATAC-seq). The data was reduced to only properly
1138 paired reads using "samtools view" with the flag "-f 3". The library complexity was
1139 estimated and the data sets were subsampled to reach a similar complexity as described
1140 previously[79]. Data shown in Fig. 5h and Extended Data Fig 7b were not subsampled because
1141 complexity was nearly identical. PCR duplicates were removed using the RemoveDuplicates
1142 function from Picard Tools (version 2.19.0). Read mates were fixed using samtools fixmate.
1143 BigWig files were calculated using BamCoverage (Deeptools version 3.5.2) with CPM
1144 normalisation

1145 For peak calling the bam files were converted to BEDPE files and the Tn5 shift was corrected
1146 by running a bash script provided at https://github.com/reskejak/ATAC-seq
1147 (bedpeTn5Shift.sh). Files were then converted to minimal bed format and peaks were called
1148 using Macs2 (version 2.1.1)[77] callpeak function in broad mode with broad-cutoff set to 0.05.
1149 Peaks overlapping with ENCODE blacklisted regions[78] were removed. Consensus peaks for
1150 each condition were defined as the intersect of peaks from both biological replicates. Venn
1151 diagrams were generated using the ChIPPeakAnno package[80].
1152
1153
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1157

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