Rapid degradation and 3D CLEM of condensin reveal chromatin compaction uncoupled from chromosome architecture in mitosis

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Abstract:

The requirement for condensin in chromosome formation in somatic cells remains unclear as imperfectly condensed chromosomes do form in conventional condensin-depleted cells. Here we have dissected the role of condensin at different stages of vertebrate mitosis by combining auxin-mediated rapid depletion of condensin subunit SMC2 with chemical genetics to obtain near-synchronous mitotic entry of chicken DT40 cells. We analysed the outcomes by live and fixed-cell microscopy methods, including 3D correlative light and serial block face scanning electron microscopy. Following rapid depletion of condensin, chromosomal defects were obvious. The chromatin was compacted normally, but formed a single mass of mitotic chromosomes clustered at one side of a bent mitotic spindle. Cultures arrest at prometaphase, eventually exiting mitosis without segregating chromosomes. Experiments titrating the auxin concentration suggest a previously unsuspected dual role of condensin, as different condensin levels are required for anaphase chromosome segregation and formation of a normal chromosome architecture.

1 INTRODUCTION:

2 As cells enter mitosis, the chromatin is compacted 2-3-fold in volume as it
3 undergoes a dramatic re-organisation (Lleres et al., 2009; Martin and Cardoso, 2010;
4 Vagnarelli and Earnshaw, 2012). Rod-shaped mitotic chromosomes form and
5 chromosome scaffold proteins are localised along the sister chromatid axes (Paulson
6 and Laemmli, 1977; Lewis and Laemmli, 1982; Earnshaw et al., 1985; Gasser et al.,
7 1986; Saitoh et al., 1994; Samejima et al., 2012). These proteins include
8 topoisomerase II α , chromokinesin KIF4 and SMC2 (Structural Maintenance of
9 Chromosomes 2), a core subunit of both condensin complexes (Earnshaw et al.,
10 1985; Gasser and Laemmli, 1986; Saitoh et al., 1994; Hirano and Mitchison, 1994;
11 Hirano et al., 1997; Ono et al., 2003; Samejima et al., 2012).

12 Loss of condensin leads to chromosome mis-segregation with masses of
13 lagging chromosomes blocking cytokinesis in all the experimental systems tested
14 including yeasts, *D. melanogaster*, nematodes, and vertebrate cells (Saka et al.,
15 1994; Steffensen et al., 2001; Hagstrom et al., 2002; Hudson et al., 2003) (Houlard et
16 al., 2015). Mitotic chromosome structural defects are also commonly observed.
17 However, the extent of those defects varies between different experimental systems
18 (Hirano and Mitchison, 1994; Hirano et al., 1997; Saka et al., 1994; Bhat et al., 1996;
19 Steffensen et al., 2001; Hagstrom et al., 2002; Somma et al., 2003; Hudson et al.,
20 2003; Coelho et al., 2003; Vagnarelli et al., 2006; Maddox et al., 2006; Samoshkin et
21 al., 2009). At one extreme, immuno-depletion of condensins from *Xenopus* egg
22 extracts results in the formation of a decondensed chromosome mass, indicating
23 that condensins are essential for assembly and maintenance of mitotic chromosome
24 structure (Hirano and Mitchison, 1994; Hirano et al., 1997). *S. pombe* temperature-
25 sensitive mutants of condensin subunits also fail to condense chromosomes under
26 restrictive conditions (Saka et al., 1994; Sutani et al., 1999). Meiotic I oocytes
27 depleted of condensin subunits showed massively deformed chromosomes (Houlard
28 2015).

29 At the other extreme, vertebrate cells depleted of condensins using
30 conditional knockouts or siRNA exhibit relatively moderate defects (Hudson et al.,
31 2003; Ono et al., 2003; Ono et al., 2004; Vagnarelli et al., 2006; Samoshkin et al.,
32 2009). Individual chromosomes are seen, but they are wider and appear to lack the

33 structural rigidity seen in wild type chromosomes. These inconsistent phenotypes
34 among different experimental systems pose a “condensin paradox” (Gassmann et al.,
35 2004), suggesting that condensin might not be universally required for mitotic
36 chromosome formation.

37 In the *Xenopus* egg extract experiments, condensin was immuno-depleted
38 before the addition of sperm DNA. In the case of *S. pombe*, condensin was rapidly
39 inactivated by a temperature shift. In contrast, vertebrate condensin was gradually
40 lost by natural turnover over more than one cell cycle after synthesis of new protein
41 was halted. Thus, the milder defects in chromosome assembly seen in vertebrates
42 might be explained by cellular adaptation to the gradual loss and/or incomplete
43 depletion of condensin [see e.g. (Wood et al., 2016)]. Furthermore, the various
44 mitotic defects observed could result from a mixture of primary and secondary
45 effects or even non-mitotic functions of condensin (Hirano, 2016). Together, these
46 observations suggested that rapid depletion of condensin in vertebrate cells might
47 more accurately reveal its true mitotic function(s).

48 This rapid depletion is now possible using an Auxin Inducible Degron (AID)
49 system (Nishimura et al., 2009; Kanemaki, 2013). The plant hormone auxin enhances
50 the affinity of the plant-specific F-box protein OsTIR1 for the AID tag AtIAA17. In the
51 presence of auxin, tagged target proteins become poly-ubiquitylated and are
52 degraded rapidly via the ubiquitin-proteasome pathway. It can take as little as 1 h to
53 deplete a target protein in vertebrate cells. Furthermore, the AID system allowed us
54 to study cells partially depleted of condensin by titrating the amount of auxin
55 (Nishimura 2009). This is difficult or impossible using the TEV protease cleavage
56 mediated condensin inactivation system.

57 A fundamental difficulty with studying mitotic chromosome formation is that
58 chromosome morphology changes on a minute-by-minute basis as cells enter
59 mitosis. However, prophase cells comprise less than 1 % of the population in
60 asynchronous cultures. Thus, cell synchrony protocols are needed. Importantly,
61 commonly used nocodazole or colcemid treatments are not suitable for studying
62 chromosome morphology, as both treatments delay cells in prometaphase and
63 induce hyper-condensation of the chromosomes.

64   An alternative strategy is to synchronise cultures before they enter mitosis.
 65   Regulated CDK1 activity is essential for mitotic entry and progression. Manipulation
 66   of this activity can be used to enrich for mitotic cells (Nurse and Bissett, 1981);
 67   (Vassilev et al., 2006; Smith et al., 2011). To this end, an analogue sensitive cyclin
 68   dependent kinase 1 (CDK1^{as}) system was established to specifically inhibit CDK1
 69   activity (Hochegger et al., 2007). CDK1^{as} has a mutation in the ATP binding pocket
 70   allowing the binding of 1NMPP1, an ATP analogue with a bulky side chain (Bishop et
 71   al., 2000). Addition of 1NMPP1 blocks CDK1^{as} cells in late G2, and upon washout of
 72   the drug, the cells swiftly enter mitosis.

73   Here we have used rapid degradation of SMC2 in highly synchronized
 74   cultures coupled with 3D-CLEM (correlative light and electron microscopy) to probe
 75   the function of condensin in mitotic chromosome structure and segregation. We find
 76   that mitotic chromatin is compacted to the normal extent though the intrinsic
 77   chromosome architecture is faulty in condensin-depleted cells. We also find that
 78   differing levels of condensin are required for normal chromosome architecture,
 79   sister chromatid axis formation and chromosome segregation at anaphase.

80

81 RESULTS:

82 SMC2-AID-GFP cell line to study the mitotic roles of condensin

83 Defining the roles of condensin in vertebrate cells during mitosis has been
84 difficult due to relatively mild defects observed following depletion of condensin
85 subunits in mitotic cells of numerous species, compared to defects seen *in vitro*, in
86 yeasts, in meiotic mouse oocytes and in fly embryos. This could be explained by
87 variable levels of residual condensin in different experimental systems or by the cells
88 adapting to the gradual loss of condensin. We hypothesized that rapid depletion of a
89 key condensin subunit might resolve this issue. In order to test this hypothesis, we
90 decided to modify SMC2 (condensin I and II common subunit) to be rapidly depleted
91 in chicken DT40 cells. This is because both condensin I and II have important roles in
92 mitotic chromosome assembly in chicken DT40 cells similar to human cultured cells
93 (Ono 2003, Green 2012) and also we could directly compare the defects with those
94 of our chicken DT40 conditional SMC2 knockouts (SMC2^{ON/OFF}) (Hudson 2003).

95 Taking advantage of an inducible degron (AID) system, we established the
96 SMC2-AID-GFP cell line expressing a SMC2 cDNA fused with a minimal AID degron
97 and GFP tag as the sole source of SMC2 together with the auxin-responsive F-box
98 protein TIR1 (Figure 1A and see Materials and Methods). SMC2-AID-GFP cells
99 proliferated well only in the absence of auxin (Figure 1B). In contrast, addition of
100 auxin did not affect the growth rate of wild type cells. Mitotic index (MI), mitotic
101 profile and cell cycle profile of the SMC2-AID-GFP cells were similar to the ones of
102 wild type cells without auxin (Figure 1-figure supplement 1A, Figure 2-figure
103 supplement 1C). SMC2-mAID-GFP was expressed at a level similar to the endogenous
104 SMC2 of wild type cells (Figure 1C) and localised correctly along the axis of sister
105 chromatids in living cells (Figure 1D). Prometaphase chromosomes of SMC-AID-GFP
106 cells appeared indistinguishable from those of wild type cells in the absence of auxin
107 (Figure 1G, Figure 1-figure supplement 1B).

108

109 Chromosomes condense into a single mass following rapid SMC2 depletion

110 Auxin addition induced the rapid depletion of SMC2-mAID-GFP in
111 asynchronous cell cultures. After a 3 h auxin treatment, SMC2-mAID-GFP fell to
112 approximately 5% of the wild-type level, the number of GFP-positive cells declined,

113 and the GFP signal was lost (Figure 1C, E, Figure 1-figure supplement 1C). Chromatin
114 of mitotic cells depleted of SMC2 clustered in a compact mass in which single
115 chromosomes could not be resolved (Figure 1E-G, Figure 1-figure supplement 1B).
116 The chromosome morphological defects seen in SMC2-AID-GFP cells treated with
117 auxin were much more severe than those in SMC2^{OFF} cells treated with doxycycline
118 for 30-34 h. SMC2^{OFF} (conventional conditional knockout) chromosomes were wider
119 and fuzzier than controls, but individual chromosomes were still visible (Figure 1G,
120 2Ai, Figure 1-figure supplement 1B). Furthermore, SMC2^{OFF} cells underwent aberrant
121 anaphase with chromosome bridges as previously reported (Figure 1Fiv). In previous
122 reports, chromosome mis-segregation at anaphase was the most prominent
123 phenotypic defect after condensin depletion or inactivation (Saka et al., 1994;
124 Steffensen et al., 2001; Hagstrom et al., 2002; Hudson et al., 2003). In contrast, we
125 were surprised to observe no anaphase or telophase cells following rapid SMC2
126 depletion (Figure 2C).

127   The strong chromosome phenotype observed after rapid condensin depletion
128 made it difficult to determine the stage of mitosis at which the cells arrested. We
129 therefore stained a sub-clone of SMC2-AID-GFP cells, expressing CENP-H-GFP from
130 the endogenous allele, with antibodies to histone H3S10ph and INCENP following
131 treatment with vehicle (ethanol) or auxin for 3 h. The signal intensity of H3S10ph is
132 normally high on prometaphase and metaphase chromosomes and progressively
133 decreases after anaphase onset. INCENP is a scaffolding and regulatory subunit of
134 the chromosome passenger complex that localises on chromosome arms and
135 centromeres in early mitosis, then transfers to the central spindle and cleavage
136 furrow during mitotic exit (Carmena et al., 2012).

137   The H3S10ph level on the SMC2-depleted chromosome mass resembled that
138 on control prometaphase or metaphase chromosomes (Figure 1Ei,iii, 2Aii,iii).
139 Furthermore, INCENP localised on the SMC2-depleted chromosome mass and not on
140 the spindle (Figure 2-figure supplement 1A). These observations suggest that after
141 auxin treatment, SMC2-depleted cells with chromosome clusters were in early
142 mitosis.

143   These results in SMC2-AID-GFP cells treated with auxin revealed that
144 condensin is required to shape and resolve individual mitotic chromosomes in

145 cultured vertebrate cells. In addition, phenotypic differences between SMC2^{OFF} cells
146 and SMC2-AID-GFP cells depleted of SMC2 supported our hypothesis that the milder
147 defects observed in previously reports were due to the gradual loss of condensin.

148

149 **Use of chemical genetics to obtain mitotically synchronised cells lacking condensin**

150 In order to obtain DT40 cultures progressing through mitosis in a
151 synchronous wave, we introduced a CDK1^{as} cDNA into SMC2-AID-GFP cells and then
152 disrupted the endogenous CDK1 alleles using CRISPR/Cas9 technology. 1NMPP1, a
153 bulky ATP analogue, inhibits the mutant CDK1^{as} but no other kinases in these cells.
154 As expected, 1NMPP1 did not inhibit cells from progressing through G₁, S and G₂
155 phases, however, it prevented G₂ cells from entering mitosis, and forced pre-existing
156 mitotic cells to exit mitosis prematurely (Hocheegger et al., 2007). No mitotic cells
157 were detected 30 min after addition of 1NMPP1 (Figure 2-figure supplement 1B).
158 Subsequent washout of 1NMPP1 from the arrested G₂ cells triggered a rapid and
159 near synchronous wave of mitotic entry and exit (Figure 2C, Figure 2-figure
160 supplement 1C).

161 Introduction of the CDK1^{as} circuit into SMC2-AID-GFP cells (termed SMC2-
162 AID-GFP/CDK1^{as} cells) allowed us to investigate the effects of depleting condensin
163 either just prior to mitosis or during a mitotic arrest while avoiding confounding
164 effects from previous cell divisions.

165 A typical experimental time line using SMC2-AID-GFP/CDK1^{as} cells is
166 illustrated in Figure 2B (see Materials and Methods). All control cells (no auxin) were
167 in interphase at T= 0 min. The mitotic index of the control cells increased gradually
168 from T= 15 min, peaked at T= 60 min, then decreased thereafter (Figure 2C).
169 Prophase cells were observed at T= 15 min but were mostly gone by 30 min. Most
170 mitotic cells at T= 30 - 60 min were in prometaphase or metaphase (Figure 2C). Cells
171 in anaphase and telophase were seen at T= 60 – 120 min. Furthermore, G₁ cells
172 were detected 1 h after release from a 1NMPP1 block by DNA content analysis
173 (Figure 2-figure supplement 1C). Thus, mitosis lasts about one hour in control cells
174 after release from a 4 h 1NMPP1 block.

175

**SMC2-depleted cells accumulate at abnormal prometaphase then exit mitosis
without segregating chromosomes**

Control cells exhibited prophase chromosome condensation within 15 min of the 1NMPP1 washout (Figure 2C). In contrast, SMC2-depleted cells did not show chromatin condensation until after nuclear envelope breakdown, as previously reported with RNAi or conditional knockouts of condensin (Hudson et al., 2003; Hirota et al., 2004; Ono et al., 2004).

The lack of prophase condensation could explain the slightly lower mitotic index of SMC2-depleted cells at T= 15 min (Figure 2C). However, the mitotic index caught up with that of control cells by T= 30 - 60 min and continued to increase throughout the time course. No recognizable anaphase or telophase was observed in SMC2-depleted cells over the 2 h time course of this experiment confirming the results in asynchronous culture (Figure 2C, D). Nevertheless huge polyploid interphase and mitotic cells were seen following 2 days auxin treatment (Figure 2-figure supplement 1E), suggesting somehow these cells exited the previous mitosis.

Live cell imaging of SMC2-depleted cells showed that these cells exited mitosis without properly segregating chromosomes (Figure 2E) although control cells did (Figure 2-figure supplement 1D). This can explain why we did not observe anaphase or telophase cells following SMC2-depletion.

SMC2-depleted cells have a malformed mitotic spindle with the chromosome mass to one side

Control chromosomes congress to the middle of a bipolar spindle during late prometaphase and metaphase (Figure 1E, 2Di, 3A, 3B, Figure 3-figure supplement movie 1). In contrast, the single amorphous mass formed by SMC2-depleted chromosomes appeared to be largely excluded from the spindle. A few chromatin fibers protruded from this mass and extended into the spindle with centromeres at their tips (Figure 2Di, 3A, Figure 3-figure supplement movie 2). This suggests that chromosome arms did not follow as their centromeres congressed to the spindle midzone.

The mitotic spindle in SMC2-depleted cells was bipolar but often twisted or asymmetric (Figure 1E, 2D, 3A, 3B, Figure 3-figure supplement movie 2). Mitotic

208 spindles and chromosomes in SMC2-depleted cells did not position properly
 209 between the two centrosomes, which were visualised using PACT-RFP
 210 (pericentrin/AKAP450 centrosomal targeting - Figure 3A, B). In order to quantify the
 211 relative positions of chromosomes relative to the mitotic spindles, angles between
 212 chromosomes and centrosomes in late prometaphase or metaphase cells were
 213 measured using SMC2-AID-GFP/CDK1^{as} cells expressing PACT-RFP (see Materials and
 214 Methods). The angle between the centre of mass of the chromosomes and the two
 215 centrosomes was between 155-180 degrees in most control cells (18 out of 22)
 216 (Figure 3B,C). In contrast, this angle was less than 90 degrees in nearly half of the
 217 SMC2-depleted cells (13 out of 27).

218 Forces exerted by spindle attachments exacerbated the structural defects of
 219 mitotic chromosomes and altered the relative position and shape of the
 220 chromosomes and spindle. When microtubules were depolymerized by nocodazole
 221 treatment in control cells, rod-shaped individual mitotic chromosomes could be
 222 recognized (Figure 3D). In contrast, individual chromosomes could not be resolved in
 223 SMC2-depleted cells treated with nocodazole although the chromosome mass
 224 became more symmetrical and protruding chromatin fibers disappeared.

225 CENP-I is a kinetochore protein whose depletion by RNAi causes defects in
 226 kinetochore assembly, visualized as decreased CENP-T levels at kinetochores (Figure
 227 3E-G). Microtubules do not bind tightly to such compromised kinetochores
 228 (Nishihashi et al., 2002; Hori et al., 2008a). In CENP-I-depleted cells, the
 229 morphologies of SMC2-depleted chromosomes were still aberrant, however the
 230 chromosome mass was symmetrical and overlapped the mitotic spindle, which also
 231 adopted a symmetrical shape (Figure 3G).

232 These experiments confirm that condensin is required to shape mitotic
 233 chromosomes and reveal that mitotic spindle forces further deform the amorphous
 234 SMC2-depleted chromosomes, presumably because SMC2-depleted chromosomes
 235 lack structural integrity in arms and in pericentromere regions as reported previously
 236 (Oliveira et al., 2005; Gerlich et al., 2006; Bouck and Bloom, 2007; Ribeiro et al.,
 237 2009). We also confirmed that lack of structural integrity in SMC2-depleted
 238 chromosomes results in mispositioning of the chromosome mass and spindle
 239 malformations (Wignall SM 2003, Ono T 2004).

240

241 **SMC2-depleted kinetochores fail to make stable attachments to microtubules**

242 The CENP-H-GFP signals on SMC2-depleted kinetochores that stretched out
243 from the chromosome mass were round (21 out of 21 centromeres) and resembled
244 those in control metaphase cells, where correlative light and electron microscopy
245 (CLEM) revealed a typical trilaminar kinetochore structure with attached
246 microtubules (Figure 4A arrows, B). In contrast, the CENP-H-GFP signal on SMC2-
247 depleted kinetochores inside or at the edge of the chromosome mass was often
248 deformed (Figure 4C yellow arrowheads, see also Figure 2A and Figure 3-figure
249 supplement movie 2). Indeed the trilaminar structure was less evident following
250 SMC2 depletion (Figure 4A, B). The shape of kinetochores may be distorted due to
251 lack of structural integrity of the underlying chromatin as previously suggested
252 (Samoshkin 2009).

253 Strong MAD2 signals were seen in almost all SMC2-depleted late
254 prometaphase cells (24 out of 25 cells at 1 h after release from 4 h 1NMPP1 block)
255 (Figure 4C). In contrast, approximately half of control late prometaphase cells
256 showed strong MAD2 signals (13 out of 25 cells) and other cells showed only weak
257 MAD2 signal (11 out of 25 cells). This suggested that these MAD2 positive
258 centromeres lack stable bipolar attachments and that the prometaphase
259 accumulation of SMC2-depleted cells (Figure 2C) might be due to an unsatisfied
260 spindle assembly checkpoint (SAC).

261 Indeed, silencing of the SAC by Mad2 RNAi caused both SMC2-depleted and
262 control cells to enter anaphase with unaligned chromosomes (Figure 4-figure
263 supplement 1). Mitotic exit was confirmed by INCENP staining on the central spindle
264 and by reduced Cyclin B2 staining (Figure 4-figure supplement 1A, Bi,iii,iv).
265 Approximately 80% of SMC2-depleted cells treated with MAD2 siRNA showed highly
266 uneven chromosome segregation with chromosome bridges and centromeres
267 trapped in the intercellular bridge (Figure 4-figure supplement 1A, B, D). Live cell
268 imaging showed that all SMC2-depleted daughter cells treated with MAD2 siRNA
269 fused after an abortive cytokinesis (Figure 4-figure supplement 1E).

270 Thus, SMC2-depleted kinetochores can attach to microtubules but are
271 defective in forming stable amphitelic attachments.

272

273 **SMC2 is required for intrinsic chromosome organization but not for mitotic**
 274 **chromatin compaction**

275 To better understand the consequences of rapid SMC2 depletion on
 276 chromosome morphology we performed an ultrastructural analysis using 3D-CLEM
 277 with SBF-SEM (serial block face scanning electron microscopy) and digital
 278 reconstructions (Booth et al., 2016).

279 To establish a default set of control parameters, the parental wild type DT40
 280 cell line was first analysed. The entire chromosome complement of a metaphase
 281 control cell, was reconstructed from 140 serial sections and modeled using Amira
 282 (Figure 5A). The presence of two pairs of centrioles surrounded by PCM and flanking
 283 the aligned chromosomes confirmed that the cell was indeed in metaphase. Using
 284 separation algorithms we identified 74 discrete chromosome units (Figure 5Av-vi),
 285 with a combined volume of $75 \mu\text{m}^3$ and surface area of $424 \mu\text{m}^2$ (Figure 5D). The
 286 normal DT40 karyotype has 80 chromosomes, though scoring of the smallest
 287 microchromosomes can be difficult (Sonoda et al., 1998; Molnar et al., 2014).

288 A similar analysis was next performed on a cell following rapid SMC2
 289 depletion (Figure 5B). Remarkably, despite the clear disorganization of the chromatin
 290 mass, it was possible to resolve 84 chromosome units (Figure 5C), with a combined
 291 volume of $71 \mu\text{m}^3$ and surface area of $931 \mu\text{m}^2$ (Figure 5D). Interestingly, although
 292 both the chromosome number and combined volumes were similar (74 vs 84 and $75 \mu\text{m}^3$
 293 vs $71 \mu\text{m}^3$), the surface area had more than doubled following SMC2 depletion
 294 ($424 \mu\text{m}^2$ vs $931 \mu\text{m}^2$). This suggests that chromatin compaction measured as total
 295 chromatin volume was near normal, but that the shape of the chromosomes as
 296 revealed by the surface area was severely compromised. By ranking the
 297 chromosomes according to their volumes, we could confirm that individual
 298 chromosomes from the depleted cell had a similar volume, but a far greater surface
 299 area than wild-type chromosomes (Figure 5D).

300 This analysis confirms that, as previously suggested (Vagnarelli et al., 2006),
 301 condensin has a key role in establishing a normal mitotic chromosome architecture,
 302 but is not essential for volume-wise reduction of chromatin (chromatin compaction)
 303 from interphase to mitosis.

304

305 **Condensin is essential for normal intrinsic architecture of condensed mitotic**
 306 **chromosomes**

307 Use of the intrinsic mitotic chromosome structure (IMCS) assay previously
 308 established that condensin is required to establish a normal mitotic chromosome
 309 architecture (Hudson et al., 2003) (Figure 6A). In this assay, cells were treated with
 310 nocodazole for 12 h before exposure to a hypotonic buffer (Figure 6-figure
 311 supplement 1A). To test condensin's role in establishing or maintaining the structural
 312 memory of mitotic chromosomes, respectively, cells were treated with auxin either
 313 for the entire 12 h (SMC2 depleted before mitotic chromosome assembly) or for the
 314 last 2 h (SMC2 depleted after mitotic chromosome assembly). Auxin was omitted
 315 from controls so that SMC2-mAID-GFP was continuously present.

316 For the IMCS assay, chromosomes were subjected to two cycles of unfolding
 317 and refolding, then their shape was scored in one of three categories: reformed,
 318 partially reformed or disorganised (Figure 6A, B). Approximately 90 % of control cells
 319 (minus auxin) showed either reformed or partially reformed chromosomes (Figure
 320 6B, C). In contrast, cells depleted of SMC2 prior to chromosome assembly (auxin for
 321 12 h) exhibited only disorganized chromatin after this treatment. Remarkably, a
 322 similar result was obtained for > 90 % of cells depleted of SMC2 only after mitotic
 323 chromosome formation (auxin for 2 h). Depletion of SMC2-mAID-GFP after auxin
 324 addition was confirmed by immunoblot analysis (Figure 6-figure supplement 1B, C).
 325 Thus, the structural memory of mitotic chromosomes is not maintained in the
 326 absence of condensin, even when SMC2 is degraded *after* mitotic chromosomes
 327 have formed.

328 We next exploited the ability of SMC2-AID-GFP/CDK1^{as} cells to enter mitosis
 329 in a synchronous wave in order to follow the process of chromosome formation in
 330 the presence or absence of condensin. We also characterized chromosomes
 331 depleted of SMC2 during mitosis (Figure 6D, Figure 6-figure supplement 2A).

332 All cells in the culture were in interphase at the end of a 13 h 1NMPP1
 333 treatment ($T \approx 0.5$ h) (Figure 6E). Approximately 80% of those cells entered mitosis
 334 within 1h after 1NMPP1 washout ($T = 1.5$ h). The mitotic index then remained almost
 335 unchanged until nocodazole washout ($T = 4.5$ h).

336 Mitotic chromosomes were readily visible in control cells during nocodazole
337 treatment (Figure 6Ei, 6Gi: T= 1.5 - 4.5 h). Chromosomes began to align at a
338 metaphase plate by 30 minutes after nocodazole washout at T = 4.5 h (Figure 6Ei).
339 Most of these cells exit mitosis at T= ≤6 h, as revealed by a reduction in Cyclin B2
340 levels (Figure 6-figure supplement 2C, Ei, Fi).

341 Depletion of SMC2 after mitotic chromosome formation revealed that
342 condensin is required to maintain chromosome morphology in mitosis. Rod-shaped
343 chromosomes were clearly visible prior to auxin addition at T= 1.5 h, but became
344 much less distinct at T= 2.5 h, within 1 h of auxin addition (Figure 6Eii). After
345 hypotonic treatment, chromosomes appeared normal at T= 1.5 h, but were
346 completely disorganised within 1 h of auxin addition at T= 2.5 h (Figure 6Gii, 6Hii).

347 For cells in which SMC2 had been depleted prior to mitotic entry, mitotic
348 chromosome structure was defective at all time points following release from the
349 1NMPP1 block (T= 1.5 – 4.5 h - Figure 6Eiii). Without hypotonic treatment, these
350 SMC2-depleted mitotic chromosomes appeared as a condensed ball. In cells treated
351 with hypotonic buffer prior to fixation, SMC2-depleted mitotic chromosomes formed
352 a disorganized mass of dispersed chromatin at all time points (Figure 6Giii, 6Hiii).

353 The morphology of SMC2-depleted mitotic chromosomes and subsequent
354 chromosome mis-segregation defects were indistinguishable regardless of whether
355 SMC2 was depleted prior to mitotic entry or during mitosis after chromosomes had
356 formed. This confirms for somatic cells the early observation (Hirano and Mitchison,
357 1994) and recent studies with mouse oocytes and Drosophila embryo (Houlard 2015,
358 Piskadlo 2017), that condensin is necessary to maintain the structure of mitotic
359 chromosomes throughout mitosis.

360

361 **Titration of condensin levels reveals differential requirements for SMC2 in mitotic** 362 **chromosome formation and chromosome segregation**

363 Condensin is required both for maintenance of chromosome architecture and
364 for sister chromatid segregation at anaphase. To further explore the functional link
365 between these two processes, we used auxin treatments to produce cultures with a
366 graded series of condensin concentrations. This was possible because the abundance

of an AID tagged protein can be manipulated by varying the auxin concentration (Nishimura et al., 2009).

Auxin concentrations yielding a range of partial depletions of SMC2-mAID-GFP were selected based on data obtained from flow cytometry, microscopy, and immunoblotting analysis (Figure 7A, B). SMC2-AID-GFP/CDK1^{as} cells were treated with 1NMPP1 for 4 h and with various concentrations of auxin for 3 h. After release from 1NMPP1 to allow entry into mitosis, cells were fixed every 30 min and analysed as in Figure 2. Auxin was omitted from wash and release media apart from 125/250 μ M auxin treated cells because we noticed that in mitotic cells even low auxin concentrations eventually resulted in depletion levels equivalent to those seen with 125/250 μ M auxin treatment. This is likely due to the reduction of protein synthesis during mitosis, such that even a low degradation activity could eventually deplete the AID-tagged protein.

Immunoblot analysis showed that cellular levels of SMC2 fell to ~75% or ~45% of those in wild-type cells after addition of 1.95 μ M or 7.81 μ M of auxin, respectively (Figure 7A). (The absolute levels vary between experiments but the relative levels are preserved.) Reduction of SMC2 by 25% had little impact on either the morphology or segregation of mitotic chromosomes (Figure 7C). However, chromosome segregation defects were observed when SMC2 fell to ~55 % of wild type levels. In those cells, prometaphase chromosomes were slightly wider but individual rod-shaped chromosomes were still discernible. Despite this near normal morphology, some chromatin bridges were observed when the cells entered anaphase and telophase.

We next asked whether the observed defects in sister chromatid segregation were due to defects in the intrinsic structure of the chromosomes or to another function of condensin. We exposed SMC2-AID-GFP/CDK1^{as} cells to 1NMPP1 for 4 h plus various concentrations of auxin for 3 h, then washed out the 1NMPP1 to allow cells to enter mitosis (Figure 8A). At 45 min after 1NMPP1 washout (when cells were in prometaphase), cells were subjected to the IMCS assay as in Figure 6A. Later, at 90 min after 1NMPP1 washout (when cells were in anaphase or telophase), cells were fixed and stained to examine sister chromatid segregation. For cells treated with 125

398 μ M auxin we did not observe typical anaphase or telophase cells, so we instead
399 scored chromatin bridges at cytokinesis.

400 This experiment revealed very clear differences in the threshold
401 requirements for condensin in chromosome architecture and sister chromatid
402 segregation. In the IMCS assay, mitotic chromosome structures reformed in 90% of
403 cells when SMC2 protein levels had fallen to 60% of wild type (15.63 μ M auxin –
404 Figure 8B, D). In contrast, only 45% of cells successfully segregated chromosomes
405 without chromosome bridges under these conditions (Figure 8C, D).

406 Collectively, these results reveal that higher levels of condensin are required
407 for sister chromatid resolution and anaphase chromosome segregation than for
408 formation of a normal mitotic chromosome architecture, as assayed by the IMCS
409 assay.

410

411 Partial depletion of condensin disrupts the chromosome axes

412 Use of the auxin titration series revealed that even under conditions of
413 partial condensin depletion where the IMCS assay reports that chromosome
414 architecture is near normal, the organization of the chromosome axes is strongly
415 affected. When condensin levels have been depleted by as little as 50%, the
416 distribution of condensin and topo II α is disrupted and the proteins appear to diffuse
417 throughout the chromosomes. This cannot be observed in 125 μ M auxin, where
418 condensin is effectively gone and topoisomerase II α is no longer visible on the
419 chromatid (Figure 9A). Diffuse chromosomal localisation of topoisomerase II α has
420 been reported previously following conventional condensin depletion (Hudson 2003,
421 Coelho 2003). The anaphase bridges observed under partial depletion of SMC2 could
422 be explained by incomplete decatination and/or possibly recatination of sister
423 chromatids due to disruption of chromosome axis and diffuse localisation of
424 topoisomerase II α as recently reported (Piskadlo 2017). Furthermore, it could explain
425 why the chromosome segregation defects have been observed as the most
426 prominent and universal defect when condensin depletion occurred with slower
427 kinetics.

428

429 DISCUSSION

430 Mitotic chromosome condensation is a complex process, involving removal of
431 interphase chromatin structures (TADs and compartments), two-to-three-fold
432 compaction of the chromatin, formation of rod-shaped structures and sister
433 chromatid resolution (Vagnarelli, 2012; Naumova et al., 2013). The role(s) of
434 condensin in these processes remain unclear. Here we show that condensin is not
435 required for mitotic chromatin volume compaction and that different threshold
436 levels of condensin are required for establishment of mitotic chromosome
437 architecture and for chromatid segregation in anaphase.

438 There has been a long-standing controversy as to what are the primary mitotic
439 defects after condensin depletion. Prior to the discovery of condensin, it was
440 reported that SMC proteins were needed to both assemble and maintain mitotic
441 chromosomes in *Xenopus* egg extracts (Hirano and Mitchison, 1994). A recent study
442 of mouse meiotic cells and *Drosophila* embryos reached a similar conclusion
443 (Houlard et al., 2015), but in between, many studies of somatic cells reported that
444 rod-shaped chromosomes could form even when condensin had been significantly
445 depleted (Bhat et al., 1996; Steffensen et al., 2001; Hagstrom et al., 2002; Somma et
446 al., 2003; Hudson et al., 2003; Coelho et al., 2003; Vagnarelli et al., 2006; Maddox et
447 al., 2006; Samoshkin et al., 2009). Interestingly, those studies agreed that although
448 chromosomes could form in cells depleted of condensin subunits, anaphase sister
449 chromatid separation was seriously impacted and prominent chromatin bridges
450 were commonly seen.

451 The present study combined use of auxin inducible degron (AID) technology for
452 rapid depletion of condensin with CDK1^{as} alleles to obtain improved synchrony of
453 mitotic entry. SMC2-AID-GFP/CDK1^{as} cells showed more homogenous and more
454 severe defects than those seen in SMC2^{OFF} conditional knockout cells. Interestingly,
455 residual levels of SMC2 were similar in both systems (5-7 % of wild type cells).
456 Importantly, the AID system appears to function particularly well in somatic mitotic
457 cells, where protein translation is reduced. Thus, the residual levels of condensin in
458 mitotic cells may be lower compared to those in G2 phase.

459 We hypothesize that previous discrepancies in chromosome morphology
460 observed with differing experimental protocols did not simply reflect the extent of

condensin depletion, as previously speculated, but instead the time taken to deplete condensin, and the time allowed for cells to up-regulate compensatory pathways.

Use of SMC2-AID-GFP/CDK1^{as} cells allowed us to obtain $>10^8$ cells with $\geq 80\%$ purity in G2, prophase and prometaphase without microtubule inhibitors. We could distinguish the roles of condensin at mitotic entry, during metaphase and during mitotic exit because SMC2 could be depleted just prior to mitotic entry and also within mitosis in a time restricted manner. We found that mitotic chromosomes depleted of condensin after their assembly were morphologically and structurally indistinguishable from chromosomes assembled in the absence of condensin. This is consistent with observations in the *Xenopus* egg extract system (Hirano and Mitchison, 1994). It is tempting to speculate that one essential role of condensin is to lock-in the architecture of mitotic chromosomes and prevent other factors such as topoisomerase II α from promoting aggregation or random (de)catenation of the chromatin.

Two recent studies showed that meiotic chromosomes in mouse oocytes or mitotic chromosomes in early *Drosophila* embryos rapidly depleted of condensin lost structural integrity and were morphologically aberrant (Houlard et al., 2015) (Piskadlo 2017). Importantly in both of these studies and in the present work, the resulting chromatin did not look like interphase, but was highly compacted although the usual rod-like morphology was lost. The three studies thus clearly show that condensin is essential for shaping and maintaining individual mitotic chromosomes *in vivo* in embryos and in somatic cells. These results are consistent with the original studies with *Xenopus* egg extract systems and with studies in *S. pombe* (Saka et al., 1994). Thus, this role of condensin is evolutionally conserved.

In cells rapidly depleted of SMC2, individual chromosomes could not be distinguished by conventional fluorescence microscopy within the single mass of mitotic chromatin. Indeed, the first impression from conventional electron microscopy was of a shattered mass of condensed chromatin. However, ultra-structural analysis using 3D-CLEM with SBF-SEM and digital reconstructions (Booth et al., 2016) successfully resolved the chromosome mass into units that apparently correspond to individual chromosomes. Moreover, the total volume of SMC2-

492 depleted chromosomes was similar to that of wild type chromosomes at metaphase
493 even though the surface area was >4-fold greater.

494 This result confirmed our previous hypothesis that condensin is dispensable for
495 mitotic chromatin volume-wise compaction, but required for structural organization
496 of the condensed chromosomes (Vagnarelli et al., 2006; Samejima et al., 2012).
497 Surprisingly, mitotic chromosomes can still form after conventional depletion of all
498 the three major components of the mitotic chromosome scaffold (condensin,
499 topoisomerase II α and KIF4A) (Samejima et al., 2012). Furthermore, extensive
500 proteomic studies failed to identify any other major structural components of
501 mitotic chromosomes with a likely role in promoting mitotic chromatin compaction
502 (Ohta et al., 2010; Samejima et al., 2015). These results and other studies suggest
503 that chromatin compaction in mitosis is mediated by factors intrinsic to the
504 chromatin, possibly including histone post-translational modifications (Markaki et al.,
505 2009; Georgatos et al., 2009) (Zhiteneva et al., 2017).

506 In addition to the obvious effects on chromosome architecture, rapid depletion
507 of SMC2 led to spindle defects that included abnormal exclusion of chromosomes
508 from the mitotic spindle as well as the shape of the mitotic spindle itself. One
509 characteristic of condensin depletion is the presence of thin chromatin fibers as
510 centromeres stretch out from the bulk of the chromatin. Condensin is essential for
511 establishing the proper elasticity of centromeric heterochromatin, an important
512 determinant of kinetochore movements under tension (Ribeiro et al., 2009;
513 Samoshkin et al., 2009; Jaqaman et al., 2010). The stretched fibers correspond to
514 centromeric heterochromatin that becomes abnormally compliant after condensin
515 depletion. This heterochromatin is stretched by spindle forces acting on
516 kinetochores. Interfering with microtubule attachments at kinetochores restores, to
517 some extent, the chromosome morphology.

518 Regarding the shape of the spindle, it has long been known that tension
519 stabilises kinetochore-microtubule attachments (Nicklas and Koch, 1969; Akiyoshi et
520 al., 2010) (Miller MP et al., 2016). We postulate that either the lack of tension,
521 compromised kinetochore structure, or possibly mis-localised aurora B kinase
522 activity due to the loss of condensin affects kinetochore microtubule attachments.
523 Furthermore, lack of structural integrity in chromosomes appeared to cause their

incorrect positioning relative to centrosomes and the mitotic spindle. It is tempting to speculate that the centromeres are under force from both the mitotic spindle and the bulky chromosome mass. This may in turn produce the abnormal mitotic spindle morphology observed in SMC2-depleted cells and possibly promote incorrect kinetochore-microtubule attachments (Saunders and Hoyt, 1992) (Wignall SM 2003, Ono T 2004).

Availability of an AID degron allele allowed us to examine the effects of creating a hypomorphic series in which levels of SMC2 were lowered to differing extents. The residual amounts of AID tagged proteins are inversely correlated with the auxin concentration (Nishimura et al., 2009). Use of a titration series allowed us to demonstrate that higher levels of condensin are required for successful anaphase sister chromatid separation than for establishing the metaphase chromosome architecture as defined by our IMCS assay. In other experiments, cells rapidly depleted of either condensin I or II complex alone still form morphologically distinct individual mitotic chromosomes but exhibit chromosome segregation defects, indicating the requirement for a higher amount of condensin at anaphase (data not shown). This could explain why severe mitotic chromosome assembly defects were not reported in the experimental systems with gradual depletion of condensin. Failures in cytokinesis during previous cell divisions could have interfered with the observation of morphological defects at the next mitosis.

Why is the threshold amount of SMC2 higher for segregating chromosomes? Partially lowering condensin levels appears to disrupt the orderly distribution of condensin and topo II α along the chromatid axes. This altered distribution of the two proteins may either cause anaphase chromosomes to become mechanically more fragile or interfere with the decatenation of sister chromatids. The existence of different threshold levels of condensin is consistent with the idea that condensin's roles in chromosome condensation and sister-chromatid separation are different (Sullivan et al., 2004; D'Amours et al., 2004).

In summary, we show here that the architecture of mitotic chromosomes in somatic cells is not static but must be maintained by the action of condensin throughout mitosis. In the absence of condensin, chromatin appears to be fully compacted in volume but lacks a normal architecture. Rigidity of mitotic

556 chromosomes is essential for proper kinetochore and microtubule attachments and
557 also for correct shaping and positioning of the mitotic spindle. Wild-type levels of
558 condensin are particularly critical to ensure the completion of sister chromatid
559 separation in anaphase even though the bulk of sister chromatid resolution normally
560 occurs during prophase (Nagasaka et al., 2016).
561

562 MATERIALS AND METHODS

563 Cell culture, transfection, siRNA

564 Chicken DT40 (B lymphoma) cells were cultured in RPMI1640 medium
565 supplemented with 10 % fetal bovine serum and 1% chicken serum at 39°C in 5% CO₂
566 in air.

567 Stable transfection of DT40 cells was performed as described previously
568 (Samejima et al., 2008). We utilized Neon setting 5 (ThermoFisher Scientific) to
569 transiently transfect plasmid DNA (4-10 µg) or siRNA oligonucleotides (2 µl of 100
570 µM) into 2-4 million cells suspended in 100 µl buffer R from the Neon kit. MAD2
571 siRNA duplex oligonucleotides were synthesized: (5'-
572 GAAAGCCAUUCAGGAUGAAAUUCGA -3') and (5'-
573 UCGAAUUUCAUCCUGAAUGGCUUUC -3') (ThermoFisher Scientific). Control
574 oligonucleotide (AllStars Negative Control siRNA) was purchased from QIAGEN.

575

576 Cell lines

577 *SMC2 conditional DT40 knockout (SMC2^{ON/OFF}) cells*: SMC2^{ON/OFF} cells were
578 previously established (Hudson et al., 2003). In brief, the endogenous *SMC2* gene
579 locus (exon 1-6 including the start codon) was disrupted by Histidinol resistance
580 cassette. These cells depend on the expression of a GgSMC2 cDNA driven by a
581 tetracycline suppressible promoter (SMC2^{ON}). Addition of doxycycline stops the
582 expression of SMC2 protein. SMC2 protein was depleted in the cells treated with
583 doxycycline for > 30 h (SMC2^{OFF}).

584 *SMC2-AID-GFP DT40 cells*: SMC2-AID-GFP cells were based on the SMC2^{OFF} cells
585 which were constantly cultured in the presence of doxycycline (0.5 µg/ml) to so that
586 non-tagged GgSMC2 protein was not present in the cells. GgSMC2 cDNA fused with
587 minimal AID (mAID) tag (AtIAA17⁶⁵⁻¹³²) (Natsume et al., 2016) and GFP tag (SMC2-
588 mAID-GFP) at the C-terminus was driven by a 3.8 kb fragment of the GgSMC2
589 promoter (Hudson et al., 2003) . SMC2-mAID-GFP was present as the sole source of
590 SMC2 protein in SMC2-AID-GFP cells (Figure 1A). A CMV promoter drove the
591 expression of plant specific F-box protein OsTIR1 linked to a MmDHFR cDNA by a T2A
592 peptide (synthesized at ThermoFisher Scientific and cloned into pCDNA3). 10 µM
593 Methotrexate (MTX) was used to select cells expressing MmDHFR as well as OsTIR1

at a high level. Plasmids coding SMC2-mAID-GFP and OsTIR1 were randomly integrated into the genome.

CDK1^{as} DT40 cells: CMV promoter drove the expression of the XlCdk1^{as} cDNA (Hochegger et al., 2007) linked to a puromycin resistance gene by a T2A peptide. The GgCdk1 gene was inactivated by transient transfection of plasmids encoding hCas9 cDNA (addgene #41815) and a GgCdk1 guideRNA (based on addgene #41824). The target sequence of the guide RNA was AAAATACGTCTAGAAAGTG.

Where desired, a CENP-H-GFP targeting vector (gift of Yasunari Takami, University of Miyazaki) was used to tag the endogenous CENP-H locus in DT40 cell lines. PACT-RFP construct is a gift of Viji Draviam (University of Cambridge).

Antibodies and drug treatments

Antibodies used for immunoblotting and indirect immunofluorescence analysis were: rabbit anti-SMC2 (1:500) (Saitoh et al., 1994), guinea pig anti-Ggtopo 2 α (1:1000) (Earnshaw et al., 1985), rabbit anti-GgCENP-T (1:1,000) (Hori et al., 2008b), and rabbit anti-MAD2 (1: 100 gift of T. Fukagawa), rabbit-anti GgcyclinB2 (gift of Eric Nigg), mouse anti- α -tubulin B512 or DM1A (1:1,000-4,000; Sigma-Aldrich), mouse anti-Cdk1 monoclonal A17 (1: 200-500; Abcam), and rabbit anti-histone H3 phospho Ser 10 (D2C8) (1:1600, Cell Signaling).

Nocodazole dissolved in DMSO was added to a final concentration of 0.5 μ g/ml (Sigma-Aldrich). Doxycycline dissolved in water was added to a final concentration of 0.5 μ g/ml (BD) unless stated otherwise. 1NMPP1 dissolved in DMSO was added to a final concentration of 2 μ M. Indole-3-acetic acid (auxin) dissolved in ethanol was added to a final concentration of 125 μ M unless stated otherwise (Fluka).

Use of CDK1^{as} system

Typically CDK1^{as} cells were treated with 2 μ M 1NMPP1 for 4 h to accumulate cells in G₂ phase. 1NMPP1 is a bulky ATP analogue that reversibly and specifically inhibits analogue sensitive kinase mutants. 1NMPP1 treatment longer than 4 h results in an increase of multipolar spindles due to centrosome amplification during the G₂ block. However, CDK1^{as} cells blocked at G₂ phase for more than 10 h could still enter mitosis synchronously after 1NMPP1 washout without any apparent problems

626 apart from the multipolar spindle. In order to release from the 1NMPP1 block, cells
627 were washed two to three times with fresh media.

628

629 Indirect immunofluorescence of fixed cells

630 Cells were fixed in pre-warmed 4 % Formaldehyde/PBS for 10 min and
631 permeabilized with 0.15 % triton for 5-10 min. Cells were blocked with 5 % BSA/PBS
632 for 30 min. 1st antibody diluted in the blocking buffer was applied to the cells for 1 h.
633 Cells were washed with PBS 3x for 5 min. 2nd antibody diluted in the blocking buffer
634 (1:500-1:1000, Alexa Fluor 488, 555 or 594, 647 (Molecular Probes, ThermoFisher
635 Scientific) was applied to the cells for 30 min. Cells were washed with PBS for 5 min
636 three times. DNA was stained with Hoechst33342 and mounted with Prolong
637 diamond (Molecular Probes, ThermoFisher Scientific).

638 **DeltaVision microscopy:** 3D datasets were acquired using a cooled CCD
639 camera (CoolSNAP HQ; Photometrics) on a wide-field microscope (DeltaVision
640 Spectris; Applied Precision) with a 100x NA 1.4 Plan Apochromat lens. The datasets
641 were deconvolved with softWoRx (Applied Precision), converted to Quick Projections
642 in softWoRx, exported as TIFF files, and imported into Adobe Photoshop for final
643 presentation.

644 **3D structured illumination microscopy (3D-SIM):** Super-resolution images
645 were acquired using structured illumination microscopy. Samples were prepared on
646 high precision cover-glasses (Zeiss, Germany). 3D SIM images were acquired on a N-
647 SIM (Nikon Instruments, UK) using a 100x 1.49NA lens and refractive index matched
648 immersion oil (Nikon Instruments). Samples were imaged using a Nikon Plan Apo
649 TIRF objective (NA 1.49, oil immersion) and an Andor DU-897X-5254 camera using
650 405, 488 and 561nm laser lines. Step size for Z stacks was set to 0.120 μ m as
651 required by manufacturers software. For each focal plane, 15 images (5 phases, 3
652 angles) were captured with the NIS-Elements software. SIM image processing,
653 reconstruction and analysis were carried out using the N-SIM module of the NIS-
654 Element Advanced Research software. Images were checked for artefacts using the
655 SIMcheck software (<http://www.micron.ox.ac.uk/software/SIMCheck.php>). Images
656 were reconstructed using NIS Elements software (Nikon Instruments) from a z stack
657 comprising at least 1 μ m of optical sections. In all SIM image reconstructions the

658 Wiener and Apodization filter parameters were kept constant. 3D datasets were
659 visualized and analysed using Imaris V8.4 (Bitplane, Oxford Instruments, Oxfordshire,
660 UK).

661 **Zeiss Airyscan microscopy:** Images of chromosome scaffold proteins on
662 chromosome spreads were obtained using the Airyscan module on a Zeiss LSM 880
663 confocal, using a x100 alpha Plan-Apochromat objective. Hoechst 33342 was
664 detected using a 405 nm Diode laser and a 420-480 nm bandpass emission filter,
665 Alexa488 was detected using the 488 nm line of an Argon laser and a 495-550 nm
666 bandpass emission filter, the Alexa555 was detected using a HeNe561 laser and a
667 570 nm long pass emission filter. Alexa647 was detected using a 633 laser and a 605
668 nm long pass emission filter. Step size for Z stacks was set to 0.145 μm . 3D datasets
669 were visualized and analysed using Imaris V8.4 (Bitplane, Oxford Instruments,
670 Oxfordshire, UK).

671

672 **Live cell imaging with Zeiss Airy microscopy**

673 Cells were centrifuged and suspended into Leibovitz's L-15 Medium (Thermo
674 Fisher Scientific) supplemented with 10% FBS and 1% Chicken serum. DNA was
675 visualised by 1 μM SiR DNA (Spirochromome). GFP was detected using the 488 nm
676 line of an Argon laser and a 495-550 nm bandpass emission filter, RFP was detected
677 using a HeNe561 laser and a 570 nm long pass emission filter. SiR DNA was detected
678 using a 633 laser and a 605 nm long pass emission filter. Step size for Z stacks was set
679 to 0.4 μm . Images were taken every 5 min. 3D datasets were visualized and analysed
680 using Imaris V8.4 (Bitplane, Oxford Instruments, Oxfordshire, UK).

681

682 **Intrinsic mitotic chromosome structure (IMCS) assay**

683 Cells were enriched in mitosis either by 12h nocodazole treatment (0.5 $\mu\text{g}/\text{ml}$)
684 (Figure 6A-C) or by 1NMPP1 treatment (Figure 8A, B, D). Auxin was added at the
685 indicated time points (Figure 8A). The cells were plated on polylysine-coated slides
686 for 30 min before the following treatments. The slides were rinsed with PBS,
687 immersed in TEEN buffer for 5 min (1 mM triethanolamine/HCl, pH 8.5, 0.2 mM
688 NaEDTA, and 25 mM NaCl), before immersion into RSB buffer for 15 min (10 mM
689 Tris/HCl, pH 7.4, 10 mM NaCl, and 5 mM MgCl_2). Following two cycles of TEEN

690 buffer/RBS buffer treatments, the cells were fixed with 4% Formaldehyde in RBS
691 buffer and mounted with Vectashield (containing DAPI). The final chromosome
692 morphologies were classified as reformed (green), partially reformed (yellow) or
693 disorganised (magenta).

694

695 **Quantitative immunoblotting**

696 Membranes were incubated with the relevant primary antibodies recognizing
697 α -tubulin (as a loading control), SMC2, and MAD2, then subsequently with IRDye-
698 labeled secondary antibodies (LI-COR Biosciences). Fluorescence intensities were
699 determined using a CCD scanner (Odyssey; LI-COR Biosciences) according to the
700 manufacturer's instructions.

701

702 **Flow cytometry analysis**

703 GFP-positive and -negative living cells were analyzed using a FACSCalibur flow
704 cytometer following the manufacturer's instructions.

705 DNA contents: Cells were fixed with ice-cold 70% ethanol for overnight. These
706 cells were rinsed with PBS then resuspended with PBS containing 100 μ g/ml RNase A
707 and 5 μ g/ml Propidium iodide and were analyzed using a FACSCalibur flow
708 cytometer following the manufacturer's instructions.

709

710 **Measurement of the angle of chromosome mass and centrosomes**

711 SMC2-AID-GFP/CDK1^{as} cells expressing PACT-RFP was treated with 1NMPP1 for
712 4 h with/without a 3 h auxin treatment. 60 min after 1NMPP1 washout, cells were
713 fixed in pre-warmed 4 % Formaldehyde/PBS for 10 min. DNA was stained with
714 Hoechst33452 and mounted with Prolong diamond (Molecular Probes, ThermoFisher
715 Scientific). Pictures of late prometaphase and metaphase cells were taken using Zeiss
716 Airy microscopy as described above. (X, Y, Z) coordinates of the centre of
717 homogenous mass of PACT-RFP signals (X_1 , Y_1 , Z_1) and (X_3 , Y_3 , Z_3), and chromosomes
718 (X_2 , Y_2 , Z_2) were obtained using Imaris V8.4 (Bitplane, Oxford Instruments,
719 Oxfordshire, UK). The angle of chromosome mass and centrosomes was calculated
720 based on the coordinates.

721

722 **SBF-SEM**

723 ***Preparation of cells***

724 Cells were seeded onto gridded dishes (MatTek) and fixed with 3 %
 725 glutaraldehyde and 1% paraformaldehyde in 0.1 M sodium cacodylate buffer for 1 h
 726 at RT. Cells were then washed with PBS 3 X 5 min and samples prepared for SBF SEM
 727 (West et al., 2010). Extra contrasting steps were introduced, compared to those used
 728 for standard TEM to reduce charging and improve the signal to noise ratio. In detail,
 729 following fixation, the cells were post fixed and stained with reduced osmium, (2 %
 730 osmium tetroxide in ddH₂O + 1.5 % Potassium Ferrocyanide in 0.1M sodium
 731 cacodylate buffer) for 1 h at RT. This was followed by 0.1 % tannic acid in ddH₂O for
 732 20 min at RT. A second osmication step (2 % in ddH₂O for 40 min at RT), preceded an
 733 overnight incubation in aqueous 1 % uranyl acetate at 4°C. The next day cells were
 734 stained with Walton's lead aspartate (0.02 M in lead nitrate + 0.03 M in aspartic acid
 735 in ddH₂O, adjusted to pH 5.5) for 30 min at 60°C. To prevent precipitation artefacts
 736 the cells were washed for a minimum of 5 x 3 min with ddH₂O between each of the
 737 staining steps described. Next, samples were dehydrated in a graded ethanol series
 738 of 30 %, 50 %, 70 %, 90 % in ddH₂O for 5 min each, followed by 2 x 5 min 100%
 739 ethanol. Samples were then infiltrated with TAAB Hard Premix resin at ratios of 1:1,
 740 2:1 and 3:1 with resin: 100 % ethanol, 30 min per incubation. Finally, samples were
 741 incubated in 100 % resin for 2 x 30 min, before embedding the whole dish in 2 mm of
 742 100 % fresh resin. Samples were cured for 48 h at 60°C.

743 ***Preparation of blocks for 3View SBF-SEM***

744 Resin is separated from the gridded dish by trimming away the excess plastic
 745 and carefully sliding a razor between the dish and the resin (Booth et al., 2013).
 746 Excess resin is removed using a junior hacksaw and scalpel before the block is
 747 mounted onto a cryo-pin, cell side up using superglue. Targeted trimming is
 748 performed using an ultra-microtome and etched coordinates (Booth et al., 2013).

749 ***SBF-SEM Imaging and Acquisition***

750 Samples were painted with Electrodag silver paint (avoiding the block face) and
 751 then coated with 10nm AuPd using a Q150T sputter coater (Quorum Technologies).
 752 The sample was inserted into the Gatan 3View sample holder and adjusted so the
 753 block face would be central in the microtome and parallel with the knife-edge.

Consecutive sectioning and imaging was performed. To obtain a typical resolution of 12 nm in x and y, a frame width of 1024 x 1024 was used. Section thickness was 60 nm over 200-400 sections.

3D reconstruction, modeling and segmentation.

3View EM stacks were annotated using Amira (FEI). Chromosomes present in every orthoslice were annotated using *masking* and *thresholding* alone (fully automated) or in combination with *magic wand* and *blow* tools (semi automated).

The modelled complement of chromosomes was segmented into discernable isolated objects using *interactive thresholding* and *separate objects* modules. Objects were separated using 3D interpretation and a neighbourhood criteria of 26 connected elements, by at least one corner, edge or face. The marker contrast range (*H-extrema*) was set between 5 and 7, depending on the sample. *Label analysis* modules were used to measure the geometry of all isolated structures. Surface renders were generated using unconstrained smoothing at levels 5-7.

1NMPP1 Analogue Synthesis:

Synthesis of the ATP analog 4-amino-1-tert-butyl-3-(1'-naphthylmethyl)pyrazolo[3,4-d] pyrimidine (1NM-PP1) was carried out in five steps as described by (Hanefeld et al., 1996) with the following modifications. The starting material was 1-naphthaleneacetic acid and the first two steps were carried out under N₂. In Step 2, triethylamine was used in place of NaH and the product 1-naphthaleneacetyl malonylnitrile was recrystallized from acetonitrile and chloroform. The enol ether product of Step 3 was purified on a silica gel column (elution with 3:1 hexane:ethyl acetate) and stored under N₂. The final product, a white solid that gave a single spot on TLC, was purified similarly and further recrystallized from ethyl acetate. Its identity was confirmed by ¹H NMR, HH COSY and high resolution mass spectrometry. ¹H NMR (270 MHz, CDCl₃) gave peaks at 1.84 (singlet, 9H), 4.75 (singlet, 2H), 4.85 (broad singlet, 2H), 7.18 (doublet, 1H), 7.38 (triplet, 1H), 7.54 (multiplet, 2H), 7.79-7.92 (multiplet, 2H), 8.22 (doublet, 1H) and 8.24 (singlet, 1H), in close agreement with published data (Bishop et al., 1999). Mass spectrometry gave a mass of 331.181712 for the molecular ion (calculated mass for

785 $C_{20}H_{21}N_5$, 331.179696). The solid compound was stored at 4°C. Aliquots were
786 dissolved in DMSO at 25 mM and stored at $\pm 20^\circ\text{C}$.
787

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796

797 **Author contributions**

798 KS, experimental design, data acquisition, analysis and interpretation, wrote the
799 manuscript; DB electron microscopy, manuscript revision; HO, MP and CW helped to
800 make cell lines. JP and LX synthesized 1NMPP1; MK contribution of new
801 experimental tools and manuscript revision; WCE, experimental design, data analysis
802 and interpretation, co-wrote the manuscript.

803

804 **Conflicts of interest**

805 The authors declare that they have no competing interests.

806

807

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FIGURE LEGENDS

Figure 1. Rapid depletion of SMC2-mAID-GFP upon auxin treatment results in a severely defective mitotic chromosome formation. (A) Diagram introducing SMC2-AID-GFP cells. Auxin addition recruits the miniAID tag to the SCF^{TR1} complex, resulting in rapid degradation of SMC2-mAID-GFP via the proteasome pathway. **(B)** Growth curve of wild type cells and SMC2-AID-GFP cells treated with either ethanol (Et: solvent) or 125 μ M auxin (Au). Error bars show standard deviation. **(C)** Immunoblot analysis of asynchronous wild type and SMC2-AID-GFP cells. SMC2-AID-GFP cells were treated with 125 μ M auxin for 0 – 3 h. SMC2 and SMC2-mAID-GFP were detected with anti-SMC2 antibody. α -tubulin was a loading control. **(D)** Live cell imaging of a SMC2-AID-GFP cell by Zeiss Airy microscope. DNA was stained with SiR-DNA. SMC2-mAID-GFP concentrated along the axis of sister chromatids. Scale bar 1 μ m. **(E)** SMC2-AID-GFP cells treated with ethanol (solvent) (i, ii) or auxin for 3 h (iii, iv) were fixed with formaldehyde and stained for α -tubulin (green), and Histone 3 phospho-Ser10 (red) and DNA (blue) . The GFP signal was undetectable (iii, iv) and the shape of mitotic chromosomes (iii) was highly abnormal in SMC2-depleted cells. Scale bar 5 μ m. **(F)** SMC2^{ON/OFF}/CENP-H-GFP cells (Vagnarelli et al., 2006) converted to CDK1^{as} were treated with doxycycline for 0 or 30 h, stained in metaphase and anaphase for DNA (blue), CENP-H-GFP (green) and α -tubulin (red). **(G)** Wild type/CDK1^{as} cells (0 or 3 h auxin treatment), SMC2-AID-GFP/CDK1^{as} cells (0 or 3 h auxin treatment) cells and SMC2^{ON/OFF}/CDK1^{as} cells (0 or 34 h doxycycline treatment) were fixed with cold Methanol/Acetic acid and stained for DNA. More pictures in Figure1-figure supplement 1B. Scale bar 5 μ m.

Figure 2. SMC2-depleted cells accumulate at prometaphase with a single chromosome mass. (A) Phosphorylation of Histone 3 Serine10 (red) was not affected by SMC2 depletion. DNA shown in blue and CENP-H-GFP in green. Interphase cells (i), prophase cells (ii) and prometaphase cells (iii). Control: SMC2^{ON}/CDK1^{as}/CENP-H-GFP cells. SMC2-depleted: SMC2-AID-GFP/CDK1^{as}/CENP-H-GFP cells. **(B)** Experimental timeline for auxin treatment. After 1NMPP1 washout, SMC2-AID/CDK1^{as} cells were fixed with 4 % formaldehyde and stained with corresponding antibodies. **(C)** Cell cycle and morphological analysis. > 200 cells were counted for

each time point for each treatment treated as in (B). Average of three independent experiments. Error bars represent standard deviation (SD). **(D)** Representative pictures of control cells and SMC2-depleted cells at different stages of mitosis. DNA (blue), CENP-T (red), α -tubulin (green). Coloured box at the bottom right corners of merge panels correspond to the stages of mitosis in (C). SMC2-depleted mitotic cells have a single chromosome mass adjacent to a malformed mitotic spindle. Stretched chromatin fiber (arrow in j, right). Normal prometaphase-like chromosomes were occasionally observed within the auxin-treated cell population but all these cells were SMC2-mAID-GFP positive. **(E)** Stills from live-cell imaging of SMC2-AID-GFP/CDK1^{as} expressing Pericentrin/AKAP450 centrosomal targeting (PACT)-RFP treated with auxin 3 - 6 h. DNA was stained with SiR-DNA. 3D image stacks were collected at 0.4- μ m z increments on a Zeiss Airy microscope every 5 mins. SMC2-AID-GFP/CDK1^{as} cells exited mitosis without apparent chromosome segregation. Chromosomes appeared to be decondensed and centrosome positioned close by in later time points (xii-xiv) Control (no auxin) is shown in Figure 2-figure supplement 1D. Scale bars 5 μ m.

Figure 3. Malformed and mis-positioned chromosomes and mitotic spindle in SMC2-depleted cells. **(A)** SMC2^{ON}/CDK1^{as}/CENP-H-GFP cells and SMC2-AID-GFP/CDK1^{as}/CENP-H-GFP (treated with auxin) were fixed with formaldehyde and stained for α -tubulin (red) and DNA (blue). Images were acquired by structural illumination microscopy. **(B)** SMC2-AID-GFP/CDK1^{as} expressing PACT-RFP was treated with 1NMPP1 for 4 h with or without auxin for 3 h. These cells were fixed with formaldehyde 1 h after 1NMPP1 washout and stained for α -tubulin (green), CENP-T (white), DNA (blue). Cells were 3D-rotated so that two PACT-RFP signals overlap (i, iii) or position in parallel (ii, iv). Note: Signals appeared to be fuzzier and stretched due to 3D-rotation. **(C)** SMC2-AID-GFP/CDK1^{as} expressing PACT-RFP was treated as (B) and stained for DNA. Angles of chromosomes and centrosomes were calculated as described in Materials and Methods. Control (n= 22), SMC2-depleted (n= 26). P value was <0.0001 (two-tailed Mann-Whitney U Test). **(D)** Control and auxin-treated cells treated with nocodazole for 1 h and fixed with formaldehyde. DNA (blue), CENP-T (green), H3S10ph (red). **(E)** Experimental timeline for CENP-I depletion. **(F, G)**

SMC2-AID-GFP/CDK1^{as} cells were transfected either with control siRNA oligo **(F)** or CENP-I oligo **(G)**. Cells were further treated with ethanol or auxin. The chromosome mass became symmetrical in SMC2-depleted cells when kinetochore binding to microtubules was perturbed. CENP-T signals were enhanced in (i) in order to visualise the position of kinetochores. Non-enhanced images are also shown (ii). DNA (blue), CENP-T (red), α -tubulin (green) Scale bars 5 μ m.

Figure 4. Compromised kinetochore-microtubule attachments in SMC2-depleted cells. **(A)** Correlative light and transmission electron microscopy of control and SMC2 depleted cells. Images show three progressive zooms of the white-boxed region, for TEM (i), light microscopy of DAPI (red) and CENP-H-GFP (green) (ii) and a correlative overlay of both the physical and optical sections (iii). Arrow in zoom 2 points to a clearly defined trilaminar structure. Scale bars A - 3 μ m, 1 μ m, 200nm for overview, zoom 1 and zoom 2 respectively. **(B)** Enlargement of kinetochores shown in Figure 4. Microtubules (yellow lines) and kinetochore (green) were overlaid on the TEM pictures, and show microtubule attachment. Scale bar 200 nm. **(C)** SMC2^{ON}/CDK1^{as}/CENP-H-GFP cells and SMC2-AID-GFP/CDK1^{as}/CENP-H-GFP (treated with auxin) were treated with 1NMPP1 for 4 h and fixed with formaldehyde 1 h after 1NMPP1 washout. Strong MAD2 staining on the centromeres inside of the SMC2-depleted chromosome mass (yellow arrowheads) is not observed on centromeres outside of the chromosome mass (white arrows). DNA (blue), MAD2 (red), CENP-H-GFP (green). Scale bar 5 μ m.

Figure 5. 3D-EM shows that condensin is required for chromosome organization, but not for chromatin compaction. WT and condensin depleted cells were prepared for 3D SBF-SEM analysis. **(A)** A control cell with all chromosomes modeled (indigo) is shown at different angles, with and without the EM orthoslice (i – iv). Centrioles (green) and Pericentriolar material (orange) are also shown. Chromosomes were separated into individual units (multi-coloured) (v-vi). **(B)** A condensin depleted cell with all chromosomes modeled (red), shown at different angles, with and without the EM orthoslice (i –iii, v-vii). Separated chromosomes traversing an EM orthoslice (iv and viii). **(C)** SMC2-depleted chromosomes were separated into discrete units. **(D)**

1126 2D scatter plot of surface area versus volume for individual chromosomes in A
1127 (control, green) and B (condensin depleted, magenta). Scale bar 5 μ m.

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1129 **Figure 6. SMC2 is required to maintain the structure of mitotic chromosomes. (A)**

1130 The intrinsic mitotic chromosome structure (IMCS) assay. Cells were blocked in
1131 mitosis with nocodazole. Removal of cations in hypotonic buffer induced the
1132 unfolding of chromatin to the level of 10 nm fibres. Addition of Mg^{2+} triggered the
1133 shrinking and refolding of chromatin. The shape of chromosomes after two cycles of
1134 unfolding and folding was classified as reformed (green), partially reformed (yellow)
1135 or disorganised (magenta). **(B)** Representative images of DAPI-stained mitotic
1136 chromosomes at the end of the assay. Scale bar 5 μ m. **(C)** Quantification of
1137 chromosomes at the end of the assay. The graph shows the mean and SD of three
1138 independent experiments with > 50 cells/each treatment counted. **(D)** Experimental
1139 procedure for determining the role of condensin in establishing and maintaining
1140 mitotic chromosome structure. Cultures were treated with 1NMPP1 for 13 h. After
1141 1NMPP1 washout, cultures were treated with nocodazole for 4 h to hold the cells in
1142 mitosis ($T = 0.5 - 4.5$ h). The mitotic index reached approximately 80 % after 1 h of
1143 nocodazole addition and did not change until nocodazole washout at 4.5 h. Cells
1144 were fixed with formaldehyde at indicated time points and stained with DAPI before
1145 > 100 cells/each treatment's time point were counted. Mean and SD of three
1146 independent experiments are shown at F and Figure 6-figure supplement 2B-D. **(E)**
1147 Cells were fixed with formaldehyde/PBS without hypotonic treatment at the
1148 indicated time points and stained with DAPI. **(F)** The amount of SMC2 remaining was
1149 measured by immunoblot analysis and normalized to the level of α -tubulin. **(G, H)**
1150 Cells were treated with 75 mM KCl at 37 $^{\circ}$ C for 5 min prior to ice-cold
1151 methanol/acetic acid fixation and DAPI staining.

1152

1153 **Figure 7. Cells partially depleted of SMC2 show chromosome mis-segregation. (A)**

1154 The amount of SMC2 remaining in asynchronous culture upon 4 h auxin treatment
1155 was measured by immunoblot analysis and normalized to the level of α -tubulin.
1156 Mean and SD of three independent experiments are shown. **(B)** Representative
1157 images of SMC2-AID-GFP cells at prometaphase upon 4 h auxin treatment with

1158 various concentrations of auxin. SMC2-mAID-GFP (green). Scale bar 5 μ m. **(C)** Effect
1159 of various auxin concentrations on mitotic progression of SMC2-AID-GFP cells.
1160 Experimental procedure as in Figure 2B. Cells were fixed every 30 min after 1NMPP1
1161 washout. Auxin was omitted from the media used for the wash and release except in
1162 the case of cells treated with 250 μ M auxin. Mean and SD of three independent
1163 experiments. > 200 cells were counted for each time point of individual treatments.
1164

1165 **Figure 8. Differing amounts of condensin are required for chromosome assembly**
1166 **and for segregation. (A)** Experimental protocol. **(B)** 45 min after 1NMPP1 washout,
1167 cells were subjected to the IMCS assay. Representative images are shown. > 100
1168 cells/each treatment were counted. Mean and SD of three independent
1169 experiments. **(C)** 90 min after 1NMPP1 washout, cells were fixed and stained with
1170 anti α - tubulin antibody and DAPI. Representative images are shown. > 20 anaphase
1171 or telophase cells/each treatment were counted. Cultures treated with 125 μ M auxin
1172 did not show anaphase/telophase cells, so cells in early cytokinesis (iv) were
1173 analysed. Mean and SD of three independent experiments. **(D)** Overlay of key
1174 phenotypes from the experiments of (B, C) plotted against the residual levels of
1175 SMC2-mAID-GFP measured by immunoblotting and normalized to the level of α -
1176 tubulin in cells treated with the indicated concentration of auxin.

1177
1178 **Figure 9 Effects of lowering condensin levels on chromosome structure and**
1179 **segregation (A)** SMC2-AID-GFP/CDK1^{as} cells were treated with 1NMPP1 for 13 h and
1180 with the indicated amounts of auxin for the final 3 h. Auxin was omitted from media
1181 used for the wash and release except for cells treated with 125 μ M auxin. 30 min
1182 after 1NMPP1 washout, cells were collected, rinsed with PBS and fixed with cold
1183 methanol/acetic acid. DNA (blue), SMC2 (red), and Top2 α (green). Scale bar 5 μ m. **(B)**
1184 Diagram summarizing the changes in protein distribution seen in (A). **(C)** Summary of
1185 the effects of differing condensin levels on mitotic DT40 cells. DNA (blue),
1186 centromeres (green), mitotic spindle (red).
1187
1188

1189 **Figure supplement:**

1190 **Figure 1-figure supplement 1. Characterisation of SMC2-AID-GFP cell line. (A)**

1191 Asynchronous wild type/CDK1^{as} cells and SMC2-AID-GFP/CDK1^{as} cells were fixed
1192 with formaldehyde and stained with DNA (blue), α -tubulin (green) and H3S10ph
1193 (red). 500 cells were counted for each sample. Mean and SD from three independent
1194 experiments are shown. **(B)** Cells were treated as Figure 1G. Scale bar 5 μ m. **(C)** Flow
1195 cytometry analysis of GFP positive cells treated with auxin for 0-3 h.

1196

1197 **Figure 2-figure supplement 1. Mitotic cells depleted of SMC2. (A)** INCENP (red)

1198 localises on chromosomes in early mitosis in SMC2-AID-GFP/CDK1^{as}/CENP-H-GFP
1199 cells both treated or not treated with auxin. Control (no auxin) and SMC2-depleted
1200 (auxin 3 h). **(B)** Asynchronous wild type/CDK1^{as} cells were treated with 1NMPP1 for 0
1201 or 30 min and stained for DNA (blue) and H3S10ph (red). **(C)** Analysis of DNA content
1202 of wild type/CDK1^{as} cells (wt) and SMC2-AID-GFP/CDK1^{as} cells using flow cytometry.
1203 Cells from asynchronously growing culture (i), 4 h block and release from 1NMPP1
1204 (ii), or 4 h block and release from 1NMPP1 with auxin (iii) were analysed as described
1205 in Materials and Methods. Positions corresponding cells with 2N DNA (blue arrow) or
1206 4N DNA (red arrow) are shown. **(D)** Stills from live-cell imaging of SMC2-AID-
1207 GFP/CDK1^{as} expressing Pericentrin/AKAP450 centrosomal targeting (PACT)-RFP
1208 (control). DNA was stained with SiR-DNA. 3D image stacks were collected at 0.4- μ m z
1209 increments on a Zeiss Airy microscope every 5 mins. SMC2-AID-GFP/CDK1^{as} cells
1210 exited mitosis (v-x). Stills of an SMC2-depleted cell (auxin) is shown in Figure 2E. **(E)**
1211 SMC2-AID-GFP cells treated with auxin for 2 days. Scale bars 5 μ m.

1212

1213 **Figure 3-movie supplement.**

1214 3D reconstruction of control (1) and SMC2-depleted cells (2) shown in Figure 3A
1215 taken by 3D-SIM microscopy. DNA (blue), CENP-H-GFP (green), α -tubulin (red).

1216

1217 **Figure 4-figure supplement 1. SMC2-depleted cells exit mitosis with highly uneven**

1218 **chromosome segregation after Mad2 RNAi (A)** INCENP concentrates on central

1219 spindle in cells exiting mitosis. Separate tubulin image is shown. Panels show (i) no
1220 auxin, control siRNA; (ii) auxin, control siRNA; (iii) no auxin, Mad2 siRNA; (iv) auxin,

1221 Mad2 siRNA. DNA (blue), CENP-H-GFP + SMC2mAID-GFP (green), INCENP (red), α -
 1222 tubulin (grey). Scale bar 5 μ m. **(B)** Cyclin B2 levels fall in cells exiting mitosis.
 1223 Separate tubulin image is shown. Panels show (i) no auxin, control siRNA; (ii) auxin,
 1224 control siRNA; (iii) no auxin, Mad2 siRNA; (iv) auxin, Mad2 siRNA. DNA (blue), CENP-
 1225 H-GFP + SMC2-mAID-GFP (green), Cyclin B2 (red), α -tubulin (grey). Scale bar 5 μ m.
 1226 **(C)** Immunoblot analysis showing MAD2 depletion by specific siRNA. **(D)**
 1227 Even/uneven chromosome segregation was scored for >50 anaphase or telophase
 1228 cells per sample. Mean and SD from three independent experiments are shown. **(E)**
 1229 Quantification of live cell imaging. Cells were transiently transfected with Histone
 1230 H2B-RFP.

1231

1232 **Figure 6-figure supplement 1. ICMS assay after nocodazole block (A)** SMC2-AID-GFP
 1233 cells were treated with nocodazole for 12 h. Auxin was added at the indicated time
 1234 points. **(B)** Depletion of SMC2 was confirmed by immunoblot analysis. **(C)**
 1235 Quantification of SMC2 relative to α -tubulin based on (B). Mean and SD from three
 1236 independent experiments are shown.

1237

1238 **Figure 6-figure supplement 2. Mitotic exit of cells depleted of SMC2. (A)**
 1239 Experimental protocol. **(B)** The relative amount of SMC2 was measured by
 1240 immunoblot analysis using anti-SMC2 and anti α -tubulin antibodies. **(C)** The relative
 1241 amount of CyclinB2 was measured by immunoblot analysis using anti-Cyclin B2 and
 1242 anti α -tubulin antibodies. **(D)** Cells were fixed with 4 % formaldehyde at the time
 1243 points indicated and stained by DAPI before counting. > 100 cells/sample were
 1244 counted. For B-D, mean and SD from three independent experiments are shown. **(E)**
 1245 Cells were fixed with formaldehyde in PBS without hypotonic treatment at the
 1246 indicated time points and stained with anti-tubulin (red), Cyclin B2 antibodies (grey)
 1247 and DAPI (blue). CENP-H-GFP + SMC2-mAID-GFP are green. Scale bar 5 μ m.

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