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1 **Impaired Condensin Complex and Aurora B kinase underlie mitotic and**
2 **chromosomal defects in hyperdiploid B-cell ALL**

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36
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49

50 **KEY POINTS:**

51

- 52 • HyperD-ALL shows a delay in early mitosis at prometaphase associated to defects in
53 chromosome alignment and segregation.
- 54 • Impaired condensin complex leads to defective AURKB, triggering chromatid cohesion
55 defects and mitotic slippage of HyperD-ALL blasts.

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57

58 **ABSTRACT**

59

60 B-cell acute lymphoblastic leukemia (B-ALL) is the most common pediatric cancer, and high-
61 hyperdiploidy (HyperD) identifies the most common subtype of pediatric B-ALL. Despite HyperD
62 is an initiating oncogenic event affiliated to childhood B-ALL, the mitotic and chromosomal
63 defects associated to HyperD B-ALL (HyperD-ALL) remain poorly characterized. Here, we have
64 used 54 primary pediatric B-ALL samples to characterize the cellular-molecular mechanisms
65 underlying the mitotic/chromosome defects predicated to be early pathogenic contributors in
66 HyperD-ALL. We report that HyperD-ALL blasts are low proliferative and show a delay in early
67 mitosis at prometaphase, associated to chromosome alignment defects at the metaphase plate
68 leading to robust chromosome segregation defects and non-modal karyotypes. Mechanistically,
69 biochemical, functional and mass-spectrometry assays revealed that condensin complex is
70 impaired in HyperD-ALL cells, leading to chromosome hypocondensation, loss of centromere
71 stiffness and mis-localization of the chromosome passenger complex proteins Aurora B Kinase
72 (AURKB) and Survivin in early mitosis. HyperD-ALL cells show chromatid cohesion defects and
73 impaired spindle assembly checkpoint (SAC) thus undergoing mitotic slippage due to defective
74 AURKB and impaired SAC activity, downstream of condensin complex defects. Chromosome
75 structure/condensation defects and hyperdiploidy were reproduced in healthy CD34+
76 stem/progenitor cells upon inhibition of AURKB and/or SAC. Collectively, hyperdiploid B-ALL is
77 associated to defective condensin complex, AURKB and SAC.

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80

81 **INTRODUCTION**

82 B-cell acute lymphoblastic leukemia (B-ALL) is characterized by the accumulation of abnormal
83 immature B-cell precursors (BCP) in the bone marrow (BM), and is the most common pediatric
84 cancer¹. B-ALL is a heterogeneous disease with distinct biological-prognostic subgroups
85 classified according to the stage at which BCPs are stalled in differentiation and
86 cytogenetic/molecular markers¹. Importantly, different biological subtypes of B-ALL have distinct
87 causal mechanisms and show different clinical outcomes¹⁻³.

88

89 High hyperdiploid B-ALL (HyperD-ALL) is the most common subtype of childhood B-ALL and is
90 characterized by the presence of 51-67 chromosomes in leukemic cells⁴⁻⁷. HyperD-ALL
91 comprises ~30% of pediatric B-ALL and usually has a favourable clinical outcome^{5,8,9}. Of note,
92 the distribution of chromosome gains is not random and preferentially show gains of
93 chromosomes X, 4, 6, 10, 14, 17, 18 and 21^{5,10}. Hyperdiploidy is an initiating oncogenic event in
94 B-ALL and secondary alterations necessary for clinical B-ALL accumulate subclonally and
95 postnatally^{5,7,11,12}. Despite hyperdiploidy is the most common B-ALL in children, very little is
96 known about its etiology and pathogenesis, and many questions about the biology of HyperD-ALL
97 remain unanswered. Despite its favourable clinical outcome, a precise knowledge of the
98 physiopathogenic mechanisms underlying HyperD-ALL is necessary because in absolute
99 numbers, the morbidity/mortality associated to HyperD-ALL still represent a clinical challenge.

100

101 HyperD-ALL is proposed to arise in a BCP *in utero*⁷. However, the causal molecular mechanisms
102 of hyperdiploidy in BCPs remain elusive. In this sense, faithful chromosome segregation is
103 essential for maintaining the genomic integrity of eukaryotic cells, and deficient chromosome
104 segregation leads to aneuploidy and cancer¹³⁻¹⁵. Three main and non-mutually exclusive
105 mechanisms, interconnected with mitosis¹⁶, underlie chromosome missegregation: (i) defects in

106 bipolar spindle formation, (ii) defects in chromosome structure and function, and (iii) defects in the
107 spindle assembly checkpoint (SAC) which controls proper mitosis until chromosomes are properly
108 attached to the spindle¹⁴⁻¹⁶. Indeed, SAC defects have been proposed to be an underlying
109 pathogenic mechanism in rare cases of ETV6/RUNX1+ B-ALL with near-tetraploid karyotypes¹⁷.
110 Therefore, abnormal mitotic control in BCP could be at the origin of hyperdiploidy in B-ALL.

111

112 Here, we used a large cohort of primary pediatric B-ALL samples (n=54) to gain insights into the
113 cellular and molecular mechanisms underlying mitotic/chromosome defects predicated to be at
114 the origin of pediatric HyperD-ALL. Our data reveal that HyperD-ALL blasts show robust
115 condensin complex defects and defective Aurora B kinase (AURKB) activity leading to abnormal
116 mitotic progression and chromosome missegregation. Functional inhibition of AURKB and the
117 SAC in normal hematopoietic stem/progenitor cells (HSPCs) reproduced hyperdiploid karyotypes
118 with abnormal chromosome structure. We conclude that defects in condensin complex, AURKB,
119 and SAC are associated to HyperD-ALL, likely representing a pathogenic mechanism.

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134 **METHODS**

135 **Pediatric B-ALL leukemic samples and cell lines**

136 Diagnostic BM samples from B-ALL pediatric patients were obtained from collaborating hospitals.
137 B-ALL diagnosis was based on FAB and WHO classifications¹. **Table S1** summarizes main
138 clinico-biological data of the patients. Fetal tissue was collected from developing embryos aborted
139 at 18 to 22 weeks of pregnancy, obtained from the MRC/Wellcome Trust Human Developmental
140 Biology Resource upon informed consent and approval by our local ethics committee. The B-ALL
141 cell lines SEM, REH and MHH-CALL-2 (DMSZ, Germany) were used for confirmatory studies.
142 This study was approved by our Institutional Review Ethics Board (Barcelona Clínic-Hospital,
143 HCB/2014/0687), and patient samples were accessed upon informed consent.

144

145 ***In vivo* expansion of B-ALL blasts**

146 All experimental procedures were approved by the Animal Care Committee of the Barcelona
147 Biomedical Research Park (DAAM7393). Primary blasts (5×10^5) were expanded *in vivo* in
148 sublethally irradiated 8-to-14-week-old non-obese diabetic/LtSz-scid IL-2Ry^{-/-} mice (NSG) upon
149 intra-BM transplantation¹⁸. PB was monitored by FACS for leukemia engraftment. Primografts
150 were sacrificed when engraftment reached 10-15% in PB, typically representing >80%
151 engraftment in BM. Blasts were isolated from BM and spleen by density-gradient centrifugation
152 for downstream analysis. For FACS analysis of leukemic engraftment, PBMCs were stained with
153 anti-human HLA-ABC-FITC, CD19-PE and CD45-APC antibodies (BD-Biosciences), and
154 analysed using a FACS Canto-II cytometer.

155

156 **Indirect immunofluorescence**

157 B-ALL cells were spun on poly-L-lysine-coated coverslips (500g, 3min) before fixation. Cells were
158 fixed and permeabilized with Triton X100-containing buffer (5min). Cells were blocked with
159 permeabilization-buffer containing 1-3%BSA (1hour, 37°C), and incubated overnight at 4°C with

160 primary antibodies (**Table S2**). Cells were washed with permeabilization-buffer, and incubated
161 (45min) with fluorophore-conjugated secondary antibodies (Jackson Labs). All antibodies were
162 diluted in blocking-buffer. Slides were mounted with Vectashield-DAPI (VectorLab). Details of
163 chromosome spreading for immunofluorescence are provided in **Supplementary Methods**.

164

165 **Confocal microscopy and image acquisition**

166 Microscope images were captured using a fully-equipped Zeiss LSM880 laser-scanning spectral
167 confocal microscope equipped with an AxioObserver Z1 inverted microscope. DAPI, Alexa-488,
168 Alexa Fluor-555 and Alexa Fluor-647 images were acquired sequentially using 405, 488, 561 and
169 633 lasers, dichroic beam splitters, emission detection ranges of 415-480nm, 500-550nm, 571-
170 625nm and 643-680nm, respectively, and the confocal pinhole was set at 1 Airy units (AU). An
171 acoustic optical beam splitter was used at the same emission detection ranges. Spectral
172 detection was performed using two photomultipliers and one central GaAsP detector used for the
173 acquisition of Alexa-647. Images were acquired in a 1024x300pixels format, zoom was set at 2,
174 pixel size at 53x53nm and dwell time at 0.51 μ sec. Z-stacks were acquired at a 300nm step size
175 to reconstruct the entire nuclei volume. Immunofluorescence signal quantification was performed
176 using FIJI-ImageJ (NIH). Details of image quantifications are provided in **Supplementary**
177 **Methods**.

178

179 Details for *in vitro* culture/expansion of B-ALL cells and HSPCs, chromosome function and
180 cytogenetic analysis, fluorescence quantification, western-blot and protein analysis, mass-
181 spectrometry (MS) assays, cell cycle and phosphor-H3S10 quantification, RT-PCR, chromatin
182 immunoprecipitation and RNA-sequencing are provided in **Supplementary Methods and Tables**
183 **S3 and S4**.

184

185 **Statistical analysis**

186 Statistical comparisons were performed using Graphpad Prism. Mean values and their standard
187 error of the mean (s.e.m) were calculated for each variable. All data was analyzed according to
188 the test indicated in the appropriate figure legends on the indicated number of experiments. Non-
189 HyperD were compared with HyperD B-ALLs. $P < 0.05$ was considered statistically significant.

190

191

192 **RESULTS**

193 **HyperD-ALL cells are low proliferative and show a delay in early mitosis**

194 Aneuploid cells typically display a gene signature characterized by an up-regulation of genes
195 involved in oxidative stress response, membrane functions and immune response regulation
196 coupled to a down-regulation of genes involved in cell proliferation and nucleic acid metabolism¹⁹⁻
197 ²¹. We first analysed the transcriptomic signature of HyperD-ALL blasts using a RNA-Seq dataset
198 from HyperD-ALL patients (n=58) and non-HyperD B-ALL patients (n=30, non-aneuploid, **Suppl**
199 **methods**)²². A Gene Ontology analysis of the 26,239 genes differentially expressed between
200 primary HyperD-ALL and non-HyperD B-ALLs confirmed an aneuploidy-like gene expression
201 signature characterized by the up-regulation of pathways associated with oxidative stress, protein
202 turn-over, cell death, immune system activation and membrane functions (**Fig 1a,b**), and down-
203 regulation of pathways associated to nucleic acid metabolism and tRNA biology (**Fig 1a,c**).
204 Consistent with this, we found that the aneuploid pediatric B-ALL cell line CALL-2 (doubled-up
205 hypodiploid karyotype: 51XX,+X,+18,+der(18)t(15;18),+21,+21)²³ exhibits a significantly lower
206 proliferative rate than the non-HyperD-ALL cell lines SEM and REH (**Fig 1d**). Mechanistically,
207 CALL-2 cells revealed a 5-fold increase in apoptosis coupled to an accumulation in G2/M (**Fig**
208 **1e,f**), indicating cell division/mitotic defects in aneuploid B-ALL cells.

209

210 To further characterize the mitotic progression of HyperD-ALL cells, we stained CALL-2, SEM
211 and REH for DAPI, tubulin, pericentrin and the anti-centromere antibody (ACA) to unequivocally
212 identify the different mitotic phases²⁴ (**Fig 1g**). Consistent with the FACS data,
213 immunofluorescence (IF) analysis revealed that CALL-2 cells accumulated in early mitosis,
214 specifically at prometaphase/metaphase, with a concomitant delay in late mitosis (**Fig 1h,i**).

215

216 **Chromosome alignment defects in prometaphase underlie the mitotic delay of HyperD-ALL**
217 **primary blasts**

218 Cancer cell lines do not phenocopy faithfully the molecular complexity of the disease.
219 Furthermore, despite CALL-2 is the only childhood HyperD-ALL cell line available, it actually
220 represents a doubled-up hypodiploid B-ALL cell line²³. We thus aimed to analyse the
221 proliferation/mitotic defects in childhood HyperD-ALL primary blasts. Because leukemic primary
222 cells fail to expand *ex vivo*²⁵⁻²⁷, we co-cultured B-ALL blasts with nestin+ fetal BM (FBM)-derived
223 MSCs which support short-term proliferation of primary B-ALL blasts without compromising clonal
224 composition²⁸ (**Fig 2a, Fig S1a,b**). Similar to cell lines, non-HyperD-ALL primary cells grown on
225 Nestin+ FBM-MSCs expanded 5-fold over a 6-day period while HyperD-ALL primary blasts failed
226 to expand at all *ex vivo* (**Fig 2b**). FACS analysis using BM diagnostic samples also showed an
227 increase in both apoptosis and frequency of G2/M cells in primary HyperD-ALL cells (**Fig 2c,d**).
228 To further link proliferative impairment with mitotic defects, we analysed the mitotic progression in
229 dividing B-ALL primary blasts expanded *in vivo* in NSG mice (**Fig 2e, S2a**). Xenografted blasts
230 were processed and IF-stained for the DNA-Kinetochorespindle staining to unambiguously
231 identify each mitotic phase (**Fig 2f**). Consistent with cell lines, HyperD-ALL primary blasts
232 accumulated in early mitosis, at prometaphase/metaphase, with a concomitant delay in late
233 mitosis (telophase and cytokinesis; **Fig 2g, Fig S2b**).

234

235 Because chromosomes align at the metaphase plate in the prometaphase-to-metaphase
236 transition, we then inspected dividing cells in early mitosis to distinguish between prometaphase
237 (non-aligned chromosomes) and metaphase (aligned chromosomes) cells. We observed a robust
238 decrease of HyperD-ALL blasts with aligned metaphase plates (**Fig 2h**), confirming chromosome
239 alignment defects in early mitosis in HyperD-ALL cells. Chromosome alignment relies on the
240 dynamics of chromosome biorentation to the spindle poles²⁹. We thus analysed the dynamics of
241 chromosome biorientation in primary B-ALL blasts by generating monopolar spindles and
242 prometaphase arrest with monastrol (a spindle bipolarity inhibitor), followed by treatment with the

243 proteasome inhibitor MG132 which further arrests dividing cells in metaphase³⁰ (**Fig 2i**).
244 Strikingly, non-HyperD-ALL primary blasts properly aligned chromosomes at the metaphase plate
245 in ~90% of the metaphases (**Fig 2j,k**). However, HyperD-ALL samples showed a massive
246 decrease in the number of cells with aligned chromosomes at the metaphase plate (~45%) (**Fig**
247 **2j,k**). Noteworthy, the defective chromosome biorientation in HyperD-ALL cells were confirmed in
248 B-ALL cell lines (**Fig S3a**). Thus, the impaired proliferation of HyperD-ALL blasts may result from
249 mitotic defects in prometaphase-metaphase due to aberrant chromosome alignment at the
250 metaphase plate.

251

252 **Chromosome misalignment defects results in chromosome missegregation and non-** 253 **modal karyotypes in HyperD-ALL blasts**

254 Because defects in chromosome alignment often result in chromosome missegregation²⁴, we
255 next analysed the rates of chromosome segregation defects, mainly lagging chromosomes and
256 anaphase bridges, in B-ALL blasts (**Fig 3a**). To overcome the accumulation of HyperD-ALL cells
257 in early mitosis, B-ALL primary blasts were treated with the cytokinesis inhibitor Blebbistatin³¹. We
258 found that HyperD-ALL primary blasts displayed a significant ~4-fold higher number of late
259 mitosis with chromosome segregation defects (**Fig 3b**).

260

261 Since chromosome missegregation leads to aneuploidy, we analysed the modal karyotype
262 distribution in 12 diagnostic B-ALL samples. We found a high chromosome stability in non-
263 HyperD B-ALL samples, with a modal chromosome number of 46 in >80% of the metaphases
264 analysed (**Fig 3c,d, Fig S4**). However, HyperD-ALL blasts showed an increased karyotype
265 instability defined by the presence of a major clone (40% of the cells) and minor clones with non-
266 modal chromosome distributions (**Fig 3c, d, Fig S4**). These results were confirmed by FISH
267 analysis for chromosomes 12 and 21 (**Fig 3e, f**). Chromosome instability in HyperD-ALL cells was

268 further confirmed in B-ALL cell lines (**Fig S3b,c**). Collectively, chromosome alignment defects
269 result in chromosome segregation defects and subsequent non-modal karyotypes in HyperD-ALL
270 blasts.

271

272 **HyperD-ALL blasts show chromosome hypocondensation and loss of centromere**
273 **stiffness due to condensin complex defects**

274 We next investigated the mechanisms leading to these mitotic/chromosome defects. IF analysis
275 of the spindle using the DNA-Kinetochore-Spindle staining showed similarly low frequency of
276 mitotic blasts with spindle abnormalities such as multipolar or disorganized spindles in non-
277 HyperD- and HyperD-ALL (**Fig 4a**). Moreover, defects in bipolar spindle formation frequently lead
278 to cytokinesis defects and tetraploidization³². Since no (near)-tetraploid cells were observed in
279 any HyperD-ALL patient (**Fig 3c,d, Fig S4**), we ruled out defects in spindle formation in HyperD-
280 ALL blasts.

281

282 We next assessed whether chromosome structure and function underlies the mitotic defects
283 observed in HyperD-ALL. We first examined the chromosome morphology of Carnoy-fixed
284 pediatric B-ALL blasts (**Table S1**). While non-HyperD-ALL metaphases mostly showed normal
285 rod-shaped chromosomes, ~60% of HyperD-ALL metaphases displayed curly-
286 shaped/hypocondensed chromosomes with irregular borders (**Fig 4b, S5a**). Importantly,
287 intrapatient comparison of diploid normal hematopoietic cells *versus* hyperD blasts confirmed that
288 chromosome hypocondensation specifically occurs in HyperD-ALL cells (10% vs 70%, **Fig S5b**).
289 Chromosome hypocondensation defects were further confirmed in B-ALL cell lines (**Fig S5c**). We
290 next scored formaldehyde-fixed B-ALL samples for chromosome structure/condensation, and
291 found significantly more metaphases containing hypocondensed and unstructured fuzzier and

292 wider chromosomes in HyperD-ALL than in non-HyperD-ALL blasts (80% vs 20%, $p<0.001$, **Fig**
293 **4c,d, S5d**), excluding an impact of the fixative on chromosome structure/condensation defects.

294

295 Condensin complexes are major components of the chromosome scaffold that regulate
296 chromosome compaction and higher-order chromatin organization during mitosis³³⁻³⁶. The
297 chromosome structure/condensation defects observed in HyperD-ALLs, prompted us to analyse
298 in chromosome spreads from primary B-ALL samples the binding pattern of SMC2, a major
299 component of condensin complexes. Non-HyperD-ALL blasts showed a normal beaded pattern
300 for SMC2, spreading along the chromatids with a centromere enrichment (**Fig 4e**). In contrast,
301 SMC2 was hardly detectable in neither chromatids nor centromeres, and showed an abnormal
302 staining pattern in HyperD-ALL blasts (**Fig 4e**). Indeed, 3D image quantification of SMC2 staining
303 revealed a significantly lower volume of SMC2 in chromosomes from HyperD-ALL blasts (**Fig 4f**,
304 **Fig S5e**).

305

306 To further characterize the defects in condensin complexes in HyperD-ALL samples, we analysed
307 by WB in PDX-expanded B-ALL cells, the distinct protein members specific for each of the two
308 human condensin complexes (complex I and II), which play a differential contribution to mitotic
309 chromosome organization/segregation (**Fig 4g**). Protein analysis confirmed the lower levels of
310 SMC2 in HyperD-ALL blasts, and revealed that both condensin complexes (CAPD2 and CAPD3)
311 were similarly affected (**Fig 4h,i**). Strikingly, however, no differences were observed at the RNA
312 level for any of the condensin complex members between HyperD-ALL and non-HyperD-ALL
313 samples (**Fig S5f**), suggesting that post-translational modifications (PTM) may underlie
314 condensin complex defects in HyperD-ALL blasts.

315

316 Compelling data strongly suggest that PTMs are essential for regulating condensin loading to
317 chromosomes^{37,38}. We thus performed MS analyses for both SMC2 acetylation and CAPD2
318 phosphorylation levels after immunoprecipitation of the condensin complexes with anti-SMC2
319 (**Fig S5g**), and found increased levels of both SMC2 acetylation and CAPD2 phosphorylation in
320 HyperD-ALL samples (**Fig 4j-l**), suggesting that PTMs regulating condensin activation may
321 represent a mechanism underlying condensin defects in HyperD-ALL blasts.

322

323 Condensin complexes set the stiffness of the centromeric chromatin required for withstand the
324 spindle pulling forces during metaphase²⁹, and SMC2 depletion results in increased
325 intercentromeric distances in metaphase chromosomes^{39,40}. To analyse centromere stiffness in
326 HyperD-ALL blasts, we measured the intercentromeric distances between sister-kinetochore
327 pairs in metaphase-arrested blasts (**Fig 4m**). For this, B-ALL blasts were metaphase-arrested
328 with chromosomes under tension (non-relaxed length) or without tension (rest length) from the
329 spindle, by using either the proteasome inhibitor MG132 or the microtubule depolymeriser
330 colcemid, respectively. HyperD-ALL blasts consistently displayed a significant longer
331 intercentromeric distance than non-HyperD-ALL blasts (**Fig 4n,o**). Collectively, PTMs of
332 condensin members may induce a defective condensin complex which leads to high-order
333 chromosome organization defects and impaired centromere stiffness/stretching at metaphase in
334 HyperD-ALL blasts.

335

336 **Kinetochores are normal in HyperD-ALL blasts**

337 The kinetochore binds microtubules at centromeres and regulates chromosome segregation⁴¹.
338 CENP-A (centromere-specific histone H3) and NDC80/NUF-2 are key centromere chromatin
339 markers of the inner and outer kinetochore plate, respectively, which control kinetochore's
340 assembly. CENP-A overlaps with the condensin complex and it is flanked by the heterochromatin

341 histone marks H3K9me3 and H3K27me3⁴²⁻⁴⁷. We thus prompted to study whether the condensin
342 defects observed in HyperD-ALL blasts are associated to centromeric chromatin defects and
343 destabilization of the kinetochore. CHIP-qPCR assays showed no differences of CENP-A,
344 H3K9me3 or H3K27me3 levels at centromeres of HyperD-ALL blasts as compared to non-
345 HyperD-ALL blasts (**Fig S6a**). Moreover, quantitative confocal microscopy analysis revealed very
346 similar levels of NUF-2 between HyperD-ALL and non-HyperD-ALL blasts (**Fig S6b,c**), indicating
347 that despite impaired centromere stiffness, the centrochromatin is not epigenetically impaired and
348 the kinetochore forms normally in HyperD-ALL blasts.

349

350 **The chromosome passenger complex (CPC) proteins AURKB and Survivin are mis-**
351 **localized from the inner-centromere in early mitotic HyperD-ALL blasts**

352 We next aimed to analyse the cellular mechanisms by which the impaired condensin complex
353 leads to mitotic/chromosome defects in HyperD-ALL blasts. We first analysed the CPC, a protein
354 complex composed by AURKB and the accessory subunits Survivin, Borealin and INCENP. The
355 CPC regulates the SAC to ensure proper kinetochore-microtubule attachment, and was shown
356 mis-localized from the inner-centromere in cells with defective condensin complex^{34,48,49}.
357 Prometaphase-arrested nonHyperD-ALL primary blasts revealed a normal chromosomal
358 distribution of both AURKB and Survivin mainly concentrated in the inner-centromere (**Fig 5a,b**).
359 However, HyperD-ALL blasts showed an aberrant chromosomal distribution of both AURKB and
360 Survivin, diffusely distributed throughout the chromosome arms rather than concentrated in the
361 inner-centromere (**Fig 5a,b**). Quantification of both AURKB and Survivin at the inner-centromeres
362 confirmed a significant decrease of both CPC proteins in centromeres of HyperD-ALL blasts (**Fig**
363 **5c,d, S7a,b**). To further characterize the localization of AURKB and Survivin, we analysed the
364 frequency of chromosomes showing either centromeric or scattered localization (**Fig 5e**), and
365 confirmed that HyperD-ALL blasts preferentially showed scattered localization throughout the

366 chromosome arms (**Fig 5f,g**). These results were reproduced using B-ALL cell lines (**Fig S7c,d**).
367 Of note, the overall chromosome-wide expression levels of both AURKB and phospho-histone H3
368 at Serine 10 (H3S10p), the major readout of AURKB activity, were significantly reduced in
369 HyperD-ALL blasts (**Fig 5h,i**). Finally, we generated condensin complex I-defective non-HyperD-
370 ALL cell lines by knocking down CAPD2, and confirmed the mis-localization of AURKB from the
371 inner-centromere (**Fig S7e,f**). Taken together, mis-localization of the CPC proteins AURKB and
372 Survivin in early mitosis represents a major mechanism linking defective condensin complex and
373 chromosome alignment/segregation defects in HyperD-ALL blasts.

374

375 **Defective AURKB is associated with loss of chromatid cohesion and SAC impairment in** 376 **HyperD-ALL blasts**

377 The confined localization of AURKB at the inner-centromere is essential for chromatid cohesion
378 and proper SAC activity^{30,50,51}. Indeed, analysis of chromosome spreads revealed that 75% of the
379 metaphases from HyperD-ALL blasts displayed “rail-road chromosomes”, a common phenotype
380 reflecting premature chromatid separation (PCS) due to reduced chromatid cohesion at
381 centromeres (**Fig 5j, S8a**). These results were reproduced using B-ALL cell lines (**Fig S8b**).

382

383 AURKB controls chromosome biorientation/alignment at the metaphase plate through
384 phosphorylation of different SAC proteins^{30,50,52,53}. We thus reasoned that AURKB defects may
385 underlie the progression towards late mitosis of HyperD-ALL blasts with mis-aligned
386 chromosomes by preventing SAC activation. To test this, we co-cultured B-ALL primografts on
387 Nestin+ FBM-MSC in the presence of nocodazol, which generates persistently unattached
388 kinetochores leading to SAC activation and mitotic accumulation (**Fig 5k**), and found that
389 nocodazol-treated HyperD-ALL blasts did not accumulate in mitosis as efficiently as non-HyperD-
390 ALL blasts (**Fig 5l,m**). These results were reproduced in B-ALL cell lines (**Fig S8c**). Cell cycle

391 analysis further confirmed that HyperD-ALL blasts do not arrest in G2/M after nocodazol
392 treatment but they accumulate in G0/G1 (**Fig 5n,o**), strongly suggesting mitotic slippage.
393 Apoptosis was not different between HyperD-ALL and non-HyperD-ALL blasts (**Fig S8d**).
394 Noteworthy, the metaphase-to-anaphase promoting regulator MAD2L2, whose loss leads to
395 accelerated mitosis and mitotic aberrations⁵⁴ was found downregulated in HyperD-ALL blasts (**Fig**
396 **5p**). Collectively, HyperD-ALL cells show chromatid cohesion defects and undergo mitotic
397 slippage most likely due to defective AURKB and impaired SAC activity, downstream of
398 condensin complex defects.

399

400 **Inhibition of AURKB and SAC in CD34+ HSPCs reproduces chromosome structure defects** 401 **and hyperdiploid karyotypes**

402 We next prompted to functionally model whether defective AURKB and impaired SAC activity
403 could reproduce the phenotype observed in HyperD-ALL primary blasts. Because hyperD-ALL
404 was shown to have a prenatal origin and pre-leukemic hyperdiploid precursors are found at
405 birth^{55,56}, we exposed fetal BM-derived CD34+ HSPCs to the AURKB inhibitor ZM447439 and/or
406 to the SAC inhibitor Reversine for 48h, and then processed cells for cytogenetics analysis (**Fig**
407 **6a,b**). Both AURKB and SAC inhibition in CD34+ HSPCs reproduced chromosome structure
408 defects observed in HyperD-ALL (**Fig 6b, S9a**). They both drastically increased (compared to
409 controls) the frequency of CD34+ HSPCs with micronuclei, a *bona fide* marker of chromosome
410 instability (**Fig 6c**), hypocondensed chromosomes (**Fig 6d**), and metaphases with PCS reflecting
411 loss of chromatid cohesion (**Fig 6e**). Such chromosome structure defects were maintained and/or
412 slighted potentiated when both AURKB and SAC were simultaneously inhibited (**Fig 6b-e**). Of
413 note, despite we could not reliably assess the karyotypes of reversine-treated CD34+ HSPCs due
414 to massive chromosome damage, AURKB inhibition resulted in ~30% of the CD34+ HSPCs
415 displaying hyperdiploid karyotypes (**Fig 6f, S9b,c**). In addition, cell cycle analysis revealed

416 massive alterations in DNA ploidy, confirming genomic imbalances in CD34+ cells upon
417 treatment with AURKB or SAC inhibitors (**Fig 6g**). The chromosome structure defects and
418 hyperdiploid karyotypes reproduced in CD34+ cells reinforce defective AURKB and SAC as an
419 underlying cellular/molecular mechanism in hyperdiploidy B-ALL.
420

421 **DISCUSSION**

422 This is the most comprehensive study to date on the cellular mechanisms underlying the mitotic
423 and chromosome defects contributing to the pathophysiology of pediatric HyperD-ALL. Here, we
424 have developed robust *in vitro* assays using nestin+ fetal BM-MSCs and *in vivo* PDX to
425 successfully expand primary B-ALL leukemic samples. Such *ex vivo* and *in vivo* expansion of B-
426 ALL leukemic samples provided enough mitotic/dividing primary blasts for addressing many
427 biological questions in a large cohort of 54 primary B-ALL samples, thus highlighting the clinical
428 relevance of our work. The only available hyperD-ALL cell line CALL-2 was used throughout the
429 study for confirmatory and gain-of-function studies, and consistently phenocopied the data
430 generated using primary HyperD-ALL primary cells. This cell line originates from a doubled-up
431 hypodiploid B-ALL²³, suggesting that the same mitotic/chromosomal defects here reported may
432 underlie the pathogenesis of hypo-diploid B-ALL patients. Future studies, however, should be
433 done in HypoD-ALL patients who are rare but clinically dismal.

434

435 We demonstrate that in contrast to nonHyperD-ALL, HyperD-ALL blasts show reduced
436 proliferative rates coupled to a delay in early mitosis at prometaphase. Such a delay in early
437 mitosis is associated to chromosome alignment defects at the metaphase plate which, in fact,
438 lead to chromosome segregation defects and non-modal karyotypes. Despite karyotype
439 heterogeneity, HyperD-ALL primary blasts show a major clone that most likely represents the
440 fittest clone after cell adaptation to aneuploidy⁵⁷. These data supports previous studies showing
441 the presence of cytogenetically different subclones in HyperD-ALL⁵⁸⁻⁶¹.

442

443 Mechanistically, HyperD-ALL primary blasts and cell lines did not show abnormalities in bipolar
444 spindle and kinetochore formation. However, they displayed important chromosome structure and
445 function defects, a major mechanism regulating chromosome segregation which is essential for
446 maintaining the genomic integrity of cells¹³⁻¹⁵. HyperD-ALL cells showed robust defects in several

447 members of the condensin complexes including SMC2 (levels reduced at chromosome scaffolds),
448 CAPD2 and CAPD3. Biochemical, functional and MS assays revealed that PTMs of condensin
449 complex proteins may represent a mechanism underlying defective condensin complexes in
450 HyperD-ALL cells. Of note, no mutations were found in condensin complex- encoding genes in
451 HyperD-ALL patients, ruling out genomic mutations as the cause of the defective condensin
452 complex in HyperD-ALL patients.

453

454 Consequently, high-order chromosome architecture defects are notorious, and include
455 chromosome hypocondensation and loss of centromere rigidity revealed by increased
456 intercentromeric distances. Consistently, a recent study has reported chromosome architecture
457 defects and lower expression of CTCF in HyperD-ALL samples⁶². Furthermore, AURKB, the
458 catalytic subunit of the CPC, and Survivin are mis-localized from the inner-centromere in early
459 mitosis, further linking defective condensin with chromosome segregation defects in HyperD-ALL
460 blasts. According to the essential localization of AURKB at the inner-centromere to protect
461 chromatid cohesion and for proper SAC activity^{30,50,51}, HyperD blasts show chromatid cohesion
462 defects as observed by PCS and impaired SAC, leading to mitotic slippage. Impaired SAC activity
463 explains why HyperD-ALL blasts proceed to late mitosis with misaligned chromosomes at the
464 metaphase plate, thus leading to chromosome segregation defects. Importantly, chromosome
465 hypocondensation and hyperdiploidy were functionally reproduced in CD34+ HSPC upon
466 inhibition of AURKB and/or SAC, reinforcing defective condensin complex, AURKB and SAC as
467 underlying cellular and molecular mechanisms in HyperD-ALL (**Figure 7**). Although they are likely
468 instrumental in the pathophysiology of the HyperD-ALL, whether these findings are causal or
469 consequential to hyperdiploidy remains an open question under investigation.

470

471 This is the first cellular and molecular in-depth characterization of the mitotic and chromosomal
472 defects of HyperD-ALL using a cohort of 54 B-ALL primary samples. It represents a highly
473 relevant study because B-ALL is the most common pediatric cancer. Studies in monozygotic
474 twins with concordant HyperD-ALL and retrospective analysis of HyperD clones in cord blood
475 indicated that HyperD clones arise prenatally⁷, and that hyperdiploidy is an initiating oncogenic
476 event generating a preleukemic clone which then requires secondary mutations to trigger a full-
477 blown leukemia⁶³. Therefore, a better mechanistic understanding of how hyperdiploidy occurs
478 and how secondary alterations are acquired becomes crucial not only to propose novel
479 therapeutic targets but also to prevent the progression/relapse of HyperD-ALL. These defects in
480 condensin complex-AURBK-SAC axis open up new avenues for modelling HyperD-ALL by
481 genetically engineering of HSPCs, which will be crucial to further address the causal contribution
482 of these defects to the origin of HyperD in B-ALL.

483

484 From a diagnostic-clinical standpoint, the high-order chromatin/chromosome structural defects
485 observed in HyperD-ALL explains very well the difficulties that clinical cytogeneticists have
486 historically encountered to obtain metaphases of standard quality from these patients, thus
487 challenging the cytogenetic diagnostic³. In addition, despite the favourable clinical outcome of
488 HyperD-ALL, unravelling the physiopathogenic mechanisms underlying HyperD-ALL is necessary
489 because in absolute numbers, the morbidity/mortality associated to HyperD-ALL still represent a
490 clinical challenge. In sum, this study sheds light on the mechanisms underlying the mitotic and
491 chromosome defects involved in the pathogenesis of HyperD-ALL and offers molecular targets
492 (Condensin complex members, CPC members -AURKB- or the SAC) for potential
493 pharmacological intervention in the most frequent molecular subtype of pediatric acute leukemia.

494

495 **AUTHOR CONTRIBUTIONS**

496 OM conceived the study, designed and performed experiments, analyzed and interpreted the
497 data, and wrote the manuscript. CB designed and performed experiments, analyzed and
498 interpreted the data. MV,LV,CML-L,PR-G,IG,HRH,FGA,JLT,ST,DP,RR and MaC performed
499 experiments and analyzed data. PB,MdB,IP,MMPI,SRP,MJC,MRO, and MiC provided patient's
500 samples and data. PM conceived the study, designed experiments, interpreted the data, and
501 wrote the manuscript.

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697

698

699 **FIGURE LEGENDS:**

700

701 **FIGURE 1: HyperD-ALL cells are low proliferative and show a delay in early mitosis. (a)**

702 Heat map of the top 50 genes more differentially expressed between HyperD (n=58) and non-
703 HyperD (n=30) B-ALL samples. **(b-c)** Top 20 statistically significant up-regulated **(b)** or down-
704 regulated **(c)** biological pathways identified using GSEA for the genes differentially expressed in
705 HyperD *versus* nonHyperD-ALL patients. Coloured bars represent normalized enrichment scores
706 (NES). p-values are shown. **(d)** 16-day proliferation curves for the indicated cell lines, n=3
707 independent experiments. **(e)** SubG0/SubG1 apoptotic levels identified by FACS for the indicated
708 cell lines, n=3 independent experiments. **(f)** Cell cycle analysis for the indicated cell lines. *Left*,
709 representative cell cycle FACS analysis. *Right*, frequency of cells in G2/M analysed, n=3
710 independent experiments. **(g)** Representative DNA-Kinetochore-spindle IF staining (DNA, ACA,
711 tubulin, and pericentrin) identifying the different mitotic phases in B-ALL cell lines. The SAC
712 identifies the transition from early to late mitosis. P: Prophase, PM: Prometaphase, M:
713 Metaphase, A: Anaphase, T: Telophase, CK: Cytokinesis. Scale bar=10 μ m. **(h,i)** Mitosis
714 progression in B-ALL cell lines. Progression from early to late mitosis **(h)**, and frequency of cells
715 at the indicated mitotic phases **(i)**, n=4 independent experiments. Graphs represent the mean,
716 and error bars represent the s.e.m. *p<0.05, **p<0.01 (Two-way ANOVA).

717

718 **FIGURE 2: HyperD-ALL primary blasts show a delay in early mitosis associated with**

719 **chromosome alignment defects in prometaphase. (a)** Schematic depicting the experimental

720 design for *ex vivo* growth of primary B-ALL blasts onto nestin+ hBM-MSCs. **(b)** *Left*,

721 representative images of primary non-HyperD and HyperD-B-ALL *ex vivo* cultures on nestin+

722 hBM-MSCs at the indicated time-points. *Right*, absolute counts of B-ALL primary blasts at the

723 indicated time-points, n=2. **(c,d)** Frequency of apoptotic (SubG0/SubG1) **(c)** and G2/M **(d)** non-

724 HyperD and HyperD-ALL primary cells from BM samples, n=3 patients of each. **(e)** Schematic

725 depicting the PDX model used to expand primary B-ALL blasts *in vivo*. **(f)** Representative DNA
726 (blue)-Kinetochores (purple)-spindle (red-green) IF staining identifying the different mitotic phases
727 in PDX-expanded B-ALLs. The SAC identifies the transition from early to late mitosis. **(g)** Mitosis
728 progression of PDX-expanded B-ALL primary cells. *Left*, progression from early to late mitosis.
729 *Right*, frequency of cells at the indicated mitotic phases, n=3 non-HyperD and n=5 HyperD PDX-
730 expanded B-ALLs. **(h)** *Left*, representative images of mitotic cells with non-aligned and aligned
731 chromosomes at the metaphase plate. *Right*, frequency of PDX-expanded B-ALL primary blasts
732 showing chromosome alignment at prometaphase/metaphase, n=4 non-HyperD and n=4 HyperD
733 PDX-expanded B-ALLs. **(i)** Schematic depicting the chromosome biorientation assay. **(j)**
734 Representative images of the DNA-Kinetochores-spindle staining in monastrol/MG132-treated
735 cells with 0 (left), 1 (middle) and >2 (right) misaligned chromosomes. **(k)** Quantification of
736 metaphase cells showing misaligned chromosomes, n=3 non-HyperD and n=3 HyperD PDX-
737 expanded B-ALLs. Graphs represent the mean, and error bars represent the s.e.m. *p<0.05,
738 **p<0.01; ***p<0.0001 (Two-way ANOVA). Scale bars=10 μ m.

739

740 **FIGURE 3: Chromosome segregation defects and non-modal karyotypes in HyperD-ALL**

741 **blasts.** **(a)** Representative DNA (blue)-Kinetochores (green)-spindle (red) IF staining identifying
742 lagging and bridge chromosomes. Yellow arrowheads depict the indicated chromosome
743 segregation defect. **(b)** Frequency of blebbistatin-treated mitotic PDX-expanded primary blasts
744 with lagging and bridge chromosomes, n=151 mitosis from 3 non-HyperD and 96 mitosis from 3
745 HyperD-ALLs. **(c)** Comparison of modal karyotypes from 50 metaphases from primary HyperD
746 (n=6) and non-HyperD (n=6) B-ALL samples. **(d)** Frequency of cells showing modal karyotype.
747 **(e,f)** FISH analysis using DNA probes for chromosomes 12 (green) and 21 (red) of 200
748 interphase nuclei from n=3 non-HyperD and 4 HyperD-ALL primary samples. **(e)** Frequency of
749 cells representing the modal clone *versus* minor clones. **(f)** Representative FISH analysis for a

750 primary non-HyperD and a HyperD-ALL. Graphs represent the mean and error bars represent the
751 s.e.m. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.0001$ (Two-way ANOVA). Scale bars=10 μm .

752

753 **FIGURE 4: HyperD-ALL blasts show chromosome hypocondensation, loss of centromere**
754 **stiffness, and defects in the condensin complex. (a)** Analysis of spindle abnormalities in B-
755 ALL primary blasts. *Left*, representative DNA-Kinetochore-spindle IF staining of mitotic cells with
756 bipolar, multipolar and disorganized spindles. *Right*, frequency of mitotic cells displaying spindle
757 defects, $n=3$ non-HyperD-ALLs ($n=251$ mitosis) and $n=3$ HyperD-ALLs ($n=251$ mitosis) PDX-
758 expanded samples. **(b)** Frequency of metaphases with hypocondensed chromosomes in primary
759 B-ALL blasts, $n=200$ metaphases from 4 Non-HyperD and $n=250$ metaphases from 5 HyperD-
760 ALLs primary samples. *Left*, representative images of normal and hypocondensed metaphase
761 chromosomes. Insets represent 3x magnifications. **(c)** Chromosome structure of formaldehyde-
762 crosslinked PDX-expanded B-ALL samples. *Left*, representative images of metaphase cells with
763 hypocondensed chromosomes. Anti-ACA staining is shown in green. *Right*, frequency of
764 formaldehyde-crosslinked metaphases showing hypocondensed or hypocondensed with
765 unstructured chromosomes in B-ALL primary samples, $n=60$ metaphases from 3 non-HyperD and
766 $n=57$ metaphases from 3 HyperD-ALLs. **(d)** Chromosome arm width using PDX-expanded B-ALL
767 samples from *c*, $n=191$ chromosomes from 3 non-HyperD and $n=143$ chromosomes from 3
768 HyperD-ALLs. **(e)** Representative IF images of metaphase PDX-expanded B-ALL blasts stained
769 with DAPI, anti-SMC2 and anti-ACA. **(f)** Quantification of the SMC2 total volume in metaphase
770 chromosomes from *e*, $n=30$ metaphases from 3 non-HyperD and 3 HyperD-ALLs. **(g)** Schematic
771 cartoon of the two human condensin complexes. **(h)** WB analysis of the indicated condensin
772 members in whole-cell lysates from PDX-expanded B-ALL samples. **(i)** Quantification of WB
773 bands from *h* normalized to actin. **(j)** Representative HPLC-ESI-MS chromatograms of the
774 indicated peptides for HyperD and non-HyperD PDX-expanded B-ALLs. **(k)** Acetylation levels of

775 SMC2 peptide SQAASILTK (m/z=480.8). **(l)** Phosphorylation levels of CAPD2 peptide
776 GPAASTQEK (m/z=524.7). Results depict the average of the peak areas from independent MS
777 experiments from 2 non-HyperD and 2 HyperD-ALL PDX-expanded blasts. **(m)** Representative
778 line-scan measurements of individual centromeres in the indicated B-ALL primary samples. DAPI
779 and ACA are depicted as a blue and red lines, respectively. Yellow arrowheads point to the
780 analysed chromosome. **(n)** Intercentromeric distance from MG132-treated PDX-expanded B-ALL
781 blasts, n=155 centromeres from 3 non-HyperD and n=119 centromeres from 3 HyperD-ALLs. **(o)**
782 Intercentromeric distance from colcemid-treated PDX-expanded B-ALL blasts, n=130
783 centromeres from 3 non-HyperD and n=111 centromeres from 3 HyperD-ALLs. Graphs represent
784 the mean and error bars represent the s.em. *p<0.05, **p<0.01; ***p<0.001; **** p<0.0001. Two-
785 way ANOVA (b,c) or t-Student (a,d,f,h,i). Scale bars=10 μ m.

786

787 **FIGURE 5: Mis-localized AURKB and Survivin from the inner centromere and loss of**
788 **chromatid cohesion and SAC impairment in HyperD-ALL blasts.** **(a,b)** Representative IF
789 staining for CENP-A and AURKB **(a)** or Survivin **(b)** in PDX-expanded B-ALL blasts. **(c,d)**
790 Quantification of the AURKB **(c)** and Survivin **(d)** fluorescence signal at the inner centromere,
791 n=30 metaphases from 3 non-HyperD and n=30 metaphases from 3 HyperD-ALL. **(e)**
792 Representative IF showing either centromeric and scattered localization of AURKB, Survivin and
793 CENP-A. **(f,g)** Frequency of PDX-expanded non-HyperD (n=3) and HyperD-ALL (n=3) blasts
794 showing centromeric *versus* scattered localization of AURKB (n=797 chromosomes from non-
795 HyperD and n=676 chromosomes from HyperD) **(f)**, and Survivin (n=964 chromosomes from non-
796 HyperD and n=814 chromosomes from HyperD) **(g)**. **(h)** Quantification of total AURKB
797 fluorescence signal from samples in c. AURKB levels are expressed relative to non-HyperD
798 blasts, which are arbitrarily set to 100. **(i)** *Left*, representative FACS staining of H3S10P. *Right*,
799 MFI of H3S10P in 3 non-HyperD and 3 HyperD-ALL samples. **(j)** *Left*, representative images of

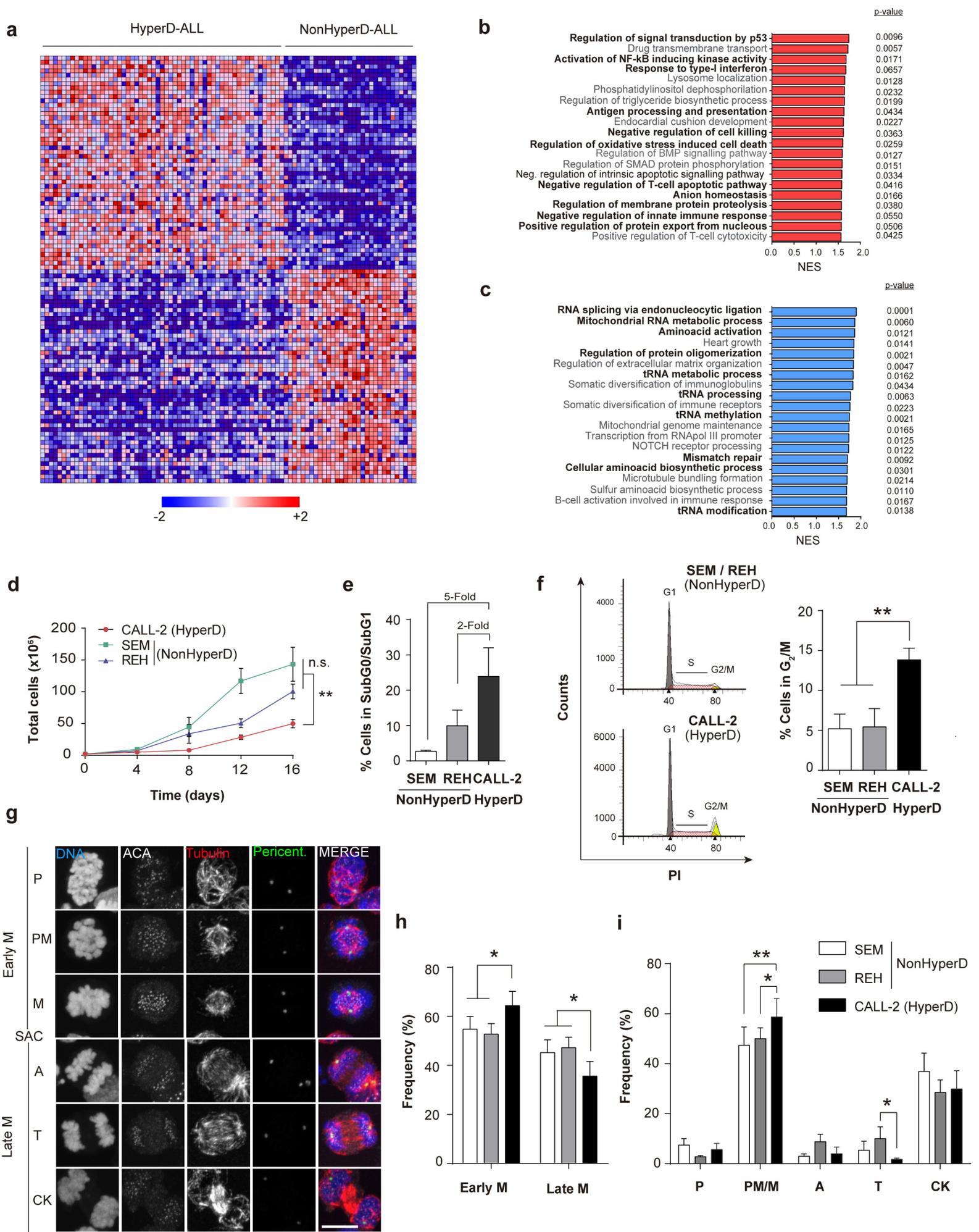
800 normal or rail-road shaped chromosomes. *Right*, frequency of metaphases showing the indicated
801 number of chromosomes with PCS; n=200 metaphases from 4 non-HyperD, n=250 metaphases
802 from 5 HyperD-ALLs samples. **(k)** Schematic depicting the workflow for functional analysis of the
803 SAC. **(l)** Representative FACS of mitotic PDX-expanded B-ALL blasts (H3S10P+CD19+ cells,
804 green) in the presence or absence of nocodazole. **(m)** Fold-change of mitotic (H3S10P+) blasts in
805 nocodazol-treated (relative to DMSO-treated) non-HyperD (n=3) and HyperD-ALL (n=3) primary
806 blasts. **(n)** Representative FACS cell cycle distribution of nocodazol- *versus* DMSO-treated PDX-
807 expanded B-ALL blasts. **(o)** Quantification of the cell cycle phases in nocodazol- *versus* DMSO-
808 treated PDX-expanded B-ALL blasts, n=3 non-HyperD and n=3 HyperD-ALL. **(p)** qRT-PCR
809 analysis of SAC proteins in B-ALL primary samples, n=9 non-HyperD and n=11 HyperD. Graphs
810 represent the mean and error bars represent the s.e.m. *p<0.05, **p<0.01; ***p<0.001; ****
811 p<0.0001 (two-way ANOVA or t-Student). Scale bars=10 μ m.

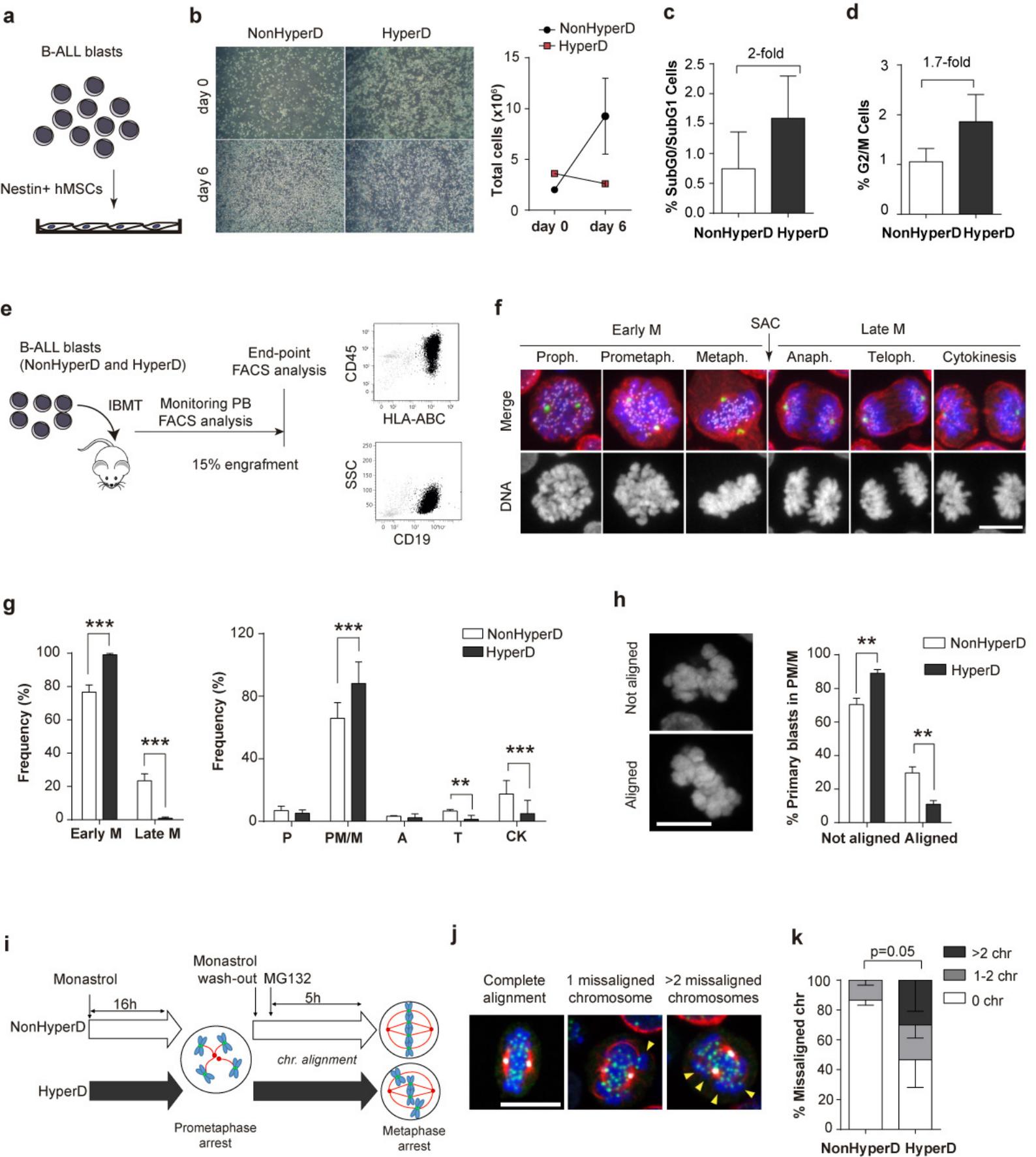
812

813 **FIGURE 6: Inhibition of AURKB and SAC in CD34+ HSPCs reproduces chromosome**
814 **structure defects and hyperdiploid karyotypes.** **(a)** Schematic depicting the workflow for
815 AURKB and SAC inhibition in CD34+ HSPCs. **(b)** Representative images of metaphase
816 chromosomes treated as indicated. **(c-f)** Frequency of metaphases with micronuclei (n=500 cells
817 per experiment) **(c)**, hypocondensed chromosomes **(d)**, PCS **(e)** and hyperdiploidy karyotype **(f)**.
818 **(g)** Representative FACS analysis showing PI staining profiles in CD34+ HSPCs treated as
819 indicated. n=150 metaphases were analysed per treatment from 3 independent experiments.
820 *p<0.05, **p<0.01 (One-tail t-Student).

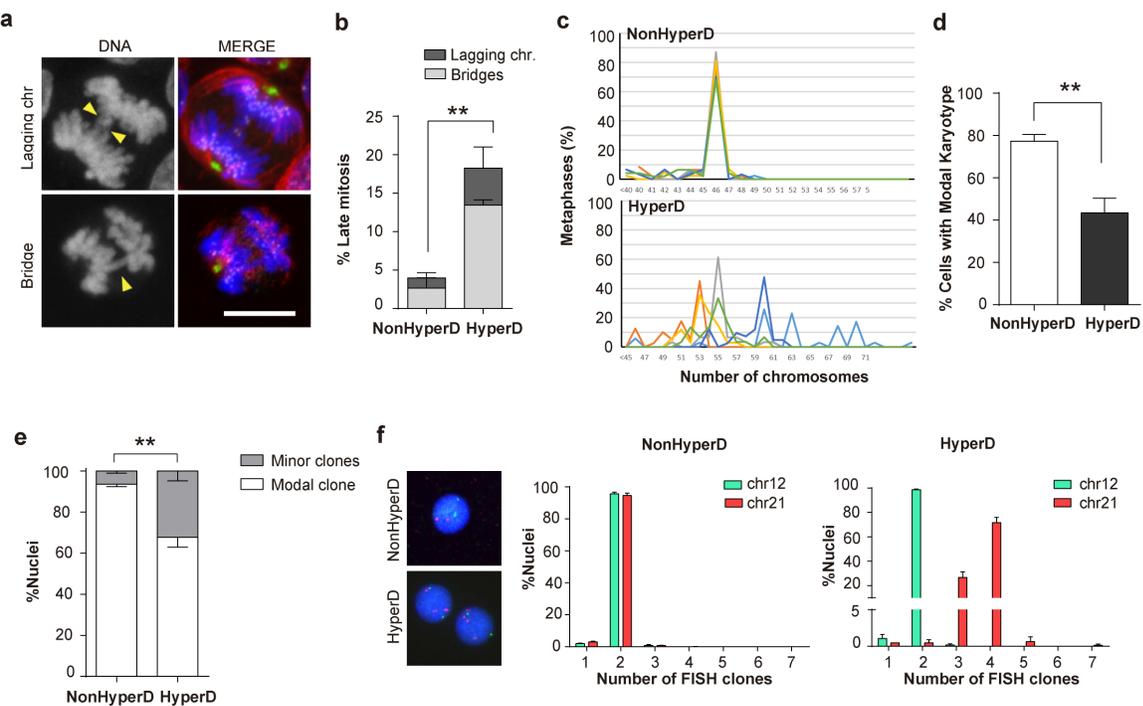
821

822 **FIGURE 7: Proposed model of the cellular mechanisms underlying the mitotic and chromosome**
823 **defects contributing to the pathophysiology of pediatric HyperD-ALL.**

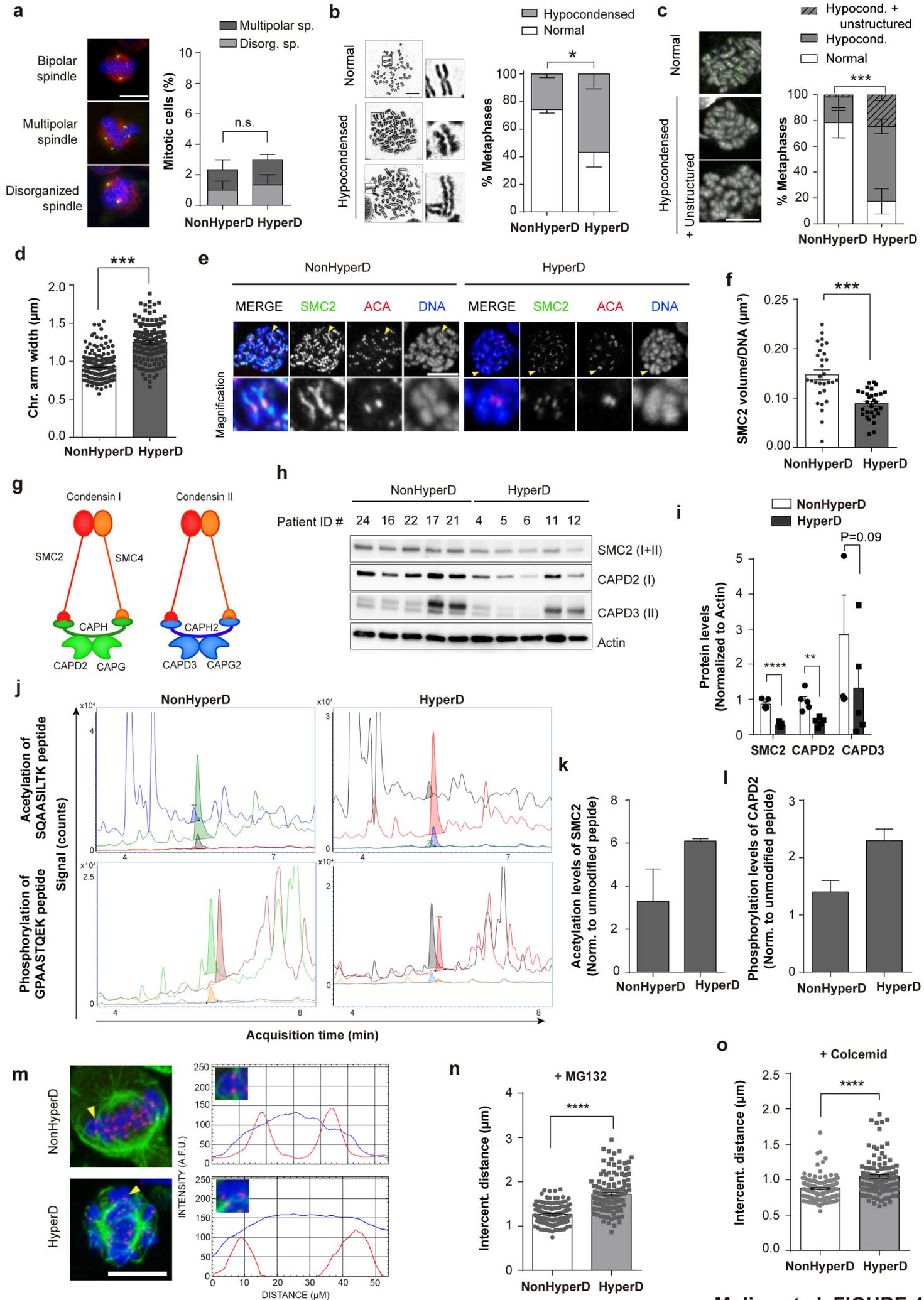


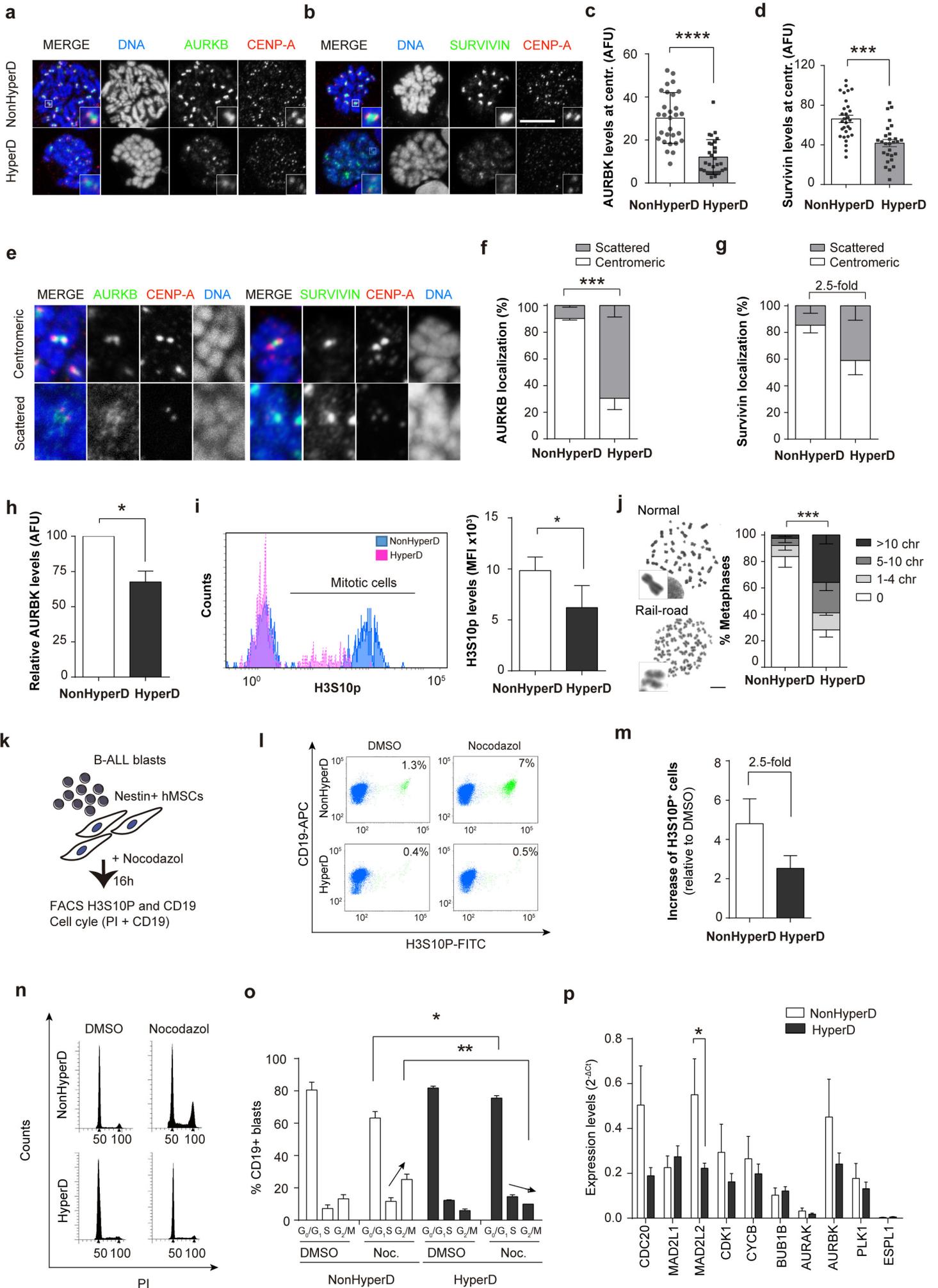


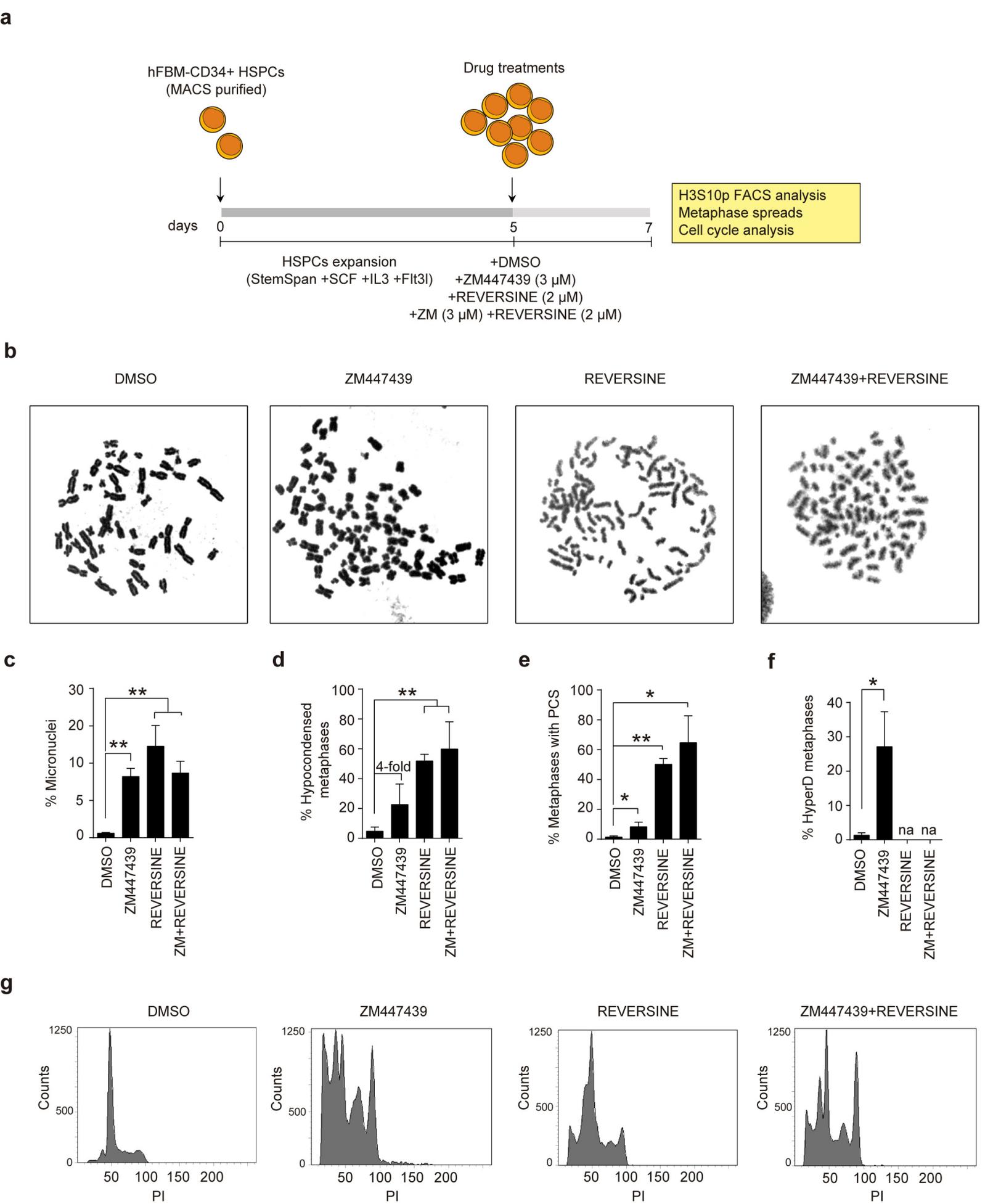
Molina et al. FIGURE 2



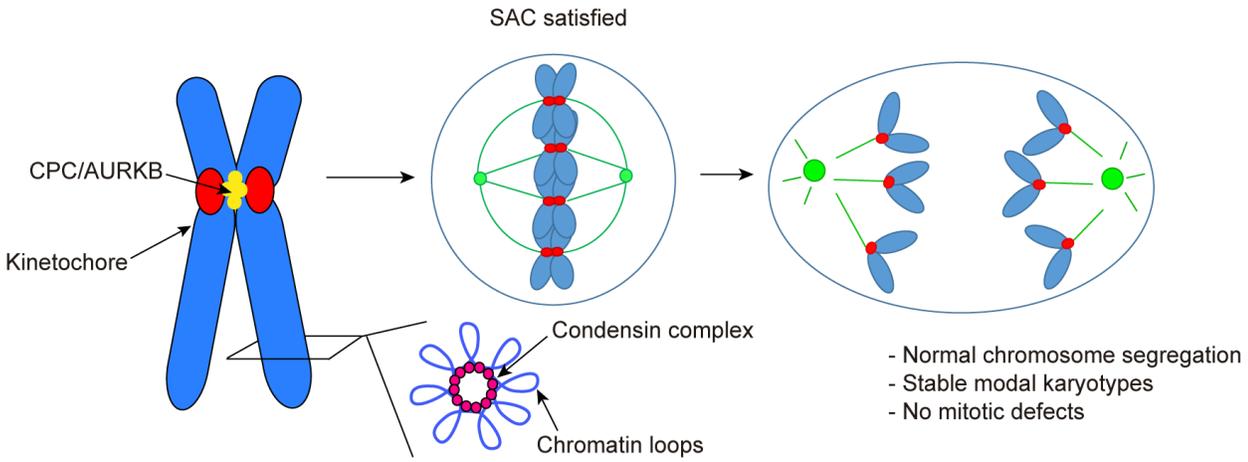
Molina et al. FIGURE 3



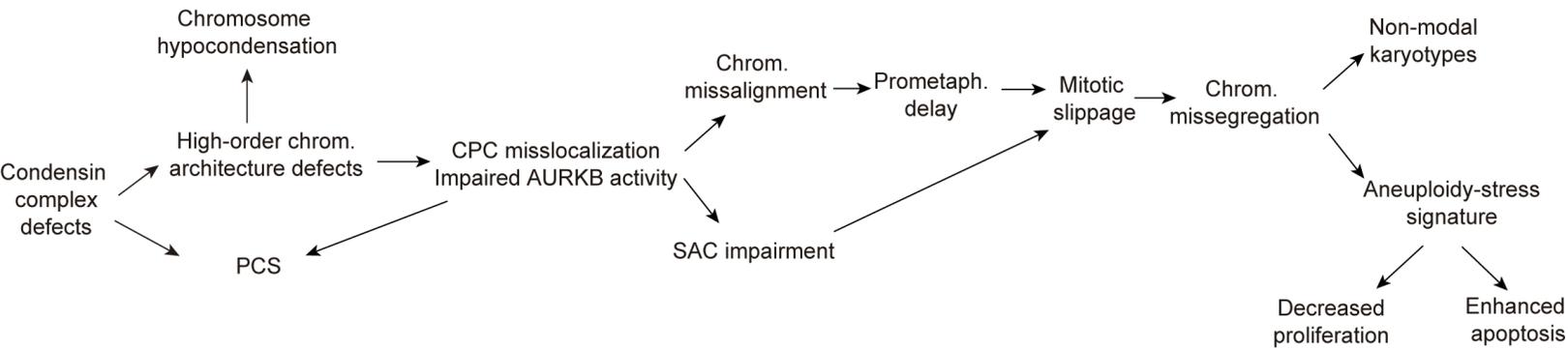
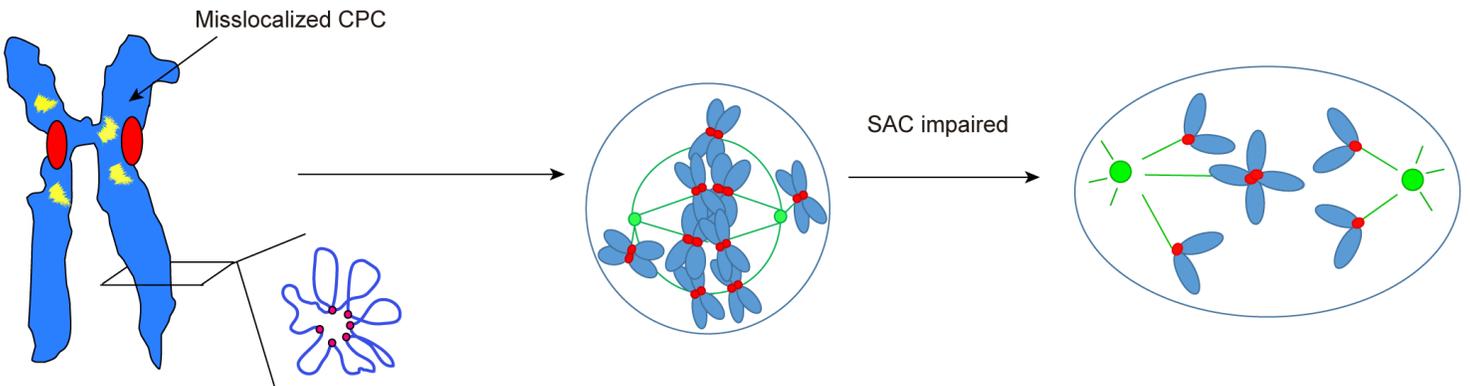




NonHyperD-ALL



HyperD-ALL



Molina et al. FIGURE 7