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1 **A cyclin-dependent kinase-mediated phosphorylation switch of disordered protein**
2 **condensation**

3
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30

31 **Abstract**

32 Cell cycle transitions result from global changes in protein phosphorylation states triggered by
33 cyclin-dependent kinases (CDKs). To understand how this complexity produces an ordered
34 and rapid cellular reorganisation, we generated a high-resolution map of changing phosphosites
35 throughout unperturbed early cell cycles in single *Xenopus* embryos, derived the emergent
36 principles through systems biology analysis, and tested them by biophysical modelling and
37 biochemical experiments. We found that most dynamic phosphosites share two key
38 characteristics: they occur on highly disordered proteins that localise to membraneless
39 organelles, and are CDK targets. Furthermore, CDK-mediated multisite phosphorylation can
40 switch homotypic interactions of such proteins between favourable and inhibitory modes for
41 biomolecular condensate formation. These results provide insight into the molecular
42 mechanisms and kinetics of mitotic cellular reorganisation.

43 **Introduction**

44 The cell cycle is driven by CDKs, which are essential to promote entry into S-phase and
45 mitosis. Two general strategies have been used to understand how CDK-dependent
46 phosphorylation brings about these transitions¹. First, top-down screens have revealed key
47 system components. Hundreds of CDK substrates²⁻⁷ and cell cycle-regulated proteins⁸ and
48 thousands of mitotic phosphorylations⁹ have been identified in this manner. Yet determining
49 their roles has lagged behind; for example, painstaking genetic analysis in yeast models was
50 required to reveal the requirement for CDK-mediated phosphorylation of two key substrates to
51 allow DNA replication^{10,11}. Second, bottom-up molecular analysis of the structural effects of
52 individual phosphorylations on single proteins provides mechanistic insight into regulation of
53 their activity¹². Both of these approaches are rendered more difficult by the fact that CDKs
54 often phosphorylate multiple sites, whose combined effects may result in a phenotype; for
55 example, multisite phosphorylation of the CDK inhibitor Sic1 in budding yeast is required to
56 prevent its degradation¹³. As such, it has proven challenging to use studies performed from
57 these different perspectives to understand global cellular behaviour.

58 Different models of CDK-mediated phosphorylation have been proposed. Specific interactions
59 between distinct CDK-cyclin complexes and sequence motifs encoded in substrates might
60 result in highly ordered phosphorylation¹⁴, yet such complex mechanisms may not be essential
61 since the cell cycle can be driven by single CDK1-cyclin complexes^{15,16}. Furthermore,

62 theoretical modelling and biochemical analysis have suggested that the mitotic control network
63 can trigger switch-like activation of CDK1¹⁷, yet approaches using fluorescent biosensors
64 imply that mitotic CDK1 activation is rather progressive¹⁸. Along with the CDK-mediated
65 ordered phosphorylation model, this suggests that mitotic phosphorylation states should change
66 progressively *in vivo*, yet mitotic reorganisation is rapid and abrupt. Thus, understanding cell
67 cycle transitions requires a description of the dynamics of global mitotic phosphorylation,
68 which is largely unknown, as well as an investigation of the biochemical effects of
69 phosphorylations, most of which remain uncharacterised. We reasoned that this would require
70 a multidisciplinary quantitative approach involving cell biology, biochemistry, bioinformatics
71 and biophysics. We aimed to generate a quantitative time-resolved map of *in vivo* cell cycle
72 phosphorylation, extract global principles of phosphorylation dynamics, perform comparative
73 analysis to assess conservation of these principles across species, and analyse effects of
74 phosphorylation by modelling, biophysical approaches and biochemical experiments.

75

76 **Results**

77 *A high-resolution map of in vivo cell cycle phosphorylation*

78 Dynamic phosphorylation states cannot be determined from cell populations¹⁹, while single-
79 cell proteomics studies^{20,21} currently have insufficient sensitivity and reproducibility for low
80 stoichiometry and dynamic targets. We wanted to identify and quantify cell cycle-regulated
81 phosphosites in a system devoid of artifacts arising from cell synchronisation^{22,23}, and with
82 temporal resolution that alternative approaches, like centrifugal elutriation²⁴ or FACS²⁵ cannot
83 provide. We thus took advantage of the naturally synchronous early cell cycles of *Xenopus*
84 *laevis* embryos^{26,27}. We performed quantitative phosphoproteomics *in vivo* using a sensitive
85 phosphopeptide enrichment strategy²⁸. We collected single embryos at 15-minute intervals
86 while recording visual cues of cell divisions, and phosphopeptides from each embryo were
87 purified and analysed by mass spectrometry (Fig. 1a). These individual embryo
88 phosphorylation states strongly correlated (Supplementary Fig. 1a). We identified 4583 high-
89 confidence phosphosites mapping to 1843 proteins (Supplementary Fig. 1b; Supplementary
90 Data 1), most being phosphoserines (Supplementary Fig. 1c). Our approach thus allowed us to
91 generate a dynamic map of protein phosphorylation from an unfertilised egg to a 16-cell
92 embryo.

93 We focused on 1032 sites on 646 proteins whose phosphorylation state changed over time
94 (hereafter denoted “dynamic phosphosites”). Gene ontology (GO) and network analysis of
95 proteins harbouring these sites revealed high functional association and interconnectivity
96 between groups of proteins involved in RNA binding and the nuclear pore complex (NPC),
97 DNA replication and chromatin remodeling, and microtubule regulation (Fig. 1b). Hierarchical
98 clustering uncovered four groups with distinct dynamic behaviour (Fig. 1c; Supplementary
99 Data 1). The levels of clusters A and B phosphosites were highest in eggs and post-fertilisation,
100 and decreased during the first round of DNA replication. GO analysis for group A highlighted
101 proteins involved in RNA regulation and nuclear organisation, including the NPC and nuclear
102 transport, chromosomal structure and segregation (Supplementary Fig. 1d), as also observed in
103 a recent study on meiosis exit ²⁹. Cluster B phosphosites were enriched in regulators of RNA
104 biosynthesis and stability, translation, actin, DNA replication and repair (Supplementary Fig.
105 1d). Dephosphorylation of these sites, which coincide with the meiosis-zygote transition, may
106 prepare the embryo for upcoming cell divisions ³⁰. Cluster C phosphosites progressively
107 increased after meiotic exit, while cluster D phosphosites had a clear oscillating signature with
108 upregulation preceding each cell division. GO analysis of cluster C showed dominance of
109 interphase cell cycle processes including DNA replication, RNA-related processes and
110 chromosome organisation (Supplementary Fig. 1d). Several sites were from
111 monophosphorylated peptides, while the multiphosphorylated forms were found in clusters A
112 or D (Supplementary Fig. 1e), further indicating that cluster C sites are from interphase. Cluster
113 D shows coordinated phosphorylation of multiple members of protein complexes involved in
114 distinct processes such as nuclear transport, RNA processing, chromatin remodelling and DNA
115 replication, suggesting a common mechanism of regulation (Supplementary Fig. 1f).
116 Importantly, phosphoproteome changes were not simply a reflection of changes in abundance
117 of the corresponding proteins (Supplementary Fig. 2), which are generally negligible during
118 *Xenopus* early development ³¹. Together, these results suggest that multisite phosphorylation,
119 rather than progressive phosphorylation, might constitute the mitotic trigger.

120 To investigate this idea further, we assigned *in vivo* embryo phosphosites to different cell cycle
121 stages by comparing with phosphorylation patterns of replicating or mitotic egg extracts (Fig.
122 1d). Replication was initiated by adding purified sperm chromatin to interphase egg extracts
123 and quantified over time by measuring radioactive nucleotide incorporation (Fig. 1e, top),
124 while mitosis was triggered by adding recombinant cyclin B and verified microscopically. To
125 compare meiotic exit with mitosis, we also used egg extracts arrested at meiotic metaphase II

126 (Cytostatic Factor, CSF-arrested). Overall, we identified 6937 phosphosites, which included
127 71% of the sites identified *in vivo* (Fig. 1f, Supplementary Data 1). 1728 sites varied between
128 S and M-phase, including 693 sites upregulated in S-phase and 1035 in mitosis (Fig. 1e,
129 Supplementary Data 1). The S-phase specific phosphosites detected in this dataset greatly
130 increase the known repertoire of phosphorylation sites upregulated during DNA replication⁹.
131 Although several DNA-replication factors, including MCM4 and RIF1, were already
132 phosphorylated in S-phase, they displayed marked multi-site phosphorylation in mitosis
133 (Supplementary Fig. 3a). GO analysis of interphase and mitotic sites revealed processes
134 enriched in *in vivo* cluster C and cluster D, respectively (Supplementary Fig. 3b).

135 We next analysed the cell cycle behaviour of the dynamic phosphosites that we identified *in*
136 *vivo* (Supplementary Fig. 3c). Most embryo cluster A sites were upregulated in both CSF-
137 arrested meiotic extracts and mitotic extracts, highlighting the global similarities of meiotic
138 and mitotic M-phase, despite the additional activity of the Mos/MEK/MAP kinase pathway in
139 meiosis. In contrast, 78% of embryo cluster B sites were not mapped in extracts, suggesting
140 that these are specific to the developmental transition from meiosis to early embryogenesis. In
141 agreement, cluster B sites that mapped to extracts were generally low in mitosis. Most sites
142 that mapped from embryo cluster C were present only in S-phase, whereas a subfraction peaked
143 in mitosis. As such, this analysis revealed that clusters B and C do not strictly identify cell
144 cycle phases. In contrast, all mapped cluster D sites were absent in S-phase and high in M-
145 phase *in vitro*, showing that single embryo data can successfully identify mitotic
146 phosphorylations. Next, we assessed the behaviour of the 102 peptides detected in both singly
147 and multi-phosphorylated forms in extracts, and found that they segregated into two distinct
148 clusters corresponding to interphase and M-phase, with a strong enrichment for multisite
149 phosphorylation in M-phase (odds ratio 4.6, adjusted p-value < 10⁻⁶) (supplementary Fig. 3d).
150 The same analysis of multi-site phosphorylation on the 35 peptides detected in singly or
151 multiply phosphorylated forms in embryos revealed four clusters, which correspond to clusters
152 A-D (Supplementary Fig. 3e). Despite the small sample, multi-site phosphorylation was highly
153 enriched in meiotic metaphase cluster A (odds ratio 5.6, adjusted p-value 0.011), while two
154 peptides were present as singly phosphorylated in interphase cluster C and doubly
155 phosphorylated in mitotic cluster D. Overall, these data show that diverse biological processes
156 may share a common regulatory mechanism of multisite phosphorylation during cell cycle
157 progression.

158 *Predominance of CDK targets*

159 We further investigated our detected phosphosites to identify putative kinases responsible for
160 their dynamic phosphorylation. Analysis of kinase consensus motifs showed that proline-
161 directed (S/T-P) sites, which conform to the minimal consensus for CDKs, constitute 51% of
162 all detected phosphosites *in vivo* and 60% of dynamic sites (Supplementary Fig. 4a). Around
163 10% of all phosphosites matched the full CDK1-family consensus motif (S/TPxK/R).
164 Replicating and mitotic extracts displayed a similar trend (Supplementary Fig. 4a). Putative
165 CDK targets dominated all clusters, with 80% of sites in cluster D *in vivo* and mitotic clusters
166 *in vitro* conforming to the minimal CDK motif (Fig. 1g, Supplementary Fig. 4b, c). Consensus
167 sites of other kinases such as Aurora, Polo-like kinase (PLK), DBF4-dependent kinase (DDK)
168 and Casein kinase I and II were present to a lesser extent (Supplementary Fig. 4b, d). The
169 fraction of S/T-P sites that correspond to the full CDK consensus site motif is lower in clusters
170 A (6%) and B (11%) than in clusters C and D (26 and 21%, respectively). Many of the cluster
171 B S/T-P sites are likely mediated by MAP kinases, which are proline-directed kinases that are
172 active in meiotic M-phase but are inactivated during early embryonic cell cycles³². In
173 agreement with this idea, the MAP kinase consensus motif (GPLSP)³³ is clearly detectable in
174 cluster B (Supplementary Fig. 4c). In contrast, around half of cluster A sites mapped to extracts
175 are present in mitosis; this cluster has an unusual S/T-P motif that may correspond to a new
176 class of CDK sites phosphorylated with distinct kinetics from cluster D sites.

177 Few direct CDK substrates have been characterised in *Xenopus*, but we surmised that CDK
178 sites are likely conserved between vertebrates. Thus, to further analyse the proportion of
179 dynamic sites dependent on CDKs, we manually curated a set of 654 human CDK1-subfamily
180 targets (Supplementary Data 2; see Methods for sources). 304 of these have *Xenopus*
181 homologues among the 1843 phosphoproteins we detected, and 149 were present among the
182 646 proteins with dynamic phosphosites in *Xenopus* embryos (Fig. 1h). Thus, the
183 predominance of CDK motifs among dynamic phosphosites reflects a high proportion of *bona*
184 *fide* CDK substrates. This is a conservative estimate, since we only considered proline-directed
185 sites as CDK motifs, although we found that 10-20% of human and yeast CDK substrates
186 (Supplementary Data 2; see Methods for sources) were non-proline-directed (Supplementary
187 Fig. 4e), confirming a recent finding³⁴. These data reinforce the dominant role of CDKs in cell
188 cycle-regulated phosphorylation.

189 *Mechanisms generating synchronous mitotic phosphorylation in vivo*

190 Since contrasting models of CDK activity predict either switch-like or progressive dynamics
191 of substrate phosphorylation, we determined the *in vivo* dynamics of mitotic phosphorylation

192 of individual phosphosites at extremely high-time resolution. We focused on 64 cluster D sites
193 from diverse proteins, 60 of which conform to CDK minimal consensus motifs. We analysed
194 these sites in single embryos every 180-seconds using quantitative targeted phosphoproteomics
195 ³⁵⁻³⁷ by parallel reaction monitoring ³⁸ (Fig. 2a), and thus obtained a highly resolved
196 quantitative description of mitotic phosphorylation of different protein complexes *in vivo*. This
197 revealed an exceptionally synchronous transition from low to maximal phosphorylation of all
198 phosphosites preceding each cell division (Fig. 2b, c). The latter is as close to switch-like
199 behaviour (an all-or-nothing response to a small change in regulator activity) as can be
200 expected given the spatial metachronicity of mitotic entry in early *Xenopus* embryos ^{39,40}.
201 Therefore, any differences in affinities of CDKs for substrates *in vitro* ¹⁴ do not translate into
202 major timing differences in mitotic phosphorylation *in vivo*.

203 Highly synchronous mitotic phosphorylation of diverse proteins did not require oscillation of
204 CDK1-Y15 inhibitory phosphorylation, which was downregulated over time (Fig. 2d), as
205 previously reported ⁴¹, consistent with lack of oscillating phosphorylation of the CDK1-Y15-
206 regulatory enzymes, CDC25 and WEE1 (Fig. 2e). In contrast, oscillating phosphorylations on
207 NIPA and the APC/C, which regulate mitotic cyclin accumulation, as well as Greatwall kinase,
208 which activates the PP2A inhibitors Arpp19/ENSA, were apparent (Fig. 2f). This suggests that,
209 in early embryos, control of mitotic cyclin levels and PP2A activity, and thus the overall
210 CDK/phosphatase activity ratio ¹⁷, may be the key determinant of substrate phosphorylation
211 timing and generate synchronous mitotic phosphorylation, whereas regulated CDK1-Y15
212 phosphorylation is not essential (Fig. 2g). This is consistent with the self-sufficiency of futile
213 cycles in generating switch-like network output in the absence of allosteric regulation ⁴², and
214 suggests that multiple layers of regulation may have evolved to ensure robustness of the
215 dynamics.

216 *The cell cycle phosphoproteome is intrinsically disordered*

217 We next investigated whether the diverse dynamic phosphoproteins that we identified share
218 common structural features facilitating CDK-mediated phosphorylation, and if so, whether this
219 is conserved across species. Phosphosites in general are often located in intrinsically disordered
220 regions (IDRs) of proteins ⁴³, which is also true for yeast and mouse CDK sites ^{44,6,7}. However,
221 previous analyses did not take into account the enrichment of serine, threonine and proline in
222 disordered regions, which is consistently predicted across the entire proteome of *Xenopus*,
223 human and yeast (Supplementary Fig. 5a). Therefore, to date, it is not clear whether the
224 presence of phosphorylatable amino acids, or specifically their phosphorylation, is enriched in

225 disordered regions. We corrected for this compositional bias (see Methods), and found that
226 phosphorylatable residues in IDRs were more highly phosphorylated than those in ordered
227 regions (Fig. 3a-c). This enrichment was increased for proteins with at least one site displaying
228 dynamic phosphorylation; the same was true for human CDK substrates (Fig. 3b, c). To
229 estimate the differential phosphorylation of disordered sites globally, we calculated the ratio of
230 dynamically phosphorylated (*Xenopus*) or CDK-phosphorylated (yeast, human) to non-
231 phosphorylated serine and threonine in both disordered and structured regions (Supplementary
232 Fig. 5b; see Methods). This showed that cell cycle and CDK-regulated phosphorylation is
233 highly skewed towards disordered regions (Fig. 3d, Supplementary Fig. 5c). We then asked
234 whether this is also true for substrates of other protein kinases, again taking into account
235 compositional bias. We analysed the mitotic PLK and Aurora kinases (which control various
236 aspects of mitotic chromosome and spindle dynamics), DYRK kinases, (which promote mitotic
237 phosphorylation of several intrinsically disordered proteins [IDPs])⁴⁵, NEK kinases (which
238 have roles in centrosome duplication and various stages of mitosis), and MAP kinases (which
239 share the proline-directed S/T minimal consensus site with CDKs). For each of these, with the
240 exception of NEK kinase targets, documented phosphosites were strongly enriched in IDRs
241 (Supplementary Fig. 5c, d), supporting the idea that phosphorylation of residues in IDRs is a
242 general cellular control mechanism and is not specific to CDKs.

243 Given the preponderance of CDK substrates among cell cycle-regulated phosphosites despite
244 the evidence that many kinases may preferentially phosphorylate IDRs, we wondered whether
245 CDK substrates might be more disordered than phosphoproteins in general. We therefore
246 determined the percentage of disordered residues of proteins in our datasets, compared to the
247 rest of their respective phosphoproteomes (Supplementary Data 3). This revealed that, on
248 average, both *Xenopus* dynamic phosphoproteins and human and yeast CDK substrates contain
249 approximately twice the proportion of disordered amino acids as all other phosphoproteins
250 (Fig. 3e, Supplementary Fig. 5e), putting them among the top quartile of proteins with the most
251 disorder in the proteome. Furthermore, targets of most cell cycle kinases except NEK are
252 significantly more disordered than targets of MAP kinase (Fig. 3f), whose phosphosites are
253 also proline-directed and preferentially located in IDRs (Supplementary Fig. 5d). This suggests
254 that several cell cycle kinases have evolved to phosphorylate some of the most disordered
255 proteins in the proteome.

256 *Enrichment of MLO components among CDK substrates*

257 We surmised that the critical importance of intrinsic disorder might underlie a common
258 mechanism of phosphoregulation of diverse proteins during the cell cycle. We noted that IDPs
259 are key components of membrane-less organelles (MLO), many of which (*e.g.* Cajal bodies,
260 nucleoli, nuclear pore complexes, splicing speckles) show cell cycle-regulated assembly or
261 disassembly and are thought to arise by phase separation (PS)⁴⁶ that can be controlled by
262 phosphorylation^{45,47–49}. We thus hypothesised that CDKs might regulate PS during the cell
263 cycle. We analysed available data on cellular localisation for each of our curated human CDK
264 substrates, and found that 257 (39.2%) are present in MLOs (Fig. 4a). We then manually
265 curated an MLO proteome from human proteomics studies and asked whether proteins
266 undergoing cell cycle-regulated phosphorylation are enriched in these compartments
267 (Supplementary Data 4; see Methods for sources). Indeed, homologues of 204 dynamic
268 *Xenopus* phosphoproteins (31.6%) localise to MLOs, as do 73 of the 149 proteins (50%) that
269 show dynamic phosphorylation in *Xenopus* and are CDK substrates in human (Fig. 4a). Of the
270 latter, we studied the location of phosphosites on key IDPs of different MLOs, including coilin
271 (Cajal bodies), nucleophosmin, nucleolin and Ki-67 (nucleoli), 53BP1 (53BP1 bodies),
272 nucleoporins (NPC) and PML (PML bodies). As we anticipated, the vast majority of both
273 proline-directed phosphosites and confirmed CDK sites on these proteins were located in
274 predicted IDRs (Fig. 4b). We next investigated whether dynamically phosphorylated IDPs have
275 properties characteristic of phase separation. We first applied a machine learning classifier⁵⁰
276 to predict whether cell cycle-regulated phosphoproteins show higher propensity to phase
277 separate (PSAP score). Indeed, we observed a sharp increase in the PSAP score, from the
278 proteome to the phosphoproteome, and a further increase for dynamic phosphoproteins, with
279 the highest score for mitotic cluster D (Supplementary Fig. 6). Similarly, the PSAP score is far
280 higher amongst targets of most cell cycle kinases (CDK, Aurora, PLK, but not NEK) and
281 DYRK kinases than the overall phosphoproteome, but less so for MAP kinase substrates.

282 *CDKs regulate IDR phase separation*

283 Both stochastic and specific interactions between IDPs contribute to PS and MLO assembly
284^{46,51,52}, and protein phosphorylation can promote or inhibit PS, depending on the protein
285 sequence context^{49,53}. Although most MLOs disassemble in mitosis, there is a notable
286 exception: the perichromosomal layer (PCL), which has been hypothesised to form via PS. We
287 reasoned that the degree of CDK-mediated multisite IDP phosphorylation might constitute a
288 switch between PS promotion and inhibition. For example, the maximal CDK activity present
289 at the onset of mitosis might promote both disassembly of most MLOs and formation of the

290 PCL. Studying the effects of CDK-mediated phosphorylation in a considerable number of
291 diverse proteins is not feasible by biochemical approaches and is challenging even for
292 molecular dynamics simulations. To overcome this obstacle, we employed analytical
293 modeling. We took advantage of a newly developed mathematical theory called renormalised
294 Gaussian random phase approximation (rG-RPA)⁵⁴, that combines traditional RPA theory with
295 sequence-dependent single-chain theory using a renormalised Gaussian (rG) chain formulation.
296 This theory provides a better account of conformational heterogeneity and density fluctuations,
297 allows predictions of phase separation, and can be employed at a medium-throughput scale.
298 We supported our findings by another recent theory that computes sequence charge decoration
299 matrices (SCDM) to study conformational properties of a single IDP chain; this accounts for
300 the effects of sequence-specific electrostatic interactions on chain conformation, which dictate
301 the distance between any two amino acid residues⁵⁵⁻⁵⁷. Since single chain properties can
302 predict multi-chain physical behaviour, we expect SCDM to provide further insights into the
303 propensity of phase separation. We chose 12 IDPs representing different biological processes
304 and MLOs and containing multiple CDK phosphorylation sites; the choice of the disordered
305 regions of these proteins was mostly based on AlphaFold2⁵⁸ (see Methods; Supplementary
306 Data 5). Phosphorylation of all described CDK-sites lowered critical temperature and phase
307 separation propensity of 8 IDPs (NCL, NPM1, NUP53, ELYS, 53BP1, MCM4, MDC1, and
308 SF3B1) (Fig. 5a). These trends are consistent with SCDM maps showing decreased self-
309 association propensity (increased red regions, Fig. 5b). Conversely, for Ki-67, SRRM2, TICRR
310 and coilin, CDK-mediated phosphorylation increased critical temperature and PS tendency
311 (Fig. 5a), consistent with SCDM maps (increased blue or reduced red regions, Fig. 5b). Overall,
312 these data are in agreement with our hypothesis that CDK-mediated phosphorylation is a key
313 regulator of PS propensity of IDPs.

314 To analyse this in more detail, we focused on a model CDK substrate, Ki-67, an IDP that
315 organises heterochromatin structure⁵⁹ and perichromosomal layer formation from nucleolar
316 components in mitosis^{60,61}. Ki-67 is highly phosphorylated in mitosis by CDKs, which
317 regulates its perichromosomal localisation⁶². It contains a multivalent repeat domain of 122
318 amino acids, known as the Ki-67 repeat, each harbouring a highly conserved motif, the Ki-67
319 motif, currently of unknown function (Fig. 6a).

320 To evaluate the possibility that Ki-67 may show PS behaviour in living cells, we expressed a
321 GFP-tagged full length Ki-67 protein and compared its localisation pattern in cells with
322 different expression levels (Fig. 6b). At lower levels, Ki-67 was detectable in the nucleolus,

323 the site of endogenous Ki-67, but not in the nucleoplasm, while at higher levels, additional foci
324 appeared outside the nucleolus. This suggests that Ki-67 is partitioned by PS into dense and
325 dilute phases. At the highest levels, Ki-67 showed a virtually continuous condensed phase,
326 reminiscent of spinodal decomposition⁶³ whereby the single phase spontaneously separates
327 uniformly throughout the space into two phases, without the nucleation and growth which
328 normally characterises phase separation. In these conditions, chromatin exhibited the same
329 pattern (Fig. 6b), indicating that Ki-67 overexpression drives heterochromatinisation,
330 consistent with our earlier findings⁵⁹. We next examined whether Ki-67 mobility is consistent
331 with liquid-like behaviour by Fluorescent Recovery After Photobleaching (FRAP)
332 experiments. We investigated both the recovery of the bleached area of part of a Ki-67-positive
333 compartment, and an adjacent unbleached area of the same compartment. Rapid recovery of
334 the bleached area and preferential mixing of components from the unbleached to the bleached
335 area would be indicative of a liquid-like phase-separated compartment⁶⁴. We performed such
336 FRAP assays on cells with different levels of Ki-67, in interphase, or in mitosis where Ki-67
337 localises to the PCL. At low expression levels, the recovery half-time was of about 14 seconds,
338 while high expression levels showed a significantly slower recovery. This is consistent with
339 the emerging idea that phase separation may be coupled with percolation, whereby a percolated
340 network of molecules spanning the volume of condensates confers viscoelastic properties⁶⁵.
341 Interestingly, the recovery was extremely rapid in mitosis, of 8 seconds (Fig. 6c, d;
342 Supplementary Movies 1-3). This behaviour indicates liquid-like mixing with an impermeable
343 barrier of the phase-separated compartment⁶⁴, and suggests that the perichromosomal layer
344 may be a highly liquid-like phase (Fig. 6c, d; Supplementary Movies 1-3). We further analysed
345 the propensity of Ki-67 to undergo phase separation in cells by using the light-activated Cry2
346 “optodroplet” system⁶⁶ with full length Ki-67 or a series of deletion mutants. Full-length Ki-
347 67 localised to the nucleolus, as expected, but exposure to blue light caused rapid appearance
348 of small round foci in the nucleoplasm, which was dependent on the level of induced Ki-67
349 expression, consistent with PS (Fig. 6e). These foci showed colocalisation with nucleolin and
350 nucleophosmin (Fig. 6f), intrinsically disordered proteins and interactors of Ki-67 involved in
351 nucleolar organisation, indicating the existence of heterotypic interactions typical of phase-
352 separated MLOs.

353 We next analysed both by modelling and experiments the consequences of phosphorylation for
354 PS of Ki-67. As shown above, rG-RPA of full-length Ki-67 predicted that its complete
355 phosphorylation should promote PS (Fig. 5a), in agreement with Coarse-grained (CG)

356 molecular dynamics (MD) simulation, which showed increased compaction upon
357 phosphorylation (Fig. 7a; Supplementary Movie 4; Supplementary Data 6), and with SCDM
358 analysis, which indicated increased intra-chain interactions (Fig. 5b). We next tested effects of
359 CDK-mediated phosphorylation on Ki-67 phase separation propensity using the optodroplet
360 system in cells. To do this, we promoted the CDK-mediated phosphorylation state by treating
361 cells with okadaic acid to inhibit PP2A, that reverses CDK-mediated phosphorylation, or
362 inhibited CDKs with purvalanol A¹⁷. Even in the absence of blue light, treatment with okadaic
363 acid led to formation of new Ki-67 foci within the nucleoplasm (Fig. 7b, c), which also
364 incorporated nucleolin and nucleophosmin (Fig. 7d). Therefore, phosphorylation promotes PS
365 of Ki-67 independently of oligomerisation of the Cry2 domain. Conversely, pan-CDK
366 inhibition with purvalanol A prevented induction of foci upon illumination (Fig. 7b, c). These
367 results are consistent with phosphorylation of full-length Ki-67 promoting PS, as predicted by
368 SCDM, rG-RPA and MD. We observed similar behaviour for constructs lacking the C-terminal
369 LR domain, that binds chromatin, or the N-terminal domain, which is required for the nucleolar
370 localisation of Ki-67 (Supplementary Fig. 7a, b), indicating that PS of Ki-67 is an autonomous
371 property of the protein and is not dependent on a specific localisation on chromatin or to the
372 nucleolus.

373 A recent *in vitro* study using purified peptides corresponding to repeat domains showed that
374 partial phosphorylation by CDKs of one of the Ki-67 repeats, or phospho-mimicking amino
375 acid substitutions, promotes PS, possibly by generating charge blocks⁶⁷. However, rG-RPA
376 and SCDM analysis predicts that the effects of phosphorylation on PS propensity strongly
377 depend on the sequence. To test this, we analysed each repeat individually by rG-RPA and
378 SCDM, and indeed found that effects of phosphorylation were dependent on the particular
379 repeat studied, as illustrated by the behaviour of repeats 1, 3 and 12. Full phosphorylation was
380 predicted to enhance PS for repeat 1 but suppress it for repeat 3 (Supplementary Fig. 8a, b).
381 Furthermore, we observed that phospho-mimicking substitutions, which each add one negative
382 charge, do not recapitulate effects of phosphorylation, which adds two per site, and can even
383 have opposite effects, as seen for repeat 12 (Supplementary Fig. 8a, b). These results indicate
384 that effects of phosphorylation on PS depend on sequence context and phosphorylation
385 stoichiometry.

386 We hypothesised that, for many IDPs, full site phosphorylation, which is characteristic of
387 mitosis⁹, might have different effects on PS from the lower levels of phosphorylation typical
388 of interphase, thereby providing directionality to PS changes during the cell cycle and

389 potentially creating a switch at mitosis. To test this hypothesis, we designed a synthetic IDP
390 constituting a single “consensus” synthetic Ki-67 repeat derived from an alignment of all 16
391 Ki-67 repeats, in effect constituting a novel IDP and model CDK substrate with multiple
392 potential phosphosites (Supplementary Fig. 9a). We expected that this protein might show a
393 different behaviour from that of the native full-length Ki-67 due to its single valency and
394 distinct sequence context. Indeed, MD simulation (Supplementary Movies 5, 6) showed that
395 the fully phosphorylated single synthetic Ki-67 repeat is less compact (Supplementary Fig. 9b)
396 and has lower tendency to phase separate (Supplementary Fig. 9c). This behaviour contrasts
397 with full length native Ki-67, where phosphorylation promotes PS. We purified the synthetic
398 repeat as a GFP-tagged polypeptide and phosphorylated it *in vitro* with recombinant CDK
399 complexes. Nuclear Magnetic Resonance spectroscopy showed a reduced amide proton
400 spectral dispersion typical for an IDP and confirmed appearance of at least 7 phosphorylated
401 residues when fully phosphorylated (Fig. 8a), while we mapped 11 phosphorylation sites by
402 phosphoproteomics (Fig. 8b). We next performed *in vitro* phase separation assays and
403 compared both partially and fully phosphorylated forms of the consensus repeat by varying
404 incubation times and the activity of purified CDK complexes (Fig. 8c). The purified synthetic
405 Ki-67 repeat could form droplets, and, as predicted, this was abolished upon full
406 phosphorylation by CDK, whereas partial phosphorylation had no effect (Fig. 8d), suggesting
407 that CDK-mediated phosphorylation acts as a buffered switch for phase separation. To define
408 the properties of this switch, we analysed all 2048 combinations of the 11 phosphorylation sites
409 on this synthetic protein by rG-RPA and SCDM. Strikingly, 1-6 phosphosites enhanced PS
410 propensity while 8-11 phosphosites strongly reduced it (Fig. 8e, top), which was consistent
411 with SCDM analysis (Fig. 8e, bottom). For low and high numbers of phosphosites, the overall
412 behaviour was independent of the specific combination of phosphorylated sites, whereas 7 sites
413 could either enhance or reduce PS propensity, depending on the exact combination. Taken
414 together, our results lend support to our hypothesis whereby CDK-mediated phosphorylation
415 can generate a buffered switch for homotypic interactions that contribute to PS.

416

417 **Discussion**

418 In this multidisciplinary study, we show *in vivo* that CDK-dependent mitotic phosphorylation
419 occurs synchronously on diverse proteins whose common denominators are a high level of
420 disorder, localisation to MLOs, and multisite phosphorylation. Our data suggest that the
421 majority of cell cycle regulated phosphorylation may be controlling phase separation of

422 components of membraneless organelles. Furthermore, CDK-mediated multisite
423 phosphorylation may act as a phase separation switch promoting an abrupt transition into
424 mitosis.

425 We first exploited the naturally synchronous cell cycles of *Xenopus laevis* to characterise cell
426 cycle-regulated phosphorylation in single embryos at ultra-high resolution, allowing us to
427 distinguish between progressive phosphorylation where different substrates become
428 phosphorylated sequentially, as suggested by a model in which a complex combination of
429 features encoded in the amino acid sequence of substrates determines phosphorylation timing
430 ¹⁴, from the rapid phosphorylation that might result from the observed switch-like mitotic
431 activation of CDK1 ⁶⁸. A previous large-scale analysis of cell cycle-regulated phosphorylation
432 used cell synchronisation procedures (nocodazole, thymidine) to show that potential
433 phosphorylation sites are fully phosphorylated in mitosis ⁹, but phosphorylation dynamics
434 could not be determined. True cell cycle synchronisation of entire cell populations has been
435 deemed impossible due to differences between cells in the timing of entry into and exit from
436 the blocks ²². Thus, the rather progressive dynamics of CDK-mediated phosphorylation seen
437 from synchronisation studies in fission yeast ⁶⁹ may not reflect the behaviour occurring in
438 single cells. Indeed, a comparison of cell cycle-regulated phosphorylation sites derived from
439 synchronised cells ⁹ or from non-synchronised cells selected by flow cytometry ²⁵ revealed that
440 the synchronisation procedures used previously may artificially select for maximal
441 phosphorylation. The same study ²⁵ also showed that all identified phosphosites that peaked in
442 mitosis displayed similar cell cycle behaviour, but the resolution was not sufficient to
443 distinguish differences in kinetics between different phosphosites; this might be due to the
444 elutriation windows used that, by definition, select similar but not identically sized cells from
445 the population. Thus, our study in single embryos is unique to date, and demonstrates that all
446 mitotic phosphosites observed undergo phosphorylation simultaneously, irrespective of the
447 nature of the substrate. This is not incompatible with differences in affinity of CDK1 for
448 different substrates, since a theoretical consideration of futile cycles demonstrates how graded
449 inputs can generate all-or-nothing outputs ⁴². We further show that this switch-like behaviour
450 does not depend on regulated phosphorylation of CDK1 itself, consistent with the observation
451 that tyrosine-15 phosphorylation of CDK1 is downregulated during early embryonic cell cycles
452 ^{32,41}; instead, it can likely be accounted for by regulation by Greatwall of the phosphatase that
453 reverses CDK-mediated phosphorylation, as well as by phosphorylation of NIPA, which
454 inactivates the SCF ubiquitin ligase that degrades cyclin B in interphase ⁷⁰.

455 Second, we reveal an underlying similarity between most cell cycle kinase substrates: they
456 contain a much higher proportion of disordered residues than other phosphoproteins. While it
457 has previously been found that intrinsic disorder generally predicts phosphorylation site
458 localisation for any protein, irrespective of the kinase ⁴³, and it was previously confirmed that
459 CDK sites conform to this rule by tending to cluster in regions of proteins predicted to be
460 disordered ^{6,44}, what distinguishes cell cycle-regulated phosphorylation from other
461 phosphorylation, and what determines which kinases are involved, has remained unknown. We
462 find that substrates of CDKs, Aurora and Polo-like kinases are far more disordered than the
463 phosphoproteome average. Even when substrates of two kinase families (CDK and MAP
464 kinases) with the same minimal consensus site motif are compared, the CDK substrates have
465 close to twice the disorder of MAP kinase substrates. This further highlights the enrichment
466 for disordered proteins in cell cycle processes, noted recently ⁸. Our data also resolve a
467 circularity problem inherent in earlier work: previous observations of enrichment of CDK sites
468 in disordered regions of proteins ^{6,44} might have a trivial explanation, since the amino acids
469 constituting the consensus site for CDKs (S, T, P, K and R) are already highly enriched in
470 disordered regions. Our bioinformatic approach corrects for this bias and confirms the validity
471 of the previous observations. We propose that cell cycle kinases have been selected by
472 evolution to phosphorylate the most highly disordered proteins of the proteome, and that this
473 necessarily requires several families of kinases, to phosphorylate sites in disordered regions of
474 proteins that are either positively charged at physiological pH (CDKs, Aurora) or negatively
475 charged (PLK), while Aurora and PLK cannot phosphorylate proline-proximal sites.

476 Our observations that cell cycle kinase substrates are more disordered than other proteins and
477 that they are frequently key components of MLOs lends the hypothesis that phosphorylation of
478 these substrates might regulate MLOs themselves, consistent with the cell cycle-dependent
479 assembly and disassembly of many MLOs. We provide a medium-throughput analysis of
480 effects of phosphorylation on different CDK substrates that substantiates this hypothesis, which
481 could only be achieved by applying a theoretical approach. Molecular dynamics simulations of
482 biomolecules require large computer resources and timescales and are currently essentially
483 unfeasible for modeling phase separation. In contrast, analytical simulations are powerful tools
484 that can predict conformational properties of IDPs and average the ensemble of disordered
485 states that depend on the sequence of IDRs, with high throughput and with minimal computer
486 load ⁵⁷. Theoretical models also allow us to systematically vary different inputs, such as post-
487 translational modifications, mutations, salt concentrations and pH to explore their relative

488 contributions. Our calculations with one IDP and solvent predict binary phase diagrams, and
489 the results match our experimental data for the protein that we use to test our hypothesis, Ki-
490 67.

491 A previous study provided evidence for phospho-regulation of MLOs in mitosis but attributed
492 it to a different kinase, DYRK3⁴⁵. However, DYRK3 inhibition led to mitotic formation of
493 abnormal hybrid condensates that contained material from various MLOs (splicing speckles,
494 stress granules and pericentriolar material), but did not prevent the normal breakdown of MLOs
495 such as nucleoli, nuclear pore complexes or Cajal bodies. Our data rather implicate CDK1 as
496 the likely major MLO regulator in mitosis. This is not unprecedented since CDKs can prevent
497 phase separation of components of replication complexes⁷¹, promote nuclear pore disassembly
498⁷² and dissolve stress granules in yeast⁷³. We additionally provide evidence that CDK-mediated
499 phosphorylation can both promote or inhibit biological phase separation, depending both on
500 the sequence context and the stoichiometry of phosphorylation. The latter adds an additional
501 switch-like regulation to the onset of mitosis: passing a threshold number of phosphorylations
502 can switch from promoting phase separation to inhibiting it. Thus, our data show that CDK-
503 mediated multisite phosphorylation may act as a buffered switch for phase separation, which
504 is independent of the exact combinations of phosphorylated sites, providing a robust underlying
505 mechanism that may contribute to the abruptness of the cellular reorganisation at the entry into
506 mitosis. Interestingly, phosphosites specific to meiotic M-phase samples appeared to be the
507 most highly phosphorylated, and the most multiphosphorylated peptides were detected in these
508 samples. We speculate that such a prevalence of “hyper-phosphorylation” might reflect the
509 long term maintenance of the M-phase state in unfertilised eggs, and suggest that a more
510 detailed quantitative analysis of the threshold phosphorylation levels required to generate and
511 maintain meiotic versus mitotic M-phase might reveal interesting differences between these
512 two states.

513 Finally, we provide evidence that the perichromosomal layer of mitotic chromosomes may be
514 liquid, and we suggest a mechanism for mitotic targeting of nucleolar components to the
515 perichromosomal layer by Ki-67^{59,60} via CDK-mediated phosphorylation, which reduces PS
516 propensity of several major nucleolar IDPs, thus triggering nucleolar disassembly, while
517 simultaneously promoting PS of Ki-67 to recruit nucleolar components to chromosomes.
518

519 **Methods**

520

521 *Egg collection and in vitro fertilisation.*

522 All animal procedures and experiments were performed in accordance with national
523 animal welfare laws and were reviewed by the Animal Ethics Committee of the Royal
524 Netherlands Academy of Arts and Sciences (KNAW). All animal experiments were conducted
525 under a project license granted by the Central Committee Animal Experimentation (CCD) of
526 the Dutch government and approved by the Hubrecht Institute Animal Welfare Body (IvD),
527 with project licence number AVD80100201711044. Female *X. laevis* frogs (aged >2 years;
528 Nasco, catalog N° LM00535) were primed with 50 international units (IU) of human chorionic
529 gonadotropin at least 2 days, and no more than 7-8 days before a secondary injection with 625
530 IU to induce ovulation. Roughly 16 hours after the second injection, fresh eggs were collected
531 by pelvic massage and kept in 1x Marc's Modified Ringer's (MMR). Next, eggs were placed
532 in a petri dish and checked under the microscope to keep only those that exhibited the healthy
533 pigment pattern (dark animal pole and white vegetal pole).

534 To perform the *in vitro* fertilisation, around 1/3 of a full testis (prepared from adult *X.*
535 *laevis* male frogs, purchased from the European Xenopus Resource Centre) was cut into fine
536 pieces and mixed with in 500 µl of 1x MMR. The suspension was pipetted up and down until
537 big clumps were dissolved. Next, buffer was removed from the petri dish and the eggs were
538 collected. Once eggs were well dispersed across the dish, the sperm suspension was added. The
539 dish was then flooded with 0.1x MMR to induce fertilisation.

540

541 *Sample collection.*

542 The first time point was collected immediately before adding the sperm suspension (time 0'
543 corresponds to the unfertilised egg). Eggs were kept at room temperature (18-20°) and under a
544 dissecting microscope after fertilisation. At approximately 15 minutes, fertilised eggs
545 underwent shrinkage of the animal hemisphere and rotation within the vitelline membrane, so
546 that the animal hemisphere faced upwards. These changes are known indicators of successful
547 fertilisation, so only the eggs that underwent these changes were used for the experiment.

548 Samples were collected approximately every 15 minutes. Eggs were rapidly placed in
549 individual tubes and snap froze in liquid nitrogen, trying to preserve the phosphorylation events
550 occurring at that specific time. Since the eggs were monitored under the microscope, we were
551 able to determine if samples were collected before or after a cell division had occurred.

552

553 *Xenopus* egg extracts.

554 For frog extract preparation, adult *Xenopus* females were obtained from the Centre de
555 Ressources Biologiques Xénopes (CRB; Rennes, France), and housed in a dedicated aquatic
556 facility located at the CRBM (Approval N° B34-172-39). Animals were used following
557 regulations according to the Direction Générale de la Recherche et Innovation and the French
558 Ministry of Higher Education, Research and Innovation. All procedures were validated by the
559 animal welfare committee of the Occitanie region.

560 Interphase *Xenopus* egg extracts were prepared, and DNA replication time courses
561 performed, as described previously ⁷⁶. In brief, eggs laid overnight in 150mM NaCl were
562 dejellied in degellying buffer (29mM Tris pH 8.5, 110mM NaCl, 5mM DTT); rinsed several
563 times in High Salt Barths solution (15mM Tris pH 7.6, 110mM NaCl, 2mM KCl, 1mM MgSO₄,
564 0.5mM Na₂HPO₄, 2mM NaHCO₃), twice in MMR (5mM HEPES-KOH pH 7.6, 100mM NaCl,
565 2mM KCl, 0.1mM EDTA, 1mM MgCl₂, 2mM CaCl₂), before activation with 0.3µg/ml
566 calcimycin ionophore in MMR. Subsequently, two rinses in MMR and two more in SB (50mM
567 HEPES-KOH pH 7.6, 50mM KCl, 2.5mM MgCl₂, 5% Sucrose, 0.014% β-mercaptoethanol)
568 followed, while during the last rinse the eggs were transferred on ice and SB was supplemented
569 with protease inhibitors (10µg/ml leupeptin, pepstatin and aprotinin). Eggs were spun down at
570 200g for 1min and excess of buffer removed before being centrifuged at 16,000g, 4°C for 10
571 minutes. Protease inhibitors and 10µg/ml cytochalasin B were added to the cytoplasmic
572 fraction. Extracts were further centrifuged in SW55Ti rotor for 20min at 20k rpm (48,000g) at
573 4°C. The cytoplasmic layer was extracted with a large-bore needle and syringe, and
574 supplemented with glycerol 3% and ATP regenerating system (10mM creatine phosphate,
575 10µg/ml creatine kinase, 1mM ATP, 1mM MgCl₂) added from a 20x stock. Aliquots were
576 frozen in liquid nitrogen. Mitosis was induced in extracts by adding recombinant GST-Cyclin
577 BΔ90 (40 ng/ml).

578

579 *Cell lysis.*

580 For the cell lysis, we used a similar approach to that described by Lindeboom *et al* ⁷⁷. Briefly,
581 each sample was thawed and homogenised with 15 µL of ice-cold cell lysis buffer (20mM Tris-
582 HCl pH 8.0, 70mM KCl, 1mM EDTA, 10% glycerol, 5mM DTT, 0.125% Nonidet P-40, 1mM
583 PMSF, 1x complete EDTA-free protease inhibitor, 1xPhoStop). Samples were subsequently
584 centrifuged at max speed on a benchtop Eppendorf centrifuge. 10 µL of soluble material was

585 recovered and snap-frozen in liquid nitrogen. Samples were stored at -80°C until further
586 processing.

587

588 *Protein digestion and phosphopeptide enrichment.*

589 Cell lysates were digested using the FASP method ⁷⁸. Briefly: proteins were thawed and
590 immediately reduced and alkylated with 10mM DTT and 0.05M iodoacetamide. Next, proteins
591 were digested with Lys-C (overnight) at 37°C in a wet chamber, followed by addition of trypsin
592 and further incubation under the same conditions for 4 hours. Both enzymes were used at 1:50
593 enzyme to protein ratio (protein quantification by Bradford assay showed that each individual
594 egg provides ~20 µg of yolk free protein). For the egg extract experiment, each FASP filter
595 was loaded with 200µg of protein. Peptides were cleaned using the Oasis HLB 96 well plates
596 (Waters Corporation) and consequently subjected to phosphopeptide enrichment using Fe(III)-
597 NTA 5µL cartridges in the automated AssayMAP Bravo Platform (Agilent Technologies), as
598 described by Post *et al* ²⁸. Both flow through (peptides) and eluates (phosphopeptides) were
599 dried down and stored at -80°C until further use.

600

601 *LC-MS/MS analysis.*

602 All samples for label-free shotgun proteomics were analysed using a UHPLC 1290 system
603 (Agilent Technologies) coupled to an Orbitrap Q Exactive HF mass spectrometer (Thermo
604 Fisher Scientific). Nano flow rate was achieved using a split flow setup aided by an external
605 valve as described by Meiring *et al* ⁷⁹. Peptides were first trapped onto a pre-column (inner
606 diameter [ID] of 100 µm and 2 cm length; packed in-house with 3µm C18 ReproSil particles
607 [Dr. Maisch GmbH]) and eluted for separation into an analytical column (ID of 75 µm and
608 50cm length; packed in-house with 2.7 µm Poroshell EC-C18 particles (Agilent Technologies).
609 The latter was done using a two-buffer system, consisting of buffer A (0.1% formic acid [FA]
610 in water) and buffer B (0.1% FA in 80% ACN). Peptides were trapped during 5 minutes at 5
611 µL/min flow-rate with solvent A. For the measurement of the full proteome, we used a 155 min
612 gradient from 10 to 36% of solvent B. For the phosphoproteome, we used a 95 min gradient
613 from 8 to 32% of solvent B. Both methods included a wash with 100% solvent B for 5 minutes
614 followed by a column equilibration with 100% solvent A for the last 10 minutes.

615 The mass spectrometer was operated in data dependent acquisition (DDA) mode. Full
616 scan MS was acquired from 375-1600 m/z with a 60,000 resolution at 200 m/z. Accumulation
617 target value was set to 3e6 ions with a maximum injection time of 20 ms. Up to 15 (12 for the
618 phosphoproteome) of the most intense precursor ions were isolated (1.4m/z window) for

619 fragmentation using high energy collision induced dissociation (HCD) with a normalised
620 collision energy of 27. For MS2 scans an accumulation target value of 1e5 ions and a maximum
621 injection time of 50 ms were selected. Scans were acquired from 200-2000m/z with a 30,000
622 resolution at 200m/z. Dynamic exclusion was set at 24s for the proteome and 12 s for the
623 phosphoproteome.

624 For targeted proteomics, an EASY-nLC 1200 System (Thermo Fisher Scientific)
625 coupled to an Orbitrap Q Exactive HF was used. Peptides were separated using an EASY-
626 Spray analytical column (ID of 75µm and 25cm length; packed with 2µm C18 particles with a
627 100 Å pore size) (Thermo Fisher Scientific). Gradient lengths were shortened to 60 minutes.
628 Phosphopeptides of interest from the previous experiment were selected and heavy-labeled
629 versions were synthesised (JPT Peptide Technologies). These synthetic standards were used
630 during method development for retention time scheduling and instrument ion fill-time
631 optimisation. Additionally, synthetic heavy peptides were pooled and combined with synthetic
632 retention time peptide standards (iRT, Biognosys) to generate a spectral library, measured in
633 DDA mode using the same LC-MS setup. This spectral library provided fragment intensity and
634 retention time information for quality control assessment of targeted measurements. Samples
635 were reconstituted in 2% FA containing ~200fmol of each synthetic standard. The mass
636 spectrometer was operated in data independent acquisition mode with an inclusion list of
637 targets for parallel reaction monitoring (PRM). The list included the m/z values for the heavy
638 and light versions of the phosphopeptides. Optimal measurement parameters were determined
639 using test samples spiked with the heavy-labeled standards in order to guarantee optimal
640 sensitivity for detection of endogenous phosphopeptides. We measured the targets of interest
641 in a scheduled fashion, during a four-minute window with a 120,000 resolution, maximum
642 injection time of 246ms and an accumulation target value of 2e5 ions, to ensure maximum
643 specificity and sensitivity.

644

645 *Data processing.*

646 DDA raw files were processed with MaxQuant⁸⁰ (v1.6.0.1) using a false discovery rate (FDR)
647 <0.01. The default settings were used, with the following exceptions: variable modifications,
648 specifically methionine oxidation, protein N-term acetylation and serine, threonine and
649 tyrosine phosphorylation were selected. Cysteine carbamidomethylation was selected as a
650 fixed modification. We also enabled the 'match between runs' option with the default values.
651 Fractions were set so that matching was done only among biological replicates and samples of
652 consecutive time points. The database search was conducted against a database generated by

653 Temu *et al*⁸¹. This was particularly helpful since other publicly available databases contained
654 several incomplete and/or poorly annotated sequences, which proved to be impractical for
655 further data analysis.

656 The data was uploaded to the Perseus platform⁸² for further analysis. Briefly: decoy
657 sequences and potential contaminants were filtered out. Only high confidence localisation
658 (>0.75 localisation probability) phosphosites were conserved for further analysis. Intensities
659 were log₂ transformed and then normalised by subtracting the median intensity of each sample.
660 Biological replicates were grouped accordingly by time point; this grouping allowed us to filter
661 the data and keep only those phosphosites that could be detected in at least two out of three
662 biological replicates in any of the time points. Missing values were imputed from a random
663 normal distribution applying a downshift of 1.8 times the standard deviation of the dataset, and
664 a width of 0.3 times the standard deviation. This effectively replaced missing values at the
665 lower end of the intensity distribution. We then performed an ANOVA (Benjamini-Hochberg
666 FDR <0.05) to determine which phosphosites displayed statistically significant changes
667 through the time course. Average phosphosite intensities were grouped using a combination of
668 k-means and hierarchical clustering using the ComplexHeatmap package⁸³ in R. Protein
669 intensities were processed in a similar fashion, removing proteins that were only identified by
670 peptides that carry one or more modified amino acids.

671 Next, the full list of proteins with significantly changing phosphosites were matched
672 against the human Uniprot database using the Basic Local Alignment Search Tool (BLAST),
673 to render Uniprot identifiers that were compatible with different Gene Ontology (GO) analysis
674 tools. We used the STRING web tool⁸⁴ to gain insight into the relation amongst dynamically
675 phosphorylated proteins. The full list of phosphoproteins was uploaded and analysed using
676 default settings. Next, the protein network was loaded into Cytoscape for clustering and
677 visualisation. Proteins were clustered using GLayer community clustering⁸⁵ and enrichment of
678 GO terms per cluster was obtained using BiNGO⁸⁶ (shown in Fig. 1B).

679 GO term enrichment was also acquired individually for each group (A-D) obtained after
680 hierarchical clustering of dynamic phosphosites. For this we used STRING and filtered the
681 enriched GO terms to keep only those with p <0.01, fold enrichment >5 and a minimum of 5
682 proteins per term. The list of terms was further condensed by removal of redundant terms using
683 the Revigo web tool⁸⁷. Remaining GO terms (including BP, MF and CC) were manually
684 curated to further avoid redundancy. The final set of GO terms per cluster are shown in
685 Supplementary Fig. 1. Following the same strategy, we analysed GO term enrichment for the

686 interphase and mitotic clusters from the *in vitro* dataset separately (shown in Supplementary
687 Fig. 3).

688 PRM raw files were analysed with Skyline software⁸⁸. Signal quality for each target of
689 interest was assessed visually for all samples. Quality control of endogenous signals was done
690 by confirming the perfect co-elution of both peptide forms (heavy and light), assessing their
691 retention time and peak shape. We also used the similarity of the relative intensity of fragment
692 ions ($r_{dotp} > 0.9$) between light and heavy to exclude signals that showed poor correlation.
693 Quantifications were done with a minimum of three fragments per phosphopeptide. The data
694 was loaded into R for data cleanup and visualisation, using the Complex-Heatmap and
695 ggplot2⁸⁹ packages.

696

697 *Motif analysis.*

698 Obtained phosphopeptides were aligned by centering them around the phosphosite detected
699 and the conserved motifs for the different kinases were determined using regular expressions
700 by applying the following rules:

701 PLK: [D/N/E/Y]-X-[S/T]-[Hydrophobic / ^P]

702 AURA/AURB: [K/R]-X-[S/T]*[^P]

703 NEK: [L|M|F|W]-X-[S|T]*-[A|V|I|L|F|W|Y|M]-[K|R]

704 Casein kinase 1: [D/E]-[D/E]-[D/E]-X-X-[S/T]* or [S/T]-X-X-[S/T]*

705 Casein kinase 2: [S/T]-[S/T]*-X-[E/D/S]

706 DDK: [S/T]*-[E/D]-X-[E/D] or [S/T]*-[S/T]-P

707 PKA: R-[R/K]-X-[S/T]*-[Hydrophobic]

708 Cdk full consensus motif: [S/T]*-P-X-[K/R]

709 Cdk minimal consensus motif: [S/T]*-P

710 Where [] groups multiple amino acids for one position, ^ at the left of a certain amino acid
711 informs that it is forbidden for that position, and X represents any amino acid.

712

713 *Data collection for human and yeast CDK1 targets.*

714 Data of CDK1 substrates for *S. cerevisiae* were downloaded from online supplementary
715 information of papers describing two different studies using *in vitro* (4) and *in vivo* (26)
716 approaches, respectively. We defined high confidence yeast CDK1 targets as the intersection
717 of both datasets. Other phosphorylations detected in both studies for which there was no
718 evidence for CDK1 involvement were considered as the non-CDK1-mediated
719 phosphoproteome (universe). For human CDK1 subfamily targets, we extracted information

720 available in the PhosphoSitePlus database ⁹⁰. An additional step of manual curation from the
721 following studies ^{4,5,62,91–106} was performed to obtain a high confidence human CDK1
722 subfamily targets dataset. The phosphoproteome universe was constructed with all the
723 phosphorylated proteins deposited in the PhosphoSitePlus database after subtraction of the
724 CDK1 subfamily targets. Data is available in Supplementary Data 2.

725

726 *Data collection for MLO proteomes.*

727 Data from proteomics studies of the composition of MLOs characterised by liquid-liquid phase
728 separation was obtained from the following sources: stress granules ^{107,108}, nuclear speckles
729 ^{108,109}, PML nuclear bodies ^{108,110}, P-bodies ¹¹¹, nucleoli ^{112,113}, nuclear pore complexes ¹¹⁴, Cajal
730 bodies ^{108,115}, Super-enhancer-Mediator condensates ¹¹⁶. Data is available in Supplementary
731 Data 4.

732

733 *Prediction of intrinsically disordered regions.*

734 For the UniProt proteomes of human, yeast and *Xenopus laevis*, disorder information was
735 fetched from MobiDB ¹¹⁷ with the exception of SPOT disorder predictor, which was calculated
736 for all the proteins of each dataset. For *Xenopus* proteomics studies, we used the available
737 standalone software of IUPred, VSL2B, and SPOT to predict IDRs in all the proteins of the
738 database. Data is available in Supplementary Data 3.

739

740 *Differential disorder composition.*

741 For the three organisms analysed (*Xenopus*, human and yeast), the amino acid composition for
742 the entire phosphoproteome and for the disordered regions of the phosphoproteome was
743 calculated. For each amino acid, we estimated the differential disorder composition with the
744 equation:

$$745 \text{(Comp. Disorder – Comp. Phosphoproteome) / Comp. Phosphoproteome.} \quad (1)$$

746 Positives values show aminoacids enriched in disordered regions while negative values
747 represent aminoacids depleted in disordered regions.

748

749 *Bioinformatic and statistical analysis of disorder and phosphorylation.*

750 All the statistical analysis was performed with the R programming language ([https://www.r-](https://www.r-project.org/)
751 [project.org/](https://www.r-project.org/)) using R studio as an integrated development environment (available at
752 <https://rstudio.com/>). The packages Tidyverse ⁸⁹ and Bioconductor ¹¹⁸ were used for cleaning,
753 manipulation, and graphical representation of the data. Sequence logos were generated using

754 the information content as described ¹¹⁹. IUPred scores were plotted with an *ad hoc* designed
755 script, available upon request.

756 For the contingency table analysis, the disordered regions of CDK targets and dynamic
757 phosphoproteins were calculated with three predictors (IUPred, VSL2B, and SPOT). For each
758 combination of disorder predictor and phosphorylation dataset, a two-by-two table with the
759 counts of phosphorylatable residues (Ser/Thr) phosphorylated or not, either located in IDRs or
760 in structured regions (Supplementary Fig. 6B), was generated. Each table was then analysed
761 with the Fisher test for obtaining the odds ratio and the associated P-value.

762 The source code for all the analysis conducted in this publication is available upon request.

763

764 *Calculation of SCDMs.*

765 Elements (i,j) of SCDM were calculated using equation 1 of Huihui and Ghosh ⁵⁶. In this coarse
766 grain model, each amino acid is considered a point with a charge $q = -1$ for Aspartic and
767 Glutamic acid, charge $q = 1$ for Arginine and Lysine, charge $q = 0$ for all other amino acids.
768 Phosphorylation is modeled by replacing neutral charge of Serine, Threonine to $q = -2$ to mimic
769 the effect of double negative charge of phosphate groups. SCDM maps were made visually
770 continuous between neighboring (i,j) pairs using spline-16 interpolation.

771

772 *Calculation of rG-RPA.*

773 Theoretical predictions of phase diagrams were made using a newly developed theory called
774 renormalised-RPA (rG-RPA) ⁵⁴. This theory explicitly accounts for sequence of charges in
775 the protein and builds temperature dependent phase diagrams yielding coexistence curves of
776 protein-dense and protein-dilute phases. At the present time, rG-RPA is the only
777 mathematical theory that reproduces known experimental and simulation results for both
778 polyampholytes and polyelectrolytes as a function of sequence charge (not just the
779 composition). It is an approach based on Hamiltonian (energy function) and hence do not
780 invoke any ad-hoc charge patterning terms and directly predicts full phase diagram. We used
781 the sequence of charges following the same protocol used for calculating SCDM for the
782 phosphorylated and non-phosphorylated proteins. In addition to the entropic and electrostatic
783 interaction terms used in rG-RPA, a generic Flory-Huggins type of mean-field interaction:

$$784 \chi = \epsilon / kT \quad (2)$$

785 term was added to the free energy. This interaction term models hydrophobic, cation-pi and
786 all other non-electrostatic interactions that may also drive phase separation. A common value

787 of $\epsilon = 2$ kT was used for all sequences for simplicity. The phase diagrams are given in terms
788 of a dimensionless temperature T^* . It is not possible to predict exact temperatures of phase
789 transition. However, the relative propensity to phase separate between two sequences can be
790 predicted by comparing phase diagram in this non-dimensional temperature unit. Since the
791 goal of the study is to investigate the electrostatic role of phosphorylation (induced by CDK)
792 in phase separation, we used the same value of ϵ for both the phosphorylated and non-
793 phosphorylated sequences. The generic choice of $\epsilon = 2$ kT is reasonable based on transfer
794 energy of solvophobic amino acids¹²⁰, and any other non-electrostatic interaction such as pi-
795 pi, cation-pi that may drive phase separation. For Ki67 consensus sequence and individual
796 repeat units, we used a slightly higher value of $\epsilon = 2.5$ kT since experiments required
797 crowding to induce phase separation. Overall trends are insensitive -- even though the relative
798 degree of differences in phase separation propensity may change -- if higher values of ϵ are
799 used, indicating robustness of the predictions of increased or suppressed propensity to phase
800 separate due to CDK-mediated phosphorylation. Codes for rG-RPA calculation are uploaded
801 in Github [<https://github.com/MaxCalLab/IDPTheory/tree/main/rG-RPA>].

802

803 *Selection of IDRs for SCDM and rG-RPA calculations.*

804 Intrinsically disordered region (IDR) selection for each protein was accomplished using the
805 following protocol. Regions of high disorder were identified primarily using the AlphaFold 2
806 (AF2) predicted local-distance difference test (pLDDT) – a measure of confidence on the
807 residue level^{58,121,122}. Recent work has suggested that residues with very low pLDDT scores
808 (pLDDT < 50) are likely to be disordered¹²³. In cases where no published structure existed in
809 AF2, the IUPred2A score was used and IDRs were chosen as the longest region with a score
810 above 0.5 (scores above 0.5 indicate intrinsic disorder)^{124,125}. For proteins with an AF2 entry,
811 the choice of IDR was made for the longest region where the majority of residues (more than
812 60%) had pLDDT scores below 50. This was done by extracting raw pLDDT values from the
813 mmCIF files available for each protein entry on AF2. Further refinement of IDR selection can
814 be accomplished by finding the longest ordered region (LOR), *i.e.* the longest stretch of
815 consecutive residues with pLDDT values above 50, within the IDR. If the individual LOR
816 represented more than 15% of total residues within the IDR, the LOR and following IDRs were
817 not included in the parent IDR assignment. The choice of 15% used as a threshold is arbitrary
818 and may be adjusted.

819

820 *Coarse-grained force field.*

821 We adopted a one-bead-per-residue coarse-grained (CG) model that has been shown to capture
822 the structural and phase separation properties of flexible proteins as a function of their sequence
823 ¹²⁶ and phosphorylation pattern ¹²⁷. In this framework, bonded interactions between
824 neighboring residues were modeled using a harmonic potential with a constant of 1000
825 kJ/mol/nm² and a bond length of 0.38 nm. Electrostatic interactions between charged residues,
826 i.e. Asp, Glu (-1e); Lys, Arg (+1e), His (+0.5e); ph-Ser, ph-Thr (-2e), were computed with a
827 screened Coulomb potential, using a Debye-Huckel length of 1 nm. Short-range interactions
828 were modeled by Lennard-Jones (LJ) potentials defined by

829

$$830 V_{ij}(r) = 4\lambda_{ij}\epsilon((\sigma_{ij}/r)^{12} - (\sigma_{ij}/r)^6) \quad (3)$$

831

832 where values of λ_{ij} , ϵ , and σ_{ij} used reported values^{126,127} with the exception of λ_{ArgArg} that was
833 set equal to 0.01, instead of 0.00, in order not to neglect excluded volume effects while using
834 LJ functional form. In the simulation of full-length Ki-67, the conformation of the N-terminal
835 folded domain (1-128) was restrained by an elastic network based on experimental structure
836 (PDB: 1R21) with a distance cutoff of 2 nm and an elastic constant of 5000 kJ/mol/nm². All
837 MD simulations were performed with GROMACS 2018.3 ¹²⁸. Simulations were run in the NVT
838 ensemble, controlling the temperature by means of a Langevin thermostat with a friction
839 constant of 25 ps⁻¹ and a time step of 10 fs.

840

841 *Single chain MD simulations of full-length Ki-67.*

842 The structure with PDB id 1R21 (<https://www.rcsb.org/structure/1r21>) was used to model the
843 conformation of the first 128 residues of the full-length Ki-67, while TraDES ^{129,130} was used
844 to generate the initial conformation of the remaining disordered part of the non-
845 phosphorylated molecule. The models were then joined using UCSF Chimera 1.14 ¹³¹. The
846 initial configuration for the phosphorylated full-length Ki-67 was obtained by ‘mutating’ (i.e.
847 using parameters that have been tuned specifically for ph-Ser/ph-Thr) the amino acids in the
848 non-phosphorylated structure. Initial configurations were inserted in a cubic simulation box
849 with a side of 100 nm with periodic boundary conditions. We employed a Parallel Tempering
850 (PT-MD) scheme, with eleven replicas in the 300-400K range in order to enhance the
851 conformational sampling and obtain a reliable estimate of the radius of gyration of the protein

852 as a function of the temperature. PT-MD simulation ran for $1e8$ steps, attempting Monte
853 Carlo exchanges between neighboring replicas every $1e2$ steps and saving snapshots every
854 $1e4$ steps for further analysis. This simulation length is sufficient to converge the observables
855 of interest, in particular the radius of gyration of the polymer chains (see Supplementary
856 Figure 10). Data are provided in Supplementary Data 6.

857

858 *Single chain MD simulations of consensus repeats.*

859 The initial extended configuration of the non-phosphorylated consensus repeat of Ki-67 was
860 generated with the tLEAP tool available in AmberTools18¹³² while the phosphorylated
861 structure was generated by ‘mutating’ (i.e. using parameters that have been tuned specifically
862 for ph-Ser/ph-Thr) the residues in the non-phosphorylated configuration. PT-MD simulation
863 followed the same protocol employed for the simulations of full-length Ki-67, with the only
864 difference being the range of temperatures, between 200K and 500K, with intervals of 10K.
865 Data are provided in Supplementary Data 6.

866

867 *Phase Coexistence MD simulations.*

868 Phase coexistence simulations of monomers and dimers of the non-phosphorylated consensus
869 repeat and of monomers of the phosphorylated consensus repeat were carried out employing
870 the slab method¹²⁶. Initial configurations of the slab simulations for each system were
871 generated by inserting 200 copies of the respective molecules in a $20 \times 20 \times 30$ nm box in
872 random positions and orientations with the *gmx insert-molecules* tool available in
873 GROMACS 2018.3¹²⁸, and extending the simulation box in the z direction to 200 nm.
874 Simulations were run for $1.5e8$ steps, saving frames every 10000 steps for further analysis,
875 discarding the first $0.5e8$ steps of the simulations as equilibration. This simulation length is
876 sufficient to equilibrate the density profiles along the z coordinate of the system used to
877 evaluate the equilibrium densities (see Supplementary Figure 11). Data are provided in
878 Supplementary Data 6.

879

880 *Estimation of theta temperatures.*

881 Coil-to-globule transition temperatures (T_θ) were estimated for non-phosphorylated full-length
882 Ki-67 and the monomer of the non-phosphorylated consensus repeat from single chain PT-MD
883 simulations, following a reported approach¹³³. Intramolecular distances as a function of the
884 chain separation $|i-j|$ were computed for each temperature replica and fitted with the following
885 expression:

886

$$887 \quad R_{ij} = 0.55|i - j|^\nu \quad (4)$$

888

889 where the scaling exponent ν is the fitting parameter. We then determined T_0 as the temperature
890 corresponding to $\nu = 0.5$

891

892 *Estimation of binodal curves.*

893 Density profiles from phase coexistence simulations were estimated by means of the gmx
894 density tool available in GROMACS 2018.3, centering the dense phase in the middle of the z-
895 axis. The concentration of the diluted phase was computed by averaging the density in the box
896 at $0\text{nm} < z < 60\text{nm}$ and $140\text{nm} < z < 200\text{nm}$, while the concentration of the dense phase was
897 obtained by averaging the density at $90\text{nm} < z < 110\text{nm}$. Following the approach of¹²⁶ the critical
898 temperatures, T_c , for the three systems investigated with phase coexistence simulations, have
899 been evaluated by fitting the following equation:

900

$$901 \quad \rho_H - \rho_L = A(T_c - T)^{0.325} \quad (5)$$

902

903 *Human opto-Ki-67 plasmid construction.*

904 pCDNA5_FRT_Ki67-FL(or repeats+LR or dLR)-mCherry-Cry2 was generated by PCR
905 amplification of human Ki67 from pcDNA5-FLAG-GFP-Ki-67 plasmid⁵⁹. The following
906 primers were used for the cloning:

907 full-length Ki-67:

908 1st fragment (2501bp) F1 Fwd taagcttggtaccatcgatgatgtggcccacgagacgcc F1 Rev

909 ttctctaataactgcccgtcttaaggagg

910 2nd fragment (5314bp) F2 Fwd gacggcagtgtattagagaaaatggaaacgtagc F2 Rev

911 tgggacgtgtcttggggcatctctttgtg

912 3rd fragment (1955bp) F3 Fwd atgcccgaagacacgtcccaggaaagaag F3 Rev

913 taccctcctgccttagacaccatgataatattctcactgtccctatgac

914 Repeats+LR construct :

915 1st fragment (2501bp) F1 Fwd taagcttggtaccatcgatgatgtggcccacgagacgcc F1 Rev

916 ttctctaataactgcccgtcttaaggagg

917 2nd fragment (6285bp) F2 Fwd gacggcagtgtattagagaaaatggaaacgtagc F2-r+LR Rev

918 taccctcctgccttagacaccatgattggtgtttgagagactcttgaagctg

919 dLR construct :

920 1st fragment (4812bp) dLR-F1 Fwd taagcttgggtaccatcgatgatgactaaaatgccctgccagtcattacaacc F2

921 Rev tgggacgtgtcttggggcatctctttgtg

922 2nd fragment (1955bp) F3 Fwd atgcccgaagacacgtcccaggaaagaag F3 Rev

923 tctctctcgcctttagacaccatgataatatcttcaactgtcctatgac

924 The three fragments were cloned into the AflII/KpnI digested pCDNA5_FRT_TO_TurboID-
925 mCherry-Cry2 (Addgene plasmid # 166504) using the In-Fusion HD Cloning Kit protocol.

926

927 *Generation of Flp-In T-REx 293 opto-Ki67 cell lines.*

928 Flp-In T-REx 293 (Termo Fisher Scientific, Darmstadt, Germany) cell line was grown under
929 standard conditions (37°C, 5% CO₂) in Dulbecco's modified Eagle's medium (Merck-Sigma-
930 Aldrich, D5796). The medium was supplemented with 10% fetal bovine serum (FBS), 100 µg/
931 ml Zeocin and 15 µg/ml Blasticidin. One million HEK-293 T-REx cells were plated in a 6-well
932 plate 24 hours before transfection. The next day, 500 ng of each optoKi67 expression plasmid
933 is combined with 3.5 µg pOG44 encoding the Flp recombinase. Transfection was made with 8
934 µl Lipofectamine 2000 according to the manufacturer's instructions. 48 hours post transfection,
935 cells were transferred to a 100 mm petri dish. On the next day, selection was performed by
936 adding hygromycin B at a final concentration of 50 µg/mL. Around 14 days after selection,
937 clones were pooled and expanded. The cells were tested for the expression of the construct by
938 immunofluorescence. Flp-InT-Rex 293 opto-Ki67 stable cell lines were maintained with 15
939 µg/mL Blasticidin and 15 µg/mL Hygromycin.

940

941 *Overexpression of GFP-Ki-67.*

942 HeLa cells were plated at the density of 150×10^3 cells per well in 6-well plate, containing 10mm
943 cover slips. Cells were transfected the following day with GFP-Ki-67⁵⁹ using FuGene reagent
944 following the manufacturer's instructions, at 3:1 FuGene/DNA ratio. Cells were fixed with
945 3.7% formaldehyde 24h or 48h later, stained with the indicated antibodies and mounted on
946 slides with Prolong Gold with DAPI (Invitrogen, #P36935). Images were taken with Leica
947 Thunder upright microscope using a 100x objective, with concurrent Computational Clearing.

948

949 *Fluorescence recovery after photobleaching (FRAP).*

950 HeLa cells were seeded in glass bottom plates and 24h after they were transfected with a Ki-
951 67-GFP tagged plasmid⁵⁹ with lipofectamine 2000 in Optimem serum-reduced medium

952 following the manufacturer's recommendations. FRAP was performed 24h or 48h after
953 transfection in a confocal Zeiss LSM980 NLO microscope at 37°C and 5% CO₂. Image
954 acquisition was made with 488nm laser and photobleaching was performed with the same laser
955 and 75-80% power. Multiple regions per cell were photobleached. Two images were acquired
956 pre-photobleaching and every 1s for 100s. Average fluorescence intensity values over time
957 were obtained for each region, for the corresponding contiguous unbleached regions and for
958 the nucleus, with the FIJI plugin "create spectrum jru v1". Cells and/or regions that moved
959 were excluded from the analysis. Analysis was continued in R. Correction for non-intentional
960 acquisition photobleaching was performed by dividing intensity values of the photobleached
961 region or the contiguous unbleached region by the average of the whole nucleus for each time
962 point. Normalisation was performed by setting the intensity values pre-photobleaching (time =
963 -1s) as the maximum. Normalised average fluorescence recovery curves of the photobleached
964 regions and unbleached regions of each cell were plotted in the same graph. The average
965 intensity values over time of the photobleached regions were fitted with the following non-
966 linear regression equation from the frapplot library in R:

967

$$968 \quad y = c - a \cdot e^{b \cdot t} \quad (6)$$

969 Where:

970 $c = y_{\max}$ = maximal intensity value

971 $c - a = y_{\min}$ = minimal intensity value

972 $b = -k$

$$973 \quad T - half = \frac{1}{k \cdot \ln(2)} \quad (7)$$

974

$$975 \quad \text{Total recovery} = TR = \frac{y_{\max} - y_{\min}}{1 - y_{\min}} \quad (8)$$

976

977

978 Each cell was classified by the type of Ki-67 expression: mitosis (perichromosomal layer),
979 interphase (low-medium levels of Ki-67 expression) and high expression in interphase. The
980 half-time and percentage of recovery values were plotted in R with the ggplot2 library.
981 Statistical significance was calculated with the Wilcoxon test in R.

982

983 *Opto-Ki-67 activation.*

984 Cells were plated in DMEM on coverslips a day prior to activation. Expression of opto-Ki67
985 was induced with 2 µg/ml doxycycline for 16 hours. For light activation, plates were transferred
986 into a custom-made illumination box containing an array of 24 LEDs (488nm) delivering 10
987 mW/cm² (light intensity measured using a ThorLabs-PM16-121-power meter). Cry2 activation
988 was induced using 4 min of blue light cycles: 4s On followed by 10s Off. Cells were fixed with
989 4% paraformaldehyde (PFA) for 15 min at RT, counterstained with Hoechst (Invitrogen, Cat
990 H21491) in PBS-TritonX (0.2%), and mounted on glass slides using Prolong Gold antifade
991 reagent (Invitrogen, Cat P36930). Where indicated, cells were counterstained with antibodies
992 and images were captured using a 63x objective (NA 1.46 oil).

993 To check the effect of inhibitors on Ki-67 foci formation, the cells were incubated for 1h with
994 0.5 µM okadaic acid, 5 µM purvalanol A or vehicle (DMSO) for 1 hour prior to light activation
995 and fixation. Foci number was analysed using FIJI Software and statistical significance was
996 assessed by one-way ANOVA on ranks (Kruskal–Wallis test) and pairwise post-hoc
997 comparisons using the Mann–Whitney test. P-values were adjusted by the Benjamini-
998 Hochberg method. Plots were generated using the ggplot2 library in R.

999

1000 *Ki-67 consensus repeat DNA construct.*

1001 The cDNA sequence coding for Ki-67 consensus repeat (Ki67-CR) was ordered from IDT®
1002 gene synthesis. It was subsequently cloned into pDB-GFP plasmid between HindIII and XhoI
1003 sites to obtain the pDB-GFP-Ki-67-CR vector. In this construct, Ki-67-CR was fused with a
1004 (his)₆-GFP N-terminal tag. GFP sequence is followed by the HRV 3C (3C) protease
1005 recognition site (Leu-Glu-Val-Leu-Phe-Gln/Gly-Pro). Specific cleavage can occur between
1006 Gln and Gly, with Gly-Pro remaining at the C terminus Ki-67-CR without any tag.

1007

1008 *Ki-67 consensus repeat expression and purification.*

1009 The pDB-GFP-Ki-67-CR plasmid was transformed into *E. coli* BL21(DE3); transformed cells
1010 were grown overnight at 25°C in N-5052 auto-induced medium¹³⁴, supplemented with 50

1011 $\mu\text{g/ml}$ kanamycin and 15N NH_4Cl . Cells were harvested by 20 min centrifugation at 6000g at
1012 4°C . The pellet was resuspended in 20 mM Tris-HCl pH 7.5, 300 mM NaCl and 2mM DTT
1013 (buffer A) and stored at -80°C . Cells were supplemented with a Complete® EDTA free tablet
1014 (Roche), lysed by sonication, insoluble proteins and cell debris were sedimented by
1015 centrifugation at 40000g at 4°C for 30 min. Supernatant was supplemented with imidazole to
1016 5 mM final and loaded onto 5ml gravity affinity columns (Ni Sepharose Excel 5ml, Cytiva),
1017 equilibrated with buffer A. Columns were washed with 50 ml of buffer A and proteins were
1018 eluted with a one-step gradient of buffer B (buffer A containing 500 mM imidazole). The peak
1019 fractions were analysed by SDS-PAGE. Fractions containing tagged Ki-67-CR were pooled
1020 and dialysed overnight at 4°C against buffer C (50 mM Bis-Tris pH 6.7, 50 mM NaCl, 2 mM
1021 DTT). The dialysed protein was then loaded on a Superdex S200 16/60 (HiLoad 16/600
1022 Superdex 200pg, Cytiva) equilibrated with buffer C. Fractions containing the protein of interest
1023 were analysed by SDS-PAGE and pooled. The purified GFP-Ki-67-CR protein was
1024 concentrated to 5 mg/ml with Vivaspin centrifuge concentrator (Sartorius Stedim Biotech).

1025

1026 *In vitro Ki-67 peptide phosphorylation assay.*

1027 Protein was desalted by using PD10 Mini-Trap column in 50 mM Hepes 7.5, 50 mM NaCl, 2
1028 mM DTT. Phosphorylation reaction was performed in a total volume of 220 μl , and contained
1029 140 μl of GFP-Ki-67-CR (400 μg), 5mM MgCl_2 , 500 μM ATP, 50mM β -glycerophosphate,
1030 recombinant CDK1-cyclin B/CKS1 (5 μg ; gift from Jane Endicott and Tony Ly), or
1031 recombinant GST-CDK2-cyclin A (ProKinase) in 50 mM Hepes pH 7.5, 50 mM NaCl, 2 mM
1032 DTT. Reaction was incubated at 30°C for 18 hours and stopped by adding 10 mM EDTA. It
1033 was subsequently desalted by using PD10 Mini-Trap column in 50 mM Bis-Tris pH 6.7, 50
1034 mM NaCl, 2 mM DTT, and used to perform NMR experiments, Phos-tag SDS-PAGE and
1035 phosphoproteomics.

1036

1037 *NMR experiments and data analysis.*

1038 All NMR samples contained final concentrations of 10% D_2O and 0.5 mM 4,4-dimethyl-4-
1039 silapentane-1-sulfonic acid (DSS). Experiments were performed at 293 K on a Bruker Avance
1040 III spectrometer equipped with a cryogenic triple resonance probe and Z gradient coil,
1041 operating at a 1H frequency of 700 and 800 MHz. 15N-HSQC was acquired for each sample
1042 in order to determine amide (1HN and 15N) chemical shifts of non-phosphorylated and
1043 phosphorylated GFP-tagged Ki-67-CR. 15N-HSQC spectra were acquired for respectively
1044 non-phosphorylated (and phosphorylated) proteins at 800 (700) MHz using 32 (128) scans, 128

1045 (256) increments and a spectral width of 22.5 ppm in the indirect dimension. All spectra were
1046 processed with TopSpin v3.5 (Bruker Biospin) and analysed using CCPN-Analysis software
1047 ¹³⁵. Chemical shifts were referenced with respect to the H₂O signal relative to DSS using
1048 the 1H/X frequency ratio of the zero point according to Markley et al ¹³⁶.

1049

1050 *Total internal reflection fluorescence (TIRF) experiments.*

1051 In order to measure the ability of the phosphorylated and non-phosphorylated Ki-67 repeat to
1052 liquid-liquid phase separate, 50 µM protein was prepared in 50 mM Bis-Tris pH 6.7, 50 mM
1053 NaCl, 2 mM DTT. Dextran was added just before preparing samples on circular glass
1054 coverslips (2.5 cm, 165 µm thick, Marienfeld). Coverslips were cleaned with a 15 min cycle of
1055 sonication with ultrasounds in 1M KOH, followed by a second cycle of sonication in deionized
1056 water. Samples were deposited into wells of Press-to-seal silicone isolater with adhesive
1057 (Invitrogen), and covered with a second coverslip to avoid evaporation. Images were acquired
1058 with a custom-made TIRF microscope using a LX 488-50 OBIS laser source (Coherent). Oil
1059 immersion objective with a 1.4 numerical aperture (Plan-Apochromat 100x, Zeiss) was used.
1060 Fluorescence was collected with an EmCCD iXon Ultra897 (Andor) camera. The setup
1061 includes a 1.5x telescope to obtain a final imaging magnification of 150-fold, corresponding to
1062 a camera pixel size of 81.3 nm. Fluorescence images at different time points were obtained by
1063 averaging 150 individual images, each acquired over 50 ms exposure time.

1064

1065 *Phos-tag SDS-PAGE.*

1066 For Phos-tag SDS-PAGE (12.5% Phos-tagTM SuperSepTM pre-cast gel 50 µmol/L; Fujifilm
1067 Wako Chemicals #193-16571), 500 ng of unphosphorylated and 500 ng of CDK1-cyclin B-
1068 CKS1 phosphorylated GFP-Ki-67-CR protein were loaded. SDS-PAGE was performed
1069 following standard protocol, with the exception of two additional washes 20 min each in
1070 transfer buffer containing 10 mM EDTA, followed by a wash in transfer buffer, preceding wet
1071 transfer.

1072

1073 *Antibodies*

1074 The following commercial antibodies were used in this study:

1075 MeCP2 (Abcam, ab253197; IF, 1:500)

1076 H3K9me3 (Abcam, ab8868; IF, 1:500)

1077 Nucleolin (Abcam, ab22758; IF, 1:1000)

1078 Nucleophosmin (Abcam, ab183340; IF, 1:1000)

1079 GFP (Chromotek, PABG1; Western blot, 1:10 000)
1080 Alexa Fluor 568 conjugated goat anti-rabbit (Invitrogen, A11011; IF, 1:1000)
1081 Goat anti-rabbit IgG (H+L) HRP (Thermo Fisher, # 32260; WB, 1:10 000)

1082

1083 *Mapping of Ki-67-CR phosphorylation sites by mass spectrometry*

1084 6.5 µg of GFP-Ki-67-CR protein phosphorylated by CDK1-cyclin B-CKS1 were digested in a
1085 FASP filter as described earlier for the other samples. Peptides were subsequently cleaned
1086 using C18 cartridges and phosphor-enriched using Fe(III)-NTA cartridges in the AssayMAP
1087 Bravo. Phosphopeptides were measured in a technical duplicate, acquiring in DDA mode with
1088 an Ultimate 3000 uHPLC system coupled to an Orbitrap Exploris 480 (Thermo Fisher
1089 Scientific) during a 60 minutes gradient. Raw files were searched against the Ki-67-CR
1090 sequence using MaxQuant with the same parameters as applied to the other phosphoproteomics
1091 experiments.

1092

1093 **Data availability**

1094 The mass spectrometry shotgun proteomics data generated in this study have been deposited
1095 in the ProteomeXchange Consortium via the PRIDE ⁷⁴ partner repository with the dataset
1096 identifier PXD023310
1097 [<https://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PX023310>]. The
1098 targeted proteomics data generated in this study have been deposited via Panorama ⁷⁵ with the
1099 identifier PXD026088
1100 [<https://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PX026088>].

1101 The following public databases were used in this study: UniProt [<https://www.uniprot.org/>];
1102 AlphaFold [<https://alphafold.ebi.ac.uk/>]; PhosphositePlus
1103 [<https://www.phosphosite.org/homeAction.action>]; Protein Data Bank
1104 [<https://www.rcsb.org/>]. Source data are provided with this paper.

1105

1106 **Code availability**

1107 Codes for rG-RPA calculation are uploaded in Github
1108 [<https://github.com/MaxCalLab/IDPTheory/tree/main/rG-RPA>]. Codes for phosphorylation
1109 and disorder analysis developed in this study are uploaded in Github
1110 [<https://github.com/gero007/CDK-mediated-phosphorylation-of-IDRs>].

1111

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1414 EAG, DC, MPa, AB, NS, LK and GD performed experiments and interpreted the data. MV
1415 and AC supervised GvM and EAG, respectively. PB provided advice. JMV, LK, GD, KG, DF
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1418

1419 **Figure legends**

1420 **Figure 1. The time-resolved phosphoproteome from a single-cell to a 16-cell embryo and**
1421 **its cell cycle assignment. a.** Schematic representation of the workflow. Single *Xenopus* eggs
1422 and embryos were collected followed by cell lysis, protein digestion, phosphopeptide
1423 enrichment and high-resolution proteomics analysis. **b.** STRING network of functionally
1424 associated proteins undergoing dynamic phosphorylation (each node represents a protein).
1425 Vicinity clustering reveals three main groups (yellow, blue and orange) with a high degree of
1426 association. Radar plots show the corresponding GO terms (Benjamini-Hochberg adjusted p
1427 value <0.05) for each group (axes show $-\text{Log}_{10}(\text{adj p value})$ for each GO term). **c.**
1428 Hierarchical clustering of significantly changing phosphosites (ANOVA, Benjamini-
1429 Hochberg correction, FDR 0.05), reveals 4 clusters with distinct regulation (A-D). Dashed
1430 boxes in clusters A and D are zoomed-in to highlight dynamic phosphorylation patterns
1431 (dashed lines depict the time points of cell division). Time point 0h00 corresponds to the
1432 unfertilised egg. **d.** Scheme of the experiment in the *Xenopus* egg extract. **e.** Top:
1433 quantification of DNA replication in each biological replicate. Below: Hierarchical clustering
1434 of dynamic phosphosites (ANOVA, Benjamini-Hochberg correction, FDR 0.05) reveals
1435 differential regulation of phosphosites during S-phase and mitosis. **f.** Overlap between *in vivo*
1436 (embryo) and *in vitro* (egg extract) phosphoproteomics. **g.** Proportion of phosphosites
1437 according to their potential upstream kinase for each cluster in the *in vivo* (left) and *in vitro*
1438 (right) experiments; DDK, Dbf4-dependent kinase, AURK, Aurora kinase. **h.** Circle plots
1439 presenting enrichment of homologues of human CDK substrates among *Xenopus*

1440 phosphoproteins detected *in vivo* and those with dynamic phosphosites. Source data are
1441 provided as a Source Data file.

1442

1443 **Figure 2. Switch-like mitotic phosphorylation *in vivo*.** **a.** Schematic representation of the
1444 workflow for high-time resolution analysis of mitotic phosphosites. Samples were collected
1445 over two cell divisions and enriched phosphopeptides were subjected to targeted proteomics
1446 analysis. **b.** Heat map shows a highly synchronous wave of phosphorylation preceding each of
1447 the two cell divisions. Dashed lines depict times when cell divisions were recorded. **c.** Single
1448 phosphosite plots from selected proteins related to different biological processes. Each dot
1449 represents a biological replicate (n=3). Dashed lines depict times when cell divisions were
1450 recorded. **d.** Single phosphosite plot of CDK1 inhibitory phosphorylation (Y15). **e-g.**
1451 Phosphorylation dynamics of the CDK1-oscillator network. Single phosphosite plots of CDK1
1452 regulators measured by shotgun (**e**) or targeted (**f**) phosphoproteomics. **g.** CDK1-oscillator
1453 network: our data suggest that control of cyclin levels via positive (*e.g.* NIPA ubiquitin ligase)
1454 and negative (*e.g.* APC, anaphase-promoting complex) feedback loops, accompanied by
1455 protein phosphatase 2A (PP2A) inactivation via Greatwall kinase (GWL), can generate
1456 oscillation of CDK1 activity during early cell divisions. CDK1-Y15 regulation via feedback
1457 loops consisting of CDC25 phosphatase and Wee1-like protein kinase 1-A (WEE1A; greyed
1458 out) seems to be less important for switch-like mitotic phosphorylation after the first cell
1459 division. Source data are provided as a Source Data file.

1460

1461 **Fig. 3. The cell cycle phosphoproteome is characterised by intrinsic disorder and MLO**
1462 **components.** **a.** Scheme illustrating hypothetical enrichment of phosphorylation in disordered
1463 regions when taking into account amino acid compositional bias. **b.** Scatter plot of expected vs
1464 observed phosphorylated Ser/Thr for each protein of human and *Xenopus* phosphoprotein
1465 datasets. FDR thresholds of 5% and 1% are marked in yellow and red respectively. Circles:
1466 proteins with at least one dynamic phosphorylation in *Xenopus*, or human CDK1 subfamily
1467 substrates, respectively. **c.** Boxplots showing expected vs observed phosphorylated Ser/Thr in
1468 IDRs (IUpred) among all phosphoproteins detected (left), phosphoproteins with at least one
1469 dynamic phosphosite (middle), and dynamic phosphoproteins also detected as CDK1
1470 subfamily targets in humans (right). Distributions were compared with the paired Wilcoxon
1471 signed-rank two-sided test. All phosphoproteins (1843), $p < 2.2e-16$; Dynamic (646), $p < 2.2e-16$;
1472 Dynamic and human CDK target (147), $p = 1.0e-14$. Boxplots center, median; lower and upper

1473 edges, 25% and 75% quartiles, respectively. Whiskers, data with the largest or smallest values
1474 not further than 1.5* interquartile range (IQR) from the upper or lower box limits, respectively.
1475 Beyond these values, data were not plotted, for clarity, but were included in the statistical
1476 analysis. **d.** Plots showing the common Odds Ratio of Ser/Thr phosphorylation in structured
1477 and disordered regions calculated with the Fisher's test (see Supplementary Fig. 5b, c for the
1478 statistical analysis scheme and exact p values). For all organisms, the disordered regions were
1479 calculated with three different disorder predictors. The disordered fraction is presented in a
1480 colour scale. **e.** Violin plots of the distribution of disordered residues per protein for CDK
1481 targets vs the rest of the phosphoproteome for human and yeast, and dynamic phosphoproteins
1482 vs the rest of the phosphoproteome for *Xenopus*. Intrinsic disorder was calculated with three
1483 different predictors (IUPred, SPOT, and VSL2b). Statistical significance was evaluated with
1484 the Wilcoxon–Mann–Whitney signed-rank two-sided test: *Xenopus* (CDK targets, 646, Non-
1485 CDK targets, 1198): IUPred $p=2.36e-13$, SPOT $p=4.83e-13$, VSL2b $p=5.78e-10$; Human
1486 (CDK targets, 16167, Non-CDK targets, 2153): IUPred $p<2.2e-16$, SPOT $p<2.2e-16$, VSL2b
1487 $p<2.2e-16$; Yeast (CDK targets, 100, Non-CDK targets, 2153): IUPred $p<2.2e-16$, SPOT
1488 $p<2.2e-16$, VSL2b $p<2.2e-16$. Boxplot parameters as in c. **f.** Violin plot (left) showing the
1489 distribution of disordered residues per protein (as estimated with SPOT) for CDK, MAPK,
1490 Aurora, PLK, NEK and DYRK kinase targets vs the rest of the phosphoproteome for human
1491 targets. Statistical significance was assessed by Kruskal-Wallis ANOVA, and pairwise
1492 comparisons were performed with Dunn's two-sided post-hoc tests. The adjusted p-values
1493 (Benjamini-Hochberg) are shown in a tile plot (right). Boxplot parameters as in c. Source data
1494 are provided as a Source Data file.

1495

1496 **Fig. 4. Cell cycle-regulated phosphorylation of key MLO proteins.** **a.** Human CDK1
1497 subfamily targets, *Xenopus* dynamic phosphoproteins, and the intersection of both sets, that are
1498 present in our manually curated proteome of membraneless organelles (MLOs; NPC, nuclear
1499 pore complex; PML, promyelocytic leukemia protein). **b.** Diagrams of IUPred scores over the
1500 length of human CDK targets identified as primary components of MLOs in different studies,
1501 and their *Xenopus* homologues in this study. Regions with scores >0.5 (orange) are considered
1502 to be disordered, and <0.5 (grey) structured. Blue vertical lines indicate Ser and Thr residues;
1503 yellow circles, known Ser/Thr-Pro phosphosites (human) and non-dynamic phosphosites
1504 (*Xenopus*); green circles, confirmed CDK1 subfamily phosphorylations (human) and dynamic

1505 phosphorylations (*Xenopus*), from both embryos and egg extracts. Source data are provided as
1506 a Source Data file.

1507

1508 **Fig. 5. CDK-mediated phosphorylation regulates phase separation propensity of major**
1509 **MLO components. a.** Temperature-density phase diagrams for the phosphorylated (P, in
1510 orange) and non-phosphorylated (Non-P, in green) forms of a selection of human CDK targets
1511 and major MLO components (the disordered regions analysed are indicated). Any point within
1512 the coexistence region (bounded by the solid line) will phase separate into a dilute and dense
1513 phase whose density is given by the values on the phase boundary. Points within the spinodal
1514 line (in dash) will spontaneously phase separate into dilute and dense phases without going
1515 through the process of nucleation. Circles denote critical temperature and density which is also
1516 the location where the coexistence and spinodal curves meet. For temperature above the critical
1517 value, there is no phase separation. **b.** Sequence Charge Decoration Matrix (SCDM) maps for
1518 the proteins in (a), depicting the contribution of electrostatic interaction dictating the distance
1519 between two amino acid residues *i* and *j* (shown in *x* and *y* axes). The values of SCDM for
1520 different residue pairs (*i,j*) are shown using colour schemes with red and blue denoting positive
1521 (repulsive) and negative (attractive) values, respectively. The lower and upper triangles
1522 indicate SCDM map for the unphosphorylated (non-P) and phosphorylated (P) sequences,
1523 respectively. Confirmed and putative (Ser/Thr-Pro) CDK phosphorylation sites were taken into
1524 account for the analysis (in both a and b) and are indicated with red circles.

1525

1526 **Fig. 6. The model CDK substrate Ki-67 forms biomolecular condensates in cells.**

1527 **a.** Scheme of the human Ki-67 protein (FHA, forkhead-associated domain; PP1, PP1
1528 phosphatase-binding domain; CD, conserved domain; LR, leucine arginine-rich domain; aa,
1529 amino acid). Highlighted, Ki-67 repeat consensus motif. **b.** HeLa cells expressing full-length
1530 GFP-tagged Ki-67 at different levels show evidence for phase separation; at low levels Ki-67
1531 is predominantly nucleolar but also forms foci in nucleoplasm that recruit heterochromatin, as
1532 indicated by H3K9me3 staining; at high levels Ki-67 partitions the entire nucleus into two
1533 phases, a Ki-67-dense phase that induces global heterochromatin formation marked by
1534 H3K9me3 and MeCP2. DNA was stained with DAPI. Colocalisation between DAPI (blue),
1535 GFP-Ki-67 (green), and either MeCP2 or H3K9me3 (red) is shown, right. Scale bar, 10 μ m;
1536 *n*=2 independent experiments. **c.** FRAP of Ki-67 shows liquid-like behaviour. Left:

1537 representative images of cells expressing different levels of Ki-67 in interphase (top, middle)
1538 and in mitosis where Ki-67 localises to the perichromosomal layer (bottom) showing bleached
1539 regions and contiguous non-bleached regions; just before (left) and after (right) bleaching.
1540 Scale bars, 3 μ m. Insets: images of Ki-67 fluorescence at different recovery times after
1541 bleaching. Scale bars, 0.5 μ m. Right: average fluorescence intensity values over time for
1542 bleached (dark blue) and unbleached (teal blue) regions. Orange, non-linear regression fitting
1543 of the data; n=2 independent experiments. Each line represents the dynamics of one cell, in
1544 which several regions were photobleached at time 0 and the intensity values over time were
1545 analysed, and a corresponding number of regions was not bleached but analysed (mitosis, 4
1546 regions; interphase, 2 regions; interphase with high expression, 7 regions). The mean values of
1547 all replicates were plotted as normalised intensity vs time, grey error bars, standard deviation.
1548 **d.** Left: boxplot of the average recovery half-time for each cell, grouped by category of Ki-67
1549 expression: mitosis (n=4), interphase medium-low expression (n=19) and interphase high
1550 expression (n=6). Right: boxplot of the average percentage of recovery for each cell, grouped
1551 by category of Ki-67 expression: mitosis (n=4), interphase medium-low expression (n=19) and
1552 interphase high expression (n=6). **= p<0.01 (Wilcoxon test, two-sided); t-half: interphase vs
1553 mitosis, p=0.123; interphase-HiExpr vs mitosis, p=0.014; interphase-HiExpr vs interphase,
1554 p=0.008; %recovery: interphase vs mitosis, p=0.005; interphase-HiExpr vs mitosis, p=0.014;
1555 interphase-HiExpr vs interphase, p=0.589. **e.** Optogenetic induction of Ki-67 biomolecular
1556 condensates. Representative fluorescent images of HEK-293 cells expressing opto-Ki-67 (FL)
1557 construct, induced by the indicated concentrations of doxycycline (Doxy), before (Light Off)
1558 and after (Light On) exposure to blue light. DNA was stained with Hoechst 33258; n=2
1559 independent experiments. **f.** Left, representative fluorescent images of U2OS cells expressing
1560 opto-Ki-67 construct (full length protein) before (Light Off) and after (Light On) exposure to
1561 blue light. Additional staining for nucleolar proteins nucleolin (NCL, top) and nucleophosmin
1562 (NPM1, bottom) was performed and colocalisation with Ki-67 assessed (right; Ki-67, red; NCL
1563 or NPM1, green). DNA was stained with Hoechst 33258; scale bars, 10 μ m; n=2 independent
1564 experiments. Source data are provided as a Source Data file.

1565

1566 **Fig. 7. Phosphorylation promotes phase separation of Ki-67.** **a.** Coarse-grained single-chain
1567 MD simulations for full chain Ki-67 showing dependency of the radius of gyration (Rg) on the
1568 simulation temperature. The reference temperature is the θ temperature of the non-
1569 phosphorylated molecule. Error bars correspond to the standard error of the mean obtained with

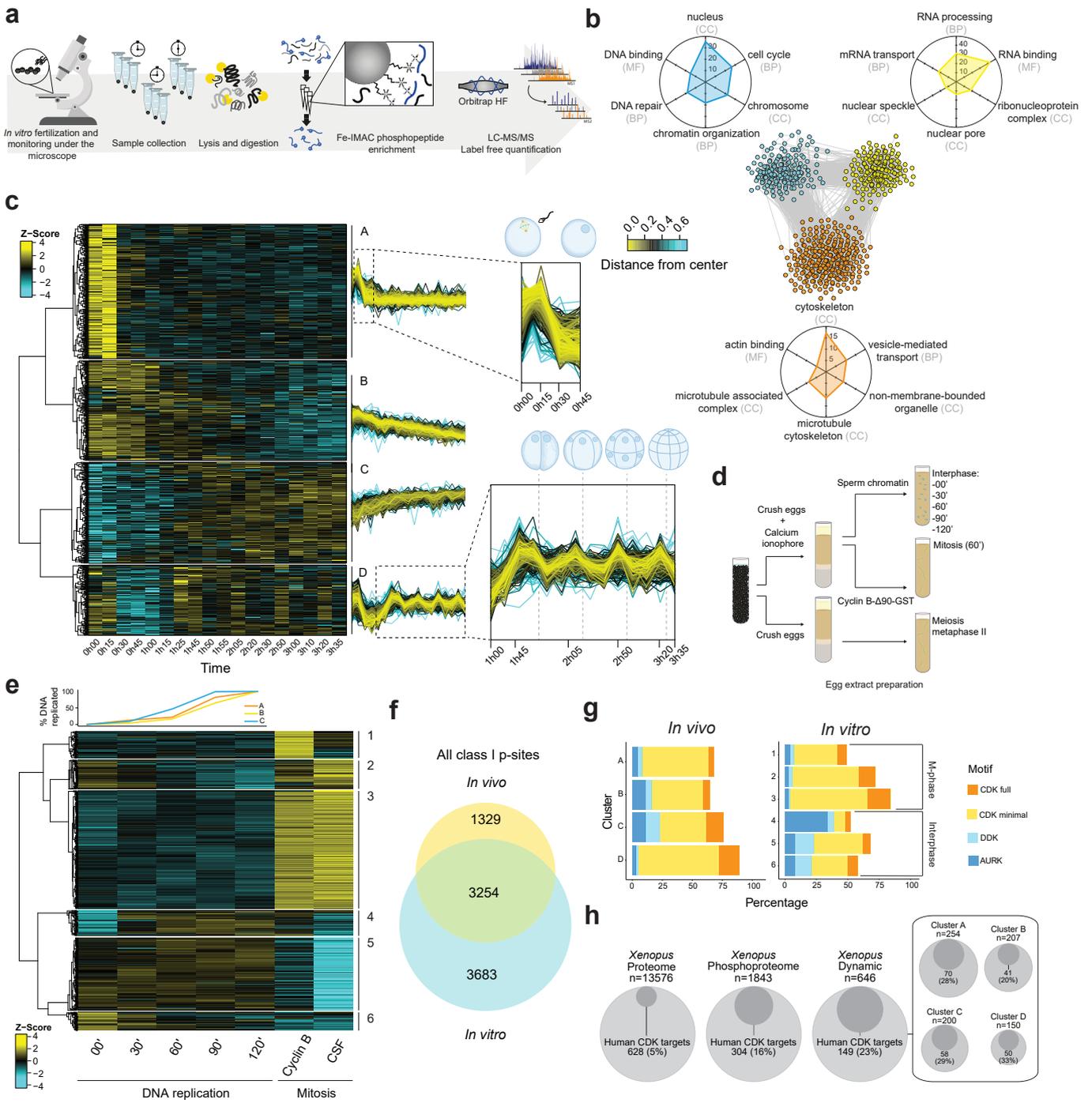
1570 block analysis by subdividing the trajectory into 10 non-overlapping blocks. **b.** Representative
1571 fluorescent images of HEK-293 cells expressing opto-Ki-67 (FL) construct before (Light Off)
1572 and after (Light On) exposure to blue light. Cells were pretreated for 1h with either vehicle
1573 (DMSO), 0.5 μ M okadaic acid (OA), to inhibit protein phosphatase 2A, or 5 μ M purvalanol A
1574 (PA), to inhibit CDKs. DNA was stained with Hoechst 33258; scale bars, 10 μ m; n=3
1575 independent experiments. **c.** Violin plot presenting quantification of results from (b); the
1576 number of foci per nucleus was counted (\geq 100 nuclei per condition). Statistical significance
1577 was assessed by one-way ANOVA on ranks (Kruskal–Wallis test) and pairwise post-hoc
1578 comparisons using the Mann–Whitney two-sided test. P-values were adjusted by the
1579 Benjamini-Hochberg method: DMSO Off vs DMSO On, $p=2.1e-14$; OA On vs DMSO Off,
1580 $p=0.315$; OA On vs DMSO Off, $p=8.6e-14$; OA Off vs DMSO On, $p=0.023$; OA Off vs DMSO
1581 On, $p=5.3e-11$; OA Off vs OA On, $p=0.186$; PA On vs DMSO On, $p=2.0e-15$; PA On vs
1582 DMSO Off, $p=0.642$; PA On vs OA On, $p=2.1e-14$; PA On vs OA Off, $p=1.8e-11$; PA Off vs
1583 DMSO On, $p<2e-16$; PA Off vs DMSO Off, $p=7.0e-05$; PA Off vs OA On, $p<2e-16$; OA Off
1584 vs OA Off, $p<2e-16$; PA Off vs PA On, $p=0.001$. Boxplots center, median; lower and upper
1585 edges, 25% and 75% quartiles, respectively. Whiskers, data with the largest or smallest values
1586 not further than 1.5* interquartile range (IQR) from the upper or lower box limits, respectively.
1587 Beyond these values, data were not plotted, for clarity, but were included in the statistical
1588 analysis. **d.** Phosphorylation-induced Ki-67 foci are biological condensates. Cells were treated
1589 for 1h with either vehicle (DMSO) or 0.5 μ M okadaic acid (OA), to inhibit protein phosphatase
1590 2A. Additional staining for nucleolar proteins nucleolin (NCL) and nucleophosmin (NPM1)
1591 was performed and colocalisation with Ki-67 foci assessed (right; Ki-67, red; NCL or NPM1,
1592 green). DNA was stained with Hoechst 33258; scale bars, 10 μ m; n=2 independent
1593 experiments. Source data are provided as a Source Data file.

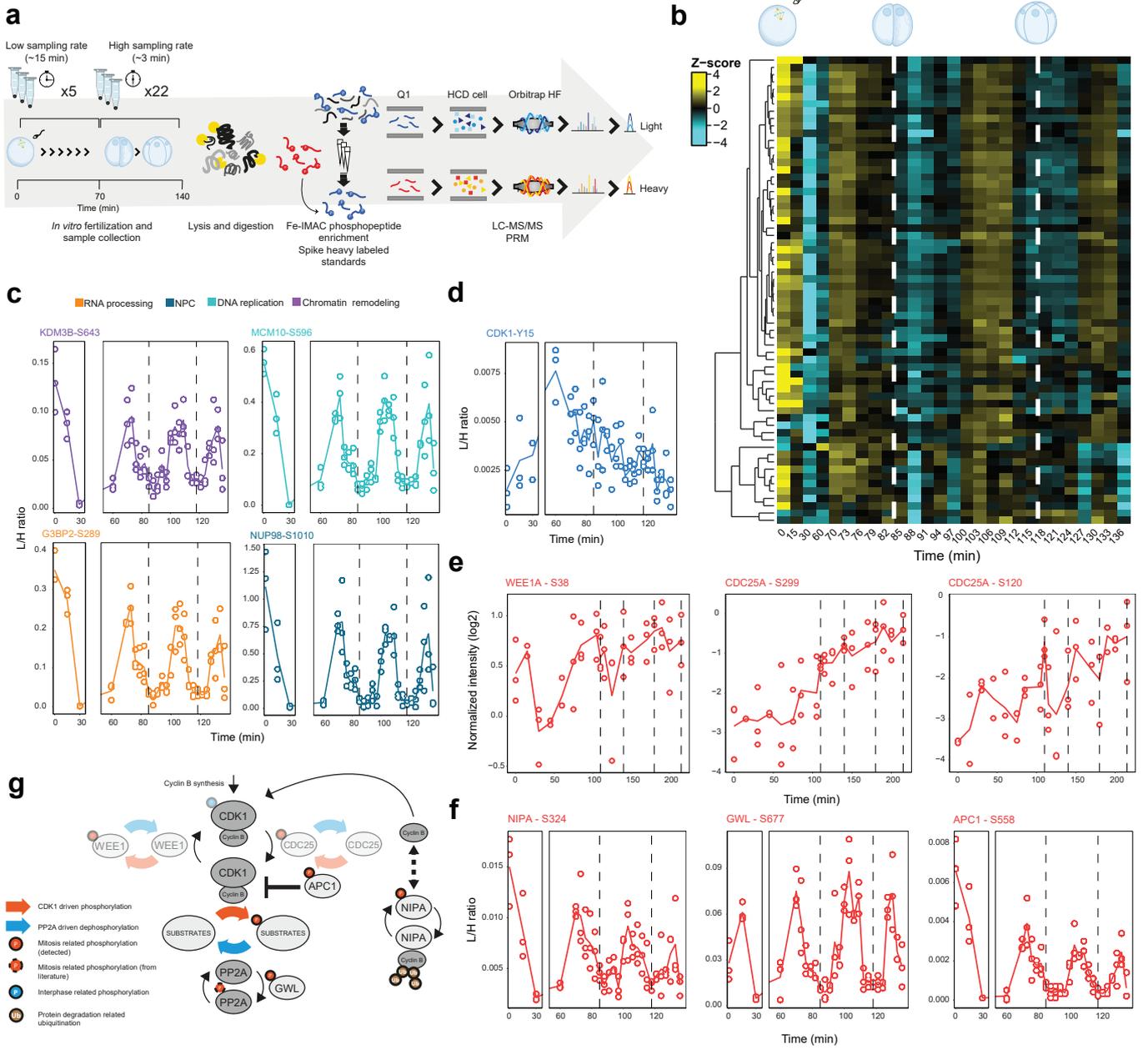
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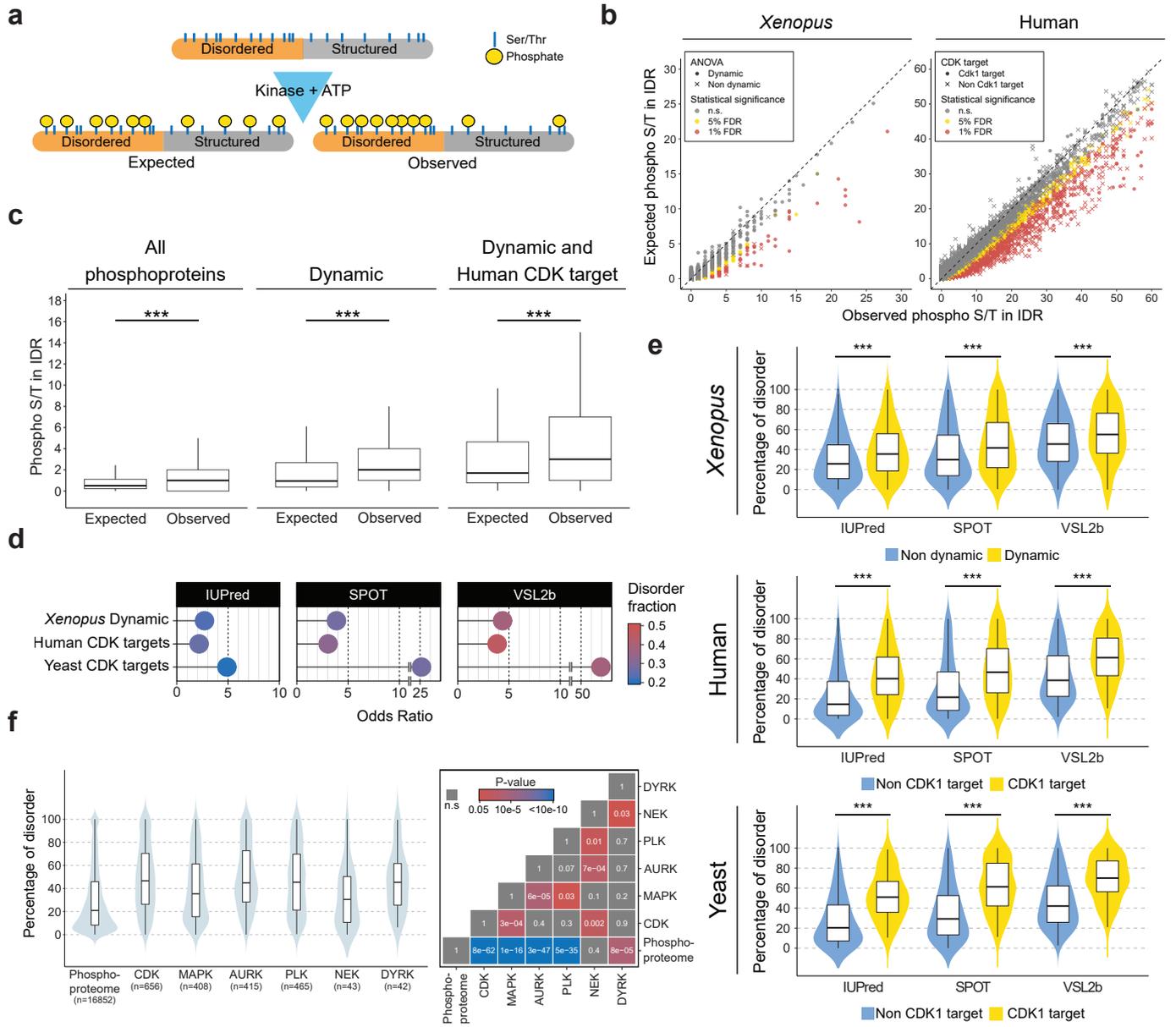
1595 **Fig. 8. CDK-mediated phosphorylation can generate a buffered phase separation switch.**

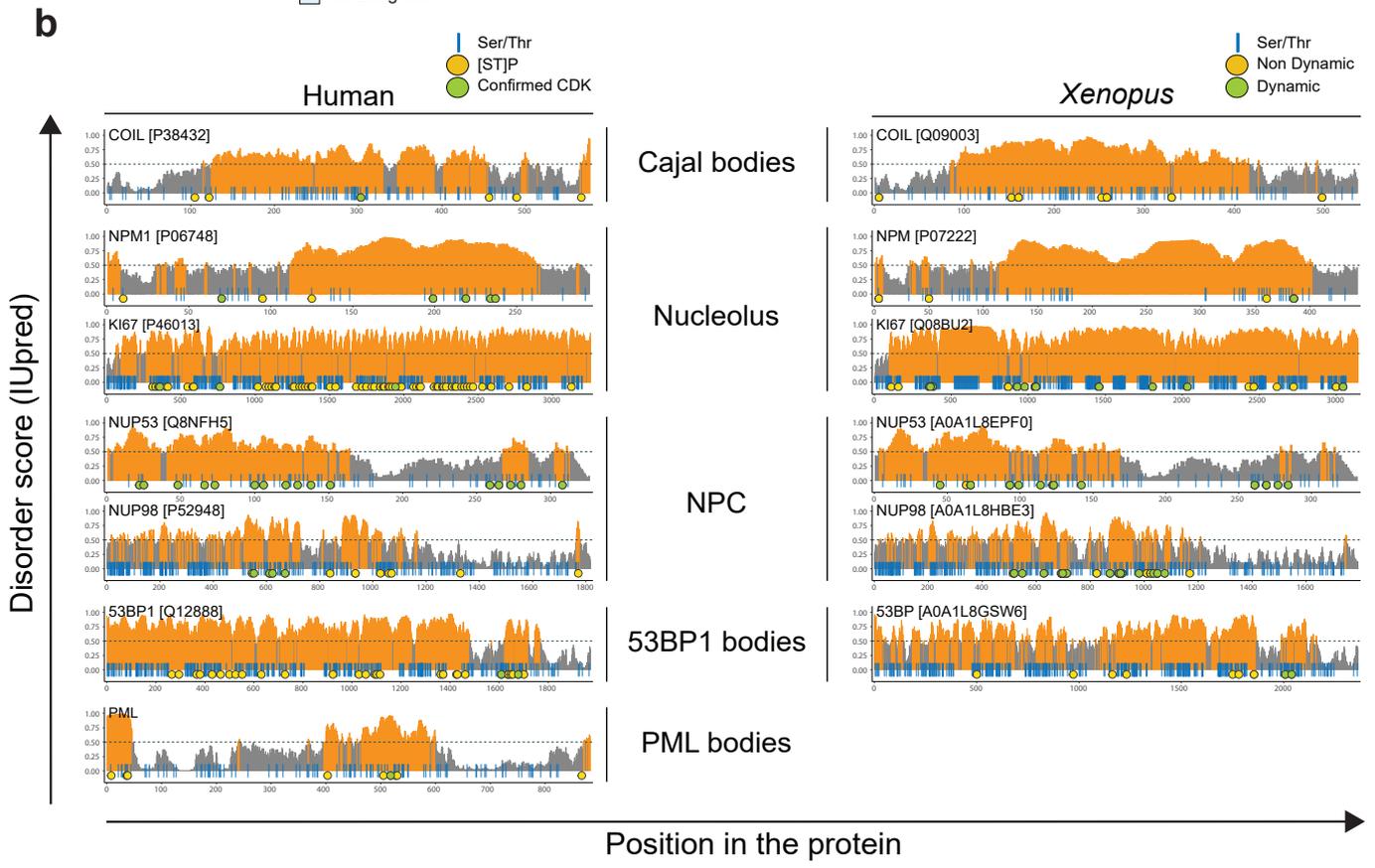
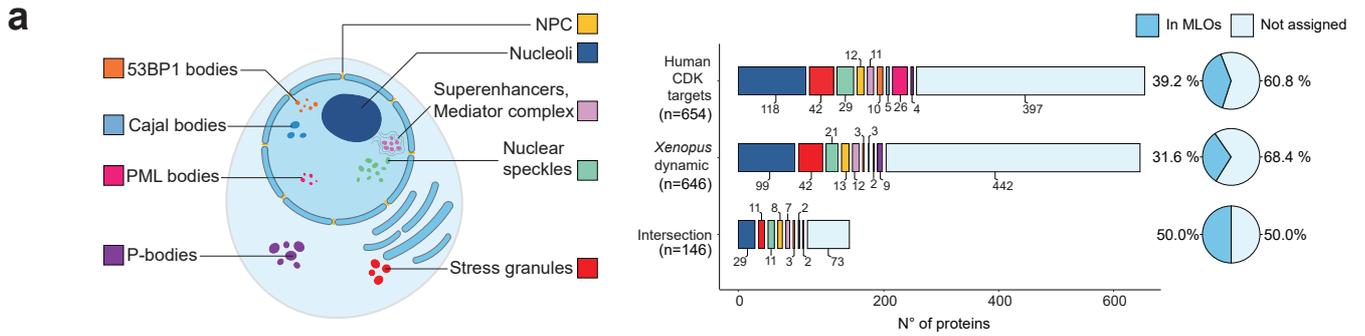
1596 **a.** Overlaid NMR ^1H - ^{15}N HSQC of unphosphorylated (blue) and CDK-phosphorylated (red)
1597 GFP-tagged Ki-67 consensus repeat. Each cross-peak corresponds to one residue. The seven
1598 new deshielded cross peaks (highlighted by a black flag) appearing above 8.5 ppm in ^1H
1599 correspond to phosphorylated serines or threonines (^1H downfield chemical shift perturbation
1600 on phosphorylated Ser/Thr residues due to phosphate electronegativity). Non phosphorylated
1601 Ser/Thr residues are surrounded by a black oval. **b, c.** GFP-Ki-67 consensus repeat was
1602 phosphorylated *in vitro* using recombinant CDK1-cyclin B-CKS1 or CDK2-cyclin A protein

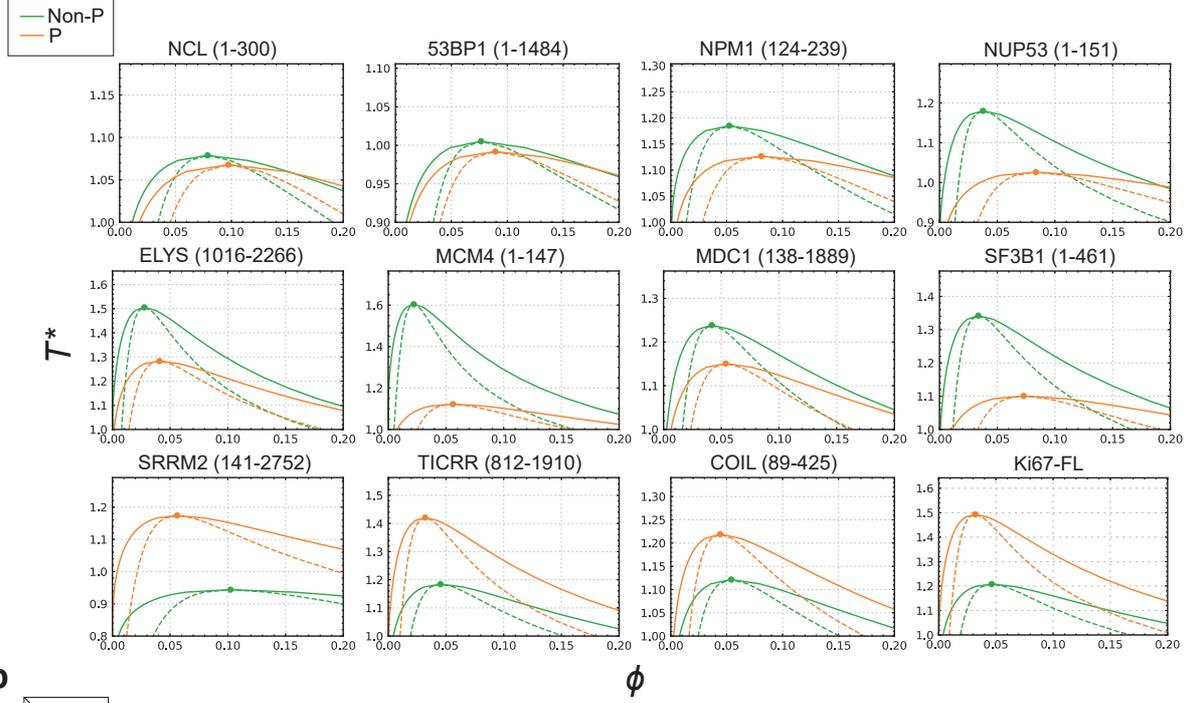
1603 complexes and the phosphosites were mapped by mass-spectrometry (b) and the stoichiometry
1604 of phosphorylation was analysed by Phos-Tag SDS-PAGE (c) (amidoblack staining was used
1605 as loading control); n=1 experiment. **d.** Representative fluorescence images of *in vitro* phase
1606 separation assay with purified GFP-tagged Ki-67 consensus repeat (CR), non-phosphorylated
1607 (non-P) or *in vitro* phosphorylated (P) with recombinant CDK1-cyclin B-CKS1 or CDK2-
1608 cyclin A, at indicated time points; scale bars, 10 μ m; n = 2 independent experiments, with three
1609 fields recorded for each condition. **e.** Top, temperature-density phase diagrams for the
1610 consensus repeat sequence of Ki-67. Critical temperature and density (blue circles) were
1611 computed for all possible 2048 sequences that arise from different degrees of phosphorylation.
1612 For a given degree of phosphorylation there are multiple possible sequences, of which two
1613 were chosen, corresponding to the highest and lowest values of the critical point. For these,
1614 temperature-dependent (in reduced unit) phase diagrams (solid orange) and spinodal lines
1615 (dashed orange) are shown along with the unmodified sequence (in green). Only critical points
1616 (blue circles) are presented for all the other sequences for a given stoichiometry/degree of
1617 phosphorylation. Bottom, SCDM maps of the unmodified sequence (Non-P, lower triangle)
1618 and a specific phosphorylated sequence (P, upper triangle). The phosphorylated sequence for
1619 a given stoichiometry (degree of phosphorylation) was chosen by selecting the sequence with
1620 the corresponding critical temperature and density closest to the average of the highest and
1621 lowest critical points. SCDM and Phase diagrams are consistent and show that phosphorylation
1622 can lower propensity to phase separate when eight or more sites are phosphorylated, contrary
1623 to sequences where six or less sites are phosphorylated. Source data are provided as a Source
1624 Data file.









a**b**