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A CDK-mediated phosphorylation switch of disordered protein condensation

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A CDK-mediated phosphorylation switch of disordered protein condensation

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27 Cell cycle transitions arise from collective changes in protein phosphorylation states
28 triggered by cyclin-dependent kinases (CDKs), but conceptual and mechanistic
29 explanations for the abrupt cellular reorganisation that occurs upon mitotic entry are
30 lacking. Specific interactions between distinct CDK-cyclin complexes and sequence
31 motifs encoded in substrates might result in highly ordered phosphorylation¹, while
32 bistability in the mitotic CDK1 control network can trigger switch-like phosphorylation².
33 Yet the dynamics of mitotic phosphorylation has not been demonstrated *in vivo*, and the
34 roles of most cell cycle-regulated phosphorylations are unclear. Here, we show evidence
35 that switch-like phosphorylation of intrinsically disordered proteins (IDPs) by CDKs
36 contributes to mitotic cellular reorganisation by controlling protein-protein interactions
37 and phase separation. We studied protein phosphorylation in single *Xenopus* embryos
38 throughout synchronous cell cycles, performed parallel assignment of cell cycle phases
39 using egg extracts, and analysed dynamics of mitotic phosphorylation using quantitative
40 targeted phosphoproteomics. This provided a high-resolution map of dynamic
41 phosphosites from the egg to the 16-cell embryo and showed that mitotic phosphorylation
42 occurs on entire protein complexes involved in diverse subcellular processes and is
43 switch-like *in vivo*. Most cell cycle-regulated phosphosites occurred in CDK consensus
44 motifs and located to intrinsically disordered regions. We found that substrates of CDKs
45 and other cell cycle kinases are significantly more disordered than phosphoproteins in
46 general, a principle conserved from yeast to humans, while around half are components
47 of membraneless organelles (MLOs), whose assembly is thought to involve phase
48 separation. Analytical modelling predicts modulation of homotypic IDP interactions by
49 CDK-mediated phosphorylation, which was confirmed by biophysical and biochemical
50 analysis of a model IDP, Ki-67. These results highlight the dynamic control of intrinsic
51 disorder as a conserved hallmark of the cell cycle and suggest a mechanism for CDK-
52 mediated mitotic cellular reorganisation.

53 Main

54 To explain behaviour of complex systems, such as the cell cycle, two general strategies have
55 been used³: top-down identification of system components, such as screens which have
56 identified hundreds of CDK substrates⁴⁻⁹ and cell cycle-regulated proteins¹⁰, and bottom-up
57 molecular analysis of the structural effects of individual phosphorylations on single proteins¹¹.
58 Yet it has proven challenging to use studies performed at such different scales to reconcile
59 different models of CDK-mediated phosphorylation. We reasoned that understanding how

60 thousands of mitotic phosphorylations¹² bring about an ordered cell cycle transition would
61 require a multidisciplinary quantitative approach involving cell biology, biochemistry,
62 bioinformatics and biophysics. A *sine qua non* is a time-resolved map of *in vivo* cell cycle
63 phosphorylation in a system devoid of artifacts arising from cell synchronisation^{13,14}, and with
64 temporal resolution that alternative approaches, like centrifugal elutriation¹⁵ or FACS¹⁶ cannot
65 provide. Dynamic phosphorylation states cannot be determined from cell populations¹⁷, while
66 single-cell proteomics studies^{18,19} currently have insufficient sensitivity and reproducibility for
67 low stoichiometry and dynamic targets.

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

69 We took advantage of the naturally synchronous early cell cycles of *Xenopus laevis*
70 embryos^{20,21} to perform quantitative phosphoproteomics *in vivo*, using a sensitive
71 phosphopeptide enrichment strategy²². We collected single embryos at 15-minute intervals
72 while recording visual cues of cell divisions. Phosphopeptides from each embryo were purified,
73 separated by nano-LC and analysed by mass spectrometry (Fig. 1a). We identified 4583 high-
74 confidence phosphosites mapping to 1843 proteins (Extended data Fig. 1a; Data S1), most
75 being phosphoserines (Extended data Fig. 1b). Individual embryo phosphorylation states
76 strongly correlated (Extended data Fig. 1c). We thus generated a dynamic map of protein
77 phosphorylation from an unfertilised egg to a 16-cell embryo.

78 We focused on 1032 sites whose variation in phosphorylation over time was statistically
79 significant (hereafter denoted “dynamic phosphosites”) which occurred on 646 proteins. Gene
80 ontology (GO) and network analysis revealed high functional association and interconnectivity
81 between groups of proteins involved in RNA binding and the nuclear pore complex (NPC),
82 DNA replication and chromatin remodeling, and microtubule regulation (Fig. 1b). Hierarchical
83 clustering uncovered four distinct groups that reflect cell cycle-regulated behaviour (Fig. 1c;
84 Data S1). The levels of clusters A and B phosphosites were highest in eggs and post-
85 fertilisation, and decreased during the first round of DNA replication, suggesting that
86 dephosphorylation of these sites may prepare the zygote for upcoming cell divisions²³. GO
87 analysis for group A highlighted proteins involved in RNA regulation and nuclear organisation,
88 including the NPC and nuclear transport, chromosomal structure and segregation (Extended
89 data Fig. 1d), as also observed in a recent study on meiosis exit²⁴. Cluster B phosphosites were
90 enriched in regulators of RNA biosynthesis and stability, translation, actin, DNA replication
91 and repair (Extended data Fig. 1d). Cluster C phosphosites progressively increased after
92 meiotic exit, while cluster D phosphosites had a clear oscillating signature with upregulation

93 preceding each cell division. GO analysis of cluster C shows dominance of interphase cell cycle
94 processes including DNA replication, RNA-related processes and chromosome organisation
95 (Extended data Fig. 1d), and included phosphosites displaying a reciprocal oscillating trend
96 and a lower amplitude compared to cluster D sites. Several such sites, *e.g.* S31 of the replication
97 licensing protein MCM4, were from monophosphorylated peptides, while the
98 multiphosphorylated forms were found in cluster D (Extended data Fig. 1e). Thus, cluster C
99 contains the earliest phosphorylations of proteins that are highly phosphorylated at mitosis.
100 Cluster D shows coordinated phosphorylation of multiple members of protein complexes
101 involved in diverse processes, suggesting a common mechanism of regulation (Extended data
102 Fig. 1f). Importantly, phosphoproteome changes were not simply a reflection of changes in
103 abundance of the corresponding proteins (Extended data Fig. 2), which are generally negligible
104 during *Xenopus* early development²⁵.

105 We assigned *in vivo* embryo phosphosites to different cell cycle stages by comparing with
106 phosphorylation patterns of replicating or mitotic egg extracts (Fig. 1d). Replication was
107 initiated by adding purified sperm chromatin to interphase egg extracts and quantified over
108 time (Fig. 1e, top), while mitosis was triggered by adding recombinant cyclin B and verified
109 microscopically. We also used egg extracts arrested at meiotic metaphase II (Cytostatic Factor,
110 CSF-arrested). Overall, we identified 6937 phosphosites, which included 71% of the sites
111 identified *in vivo* (Fig. 1f, Data S1). 1728 sites varied between S and M-phase, including 693
112 sites upregulated in S-phase and 1035 in mitosis (Fig. 1e, Data S1). GO analysis of interphase
113 and mitotic sites revealed processes enriched in *in vivo* cluster C and cluster D, respectively
114 (Extended data Fig. 3a). Several DNA-replication factors, including MCM4 and RIF1, showed
115 multi-site phosphorylation specifically in S-phase (Extended data Fig. 3b). This
116 phosphoproteomics dataset greatly increases the known repertoire of phosphorylation sites
117 upregulated during S-phase¹².

118 We next analysed the cell cycle behaviour of dynamic phosphosites that we found *in vivo*
119 (Extended data Fig. 3c). Most embryo cluster A sites were upregulated in both CSF-arrested
120 meiotic extracts and mitotic extracts, highlighting the global similarities of regulation of
121 meiotic and mitotic M-phase, despite the additional activity of the Mos/MEK/MAP kinase
122 pathway in meiosis. Around half of embryo cluster B sites were present only in interphase,
123 while the rest showed a minimum phosphorylation in late S-phase, confirming their
124 dephosphorylation during the first round of DNA replication. As expected, most sites from
125 embryo clusters C and D were part of the *in vitro* S-phase and mitotic groups, respectively.

126 Therefore, single embryo data can successfully identify cell cycle-dependent phosphorylation.
127 In mitosis, as expected, monophosphorylated species are reduced because multisite
128 phosphorylation emerges (Extended data Fig. 3d; Extended data Fig. 1e).

129 **Predominance of CDK targets**

130 Analysis of kinase consensus motifs showed that proline-directed (S/T-P) sites, which conform
131 to the minimal consensus for CDKs, comprise 51% of all detected phosphosites *in vivo* and
132 60% of dynamic sites (Extended data Fig. 4a). Around 10% of all phosphosites matched the
133 full CDK1-family consensus site: S/TPxK/R. Replicating and mitotic extracts displayed a
134 similar trend (Extended data Fig. 4a). Putative CDK targets dominated all clusters, with 80%
135 of sites in cluster D *in vivo* and mitotic clusters *in vitro* conforming to the minimal CDK motif
136 (Fig.1g, Extended data Fig. 4b, c). Consensus sites of other kinases such as Aurora, Polo-like
137 kinase (PLK), DBF4-dependent kinase (DDK) and Casein kinase I and II were present to a
138 lesser extent (Extended data Fig. 4b, d). In meiotic M-phase, MAP kinases, which have the
139 same consensus motif as CDKs, are likely responsible for sites specific to embryo cluster A or
140 CSF extracts, but these kinases are inactivated during early embryonic cell cycles²⁶, suggesting
141 that most of the other dynamic proline-directed phosphorylations are due to CDKs.

142 Although few direct CDK substrates have been characterised in *Xenopus*, they are likely
143 conserved between vertebrates. We therefore manually curated a set of 654 human CDK1-
144 subfamily targets (Data S2; see Supplementary Methods for sources). 303 of these have
145 *Xenopus* homologues among the 1843 phosphoproteins we detected, and 149 were present
146 among the 646 proteins with dynamic phosphosites in *Xenopus* embryos (Fig. 1h). Thus, the
147 predominance of CDK motifs among dynamic phosphosites reflects a high proportion of *bona*
148 *fide* CDK substrates. This is a conservative estimate, since we only considered proline-directed
149 sites as CDK motifs, although we found that 10-20% of human and yeast CDK substrates (Data
150 S2; see Supplementary Methods for sources) were non-proline-directed (Extended data Fig.
151 4e), confirming a recent finding³³. These data reinforce the dominant role of CDKs in cell
152 cycle-regulated phosphorylation.

153 **Mitotic phosphorylation is switch-like *in vivo***

154 We next determined whether mitotic phosphorylation of individual phosphosites is progressive
155 or switch-like *in vivo*. We analysed dynamics of 64 cluster D sites from diverse protein
156 complexes in single embryos every 180-seconds using quantitative targeted
157 phosphoproteomics²⁷⁻²⁹ by parallel reaction monitoring³⁰, thereby obtaining a quantitative

158 description of mitotic phosphorylation *in vivo* at extremely high-time resolution (Fig. 2a). This
159 revealed parallel and abrupt upregulation of all phosphosites preceding each cell division (Fig.
160 2b, c), indicating switch-like phosphorylation of diverse protein complexes at mitotic onset.
161 This was not due to oscillation of CDK1-Y15 inhibitory phosphorylation, which was
162 downregulated over time (Fig. 2d), as previously reported³¹, consistent with lack of
163 corresponding phosphorylation of the CDK1-Y15-regulatory enzymes, CDC25 and WEE1. In
164 contrast, oscillating phosphorylations on NIPA and the APC/C, which regulate mitotic cyclin
165 accumulation, as well as Greatwall kinase, which activates the PP2A inhibitors Arpp19/ENSA,
166 were apparent (Extended data Fig. 5a). These data suggest that control of mitotic cyclin levels
167 and PP2A activity, and therefore the overall CDK/phosphatase activity ratio², suffices for
168 switch-like mitotic phosphorylation whereas regulated CDK1-Y15 phosphorylation is not
169 essential (Extended data Fig. 5b). This is consistent with the self-sufficiency of futile cycles of
170 opposing enzymes in generating switch-like network output in the absence of allosteric
171 regulation³².

172 **The cell cycle phosphoproteome is intrinsically disordered**

173 We wondered whether the diverse dynamic phosphoproteins share common structural features
174 facilitating switch-like CDK-mediated phosphorylation. Phosphosites in general are often
175 located in intrinsically disordered regions (IDRs) of proteins³⁴, which is also true for yeast and
176 mouse CDK sites^{35,8,9}. Yet previous analyses did not exclude the possibility that this is an
177 artefact due to the enrichment of serine, threonine and proline in disordered regions, which is
178 consistently predicted across the entire proteome of *Xenopus*, human and yeast (Extended data
179 Fig. 6a). We corrected for this compositional bias, and found that phosphorylatable residues in
180 IDR are indeed more highly phosphorylated than those in ordered regions (Fig. 3a-c). This
181 enrichment was increased for proteins with at least one site displaying dynamic
182 phosphorylation; the same was true for human CDK substrates (Fig. 3b, c). To estimate the
183 differential phosphorylation of disordered sites globally, we calculated the ratio of dynamically
184 phosphorylated (*Xenopus*) or CDK-phosphorylated (yeast, human) to non-phosphorylated
185 serine and threonine in both disordered and structured regions (Extended data Fig. 6b; see
186 Methods). This confirmed that cell cycle-regulated phosphorylation is largely skewed towards
187 disordered regions and that CDKs preferentially phosphorylate disordered sites (Fig. 3d,
188 Extended data Fig. 6c). We then asked whether this is also true for substrates of other protein
189 kinases. We analysed the mitotic PLK and Aurora kinases, DYRK kinases, which promote
190 mitotic phosphorylation of several IDPs³⁶, NEK kinases, which have roles in centrosome

191 duplication and various stages of mitosis, and MAP kinases, which share the proline-directed
192 S/T consensus site. For each kinase, documented phosphosites were strongly enriched in IDRs
193 (Extended data Fig. 6c, d), supporting the idea that phosphorylation of residues in IDRs is
194 kinetically favoured³⁴.

195 To explain the dominance of CDK-mediated phosphorylation in the cell cycle, we surmised
196 that their substrates might be more disordered than phosphoproteins in general. We therefore
197 determined the percentage of disordered residues of proteins in our datasets, compared to the
198 rest of their respective phosphoproteomes (Data S3). This revealed that, on average, both
199 *Xenopus* dynamic phosphoproteins and human and yeast CDK substrates contain
200 approximately twice the proportion of disordered amino acids as other phosphoproteins (Fig.
201 3e, Extended data Fig. 6e), putting them among the top quartile of proteins with the most
202 disorder in the proteome. If this reflects the importance of disordered proteins for the cell cycle
203 generally, then substrates of other cell cycle kinases might also be more disordered than other
204 phosphoproteins. Indeed, targets of most cell cycle kinases are significantly more disordered
205 than targets of MAP kinase (Fig. 3f), whose phosphosites are also proline-directed and
206 preferentially located in IDRs (Extended data Fig. 6d).

207 **Enrichment of MLO components among CDK substrates**

208 We thus reasoned that phosphorylation may have been selected to regulate the functions of
209 IDPs during the cell cycle. IDPs are key components of membrane-less organelles (MLO),
210 many of which (*e.g.* Cajal bodies, nucleoli, nuclear pore complexes, splicing speckles) are
211 thought to arise by phase separation (PS)³⁷, are disassembled in mitosis, and can be regulated
212 by phosphorylation^{36,38,39}. To corroborate our hypothesis, we analysed available data on
213 cellular localisation for each of our curated human CDK substrates. We found that 257 (39.2%)
214 are present in MLOs, including key IDPs such as coilin (Cajal bodies), nucleophosmin,
215 nucleolin and Ki-67 (nucleoli), 53BP1 (53BP1 bodies), nucleoporins (NPC) and PML (PML
216 bodies) (Fig. 3g). We then manually curated an MLO proteome from human proteomics studies
217 (Data S4; See Supplementary Methods for sources). Homologues of 204 dynamic *Xenopus*
218 phosphoproteins (31.6%) localise to MLOs, as do 73 of the 149 proteins (50%) that show
219 dynamic phosphorylation in *Xenopus* and are CDK substrates in human (Fig. 3g). The vast
220 majority of proline-directed phosphosites and confirmed CDK sites in these proteins were
221 located in predicted IDRs (Extended data Fig. 7).

222 **CDKs regulate IDR phase separation**

223 Both stochastic and specific interactions between IDPs contribute to PS and MLO
224 assembly^{37,40,41}. We hypothesised that cell cycle kinase-mediated phosphorylation might
225 modulate such interactions. We first applied a machine learning classifier⁴² to predict whether
226 cell cycle-regulated phosphoproteins have an increase in average propensity for PS (PSAP
227 score). Indeed, we observed a sharp increase in the PSAP score, from the proteome to the
228 phosphoproteome, and a further increase for dynamic phosphoproteins, with the highest score
229 for mitotic cluster D (Extended data Fig. 8a). Similarly, the propensity for PS is far higher
230 amongst targets of most cell cycle kinases (CDK, Aurora, PLK, but not NEK) and DYRK
231 kinases than the overall phosphoproteome, but less so for MAP kinase substrates.

232 Next, to better understand the biochemical effects of their cell cycle-regulated phosphorylation,
233 we analysed a selection of IDRs from CDK substrates. We applied a general heteropolymer
234 theory that uses sequence charge decoration matrices (SCDM), based on electrostatic
235 interactions only, to identify intra-chain interaction topology^{43,44}. Since this should correlate
236 with inter-chain interactions that promote PS, SCDMs provide indirect insights to propensity
237 to phase separate. Of the 12 IDPs tested, 7 (nucleolin, nucleophosmin, NUP53, ELYS, MCM4,
238 53BP1 and the splicing factor SF3B1) had SCDM maps showing visibly decreased self-
239 association propensity (increased red regions in Extended data Fig. 8b), implying reduced
240 propensity to phase separate, upon CDK-site phosphorylation. Conversely, for SRRM2, CDK-
241 mediated phosphorylation is predicted to increase intra-chain attraction (Extended data Fig. 8b)
242 and hence PS tendency. For 4 proteins (MDC1, TICRR, COILIN, and CDT1), SCDM maps
243 were inconclusive. To further analyse these trends, we calculated radius of gyration of several
244 IDRs using all-atom simulation. Effects of phosphorylation on CDT1 (28.4Å to 30.3Å), TICRR
245 (56.2Å to 57.3Å) and coilin (39 Å to 37.9 Å) were minor, while MCM4 IDR expands upon
246 phosphorylation (21.9Å to 26.3Å), consistent with SCDM analysis. Overall, these data suggest
247 that phosphorylation is a key regulator of homotypic interactions, an important element of PS
248 propensity, of most IDRs.

249 To test this hypothesis, we focused on a model CDK substrate, Ki-67, an IDP that organises
250 heterochromatin structure⁴⁵ and perichromosomal layer formation from nucleolar components
251 in mitosis^{46,47}. Ki-67 contains a multivalent Ki-67 repeat domain that is highly phosphorylated
252 in mitosis by CDKs (Fig. 4a), which regulates its perichromosomal localisation⁴⁸. SCDM
253 analysis predicted that phosphorylation of full-length Ki-67 should promote self-interaction
254 and thus PS, but this cannot be attributed to interactions of its repeat motif alone, since
255 phosphorylation of the latter is predicted to reduce homotypic interactions (Fig. 4b). In

256 agreement, coarse-grained (CG) molecular dynamics (MD) simulations (Extended data Movies
257 1 and 2) showed that the radius of gyration of full-length Ki-67 decreased upon
258 phosphorylation (Fig. 4c, left) while that of a single consensus repeat motif increased (Fig. 4c,
259 right). MD simulations also showed that PS is enhanced by increasing repeat valency and
260 counteracted by phosphorylation (Fig. 4d), consistent with SCDM analysis. To test these
261 predictions experimentally, we first used the optogenetic Cry2 “optodroplet” system⁴⁹ with full
262 length Ki-67 or a series of deletion mutants. Full-length Ki-67 localised to the nucleolus, as
263 expected, but exposure to blue light caused rapid appearance of small round foci in the
264 nucleoplasm, which was dependent on the level of induced Ki-67 expression, consistent with
265 PS (Extended data Fig. 9a). Importantly, promoting CDK-mediated phosphorylation by
266 inhibiting PP2A with okadaic acid² led to foci formation in the absence of blue light, while
267 pan-CDK inhibition with purvalanol A prevented induction of foci upon light (Fig. 4e, f). These
268 results indicate that, as predicted by SCDM and MD, phosphorylation of full-length Ki-67
269 promotes PS. Results were similar for constructs lacking the C-terminal LR domain, that binds
270 chromatin, or the N-terminal domain, which is required for the nucleolar localisation of Ki-67
271 (Extended data Fig. 9b). Finally, we purified a consensus repeat polypeptide (Extended data
272 Fig. 10a) and phosphorylated it *in vitro* with recombinant CDK complexes. Nuclear Magnetic
273 Resonance spectroscopy showed a reduced amide proton spectral dispersion typical for an IDP,
274 and confirmed appearance of 7 phosphorylated residues upon incubation with purified CDKs
275 and ATP (Fig. 4g). We mapped phosphorylation sites and intensity by phosphoproteomics and
276 Phos-Tag-SDS-PAGE, indicating stoichiometric phosphorylation (Extended data Fig. 10b, c).
277 Purified GFP-tagged Ki-67 repeat motif could phase-separate *in vitro*, and, as predicted, this
278 was abolished upon full phosphorylation by CDK (Fig. 4h). Taken together, these results
279 confirm that CDK-mediated phosphorylation is able to both promote or inhibit homotypic
280 interactions that contribute to PS, and suggest that Ki-67 may have several competing modes
281 of PS that are differentially regulated by phosphorylation. Our data suggest a mechanism for
282 Ki-67-mediated mitotic targeting of nucleolar components to the perichromosomal layer^{45,46}
283 via CDK-mediated phosphorylation, which reduces PS of several major nucleolar IDPs, thus
284 triggering nucleolar disassembly, while simultaneously promoting PS of Ki-67 bound to
285 chromatin to recruit nucleolar components.

286 In conclusion, this work reveals *in vivo* that CDK-dependent mitotic phosphorylation occurs in
287 a switch-like manner on diverse proteins whose common denominators are a high level of
288 disorder and localisation to MLOs. Furthermore, our data show that CDK-mediated

289 phosphorylation regulates homotypic interactions between IDPs, which may coordinate diverse
290 cellular processes during the cell cycle. While this is not incompatible with models in which
291 high-affinity interactions contribute to MLO formation by PS^{50,51}, it suggests that cell cycle
292 control may be less specific than previously thought.

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311 designed and interpreted experiments. JMV, HT, AH, GvM, LK and GD performed
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317 **Supplementary Materials:**

318 Materials and Methods

319 Extended data figures 1-10

320 Data S1-S4

321 Movies S1, S2

322

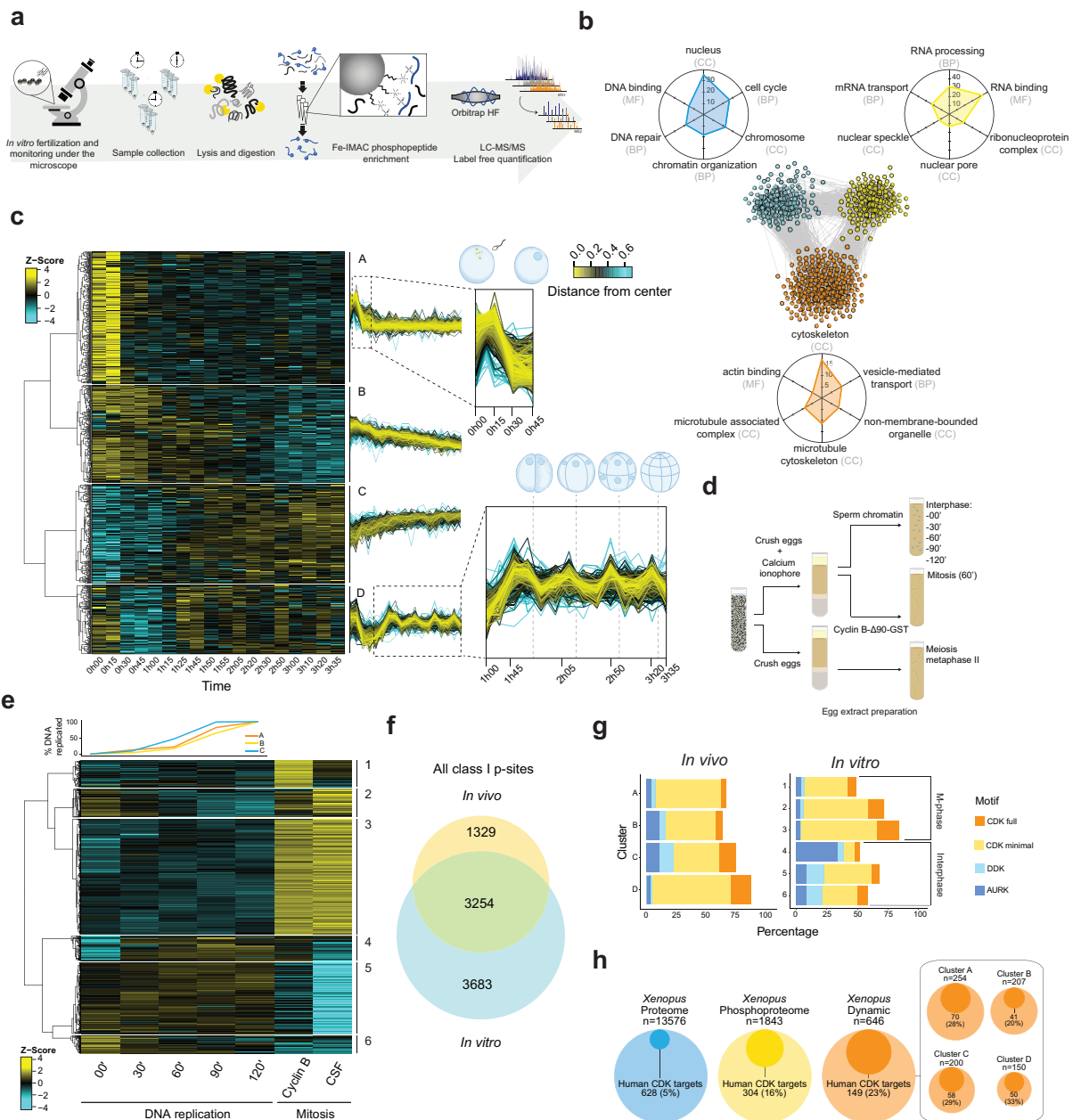
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430

431 **Figure 1. The time-resolved phosphoproteome from a single-cell to a 16-cell embryo and**432 **its cell cycle assignment. (a)** Schematic representation of the workflow. Single *Xenopus* eggs

433 and embryos were collected followed by cell lysis, protein digestion, phosphopeptide

434 enrichment and high-resolution proteomics analysis. (b) STRING network of functionally

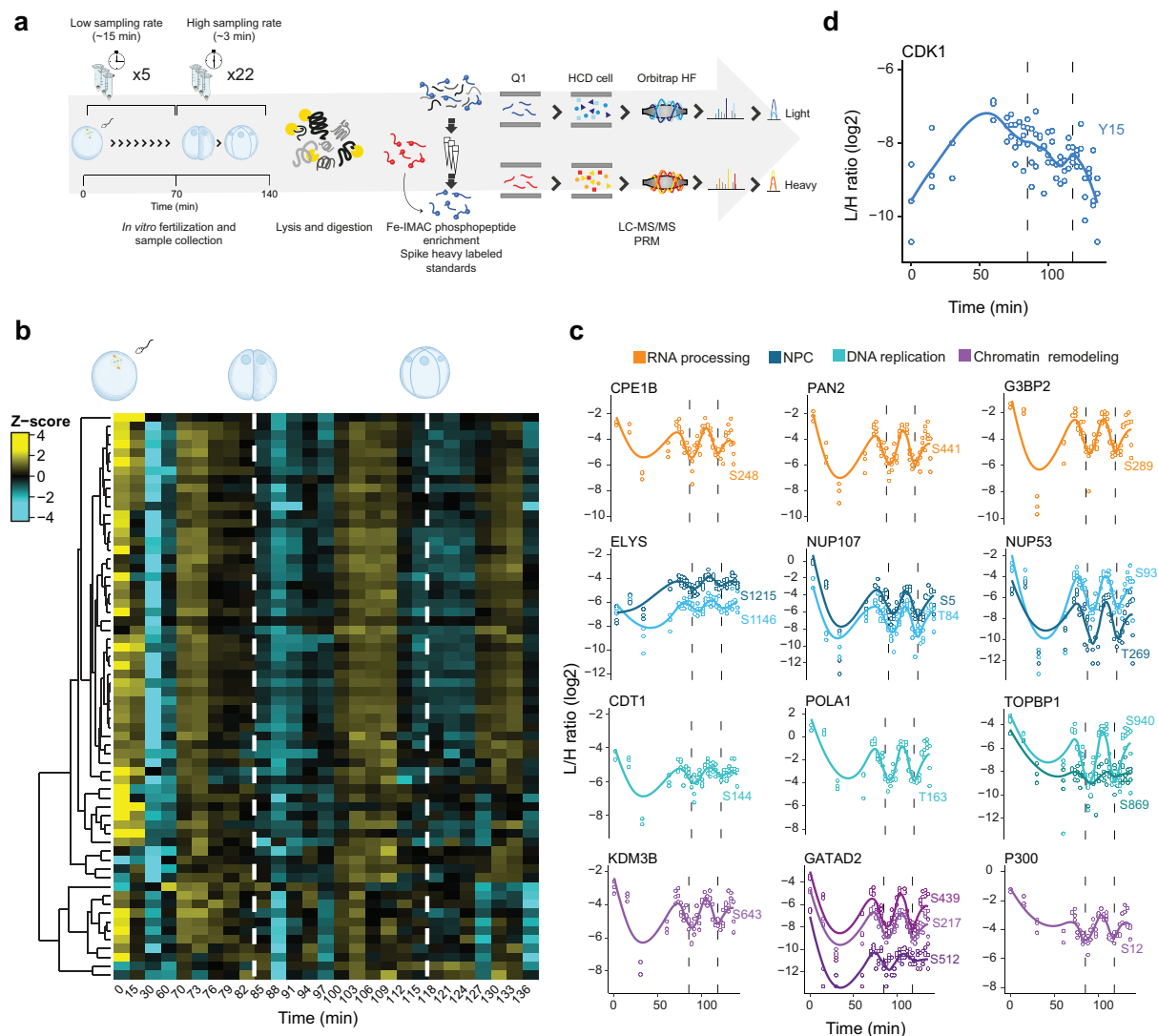
435 associated proteins undergoing dynamic phosphorylation (each node represents a protein).

436 Vicinity clustering reveals three main groups (yellow, blue and orange) with a high degree of

437 association. Radar plots show the corresponding GO terms (adjusted p value <0.05) for each

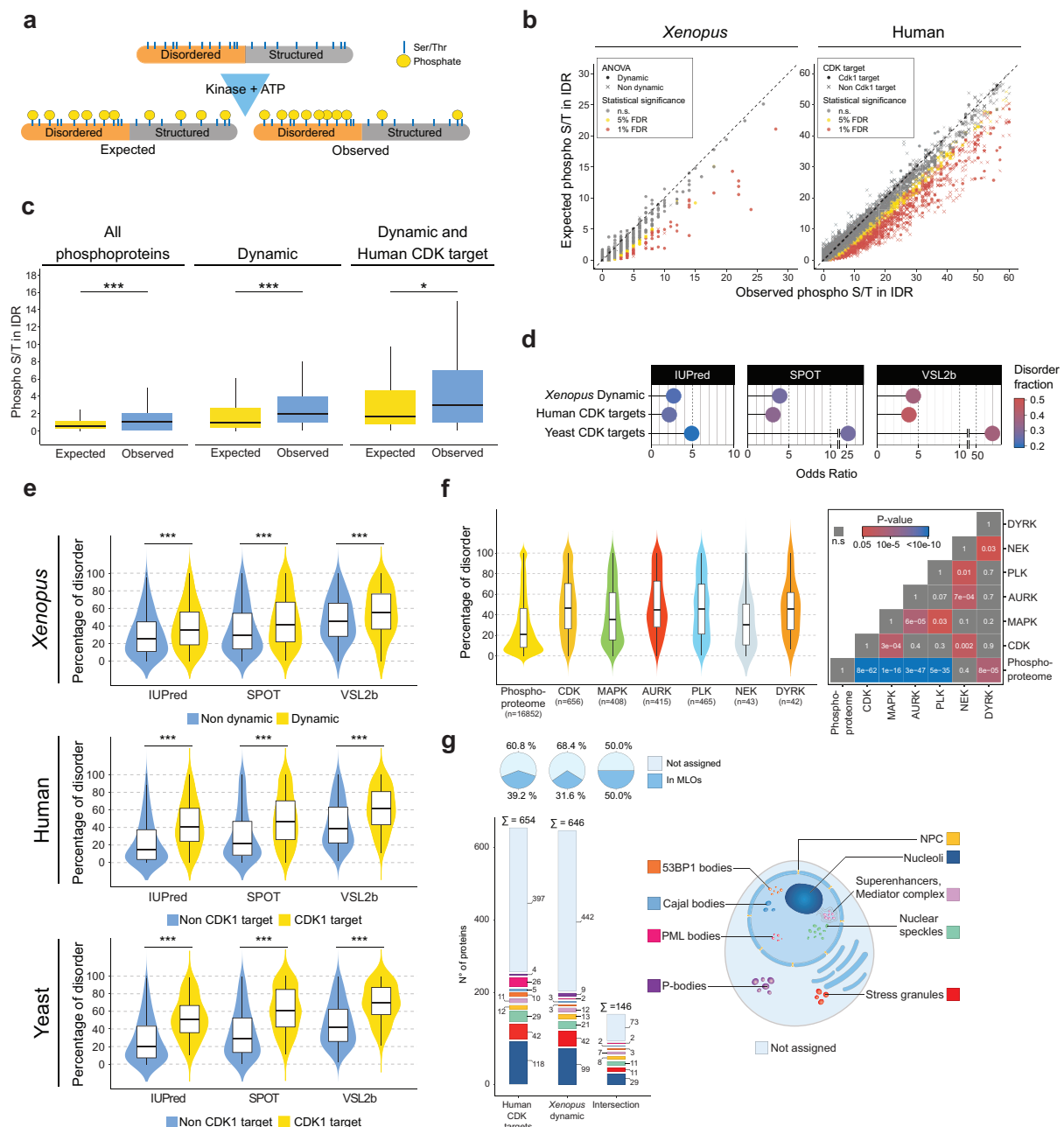
438 group (axes show $-\text{Log}_{10}(\text{adj p value})$ for each GO term). (c) Hierarchical clustering of

439 significantly changing phosphosites (ANOVA, Benjamini-Hochberg correction, FDR 0.05),
440 reveals 4 clusters with distinct regulation (A-D). Dashed boxes in clusters A and D are zoomed-
441 in to highlight dynamic phosphorylation patterns (dashed lines depict the time points of cell
442 division). (d) Scheme of the experiment in the *Xenopus* egg extract. (e) Top: quantification of
443 DNA replication in each biological replicate. Below: Hierarchical clustering of dynamic
444 phosphosites (ANOVA, Benjamini-Hochberg correction, FDR 0.05) reveals differential
445 regulation of phosphosites during S-phase and mitosis. (f) Overlap between *in vivo* (embryo)
446 and *in vitro* (egg extract) phosphoproteomics. (g) Proportion of phosphosites according to their
447 potential upstream kinase for each cluster in the *in vivo* (top) and *in vitro* (bottom) experiments.
448 (h) Circle plots presenting enrichment of homologues of human CDK substrates among
449 *Xenopus* phosphoproteins detected *in vivo* and those with dynamic phosphosites.
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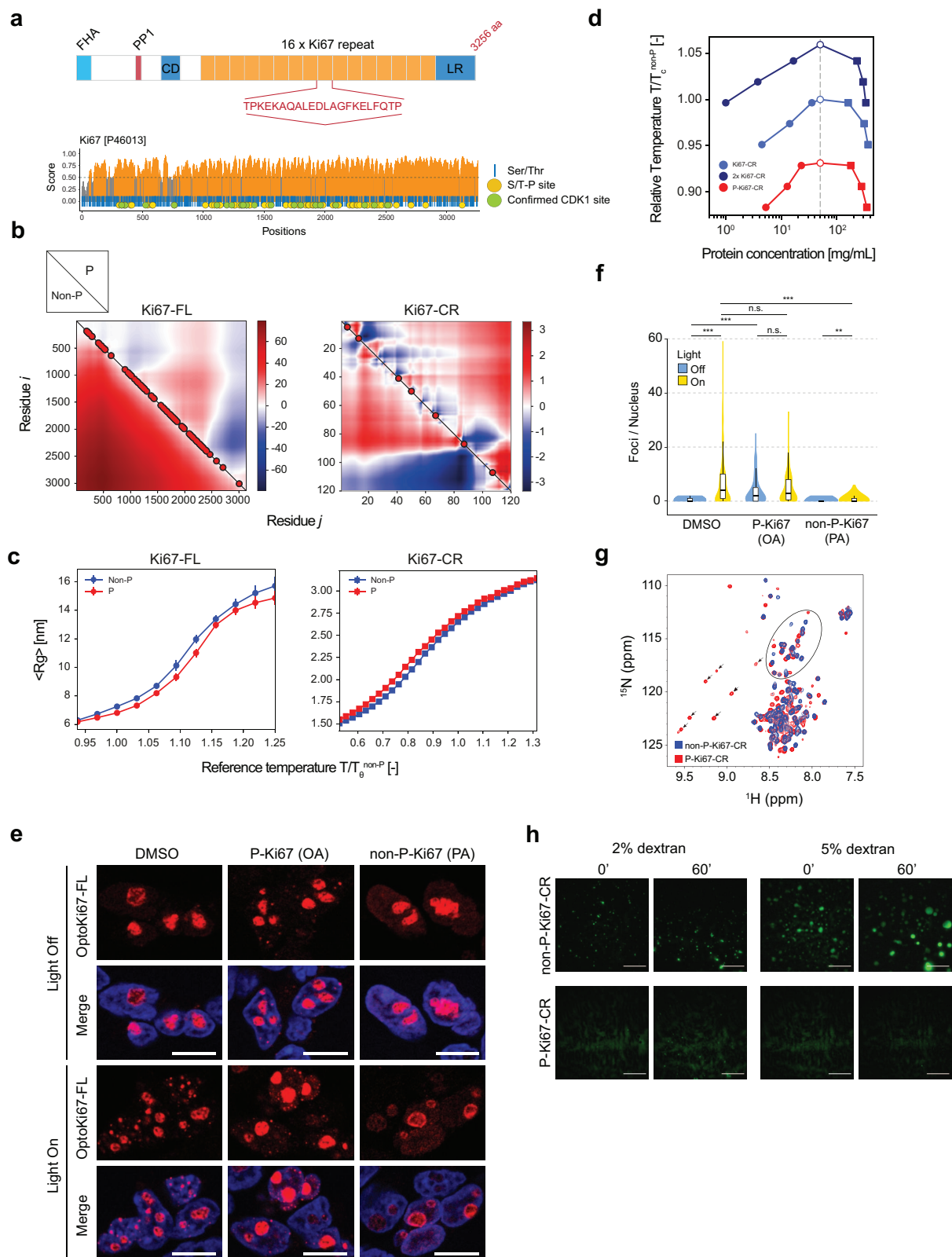
452 **Figure 2. Switch-like mitotic phosphorylation *in vivo*.** (a) Schematic representation of the
 453 workflow. Samples were collected over two cell divisions and enriched phosphopeptides were
 454 subjected to targeted proteomics analysis. (b) Heat map shows a highly synchronous wave of
 455 phosphorylation preceding each of the two cell divisions. Dashed lines depict times when cell
 456 divisions were recorded. (c) Single phosphosite plots from selected proteins. Each dot
 457 represents a biological replicate (n=3). Dashed lines depict times when cell divisions were
 458 recorded. (d) Single phosphosite plot of CDK1 inhibitory phosphorylation (Y15).



459

460 **Fig. 3. The cell cycle phosphoproteome is characterised by intrinsic disorder and MLO**
 461 **components.** (a) Scheme illustrating hypothetical enrichment of phosphorylation in disordered
 462 regions when taking into account amino acid compositional bias. (b) Scatter plot of expected
 463 vs observed phosphorylated Ser/Thr for each protein of human and *Xenopus* phosphoprotein
 464 datasets. FDR thresholds of 5% and 1% are marked in yellow and red respectively. Circles:
 465 proteins with at least one dynamic phosphorylation in *Xenopus*, or human CDK1 subfamily
 466 substrates, respectively. (c) Boxplots showing expected vs observed phosphorylated Ser/Thr
 467 among all phosphoproteins detected (left), phosphoproteins with at least one dynamic
 468 phosphosite (middle), and dynamic phosphoproteins also detected as CDK1 subfamily targets
 469 in humans (right). Distributions were compared with the Wilcoxon signed-rank test. * $p < 0.05$,

470 **p<0.01, ***p<0.001. (d) Plots showing the common Odds Ratio of Ser/Thr phosphorylation
471 in structured and ordered regions calculated with the Fisher's test (see Extended data Fig. 6b,
472 c). For all organisms, the disordered regions were calculated with three different disorder
473 predictors. The disordered fraction is presented in a colour scale. (e) Violin plots of the
474 distribution of disordered residues per protein for CDK targets vs the rest of the
475 phosphoproteome for human and yeast, and dynamic phosphoproteins vs the rest of the
476 phosphoproteome for *Xenopus*. Intrinsic disorder was calculated with three different predictors
477 (IUPred, SPOT, and VSL2b). Statistical significance was evaluated with the Wilcoxon–Mann–
478 Whitney test; ***p<0.001. (f) Violin plot (left) showing the distribution of disordered residues
479 per protein for CDK, MAPK, Aurora, PLK, NEK and DYRK kinase targets vs the rest of the
480 phosphoproteome for human targets. Statistical significance was assessed by Kruskal-Wallis
481 ANOVA, and pairwise comparisons were performed with Dunn's post-hoc tests. The adjusted
482 p-values (Benjamini-Hochberg) are shown in a tile plot (right). (g) Human CDK1 subfamily
483 targets, *Xenopus* dynamic phosphoproteins, and the intersection of both sets, that are present
484 in our manually curated proteome of membraneless organelles.



485

486

487 **Fig. 4. CDK-mediated phosphorylation regulates phase separation of a model IDP. (a)**

488 Top, scheme of the human Ki-67 protein (FHA, forkhead-associated domain; PP1, PP1

489 phosphatase-binding domain; CD, conserved domain; LR, leucine arginine-rich domain).

490 Highlighted, Ki-67 repeat consensus motif. Bottom, diagram of IUPred score over the length
491 of human Ki-67. Regions with scores >0.5 (orange) are considered to be disordered, and <0.5
492 (grey) structured. Blue vertical lines indicate Ser and Thr residues; yellow circles, known
493 Ser/Thr-Pro phosphosites; green circles, confirmed CDK1 subfamily phosphorylations. (b)
494 Sequence Charge Decoration Matrix (SCDM) maps for full length Ki-67 (FL, left) and Ki-67
495 consensus repeat (CR, right), depicting the contribution of electrostatic interaction dictating the
496 distance between two amino acid residues i and j (shown in x and y axes). The values of SCDM
497 for different residue pairs (i,j) are shown using colour schemes with red and blue denoting
498 positive (repulsive) and negative (attractive) values, respectively. The lower and upper
499 triangles indicate SCDM map for the unphosphorylated (non-P) and phosphorylated (P)
500 sequences, respectively. Confirmed and putative (Ser/Thr-Pro) CDK phosphorylation sites are
501 indicated with red circles. (c) Dependency of the radius of gyration (R_g) on the simulation
502 temperature in single-chain MD simulations for full chain Ki-67 (left) and consensus repeat
503 (right). The reference temperature is the θ temperature of the non-phosphorylated molecule for
504 full chain and consensus repeat, respectively. Reported error bars are obtained by block
505 analysis over 10 blocks. (d) Binodal curves from phase coexistence simulations of the Ki-67
506 consensus repeat sequence. For each temperature, filled circles indicate the dilute phase density
507 and squares indicate the coexisting dense phase density. Empty circles indicate the fitted
508 critical temperature (T_c) of each system. The T_c of the non-phosphorylated monomer (light blue
509 empty circle) was the reference for the normalisation of the temperature values. The light gray
510 dashed line indicates the total concentration used in the simulations. The reference temperature
511 is the θ temperature of the non-phosphorylated molecule for full chain and consensus repeat,
512 respectively. Reported error bars are obtained by block analysis over 10 blocks. (e)
513 Representative fluorescent images of HEK-293 cells expressing opto-Ki-67 (FL) construct
514 before (Light Off) and after (Light On) exposure to blue light. Cells were pretreated for 1h with
515 either vehicle (DMSO), 0.5 μM okadaic acid (OA), to inhibit protein phosphatase 2, or 5 μM
516 purvalanol A (PA), to inhibit CDKs. DNA was stained with Hoechst 33258; scale bars, 10 μm .
517 (f) Violin plot presenting quantification of results from (e); the number of foci per nucleus was
518 counted. Statistical significance was assessed by one-way ANOVA on ranks (Kruskal–Wallis
519 test) and pairwise *post-hoc* comparisons using the Mann–Whitney test. P-values were adjusted
520 by the Benjamini-Hochberg method. (g) Overlaid NMR ^1H - ^{15}N HSQC of unphosphorylated
521 (blue) and CDK-phosphorylated (red) GFP-tagged Ki-67 consensus repeat. Each cross-peak
522 corresponds to one residue. The seven new deshielded cross peaks (highlighted by a black flag)

523 appearing above 8.5 ppm in ^1H correspond to phosphorylated serines or threonines (^1H
524 downfield chemical shift perturbation on phosphorylated Ser/Thr residues due to phosphate
525 electronegativity). Non phosphorylated Ser/Thr residues are surrounded by a black oval. (h)
526 Representative fluorescence images of *in vitro* phase separation assay with purified GFP-
527 tagged Ki-67 consensus repeat (CR), non-phosphorylated (non-P) or *in vitro* phosphorylated
528 with recombinant CDK1-cyclin B-CKS1 (P), at indicated dextran concentrations and time
529 points; scale bars, 10 μm .

Supplementary Files

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- [DATAS2CDKphosphorylationdata.xlsx](#)
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