**CDK-mediated control of intrinsic disorder dominates the cell cycle phosphoproteome**

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**Abstract**

Eukaryotic cell cycle control involves phosphorylation of numerous substrates by multiple protein kinases. Yet it can be driven solely by oscillating activity of cyclin-dependent kinase 1 (CDK1)1,2. suggesting that cell cycle-regulated phosphorylations may be quantitatively or qualitatively different from other phosphorylations. Here, we tested this hypothesis *in vivo* during *Xenopus* embryogenesis, and demonstrated its validity from yeast to humans. Using a high sensitivity phosphoproteomics workflow3, we analysed phosphorylation in single *Xenopus* embryos, from which we deconvoluted the cell cycle dynamics of protein phosphorylation. We found that 747 of 1,032 (72%) dynamic phosphosites on 646 proteins were located in intrinsically disordered regions (IDRs) of proteins, and 60% were located in CDK consensus motifs. We assigned cell cycle behaviour to these sites by 180-second time-resolution targeted proteomics *in vivo* and parallel phosphoproteomics of replicating or mitotic *Xenopus* egg extracts. To estimate the proportion of CDK targets, we manually curated 659 known human CDK substrates, and found homologues of 148 *Xenopus* proteins that show dynamic phosphorylation (23%). Proteome-wide analysis revealed that *Xenopus* dynamic phosphoproteins, as well as human and yeast CDK substrates, are enriched in intrinsic disorder, and the phosphosites are predominantly located in IDRs. A large proportion of CDK substrates and proteins with dynamic phosphosites localise to membrane-less organelles, whose cell cycle-regulated assembly is thought to depend on liquid-liquid phase separation mediated by IDRs4. Together, these results suggest that phosphorylation of IDRs by CDK evolved as the predominant mechanism for regulating the cellular reorganization that occurs during cell cycle progression.

**Main text**

Cell cycle progression is presumed to arise from the collective behavior of altered protein phosphorylation states, and depends on CDK1-family cyclin-dependent kinases (CDKs). These CDKs phosphorylate hundreds of sites on diverse proteins5–8, and regulate DNA replication, mitosis, transcription, chromatin remodeling, DNA repair, the cytoskeleton, nuclear transport, protein translation, formation of a mitotic spindle and even ciliogenesis9–11. Understanding how such a combination of phosphorylations generates cell cycle order requires a global picture of cell cycle-regulated phosphosites *in vivo*, which are technically challenging to identify. Highly dynamic phosphorylation states cannot readily be determined from populations of cells12, where whole-culture synchronisation methods generate artefacts and produce populations that are not truly synchronised13. Yet although single-cell proteomics studies have identified several hundreds of proteins14,15, sensitivity and reproducibility are insufficient for low stoichiometry and highly dynamic targets such as phosphosites.

To overcome these obstacles we used an extremely sensitive phosphopeptide enrichment strategy3 to perform semi-quantitative phosphoproteomics on the highly synchronous early cell cycles of *Xenopus laevis* embryos, which consist solely of S and M-phase16,17. Importantly, proteome changes are negligible compared with phosphoproteome changes in *Xenopus* early development18. We collected individual embryos at 18 time-points separated by 15-minute intervals, while recording visual cues of cortical rotation of fertilised eggs and subsequent cell divisions. Phosphopeptides from each embryo were enriched, separated by nanoLC and analysed by high-resolution mass spectrometry (Figure 1a). Individual embryo phosphorylation states strongly correlated, demonstrating their synchrony and the robustness of our methodology (Figure 1b). We thus generated a cell cycle map of protein phosphorylation from unfertilised eggs to 16-cell embryos. This identified 4,583 phosphosites with high localisation probability (>0.75) mapping to 1,843 proteins (Figure 1c), the majority being phosphoserines (Figure 1d). Motif analysis showed that around 51% of the detected phosphosites are proline-directed (S/T-P), thus, potential CDK sites. Moreover, around 10% of all phosphosites matched the canonical CDK1-family motif S/TPxK (Supplementary Figure 1).

Next, we focused on 1,032 phosphosites whose abundance was dynamic during the time-course. Hierarchical clustering revealed distinct groups (Figure 1e) which were all enriched in proline-directed phosphosites (Supplementary Figure X). Gene ontology (GO) analysis of all dynamic sites, coupled with network analysis, revealed high functional association and interconnectivity between groups of proteins related to RNA binding and the NPC, DNA replication and chromatin remodeling, and microtubule regulation (Figure 1f). The identification of multiple components of the NPC, RNP granules, the replisome, and several chromatin remodelers suggests coordinated phosphorylation of whole protein complexes (Figure 1g). Cluster A contained phosphosites with initial high intensity that dropped at 30 minutes, correlating with the degradation of cyclin B around 15 minutes after fertilisation and exit from meiotic metaphase II. As observed in a recent study19, group A contained proteins of the nuclear pore complex (NPC) and chromatin remodeling proteins (Supplementary Figure 2a), whose dephosphorylation coincides with the rapid transition from open nuclei with condensed chromatin to an enclosed nuclear envelope. Cluster B phosphosites were of lower intensity and dephosphorylation rate, and were enriched in RNA binding proteins localising to ribonucleoprotein (RNP) granules and components of the cytoskeleton (Supplementary Figure 2b). These phosphosites were upregulated before the first cell division, during the cortical rotation and transport of maternal mRNA responsible for axis specification in the embryo, suggesting that phosphosites in clusters A and B conclude the transition from meiosis to mitosis and prepare the one-cell embryo for upcoming cell divisions20. Motif analysis revealed a predominance of CDK consensus sites, while potential target sites of other kinases such as Aurora, PLK, PKA, Dbf4-dependent kinases (DDK) and Casein kinase I and II were present to a lesser extent (Figure 1h).

Cluster C phosphosites, of which around 52% were proline-directed, generally increased over the time course, while Cluster D phosphosites, the vast majority (84%) of which were proline–directed, had a clear oscillating signature which corresponds to mitotic phosphorylation. Cluster C sites included phosphosites displaying a reciprocal oscillating trend and a lower amplitude compared to cluster D sites, suggesting that their phosphorylation from S-phase onwards. Several sites with this trend, for example MCM4 S31ph, were from monophosphorylated peptides, while the multiphosphorylated form was found in cluster D. This identifies cluster C as containing the earliest phosphorylations of proteins that are highly phosphorylated in mitosis (Supplementary Figure X). While cluster C phosphosites were less enriched in minimal CDK motifs than cluster D, both clusters had similar enrichment of the full CDK motif (14% and 17.3% respectively; Supplementary figure X), suggesting that interphase CDK phosphorylation relies more on full motifs. Cluster C was enriched in consensus sites for Dbf4-dependent kinase (DDK), (12%) and Aurora kinases/PKA (10%), indicating that the latter kinases are active well before mitosis (Figure 1h). GO analysis of clusters C and D nevertheless similarly revealed enrichment in ribonucleoproteins and RNA binding, nucleocytoplasmic transport, chromatin organisation and DNA replication (Supplementary Figure X), implying progressive control of these biological mechanisms by phosphorylation throughout the cell cycle.

Since cluster D sites peak in mitosis, we measured their dynamics at high time-resolution using quantitative parallel reaction monitoring (PRM). We chose 64 phosphosites on proteins of RNP granules, the replisome, chromatin remodelers, and NPCs. For each, we used heavy isotope-labeled phosphopeptides as internal standards. We sampled single embryos every 180-seconds, from the egg to the four-cell embryo stages, to obtain a high time-resolution quantitative description of cell cycle-related phosphorylation *in vivo* (Figure 2a). This revealed parallel upregulation of all phosphosites preceding each cell division (Figure 2b, c) and coinciding with CDK1 activation (Figure 2d), indicating highly synchronous phosphorylation of diverse proteins at mitotic onset.

To confirm and extend the attribution of phosphosites to different cell cycle stages, we compared these *in vivo* phosphorylation patternswith dynamics of protein phosphorylation in egg extracts, during a time course of DNA replication or in mitosis (Figure 3a, scheme; b, replication time-course). Replication was initiated by adding purified sperm chromatin to interphase egg extracts, while mitosis was triggered by adding recombinant cyclin B. We also used egg extracts arrested at meiotic metaphase II. Overall, we identified 6937 phosphosites, which included 71% of the sites identified *in vivo* (Figure 3c). Of these, 1425 varied between replication and M-phase, including 427 sites (40% proline-directed) upregulated in S-phase and 632 (75% proline-directed) M-phase sites (Figure 3d and Table SX). S-phase sites were also highly enriched in consensus motifs for Cdc7 and Aurora kinases (Figure 3e, f; Supplementary Figure X). Using these datasets, we analysed the cell cycle behaviour of sitesthat were also dynamic *in vivo* (Figure 3g). Embryo cluster A sites were upregulated in both meiotic and mitotic extracts *in vitro,* highlighting the functional similarities between meiotic and mitotic M-phase. Embryo cluster B sites were generally progressively downregulated during S-phase, with a subset increasing at mitosis, suggesting that their dephosphorylation is a consequence of M-phase exit at fertilisation but is not required for DNA replication. As expected, sites from embryo clusters C and D were part of the *in vitro* replicating S-phase and mitotic groups, respectively. These results substantially increase our knowledge of vertebrate cell cycle-regulated phosphorylations, and show that, irrespective of the cell cycle phase, the most abundant phosphosites that change during the cell cycle are proline-directed sites.

Our data suggest that dynamic phosphosites are enriched in CDK targets. Few direct CDK substrates have been characterised in *Xenopus*, but are likely conserved with other vertebrates. We therefore compiled a set of 659 human CDK1-subfamily substrates (Table SX), combining data from PhosphoSite Plus21 with manually curated information from several human CDK substrate screens (see Supplementary Methods for sources). 303 of these 659 CDK substrates have *Xenopus* homologues among the 1843 phosphoproteins we detected*,* 148 of which were present among the 646 proteins with dynamic phosphosites (Figure 4a). Next, we wondered whether these phosphoproteins share common structural features. Intrinsically disordered regions (IDR) generally predict location of phosphosites22, so we first computationally analysed intrinsic disorder in our entire datasets using the energy estimation-based predictor IUPred23. We indeed found that both dynamic and CDK-mediated phosphosites were often located in predicted IDR (examples in Figure 4b). This agrees with previous analyses of yeast *in vitro* CDK1 substrates or *in vivo* CDK1-dependent phosphosites, which found preferential localisation in IDRs24,25, as well as with CDK1 substrates in mice26. However, since sequence attributes of phosphorylation sites in general are similar to those found in IDRs22, this observation may partly result from the enrichment of serine and threonine in disordered regions (Figure 4c). To correct for this compositional bias and investigate intrinsic disorder among the cell cycle phosphoproteome systematically, we compared the number of phosphosites detected in predicted IDRs to that expected according to the distribution of phosphorylatable amino acids. Even after this correction, our identified phosphosites were strongly enriched in predicted IDRs, especially for proteins with at least one peptide displaying dynamic phosphorylation. The same was true for human CDK substrates (Figure 4d, e).

Next, we asked whether proteins that are phosphorylated in a cell cycle-dependent manner, and CDK substrates in particular, are more or less disordered than other phosphoproteins. Using three different disorder prediction methods, we determined the percentage of disordered residues of *Xenopus* proteins with dynamic phosphosites, as well as human CDK substrates, compared to the rest of their respective phosphoproteomes (non-cell cycle-dependent phosphorylations in our *Xenopus* dataset, and non-CDK-dependent phosphosites from the Phosphosite Plus database)*.* This revealed that both *Xenopus* dynamic phosphoproteins, and human CDK substrates, are significantly more disordered than all other phosphoproteins (Fig. 4f). To see whether this finding is conserved across the eukaryotic kingdom, we also analysed yeast, in which we could define very high-confidence CDK1 phosphosites by intersecting data obtained using chemical genetics to identify *in vitro* CDK1 substrates6 and *in vivo* CDK1-dependent phosphosites24. 100 of the 185 CDK1 substrates defined *in vitro* were also phosphorylated in a CDK1-dependent manner *in vivo*, 19 of which do not present a minimal CDK1 consensus motif (Supplementary Figure X). Thus, CDK1-mediated phosphorylation on sites other than S/TP may have been previously underestimated or disregarded. Using this dataset, we found that yeast CDK1 substrates are highly enriched in disorder compared to the non-CDK-dependent yeast phosphoproteome (Figure 4f), revealing conservation of this principle across eukaryotes. This was true using 12 different intrinsic disorder prediction methods, supporting the robustness of our conclusions (Supplementary Figure Xa, b). Almost all methods gave a lower fraction of disordered residues in the yeast proteome than in *Xenopus* and human (Supplementary Table X), consistent with an increase in disorder in more complex organisms27.

Finally, for *Xenopus* proteins with dynamic phosphosites, and for human and yeast CDK substrates, we calculated the proportion of phosphorylated to non-phosphorylated serine and threonine in disordered and structured regions. Using each of the three different disorder predictors, the ratio of these values combined over the whole dataset (the common odds ratio) was far greater than one, confirming that phosphosite enrichment in IDRs is a general feature of cell cycle-regulated and CDK-mediated phosphorylations throughout eukaryotes (Figure 4g, Supplementary table X).

Protein phosphorylation regulates cell cycle-dependent regulation of assembly and disassembly of membrane-less organelles (MLO)28–30 including NPC, nucleoli, 53BP1 bodies, Cajal bodies, PML-nuclear bodies, P-bodies, stress granules and splicing speckles31–34. MLOs are thought to form by liquid-liquid phase separation, which relies on weak electrostatic interactions between IDR31 that may be disrupted by phosphorylation. Since we observed clustering of cell cycle-regulated phosphosites in IDRs (Figure 4a), we hypothesised that changes in the net charge of these regions due to CDK mediated phosphorylation might control MLO assembly and disassembly during cell cycle progression. To see whether there is evidence that supports this hypothesis, we manually curated the MLO proteome (See Supplementary Methods for sources), and individually verified the presence of CDK substrates and dynamic phosphoproteins among this dataset. We found that at least 235 CDK substrates (35.6%) and 201 (31%) dynamicphosphoproteins are present in MLOs (Figure 4h). CDK substrates include major proteins of MLOs highly enriched in IDRs such as coilin (Cajal bodies), nucleophosmin and Ki-67 (nucleoli), 53BP1 (53BP1 bodies), nucleoporins (NPCs) and PML (PML bodies). In these proteins, as in dynamic phosphoproteins *in vivo*, the vast majority of proline-directed phosphosites were located in predicted IDRs (Supplementary Fig Xf). Moreover, of the 148 proteins that show dynamic phosphorylation in *Xenopus* and are CDK substrates in human, 73 (49%) localise to MLOs (Figure 4h). These data support the existence of an evolutionary conserved mechanism for cell-cycle control of MLOs.

In conclusion, this work reveals that CDK sites are both quantitatively and qualitatively different from other dynamic phosphosites: they are the most numerous, they occur in a coordinated manner on multiple proteins of similar complexes, and they are highly-enriched within IDRs. Since they cluster on key proteins of MLOs, we speculate that their coordinated phosphorylation by CDKs at mitosis should destabilise weak interactions between IDRs to promote MLO disassembly, thus acting analogously to a detergent that dissolves liquid phase boundaries. Detailed biochemical and structural studies will be required to verify this hypothesis.

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