**CDK-mediated control of intrinsic disorder dominates the oscillating phosphoproteome *in vivo***

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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 CDK-mediated phosphorylations may be quantitatively or qualitatively different from other cell cycle-regulated phosphorylations. Here, we tested this hypothesis *in vivo* without perturbing cell cycles. Using a high sensitivity phosphoproteomics workflow3, we analysed phosphorylation states in single *Xenopus laevis* embryos, from which we deconvoluted the dynamics of protein phosphorylation from the fertilized egg to the 16-cell embryo. Of 4583 phosphosites identified, 45% were located in CDK consensus sites, and 239 sites on 188 proteins showed cell cycle-regulated oscillation, essentially all of which were located in intrinsically disordered regions (IDRs). 180 second time-resolution targeted proteomics of a subset of these sites revealed synchronous waves of phosphorylation correlating with overall CDK activity. Moreover, 43 proteins with oscillating phosphosites were previously identified as CDK substrates. By increasing coverage of the oscillating phosphoproteome to 3023 sites using replicating or mitotic egg extracts, we identified many new interphase phosphosites and confirmed the predominance of phosphorylation mediated by CDK compared to other kinases. Proteome-wide analysis from yeast to human of experimentally confirmed CDK phosphosites revealed that they are significantly more enriched in IDRs than other phosphosites. Furthermore, many CDK substrates localise to membrane-less organelles, whose assembly during the cell cycle is regulated by phosphorylation and is thought to depend on liquid-liquid phase separation mediated by IDRs. Together, these results suggest that phosphorylation of IDRs by CDK evolved as the predominant mechanism for regulating the diverse proteins involved in different cell cycle processes, explaining how a single enzyme with limited specificity can drive the drastic changes in cellular organization that occur during cell cycle progression.

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 specific 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 synchronization is often inadequate and generates artefacts13. 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 limitations 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-phase1,2. We collected individual embryos at 18 time-points separated by 15-minute intervals, while recording visual cues of cortical rotation of fertilized eggs and subsequent cell divisions. Phosphopeptides from each embryo were enriched, separated by nanoLC and analyzed 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 dynamic map of protein phosphorylation from unfertilized eggs to 16-cell embryos. This identified 4,583 phosphosites with high localization probability (>0.75) mapping to 1,843 proteins (Figure 1c), the majority being phosphoserines (Figure 1d). Motif analysis showed that around 45% of the detected phosphosites are proline-directed (S/T-P), thus, potential CDK sites, with massive enrichment of the canonical CDK1-family motif S/TPxK (Supplementary Figure 1). In contrast, non proline-directed phosphosites are highly enriched in basic amino acids upstream and acidic residues downstream (Supplementary Figure 1).

Next, we focused on phosphosites that changed in abundance during the time-course. Hierarchical clustering revealed distinct groups (Figure 1e) which were all enriched in proline-directed phosphosites (Supplementary Figure X). Cluster A contains phosphosites with initial high intensity that drops at 30 minutes, correlating with the degradation of cyclin B around 15 minutes after fertilization and exit from meiotic metaphase II18. As observed in a recent study19, gene ontology (GO) analysis highlighted 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 are of lower intensity and dephosphorylation rate (Supplementary Figure 2b), and are enriched in RNA binding proteins localising to ribonucleoprotein (RNP) granules and components of the cytoskeleton (Supplementary Figure 2c). These phosphosites are upregulated before the first cell division, during the cortical rotation and transport of maternal mRNA responsible for axis specification in the embryo20-22. Phosphosites in clusters A and B likely conclude the transition from meiosis to mitosis and prepare the one-cell embryo for upcoming cell divisions23. Motif analysis revealed that as well as CDK, phosphosites are also potential substrates of Aurora, PLK, PKA, Dbf4-dependent kinases (DDK), Casein kinase II and CHK1/2, with additional involvement of ATR/M and NEK kinases in cluster B (Supplementary Figure X).

Cluster D displays a clear oscillating signature that correlates with early cell divisions (Figure 1e), and 84% of sites in cluster D are proline-directed. Network analysis revealed high functional association and interconnectivity between groups 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 remodeling complexes suggests coordinated oscillating phosphorylation of whole protein complexes (Figure 1g). Cluster C phosphosites, of which around 52% are proline-directed, generally increase over the time course but include phosphosites displaying a reciprocal oscillating trend and a lower amplitude compared to cluster D sites. Closer analysis shows Cluster C contains some monophosphorylated peptides, for example MCM4 S31ph, that are multiphosphorylated in cluster D. This identifies cluster C as containing the earliest phosphorylations of highly phosphorylated proteins (Supplementary Figure 4). Accordingly, GO analysis revealed an enrichment of terms similar to cluster D (Supplementary Figure 2c). Furthermore, cluster C was more highly enriched in the full CDK-consensus motif than cluster D, suggesting that early phosphorylation, when CDK activity levels are low, results from increased kinase affinity for the substrate (Supplementary figure X). Clusters C and D also both contain around 4% of consensus PLK sites, while Cluster C contains more consensus sites for Aurora kinases/PKA (10%) than cluster D (2%), indicating that substrates of these kinases are phosphorylated from S-phase onwards (Figure 1h).

To determine whether sites in cluster D are phosphorylated concurrently or consecutively within each cell cycle, we used high time-resolution phosphoproteomics (PRM). We chose 64 phosphosites on proteins of RNP granules, DNA replication, chromatin remodeling, and NPCs. We sampled single embryos every 180-seconds, from the egg to the four-cell embryo stages, and used heavy-labeled standards for each phosphopeptide analyzed, allowing quantitation of phosphosite intensities (Figure 2a). This revealed a synchronous upregulation of all sites preceding cell division (Figure 2b, c) and coinciding with CDK1 activation (Figure 2d, e). These are highly enriched in direct CDK targets, as 43 out of 188 proteins with oscillating phosphosites in cluster D have orthologues in human that have already been identified as CDK1/2 substrates (Figure 2f (pending).

To confirm and extend the attribution of phosphosites to different cell cycle stages, we compared these phosphorylation patterns with dynamics of protein phosphorylation in egg extracts during a time course of DNA replication, or in mitosis (Figure 3a, scheme). Replication was initiated by adding purified sperm chromatin to interphase egg extracts, while mitosis was triggered by adding recombinant cyclin B or by inhibiting protein phosphatases with okadaic acid (ref). We also used egg extracts arrested at meiotic metaphase II. Overall, we identified 8,506 phosphosites, of which 3,023 varied between different cell cycle stages (Supplementary Figure 5 and Table SX). About half (46%) of 427 sites upregulated in S-phase, and 83% of 632 sites upregulated in mitosis, are proline-directed, similar to the proportions in embryo clusters C and D, respectively. The other S-phase sites are highly enriched in consensus motifs for Aurora kinases and DDK/CKII (Supplementary figure X). While most (62%) of phosphosites in embryo cluster D are upregulated in mitotic extracts (Figure 3b), some phosphosites from embryo cluster C are detected during S-phase in the extract as singly phosphorylated peptides, and acquire additional phosphorylations during mitosis (Figure 3c). These results substantially increase our knowledge of cell cycle-regulated phosphorylations, and suggest that, irrespective of the cell cycle phase, the most abundant phosphosites that change during the cell cycle are CDK substrates.

We wondered which features of oscillating phosphosites direct their cell cycle-regulated phosphorylation. We tested whether they are either enriched or depleted in intrinsic disorder, which predicts phosphosites generally31. We first computationally analyzed intrinsic disorder in our entire dataset using the energy estimation-based predictor IUPred30. We then determined the number of phosphosites detected in predicted IDRs and compared it to the number of phosphosites expected according to the distribution of phosphorylatable residues (Figure 4a). Our identified phosphosites predominantly located to predicted IDRs, especially for proteins displaying dynamic regulation, with the highest enrichment in those undergoing oscillating phosphorylation (Figure 4b, c). Closer inspection of predicted CDK-substrates undergoing oscillating phosphorylation shows that they are phosphorylated almost exclusively in predicted IDRs (Figure 4d).

At first sight, these data, and previous analyses of yeast *in vitro* CDK1 substrates6 or CDK1-dependent phosphosites28 which found enrichment in IDRs 28,29, suggest that CDKs control different steps in the cell cycle by generally mediating IDR phosphorylation. However, compositional bias might lead to false conclusions since sequence attributes of phosphorylation sites in general are similar to those found in IDRs31 – thus, CDK targets might be no different from other phosphosites. Furthermore, determining the probability of any site to be in an IDR requires an estimation of the disordered fraction of the proteome. We therefore analysed intrinsic disorder in the entire proteomes, and in previously identified CDK targets, of budding yeast, *Xenopus* and human, as well as our oscillating phosphosites in *Xenopus.* To test the robustness of our conclusions, we compared 10 different prediction methods. The fraction of disordered residues in the yeast proteome ranged between X and 38% depending on the prediction method (Supplementary Figure 6a, b). With one exception (DisEMBL-HL), 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 organisms32. Serine, threonine, and proline are among the most enriched amino acids in predicted IDRs (Supplementary figure 6c), highlighting a possible evolutionary selection for phosphorylation sites in intrinsically disordered regions, as well as the danger in defining CDK sites as intrinsically disordered without correcting for compositional bias.

To define high-confidence CDK1 phosphosites *in vivo*, we first intersected yeast data obtained using chemical genetics to identify CDK1 substrates *in vitro* 3 or CDK1-dependent phosphosites *in vivo* 4. We found that around half the CDK1 substrates defined *in vitro* were also phosphorylated in a CDK1-dependent manner *in vivo*, while half of the latter sites do not present a minimal CDK1 consensus motif (S/TP), including around 20% of the intersection between the datasets (Supplementary figure 7a). This suggests that CDK1-mediated phosphorylation on sites other than S/TP may have been underestimated. All the methods predicted a significant enrichment of disordered residues among high confidence CDK1-mediated phosphosites (defined as the overlap between *in vitro* CDK1 substrates3 and CDK1-dependent phosphosites4) both when comparing to the entire proteome (Supplementary Figure 7b) or to non-CDK phosphosites (Figure 4e). CDK substrates had about twice the density of canonical and minimal CDK1 consensus motifs as non-CDK substrates (Supplementary Figure 7c). The results were similar when analysing the 436 curated human CDK1-family phosphosites from the Phosphosite database (ref), and for our experimentally-determined *Xenopus* oscillating sites on proteins that have homologues phosphorylated by CDKs in yeast or human (Figure 4e), suggesting conservation throughout eukaryotes.

As expected, ignoring compositional bias, minimal consensus motifs (S/TP) were significantly enriched in IDR even among phosphorylated proteins not identified as CDK1 substrates (Figure 4f), but the proteins with the highest density of motifs in predicted IDR were all CDK1 targets. If preferential CDK-mediated phosphorylation in IDR is simply attributable to the increased fraction of phosphorylatable amino acids in IDR, then the same should be true for CDK-independent phosphosites. To test this, we generated a contingency table of the ratio of CDK-dependent to CDK-independent phosphorylated serine and threonine residues in both ordered and disordered regions for each CDK target. We then computed a compound ratio for all CDK targets, which should be >1 if CDK phosphosites are preferentially located in IDR relative to other phosphosites. This was true for all IDR prediction methods in yeast, *Xenopus* and human (Fig 4g), confirming cross-species specific enrichment of CDK1/2-dependent phosphorylation in IDR.

IDRs are key drivers of liquid-liquid phase separation 35,36 which is thought to drive formation of membrane-less organelles (MLO) including NPC, nucleoli, Cajal bodies, PML-nuclear bodies, P-bodies, stress granules and splicing speckles36-39, which are assembled and disassembled during the cell cycle. Phase separation relies on weak electrostatic interactions between IDR that are likely disrupted by phosphorylation, a known regulatory mechanism for cell cycle-dependent regulation of MLOs40-42. By examining manual curated proteomics data from MLOs, we asked whether their components are represented among experimentally verified CDK substrates. We found that at least X (X%) CDK substrates have been identified as MLO components, which, since MLOs including highly abundant proteins of MLOs such as coilin (Cajal bodies), nucleophosmin (nucleoli), 53BP1 (53BP1 bodies) and PML (PML bodies) (Fig 4h). In these proteins, as in oscillating phosphosites *in vivo*, the CDK-phosphosite is located exclusively in predicted IDR (Fig 4i).

In conclusion, this work reveals that CDK sites are both quantitatively and qualitatively different from other oscillating phosphosites: they predominate, occur in a coordinated manner on multiple proteins of similar complexes, and are more highly-enriched within IDR. Since they also tend to cluster (refs Durbin, Holt), we speculate that their coordinated phosphorylation by CDKs at mitosis should destabilise weak interactions between IDR to promote MLO disassembly, thus acting analogously to a detergent that dissolves liquid phase boundaries. In contrast, non-proline-directed sites are enriched in highly charged residues which may form salt-bridges, minimizing the impact of the phosphorylation on local structure and requiring more specialized kinases. Detailed biochemical and structural studies will be required to verify these hypotheses.

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