**Dynamics of cell cycle phosphorylation reveals CDK-mediated control of intrinsic disorder**

**Abstract**

Eukaryotic cell cycle control involves phosphorylation of numerous substrates by multiple protein kinases, yet can be driven by a simple oscillation of cyclin-dependent kinase 1 (CDK1) activity. Previously identified phosphosites are numerous and act in diverse protein complexes and cellular locations; how they can collectively generate cell cycle order in response to a single oscillation of one kinase activity is unknown. Here, we present *in vivo* evidence that a unifying feature of cell cycle-regulated phosphorylation is its location in intrinsically disordered regions (IDR) of proteins. We used a highly sensitive phosphoproteomics workflow in *Xenopus* to study phosphorylation dynamicsin embryos upon fertilization, from single-cell to the 16-cell stage. Of the >4000 phosphosites observed, around 200 are cell cycle-regulated, and these predominantly locate to predicted IDR. High time-resolution targeted proteomics revealed a single, highly synchronous wave of IDR phosphorylation in each cell cycle, correlating with overall CDK activity, and many of the homologues of these proteins have been identified as CDK substrates in different organisms. We classified phosphosites as S-phase or mitotic based on dynamics of their phosphorylation in egg extracts, revealing a large number of new cell cycle-regulated interphase phosphosites. We compared different methods for prediction of intrinsic disorder in entire proteomes, and found that CDK phosphosites are highly enriched in IDR even when corrected for compositional bias. Our results suggest that the diverse proteins involved in different cell cycle processes evolved phosphorylation of IDR as a key regulatory mechanism, and this is also true for CDK1, explaining how a single enzyme with limited specificity can control multiple diverse processes. We propose that IDR phosphorylation may control liquid-liquid phase-separation of subcellular organelles, most of which are disassembled in mitosis.

CDK1-family cyclin-dependent kinases (CDKs) can phosphorylate hundreds of sites on diverse proteins1–4, and, along with other protein kinases, regulate DNA replication, mitosis, transcription, chromatin remodelling, DNA repair, the cytoskeleton, nuclear transport, protein translation, formation of a mitotic spindle and even ciliogenesis5–7. However, a general model of how cell cycle order arises from collective behavior of protein phosphorylation requires an understanding of the nature of cell cycle-regulated phosphosites *in vivo*, which is technically challenging. Phosphorylation is highly dynamic and acts on timescales easily diluted at the multiple-cell level8; where cell synchronization is often insufficient and may produce artefacts9. Single-cell proteomics studies have identified several hundreds of proteins10 but sensitivity and reproducibility are limiting for low stoichiometry targets.

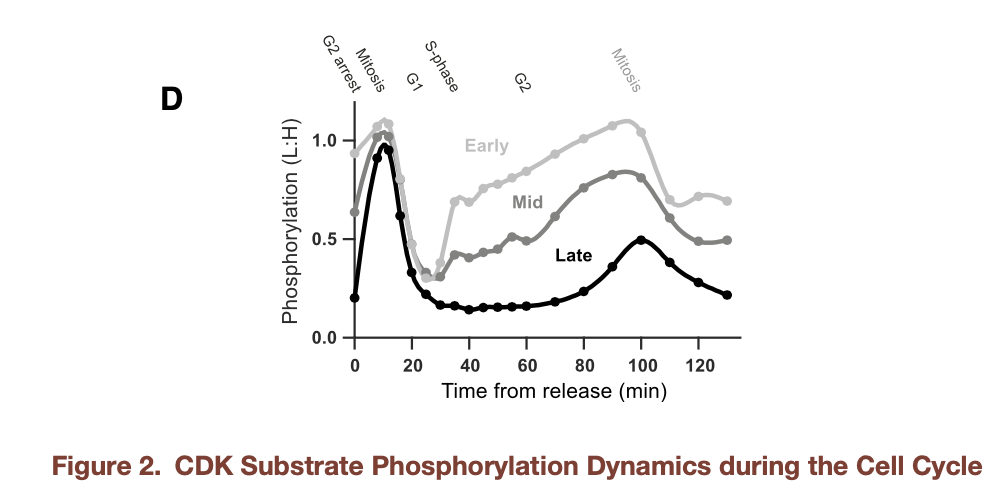
To overcome these limitations we used an extremely sensitive phosphopeptide enrichment strategy11 to perform phosphoproteomics on the highly synchronous early cell cycles of *X. laevis* embryos which consist solely of S and M-phase12,13. To study phosphorylation dynamics over the early cell divisions, we monitored fertilized eggs under the microscope, collecting samples every ∼15 minutes (Figure 1a). We correlated the visual cues of cortical rotation of fertilized eggs, and subsequent cell division, with changes in protein phosphorylation. During all stages of sample processing, each individual cell or embryo was treated as a biological replicate. Phosphopeptides were separated by nanoLC and analyzed by high-resolution mass spectrometry (Figure 1a). We collected 18 time points to generate a phosphoproteome time-lapse of early cell divisions, from a single unfertilized egg to a single 16-cell embryo. Biological replicates displayed high correlation, demonstrating the robustness of our sample preparation workflow (Figure 1b). We thus identified ∼4,500 unique phosphosites with high confidence (>0.75) (Figure 1c), the majority being phosphoserines (Figure 1d). Motif analysis showed that around half of the detected phosphosites are proline-directed, with significant enrichment of the canonical CDK1-family motif S/TPxK. Other detected motifs and their potential kinases are shown in Supplementary Figure 1.

Next, we focused on phosphosites that displayed differential regulation through our time-lapse. Hierarchical clustering of their temporal dynamics revealed distinct groups (Figure 1e). Cluster A contains phosphosites that drop in intensity at 30 minutes, correlating with degradation of cyclin B around 15 minutes after fertilization and exit from meiotic metaphase II14. Gene ontology (GO) analysis shows dephosphorylation of proteins related to the nuclear pore complex (NPC) and chromatin remodeling (Supplementary Figure 2), as also found in a recent study14. We also observed a strong decrease in phosphorylation of RNA binding proteins, likely related to the phosphorylation-driven control of mRNA expression during oocyte maturation15.

A second cluster, cluster D, displays an oscillating trend that tightly correlates with early cell divisions (Figure 1e). Network analysis of the cluster 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). Many components of the same protein complexes, including the NPC, RNP granules, the replisome and several chromatin remodeling complexes, are among these cell cycle-regulated phosphorylations, suggesting coordinated phosphorylation (Figure 1g). For example, while previous findings proposed mitotic phosphorylation of Nup98 and Nup53 as a driver of NPC dissolution in mitosis16,17, our data reveals many other nucleoporins with similarly oscillating phosphosites.

To determine whether these sites are phosphorylated concurrently, or at different times within each cell cycle, we performed quantitative phosphoproteomics with 180 second resolution from the single oocyte to the four-cell embryo stage. We used a targeted proteomics approach (PRM) to systematically measure 64 oscillating phosphosites from RNP granules, DNA replication, chromatin remodeling and NPC. We employed heavy-labeled standards for each phosphopeptide analysed, allowing accurate determination of phosphosite intensities (Figure 2a). This revealed a single synchronous wave of phosphorylation of all sites throughout the cell cycle (Figure 2b, c), as determined by phosphorylation of the CDK1 regulatory network (Figure 2c-e). These are likely mediated by CDK, as *X out of (200) oscillating phosphosites have already been identified as CDK phosphosites on the orthologues in yeast (/human / other species) (Figure 2f-x).*

In synchronized fission yeast cells, all CDK-dependent phosphosites increase in abundance in a single wave throughout the cell cycle, yet some phosphosites on proteins involved in DNA replication begin to rise in S-phase18. To see if such patterns exist in *Xenopus* early embryos, we first identified sites occurring during a time course of DNA replication, or in mitosis, in egg extracts to which sperm nuclei had been added. Mitosis was triggered either by addition of recombinant cyclin B or by inhibition of protein phosphatases with Okadaic acid. We also used egg extracts arrested at meiotic metaphase II. This identified 8500 sites of which 3000 varied between the different samples (Figure 3a and supplementary Table/dataset X). Five main clusters, A-E, were present: A, sites in interphase that are also present in mitotic extracts and become dephosphorylated during replication; B, sites that are absent in interphase, appear progressively during replication, and remain during mitosis; C, sites that are present only in interphase, but become dephosphorylated during replication; D, sites that are present in interphase and throughout DNA replication, but are absent in mitosis; E, mitosis-specific sites (Supplementary Table/dataset Y). We identified 78% of our oscillating phosphorylations, of which 114 phosphosites (62%) were significantly more abundant in the mitotic cell-free extract (Figure 3b). Of the 3000 differential sites, Y have been identified as CDK substrates in human / yeast (Fig 3c). We next analysed the behavior of these sites during embryogenesis. +*Paragraph on the behavior of these sites during embryogenesis (Figure 3d-x). What % of the different categories were present in the oscillating dataset? Are these offset oscillations or low amplitude oscillations? Explain how this fits with a model of a single wave of phosphorylation…can we draw a model like in fission yeast (see below)?*

 These data identify novel dynamic patterns of phosphorylation and a large number of new cell cycle regulated phosphosites.

We wondered which features of osciallating sites direct their cell cycle-regulated phosphorylation. Previous analysis in yeast of *in vitro* CDK1 substrates2 or CDK1-dependent phosphosites19 has suggested an enrichment in intrinsically disordered regions (IDR) of proteins 20,19. We computationally analysed intrinsic disorder in a subset of our cycling phosphosites using the energy estimation-based predictor IUPred21, and found that they appear almost exclusively located in predicted IDR (Figure 4a). To investigate this in our entire dataset, we determined the number of phosphosites detected in predicted IDRs and compared it to the expected number of phosphosites assuming uniform distribution over the protein sequence (Figure 4b). Our identified phosphosites predominantly located to predicted IDRs, especially for proteins displaying dynamic regulation, with the highest enrichment for oscillating phosphorylation (Figure 4c, d), as previously described for budding yeast CDK1-dependent phosphosites19.

At first sight, these and our data suggest that CDKs control different steps in the cell cycle by generally mediating IDR phosphorylation. However, there are two caveats. First, the disordered fraction of the proteome is not well understood; thus, the probability of any site to be in an IDR is unknown. Second, sequence attributes of phosphorylation sites in general are similar to those found in IDR22, suggesting that compositional bias might lead to false conclusions. To investigate the impact of these confounding factors, we analysed intrinsic disorder in the proteomes of budding yeast, Xenopus and human, and in previously identified CDK substrates, *using several prediction methods*. We first applied this approach to the yeast proteome. Estimations of the average disorder of proteins ranged between 10 and 50% according to the prediction method (Supplementary figure 3a, b). Among the most enriched amino acids in predicted IDR are S, T and P, highlighting the likely evolutionary selection for phosphorylation sites in intrinsically disordered regions, as well as the potential danger in defining CDK sites as intrinsically disordered (Supplementary figure 3c). Around half of yeast CDK1 substrates defined *in vitro*2 were also phosphorylated in a CDK1-dependent manner *in vivo*19, 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 3a). This suggests that CDK1-mediated phosphorylation on sites other than S/TP may have been previously underestimated. Bearing these caveats in mind, we predicted the fraction of disordered residues in CDK1 substrates and all other proteins. All three methods predicted a highly significant (p<3x10-11) enrichment of disordered residues among CDK1 sites (Figure 5a), and the latter had approximately twice as many canonical and minimal CDK1 consensus motifs as non-CDK substrates (Supplementary Figure 4a). While minimal consensus motifs (S/TP) were also significantly enriched in IDR even among proteins which were not found to be CDK1 substrates, the proteins with the highest density of sites in predicted IDR were all CDK1-dependent sites (Figure 5b). This indeed suggests that CDK1 sites are specifically enriched in IDR. To estimate this enrichment proteome-wide, we calculated the odds ratio of disordered to ordered CDK1-dependent phosphosites over other disordered to ordered serines or threonines, using stratified contingency tables. This ratio should be >1 if CDK sites are enriched in disordered regions even when correcting for compositional bias, and was 11.2 (p<2 x 10-16), confirming specific enrichment of CDK1-dependent phosphorylation in IDR.

We applied a similar approach to study disorder in the human proteome and in curated human CDK substrate sites from the PhosphoSite database. This showed *essentially similar results to yeast…(Fig 5c, d and Supplementary Figure 4b-x). W*e also analysed human homologues of the 64 selected oscillating phosphosites in *Xenopus* embryos. This showed…(Figure 5e).

*We next investigated whether a random selection of our phosphosites have previous experimental evidence for intrinsic disorder, by interrogating the protein structure database PDB for direct evidence of disorder by NMR, or absence in X-ray crystal structures (Figure 6a-x). Finally, we studied the likely physicochemical effects of IDR phosphorylation, estimating effects on local isoelectric point…and. This showed (Figure 6y-z)…and suggests that phosphorylation disrupts surface physico-chamical properties, that likely mediate protein-protein interactions.*

Among the complexes we identified whose phosphorylation oscillates through the cell cycle, many localize to non-membrane-bound organelles that display liquid-liquid phase separation (LLPS), which include NPC, nucleoli, Cajal bodies, PML-nuclear bodies, P-bodies, stress granules and splicing speckles23–26. LLPS relies on weak electrostatic interactions between IDR that are likely disrupted by phosphorylation, a known regulatory mechanism for cell cycle-dependent LLPS27–29. Our data is consistent with the hypothesis that CDKs have been selected by evolution to be low-specificity kinases capable of regulating LLPS by IDR phosphorylation. Along with differing affinities of S-phase and M-phase proteins for CDK18, this could explain how a single oscillation in activity of a single CDK-cyclin complex may be able to regulate the cell cycle.

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Figure legends

**Figure 1.** Phosphoproteomic analysis from a single-cell to a 16-cell embryo. (a) Schematic overview of the workflow. Oocytes were in vitro fertilized and monitored under the microscope during the entire time-course. For each time point, individual replicates were snap frozen in liquid nitrogen. After protein digestion, phosphorylated peptides were enriched using Fe-IMAC and analyzed by LC-MS/MS. (b) Correlation coefficient for two randomly selected time points, demonstrating high reproducibility. (c) Total number of phosphosites detected and their distribution according to the localization probability score. (d) Distribution of serine, threonine and tyrosine phosphosites identified. (e) Hierarchical clustering of significantly changing phosphosites (ANOVA, Benjamini-Hochberg correction, FDR 0.05), reveals 4 distinct clusters (A-D), showing the different regulation of phosphosites during the time course. Dashed boxes in cluster A and D mark zoomed areas to highlight dynamic phosphorylation patterns (dashed lines in zoom-in of cluster D depict the time points of cell division). (f) The center shows a STRING network of functionally associated proteins undergoing dynamic phosphorylation (each node represents a protein). Vicinity clustering of proteins in the network reveals three groups (colored yellow, blue and red) with high degree of association and connected radar plots show the respective GO terms overrepresented (adjusted p value <0.05) for each group (axis show –Log10(*adj p value*) for each GO term). (g) Proteins with known association show similar oscillatory phosphorylation. Plots highlight the dynamic trend of selected phosphosites over time, flanked by illustrations of examples of protein condensates formed by the proteins undergoing dynamic phosphorylation. Proteins highlighted in bold show at least one oscillating phosphosite in our dataset.

**Figure 2.** Oscillating phosphosites predominantly locate to IDRs. (a) Disorder tendency based on IUPRED score for selected proteins showing dynamic phosphorylation. Plots show the correlation between high disorder tendency (>0.5) and phosphosite density. (b) Diagram displays a generic protein with the observed distribution (up) and the expected distribution (down) of phosphosites over predicted ordered and disordered regions. Expected distribution is obtained by uniformly scattering phosphosites across the protein sequence. (c) Box plots show significant differences between phosphosite distribution among predicted ordered and disordered regions, becoming more evident in proteins that display dynamic phosphorylation. (d) Binomial test shows proteins with a significantly higher number of phosphorylations localized to IDRs than expected from a uniform distribution.

**Figure 3.** Targeted phosphoproteomics (PRM) at increased time-resolution reveals a single wave of phosphorylation driving early cell divisions. (a) Schematic overview of the workflow. After *in vitro* fertilization, samples were acquired with ~15 min time intervals up to minute 70’ (15 minutes before the first cell division occurred). This was followed by higher frequency sample acquisition (~3 min) surrounding the first two cell divisions. Following protein digestion and Fe-IMAC phosphopeptide enrichment, samples were spiked with heavy-isotope-labeled synthetic phosphopeptides as internal standards. The mass spectrometer was operated in PRM mode and changes in phosphorylation were quantified by calculating the ratio between the light and heavy versions of the phosphopeptide. (b) Heat map shows a tightly regulated, single wave of phosphorylation over the two cell divisions. Highlighted phosphosites from proteins related to a variety of functions during different stages of the cell cycle, show synchronized phosphorylation dynamics. Dashed lines depict the time points when cell divisions were recorded. (c) Average trend for phosphosites previously reported to be upregulated in mitosis opposes the trend of CDK1 inhibitory phosphorylation (Y15). (d) Embryonic cell cycle oscillator driving changes in protein phosphorylation through cell divisions. Detected phosphosites related to positive feedback loops: pS678 on GWL is linked to increased kinase activity and subsequent PP2A inactivation. pS344 and pS359 upregulation on human version of NIPA are related to inhibition of Cyclin B ubiquitination. Detected phosphosites related to negative feedback: pS555 on human version of APC1 is one of the phosphosites rel­­ated to increased ubiquitin-ligase activity. (e) Sequence alignment shows conservation of mitotic phosphosites regulating oscillatory phosphorylation. (f) Plots show the trend of single phosphosites during the time course. Each dot represents a biological replicate (n=3 per time point). Phosphosites under the label ‘Mitosis’ correspond to phosphorylation events previously known to be upregulated at this stage of the cell cycle; here used as markers of mitotic phosphorylation for comparison with the other phosphosites. Dotted lines depict the times when cell divisions were recorded. (g) Heat map shows upregulation of oscillating phosphosites in a *X. laevis* cell-free extract treated with two different stimulus to induce mitosis: non-degradable Cyclin B and Okadaic acid. Clear upregulation is observed when compared to cell-free extract undergoing DNA replication. Same phosphosites highlighted on (b) for comparison. (h) Proposed model. Our findings indicate CDK1 drives protein phosphorylation which regulates protein phase separation throughout the cell cycle. Frames show proposed state of the nucleoplasm and cytoplasm during interphase and mitosis, highlighting how protein phosphorylation disrupts biomolecular condensates (shown: the NPC, replication fork and RNP granules).