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

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**Abstract**

How oscillating activity of cyclin-dependent kinase (CDK)-1 alone can drive the eukaryotic cell cycle (*1*, *2*) is unknown. Here, we demonstrate *in vivo* that cell cycle-regulated phosphorylations differ quantitatively and qualitatively from other phosphorylations. We used high-resolution time-resolved phosphoproteomics in single *Xenopus* embryos, and parallel phosphoproteomics in egg extracts to confirm cell cycle behaviour. We found that 22% of 4583 phosphorylation sites on 1843 proteins were dynamic during early cell cycles, while mitotic phosphosites occurred on entire protein complexes in a switch-like manner *in vivo*. Dynamic phosphosites had two main characteristics: location to intrinsically disordered regions (IDRs) of proteins and prevalence of CDK consensus motifs. Dynamic phosphoproteins, as well as known yeast and 659 manually-curated human CDK substrates, were enriched in intrinsic disorder compared to other phosphoproteins, while many localise to cell cycle-regulated membrane-less organelles. Our results suggest that synchronous phosphorylation of IDRs by CDKs is the predominant mechanism for temporal control of cellular organisation, explaining how a single kinase can drive the cell cycle.

**Main text**

Cell cycle progression is presumed to arise from the collective behaviour of altered protein phosphorylation states, and depends on CDK1-family cyclin-dependent kinases (CDKs). These CDKs phosphorylate hundreds of sites on diverse proteins (*3*–*6*), and regulate DNA replication, mitosis, transcription, chromatin remodeling, DNA repair, the cytoskeleton, nuclear transport, protein translation, formation of a mitotic spindle and even ciliogenesis (*7*–*9*). 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 cells (*10*), where whole-culture synchronisation methods generate artefacts and produce populations that are not truly synchronised (*11*). Yet although single-cell proteomics studies have identified several hundreds of proteins (*12*, *13*), 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 strategy (*14*) to perform quantitative phosphoproteomics on the highly synchronous early cell cycles of *Xenopus laevis* embryos, which consist solely of S and M-phase (*15*, *16*). Importantly, proteome changes are negligible compared with phosphoproteome changes in *Xenopus* early development (fig. S1), as previously reported (*17*). 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 (Fig. 1A). Individual embryo phosphorylation states strongly correlated, demonstrating their synchrony and the robustness of our methodology (Fig. 1B). We thus generated a cell cycle map of protein phosphorylation from an unfertilised egg to a 16-cell embryo. This identified 4,583 phosphosites with high localisation probability (>0.75) mapping to 1,843 proteins (Fig. 1C), the majority being phosphoserines (Fig. 1D). Motif analysis showed that around 51% of the detected phosphosites were proline-directed (S/T-P), thus, potential CDK sites. Moreover, around 10% of all phosphosites matched the canonical CDK1-family motif S/TPxK/R (fig. S2A).

Next, we focused on 1,032 phosphosites whose abundance was dynamic during the time-course. Hierarchical clustering revealed distinct groups (Fig. 1E) which were all enriched in proline-directed phosphosites (fig. S2B, C). 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 nuclear pore complex (NPC), DNA replication and chromatin remodeling, and microtubule regulation (Fig. 1F). 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. GO analysis for group A highlighted proteins related to nuclear organisation, including NPC complex and nuclear transport, DNA replication, chromosomal structure and segregation (fig. S3), as also observed in a recent study (*18*). Cluster B phosphosites were of lower intensity and dephosphorylation rate, and were enriched in mRNA-binding proteins, regulators of translation and mRNA processing, as well as DNA repair factors (fig. S2). 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 divisions (*19*). 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 (Fig. 1G).

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 an oscillating signature with a clear upregulation preceding each cell division. Cluster C included phosphosites displaying a reciprocal oscillating trend and a lower amplitude compared to cluster D sites, indicating that they are phosphorylated 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 (fig. S4). This identifies cluster C as containing the earliest phosphorylations of proteins that are highly phosphorylated towards mitosis. Both clusters contained a similar number of potential CDK targets, while cluster C was also enriched in consensus sites for DDK (12%) and Aurora kinases (10%), indicating that the latter kinases are active well before mitosis (Fig. 1G). GO analysis of clusters C and D nevertheless revealed enrichment in ribonucleoproteins and RNA binding, nucleocytoplasmic transport, chromatin organisation and DNA replication (fig. S3), implying progressive control of these biological mechanisms by phosphorylation throughout the cell cycle. In cluster D, which peak around mitosis, coordinated phosphorylation of multiple members of the same protein complexes commonly occurs (Fig. 1H).

The dynamics of mitotic phosphorylation is not well understood, and may occur either in a switch-like manner due to the bistable mitotic control network (*20*), or progressively throughout S-phase and G2 (*21*). We suspected that evidence for progressive phosphorylation may be due to incomplete cell synchronisation (*11*). To see whether mitotic phosphorylation of individual phosphosites is progressive or switch-like in unsynchronised cell cycles *in vivo*, we analysed dynamics of cluster D sites 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 to obtain a high time-resolution quantitative description of cell cycle-related phosphorylation *in vivo* (Fig. 2A). This revealed parallel and abrupt upregulation of all phosphosites preceding each cell division (Fig. 2B, C) and coinciding with CDK1 activation (Fig. 2D), indicating switch-like 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 (Fig. 3A, B). 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* (Fig. 3C). Of these, 1728 varied between replication and M-phase, including 693 sites (43% proline-directed) upregulated in S-phase and 1035 (75% proline-directed) M-phase sites (Fig. 3D, Table SX). S-phase sites were also enriched in consensus motifs for DDK and Aurora kinases (Fig. 3E, F; fig. S5). Using these datasets, we analysed the cell cycle behaviour of sitesthat were also dynamic *in vivo* (Fig. 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 either present throughout S-phase, or progressively downregulated during S-phase, with a subset increasing at mitosis. As expected, most 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 Plus (*22*) 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 (Fig. 4A). Next, we wondered whether these phosphoproteins share common structural features. Intrinsically disordered regions (IDRs) generally predict location of phosphosites (*23*), so we first computationally analysed intrinsic disorder in our entire datasets using the energy estimation-based predictor IUPred (*24*). We indeed found that dynamic phosphosites were often located in predicted IDR (examples in Fig. 4B). This is consistent with previous analyses of yeast *in vitro* CDK1 substrates or *in vivo* CDK1-dependent phosphosites, which found preferential localisation in IDRs (*25*, *26*), as well as with CDK1 substrates in mice (*27*). However, since sequence attributes of phosphorylation sites in general are similar to those found in IDRs (*23*), this observation may partly result from the enrichment of serine and threonine in disordered regions (fig. S6A). Furthermore, proline is also enriched in IDR (fig. S6A), and previous screens for CDK substrates (*4*, *26*, *21*, *27*) only considered proline directed sites as CDK sites. To see whether CDK1-mediated phosphorylation on non-proline directed S/T sites may have been previously underestimated, we defined high confidence CDK1 sites in yeast by intersecting *in vitro* CDK1 substrates (*4*) and *in vivo* CDK1-dependent phosphosites (*25*). 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 (fig. S6B), indicating that CDK1 is capable of phosphorylating non-proline directed sites to some degree. Therefore, to correct for these compositional biases 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 (Fig. 4C). Even after this correction, our identified phosphosites were strongly enriched in predicted IDRs, especially for proteins with at least one site displaying dynamic phosphorylation. The same was true for human CDK substrates (Fig. 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). We found that high-confidence yeast CDK1 substrates are also highly enriched in disorder compared to the non-CDK-dependent yeast phosphoproteome (Fig. 4F), revealing conservation of this principle across eukaryotes. The same trend was observed using 13 different intrinsic disorder prediction methods, supporting the robustness of our conclusions (fig. S6C). Almost all methods gave a lower fraction of disordered residues in the yeast proteome than in *Xenopus* and human (fig. S6D), consistent with an increase in disorder in more complex organisms (*28*).

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 by CDK, or dynamic for Xenopus, 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 (Fig. 4G, fig. S6E, F).

Protein phosphorylation regulates cell cycle-dependent assembly and disassembly of membrane-less organelles (MLO) (*29*–*31*) including NPC, nucleoli, 53BP1 bodies, Cajal bodies, PML-nuclear bodies, P-bodies, stress granules and splicing speckles (*32*–*35*). MLOs are thought to form by liquid-liquid phase separation, which relies on weak electrostatic interactions between IDR (*32*) that may be disrupted by phosphorylation. Since we observed clustering of cell cycle-regulated phosphosites in IDRs (Fig. 4B), 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 analysed publicly available data on each of our curated human CDK substrates for localisation to MLOs. We also assembled an MLO proteome from human phosphoproteomics studies (Table SX; See Supplementary Methods for sources) and analysed the proportion of *Xenopus* dynamic phosphoproteins with homologues among this dataset. We found that at least 235 CDK substrates (35.6%) and 201 (31%) dynamic *Xenopus* phosphoproteins are present in MLOs (Fig. 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 and confirmed CDK sites were located in predicted IDRs (fig. S6F). Moreover, of the 148 proteins that show dynamic phosphorylation in *Xenopus* and are CDK substrates in human, 73 (49%) localise to MLOs (Fig. 4H). These data support the existence of an evolutionary conserved mechanism for cell-cycle control of MLOs.

In conclusion, this work reveals *in vivo* that cell cycle dynamic and CDK sites are both quantitatively and qualitatively different from other dynamic phosphosites: they are the most numerous, they occur synchronously on multiple proteins of similar complexes, and they are highly-enriched within IDRs. Since mitotic sites cluster on key proteins of MLOs, which should destabilise weak interactions between IDRs, their switch-like phosphorylation should promote rapid 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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**Figure legends**

**Figure 1.** **The dynamic phosphoproteome from a single-cell to a 16-cell embryo.** (A) Scheme of the experiment, see text and methods for details. (B) Correlation coefficients for two randomly selected time points. (C) Total number of phosphosites detected and their distribution according to the localisation probability score. (D) Distribution of phosphosites identified among serine, threonine and tyrosine. (E) Hierarchical clustering of significantly changing phosphosites (ANOVA, Benjamini-Hochberg correction, FDR 0.05), reveals 4 clusters with distinct regulation (A-D). Dashed boxes in clusters A and D are zoomed-in to highlight dynamic phosphorylation patterns (dashed lines depict the time points of cell division). (F) STRING network of functionally associated proteins undergoing dynamic phosphorylation (each node represents a protein). Vicinity clustering reveals three main groups (yellow, blue and orange) with high degree of association. Radar plots show the corresponding GO terms overrepresented (adjusted p value <0.05) for each group (axes show -Log10(*adj p value*) for each GO term). (G) Count of consensus phosphosite motifs detected in each of the differentially regulated phosphosite clusters (note: the sum of consensus sites exceeds the number of phosphosites due to redundancy between motif predictions). (H) Examples of proteins with known association showing similar oscillating phosphorylation. Plots highlight the dynamic trend of the cluster (grey) and selected phosphosites (orange) over time, flanked by illustrations of protein complexes formed by the proteins undergoing dynamic phosphorylation. Proteins highlighted in bold show at least one oscillating phosphosite in our dataset.

**Figure 2.** **High-resolution targeted phosphoproteomics reveals switch-like mitotic phosphorylation *in vivo*.** (A) Scheme of the experiment, see text and methods for details. (B) Heat map shows a highly synchronous wave of phosphorylation preceding each of the two cell divisions. Dashed lines depict times when cell divisions were recorded. (C) Single phosphosite plots from selected proteins. Each dot represents a biological replicate (n=3). Dashed lines depict times when cell divisions were recorded. (D) Average trend for phosphosites previously reported to be upregulated in mitosis opposes the trend of CDK1 inhibitory phosphorylation (Y15).

**Figure 3. Behaviour of the phosphoproteome during DNA replication and mitosis.** (A) Scheme of the experiment, see text and methods for details. (B) Quantification of DNA replication in each biological replicate. (C) Overlap between *in vivo* (embryo)and *in vitro* (egg extract) phosphoproteomics. (D) Hierarchical clustering of dynamic phosphosites (ANOVA, Benjamini-Hochberg correction, FDR 0.05) reveals differential regulation of phosphosites during S-phase and mitosis. (E) Count of phosphosites according to their potential upstream kinase for each cluster. (F) Dynamic trend of potential kinase targets per cluster. (G) Behavior of dynamic phosphosites detected in both the *in vivo* and *in vitro* experiments.

**Figure 4. Dynamic phosphoproteins and CDK substrates are characterised by intrinsic disorder.** (A) Circle plots presenting enrichment of homologues of human CDK substrates among *Xenopus* phosphoproteins detected *in vivo* and those with dynamic phospohosites. (B) Diagrams of IUPred scores over the length of selected proteins. Regions with scores >0.5 (red) are considered to be disordered, and <0.5 (grey) structured. Blue vertical lines indicate Ser and Thr residues; yellow circles, phosphorylated sites; green circles, dynamic phosphosites. (C) Scheme illustrating potential enrichment of phosphorylation in disordered regions when taking into account amino acid compositional bias. (D) Scatter plot of expected *vs* observed phosphorylated Ser/Thr for each protein of human and *Xenopus* phosphoprotein datasets. FDR thresholds of 5% and 1% are marked in yellow and red respectively. Crosses: proteins with at least one dynamic phosphorylation in *Xenopus*,or human CDK1 subfamily substrates, respectively. (E) Boxplots showing expected *vs* observed phosphorylated Ser/Thr among all phosphoproteins detected (left), phosphoproteins with at least one dynamic phosphosite (middle), and dynamic phosphoproteins also detected as CDK1 subfamily targets in humans (right). Distributions were compared with the Wilcoxon signed-rank test. (F) Violin plots showing the distribution of disordered residues per protein for CDK targets *vs* the rest of the phosphoproteome for human and yeast, and dynamic phosphoproteins *vs* the rest of the phosphoproteome for *Xenopus*. Intrinsic disorder was calculated with three different predictors (IUPred, SPOT, and VSL2b). Statistical significance was evaluated with the Wilcoxon–Mann–Whitney test. (G) Plot showing the -log10(p-value) *vs* the log10(common Odds Ratio) calculated with the Cochran–Mantel–Haenszel test stratified contingency tables to evaluate enrichment in IDRs of CDK-mediated phosphorylation (or dynamic phosphorylation in *Xenopus*). For all organisms, the disordered regions were calculated with three different disorder predictors. The disordered fraction for each organism and predictor is shown with a colour scale. (H) Human CDK1 subfamily targets, *Xenopus* dynamic phosphoproteins, and the intersection of both sets, that are present in our manually curated proteome of membraneless organelles.

**Supplementary figure 1.** **Proline-directed phosphosites dominate the early embryo phosphoproteome.** (A) Distribution of potential CDK targets among all detected phosphosites and dynamic phosphosites. (B) Sequence motif logo for all dynamic phosphosites and each of the differentially regulated clusters (A-D). Motifs are shown separately for proline-directed (left) and non-proline directed phosphosites (right).

**Supplementary figure 2**. **Gene ontology (GO) enrichment analysis of dynamic p-sites.** Scatter plots show the most significantly enriched (Fisher’s exact test with Bonferroni correction, p<0.05) GO (BP, MF, CC) terms for all dynamic phosphosites per cluster. Plots show the fold-enrichment of specific terms *vs* statistical significance. The size of the circles correlates with the number of proteins associated with the specific term. More details and the full list of enriched GO terms per cluster is found in Supplementary table X.

**Supplementary figure 3. Reciprocal trends of singly- and multi-phosphorylated peptides reveals early interphase and late mitotic phosphorylation, respectively.**  *In vivo* and *in vitro* dynamics of T23 and S31 of MCM4. Orange curves show the trend of T23 and S31 in the multi-phosphorylated peptide (upregulated in mitosis) while the blue curve shows the trend of S31 in the singly-phosphorylated peptide.

**Supplementary figure 4. Sequence motifs enriched in cell cycle phosphoproteomics.** Sequence motif logo for all dynamic phosphosites and each of the differentially regulated clusters (1-6). Motifs are shown separately for proline-directed (left) and non-proline directed phosphosites (right).

**Supplementary figure 6. Proteome-wide analysis of intrinsic disorder among CDK substrates and dynamic phosphoproteins (**A) Differential amino acid composition (see methods) in disordered regions for *Xenopus*, human and yeast determined with three IDR predictors. Amino acids were coloured in a rainbow fashion based on their relative abundance in each proteome. Disruptions of the rainbow pattern show specific compositional signatures for IDRs. (B) Venn diagram of observed *in vitro* (red) and *in vivo* (blue) yeast CDK targets. *In vivo* targets showing CDK minimal consensus motif phosphorylations are highlighted in yellow. (C) Violin and box plots showing the distribution of disordered residues per protein for CDK targets *vs* the rest of the phosphoproteome for human and yeast. Intrinsic disorder information of 13 different predictors was obtained from MobiDB, except for SPOT (calculated). (D) Fraction of the human, *Xenopus* and yeast proteomes predicted as disordered by the 12 methods compiled in MobiDB. (E) Scheme of the stratified 2 by 2 contingency tables analysis. Each CDK target or Dynamic phosphoprotein conforms a strata were the counts of Ser and Thr phosphorylated (by CDK or dynamic phosphorylation in Xenopus) in disordered and structured regions is stored in a 2 by 2 table. (F) Tables with the results of the Cochran–Mantel–Haenszel statistics for the CDK targets of human and yeast, and the dynamic phosphoproteins in Xenopus, using three disorder predictors.(G) Diagrams of IUPred scores over the length of human CDK targets identified as primary components of MLOs, and their *Xenopus* homologues. When Xenopus homologues do not present dynamic phosphorylation, a dynamic phosphoproteins representative of the corresponding MLO is shown. Regions with scores >0.5 (red) are considered to be disordered, and <0.5 (grey) structured. Blue vertical lines indicate Ser and Thr residues; yellow circles, known Ser/Thr-Pro phosphosites for human and detected phosphosites for Xenopus; green circles, confirmed CDK1 subfamily phosphorylations and phosphorylations showing a dynamic behavior throughout the cell cycle, for human and *Xenopus* respectively.