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The Temporal Ordering of Cell-Cycle Phosphorylation

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Cell-cycle phosphorylation is temporally ordered, at least in part, through the sequential expression of different cyclins. Recent studies by Swaffer et al. (2016) and Godfrey et al. (2017) show that intrinsic properties of the substrate proteins contribute as well: good kinase substrates tend to be phosphorylated early, and good phosphatase substrates tend to be phosphorylated late.

The cell cycle comprises the complex sequence of events through which a cell replicates its DNA and then divides to form two daughter cells. The correct temporal order of these events is essential for successful proliferation; for example, DNA replication (S phase) must occur prior to M phase, or else one or both of the daughter cells will be aneuploid. Some degree of temporal ordering is achieved by so-called checkpoints, specialized signaling pathways that monitor key cell-cycle processes and delay cell-cycle progression until these processes are completed. However, some cell cycles, famously the early embryonic cell cycles in *Xenopus laevis*, proceed without functional checkpoints, yet they still carry out S phase first and M phase second. This raises the question of how this ordering is achieved. Two recent papers, including one in this issue of *Molecular Cell* (Godfrey et al., 2017; Swaffer et al., 2016), have taken on this question, and both papers challenge the conventional wisdom.

The main events of the cell cycle are brought about by cyclin-dependent protein kinases (Cdks), which directly phosphorylate and regulate hundreds of substrate proteins, and probably indirectly regulate hundreds more. This means that the ordering of the events of the cell cycle is determined by the ordering of hundreds of phosphorylation/dephosphorylation reactions. The prevailing view has been that cell-cycle phosphorylation is temporally ordered through the existence of multiple cyclin-Cdk complexes, with some of these complexes being devoted primarily to G1 phase regulation (e.g., cyclin D-Cdk4/6 in humans and Cln3-Cdk1 in *S. cerevisiae*), some to S phase regulation (e.g., cyclin A-Cdk2 and cyclin E-Cdk2 in humans and Clb5-Cdk1 in *S. cerevisiae*), and some to M phase (e.g., cyclin B1-Cdk1 in humans and Clb2-Cdk1 in *S. cerevisiae*) (Cross et al., 2011). This puts the primary responsibility for the correct sequence of phosphorylations on the shoulders of the regulators of cyclin synthesis and degradation. There is a great deal of evolutionary and functional evidence in support of the cyclin-centric view, and there is some biochemical evidence as well. For example, Loog and Morgan showed that out of a sample of 150 budding yeast Cdk1 substrates, 14 are phosphorylated much (>10-fold) better by the S phase Clb5-Cdk1 complex than one would expect from how well they are phosphorylated by the M phase Clb2-Cdk1 complex, and these substrates include many key S phase regulators (Loog and Morgan, 2005). These findings support the idea that the temporal sequence of distinct cyclin-Cdk complexes is largely responsible for the temporal sequence of cell-cycle phosphorylations, which then ensures the proper order of cell-cycle events.

An alternative hypothesis was proposed by Paul Nurse and Bodo Stern in 1996 (Stern and Nurse, 1996). Based on the fact that, in *S. pombe*, a single B-type cyclin (Cdc13) and a single Cdk (Cdc2) were important for the regulation of both S phase and M phase, they proposed that the quantitative level of Cdc13-Cdc2 activity determined the ordering of S and M phases, with low levels of activity being sufficient to initiate S phase and higher levels being needed to initiate M phase.

This hypothesis is also the starting point for a recent paper from Matthew Swaffer and co-workers (Swaffer et al., 2016). The authors made use of a fission yeast strain containing a so-called analog-sensitive (as) allele of Cdc2, whose activity can be dialed up and down by titrating the concentration of the ATP analog 1-NmPP1. Furthermore, the Cdc2(as) was fused to a single B-type cyclin, Cdc13, with the three minor *S. pombe* cyclins Cig1, Cig2, and Puc1 and the normal Cdc2 and Cdc13 genes having all been deleted (Coudreuse and Nurse, 2010). This makes it possible to run the cell cycle with a single cyclin-Cdk complex and a controllable level of cyclin-Cdk activity. As previously shown, this strain is viable, which by itself argues against a cyclin-centric model of cell-cycle ordering (Coudreuse and Nurse, 2010). The authors now ask whether S phase regulators are still phosphorylated earlier than M phase regulators in this strain, and, if so, whether it is because they require less kinase activity for phosphorylation than the M phase substrates do.

They started by identifying 275 phosphopeptides as putative direct targets of Cdc2 by mass spectrometry. Of these, they classified 16 peptides (from 11 proteins) as being phosphorylated early in the cell cycle, 165 peptides (from 123 proteins) as being phosphorylated late, and 13 as intermediate. The early/mid proteins were phosphorylated not just

earlier but also faster than the late proteins in vivo, based on 1-NmPP1 block-and-washout experiments, and they required higher concentrations of 1-NmPP1 to block their phosphorylation than the late proteins did. These findings argue that the early/mid substrates are phosphorylated early because they can become fully phosphorylated when the Cdc13-Cdc2(as) activity is still submaximal.

This raises the question of what molecular properties might allow a cyclin-Cdk complex to phosphorylate one substrate faster than another. One factor is the identity of the phosphorylated residue. Cyclin B-Cdk1 phosphorylates serines about twice as fast as threonines in otherwise identical peptides (Suzuki et al., 2015). The nature of the immediate surrounding amino acid residues can also significantly influence the capability of Cdk1 to phosphorylate a particular site, with variations from the optimal sequence generally resulting in slower phosphorylation (Songyang et al., 1994; Suzuki et al., 2015). The substrate-enzyme interaction can be further promoted through docking motifs. For example, a hydrophobic patch on the cyclin protein can interact with an RxL motif some distance from the phosphorylation site in the substrate. And finally, the Suc1/Cks subunit of a Cdk complex can facilitate phosphorylation by binding to primed phosphoepitopes in the substrate (McGrath et al., 2013). Of these myriad possible mechanisms, Swaffer et al. presented evidence for the importance of an RxL motif in some of the early substrates. However, it seems likely that many of these various other mechanisms will prove to contribute as well.

Does this mean that the minor cyclins do not play a role in determining the order of substrate phosphorylation in *S. pombe*? No; Swaffer et al. show that the phosphorylation of the intermediate (mid) substrates is accelerated when the additional cyclins are present. However, on the whole the ordering of substrate phosphorylation is similar in the *cig1/cig2/puc1*-deleted and non-deleted strains. It is possible that other organisms rely more heavily on what is a secondary mechanism of regulation in *S. pombe*, but in fission yeast the timing of substrate phosphorylation seems to be mainly determined by how quickly substrates can be phosphorylated by Cdc13-Cdc2.

While Swaffer et al. found no evidence that phosphatases might also contribute to determining the timing of cell-cycle substrate phosphorylation, Godfrey and colleagues showed that in a different yeast, *S. cerevisiae*, a cell-cycle phosphatase does play such a role. Here they focused on the Cdc55-PP2A complex ($PP2A^{Cdc55}$), which, from work in several model systems, is thought to oppose Cdk1 phosphorylation during interphase. They first compared the cell-cycle phosphorylation of three well-characterized Cdk substrates, the early substrate Ask1 and the late substrates Sli15 and Ndd1, in strains with or without a *cdc55* deletion. The early Cdk substrate Ask1 was unaffected, but the two late substrates became phosphorylated earlier in the cell cycle, consistent with the idea that the EC_{50} values for their phosphorylation had been lowered due to the loss of $PP2A^{Cdc55}$ activity.

They then examined the global consequences of *cdc55* deletion through mass spectrometry. They found that numerous phosphopeptides were increased in abundance in the *cdc55* deletion strain, with phosphothreonine (pThr) peptides enriched relative to pSer peptides. This suggests that $PP2A^{Cdc55}$ dephosphorylates pThr faster than pSer, and, indeed, in vitro studies of PP2A have shown this to be the case (Agostinis et al., 1987). The Cdk sites that are phosphorylated late during the cell cycle were also enriched for pThr, and there was a reasonable overlap between potential $PP2A^{Cdc55}$ target sites and late substrates (27%). Mutating threonines to serines in one late substrate accelerated its phosphorylation similarly to that seen in the *cdc55* deletion. Thus, it appears that one simple determinant of when a site is phosphorylated during the cell cycle is whether it is a threonine or a serine; serine sites are preferred by Cdk1 and are phosphorylated early, and threonine sites are preferred by $PP2A^{Cdc55}$ and are phosphorylated late.

Together, these two papers make the case that, in addition to whatever contribution is made by cyclin specificity, the inherent properties of Cdk substrates help determine the order in which they are phosphorylated. Good Cdk substrates and poor $PP2A^{Cdc55}$ substrates

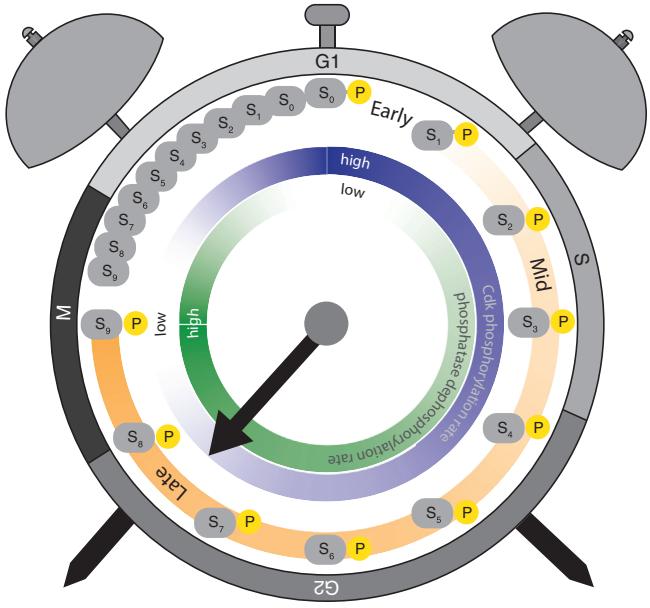

Figure 1. The Timing of Cell-Cycle Phosphorylation Is Regulated, in Part, by Intrinsic Properties of the Substrates

During the cell cycle, cyclin-dependent protein kinases (Cdks) phosphorylate a large number of different substrates (depicted here as S1 through S9). The temporal ordering of these phosphorylations helps ensure that S phase occurs prior to mitosis. Swaffer et al. (2016) found that in *S. pombe*, good substrates of Cdk1/Cdc2 (substrates with high phosphorylation rates) tend to be phosphorylated earlier in cell cycle, when Cdk1 activities are submaximal, and poor substrates tend to be phosphorylated later. Godfrey et al. (2017) found that in *S. cerevisiae*, good substrates of the phosphatase $PP2A^{Cdc55}$—proteins with threonine phosphorylation sites—tend to be phosphorylated late. On top of this regulation, the identities of the cyclins present at different times in the cell cycle contribute to the ordering of substrate phosphorylation, with the identity of the cyclin being of lesser importance in *S. pombe* and greater importance in *S. cerevisiae*.

tend to get phosphorylated first; poor Cdk substrates and good $PP2A^{Cdc55}$ substrates tend to get phosphorylated last (Figure 1). No doubt other mechanisms contribute as well to the overall ordering. For example, most Cdk substrates are multiply phosphorylated, and other things being equal, a substrate that requires many phosphorylations to have its function changed will be regulated later than one requiring fewer phosphorylations. But probably the simplest way nature could build temporal ordering into the targets of master regulators like Cdk1 and $PP2A^{Cdc55}$ would be by varying the targets' rate constants for phosphorylation and dephosphorylation, and it is nice to see that this in fact seems to be the case.

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A Finer Print Than TADs: PRC1-Mediated Domains

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Polycomb proteins are well-known epigenetic repressors with unexplained roles in chromatin folding. In this issue of *Molecular Cell*, Kundu et al. (2017) investigate the structures of PRC1-mediated domains in stem cells and probe their changes upon differentiation and in PRC knockouts.

First identified in *Drosophila* as repressors of homeotic genes (*Hox*), Polycomb repressive complexes (PRCs) are silencing machineries that are essential for proper cell differentiation and chromatin memory during development (Steffen and Ringrose, 2014). These major PRC roles spawn from their direct repression of transcription factors and signaling molecules that are critical for development.

The two main complexes, PRC1 and PRC2, have histone-modifying activities that are responsible for ubiquitylation of histone H2A at Lys119 (H2AK119ub1) and methylation of histone H3 on Lys27 (H3K27me1/2/3), respectively. PRC1 can be further divided in different sub-complexes. The canonical complex contains CBX proteins, the ubiquitin ligase RING1A/B, and the polyhomeotic (Ph)-like ortholog PHC1. Canonical PRC1 complexes are recruited to chromatin through binding of CBX proteins to the PRC2-dependent mark, H3K27me3, and by its own mark, H2AK119ub1, which is also recognized by PRC2 components. This complex interplay between the recruitment of the two major PRC complexes and their modifications highlights the complexity of PRC repression mechanisms. Moreover, it remains unclear to which extent the molecular mechanisms of PRC repression are mediated by PRC binding to chromatin leading to compaction, or through their histone marks.

Early observations that Polycomb proteins can form visible nuclear foci, called Polycomb bodies, suggested a role of Polycomb in chromatin structure (Buchenau et al., 1998). The *Drosophila* Pc (Polycomb) protein and the orthologous, mammalian CBX proteins have a chromo-domain (chromatin organization modifier) that is similar to HP1, hinting at a heterochromatin-like behavior, where self-interactions promote the assembly of larger chromatin complexes. The close liaison of PRC1 with chromatin architecture matured in the next decade. Electron microscopy studies showed in vitro compaction of nucleosome arrays by PRC1 (Francis et al., 2004). Single-cell imaging studies by fluorescence in situ hybridization showed PRC1-dependent in vivo compaction of *Hox* genes in mouse embryonic stem cells (ESCs; Eskeland et al., 2010). More