Mitotic exit: Determining the PP2A dephosphorylation program

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In mitotic exit, proteins that were highly phosphorylated are sequentially targeted by the phosphatase PP2A-B55, but what underlies substrate selection is unclear. In this issue, Cundell et al. (2016. *J. Cell Biol.* http://dx.doi.org/10.1083/jcb.201606033) identify the determinants of PP2A-B55's dephosphorylation program, thereby influencing spindle disassembly, nuclear envelope reformation, and cytokinesis.

Mitotic entry is characterized by the abrupt activation of the mitotic cyclin-dependent kinase (CDK1)–cyclin B complex. As soon as the spindle assembly checkpoint is satisfied, the anaphase-promoting complex/cyclosome, an ubiquitin E3 ligase, triggers the degradation of cyclin B, leading to a drop in CDK1 activity. In addition, phosphatase activities rise to cooperate with the declining activities of CDK1 and other kinases to reset the phosphorylation status of proteins to the general low level that is characteristic of G1 phase cells. Studies in yeast and human cells have unraveled a timely defined dephosphorylation program that coordinates and regulates events in anaphase, telophase, and cytokinesis (Bouchoux and Uhlmann, 2011; Cundell et al., 2013; Grallert et al., 2015; Qian et al., 2015). In human cells, it is known that activation of PP1 with falling CDK1 activity activates PP2A-B55 (B55 is one of the regulatory subunits of the trimeric PP2A phosphatase complex) by inactivating the Greatwall–MASTL kinase (Fig. 1 A; Heim et al., 2015; Ma et al., 2016) to reduce the phosphorylation status of the PP2A-B55 inhibitor ENSA (Gharbi-Ayachi et al., 2010; Mochida et al., 2010; Cundell et al., 2013; Heim et al., 2015; Ma et al., 2016). Although the phosphatases that coordinate mitotic exit events and how they become activated with declining CDK1 activity are established, we know little about the factors that determine the timely sequence of the dephosphorylation program of proteins, ensuring an appropriately ordered mitotic exit. In this issue, Cundell et al. decipher the determinants controlling the dephosphorylation of PP2A-B55 substrates in human cells, thereby providing important new insights into how mitotic exit events are timed.

In a remarkable body of work, Cundell et al. (2016) analyzed the phospho-proteome of synchronized mitotic HeLa cells in 5-min intervals for 45 min, identifying >23,000 phosphorylation sites for each condition. To identify PP2A-B55 substrates with high confidence, this analysis was not only done in wild-type cells but also in B55-depleted cells, to follow the logic that this second dataset would exhibit a reduction in the dephosphorylation kinetics of PP2A-B55 substrates. Conversely, the authors also depleted Greatwall kinase or the PP2A-B55 inhibitor ENSA with the idea that this would accelerate dephosphorylation of PP2A-B55 substrates. A protein phosphatase simulation model using these datasets enabled Cundell et al. (2016) to precisely determine dephosphorylation kinetics of PP2A-B55 substrates. This analysis identified nearly 3,000 phosphopeptides with PP2A-B55–dependent dephosphorylation kinetics. The PP2A-B55 substrates ranged from known substrates, such as PRC1^{pT481} and CDC20^{pS41}, to novel PP2A-regulated proteins, such as the Aurora A kinase regulator TPX2, NUMA, CENPF, the chromosomal passenger protein borealin, the chromokinesin KIF4A, and several nuclear envelope proteins (NUP153, NDC1, POM121, and NUP107).

How are all these substrates recognized by PP2A-B55? Cundell et al. (2016) identified a bipartite positively charged polybasic motif located upstream and downstream of the central CDK1 site consisting of S/TP, with a preference for threonine. Importantly, substrates that were dephosphorylated more rapidly by PP2A-B55 were more basic than substrates with slower dephosphorylation kinetics. Electrostatic interactions between negatively charged residues in PP2A-B55 and positively charged amino acids of the polybasic substrate motif may determine the kinetics of dephosphorylation. Consistent with this model, the researchers found that the initial dephosphorylation rate of PRC1^{pT481} was critically dependent on the polybasic clusters surrounding the pT481 phosphosite. Moreover, mutation of ten acidic residues in the B55 regulatory subunit to alanine greatly reduced dephosphorylation kinetics of PRC1^{pT481}. Of note, the protein ENSA is a unique PP2A-B55 substrate because it also functions as a PP2A-B55 inhibitor. Like other PP2A-B55 substrates, ENSA carries the characteristic basic patches surrounding the Greatwall phosphorylation site. However, in addition to these core features, ENSA contains a conserved tyrosine residue at position 64 next to the serine residue at position 67 that is phosphorylated by Greatwall kinase. Analysis of the dephosphorylation kinetics of ENSA mutant proteins by PP2A-B55 revealed the importance of the basic patches as well as of tyrosine 64. This dual binding to basic and aromatic residues allows for a tighter association of ENSA with B55, which explains the activity of ENSA as a PP2A-B55 inhibitor.

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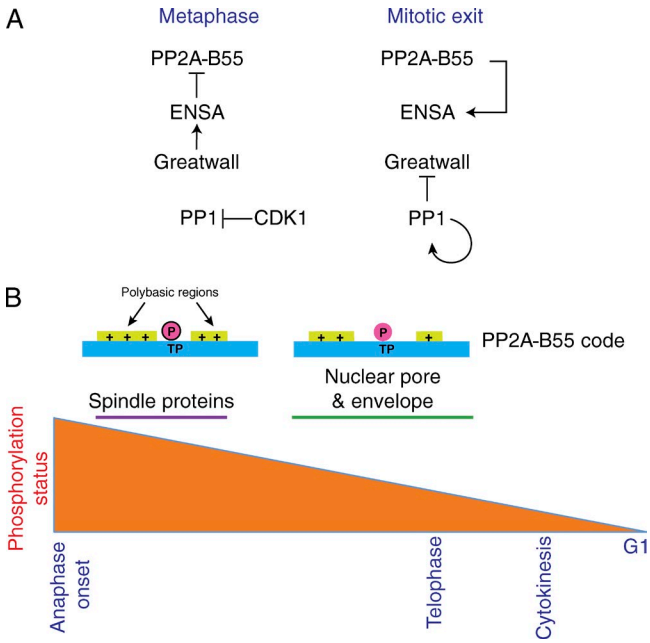

Figure 1. **Regulation of mitotic exit by a timely dephosphorylation cascade.** (A) Regulation of PP2A-B55 in metaphase and during mitotic exit. See text for details (Gharbi-Ayachi et al., 2010; Cundell et al., 2013; Heim et al., 2015; Mochida, 2015; Ma et al., 2016). (B) Model for the PP2A-B55 timer of substrate recognition according to Cundell et al. (2016). Timing of substrate dephosphorylation is determined by two polybasic regions upstream and downstream of the phosphorylated threonine residue. Early spindle proteins have a more pronounced polybasic region than later nuclear envelope proteins.

Cundell et al. (2016) observed a preference of PP2A-B55 for phosphothreonine residues (45% of the PP2A-B55 substrates are pT compared with 25% pT in the total phosphoproteome), which cannot be explained by electrostatic interactions between substrate and B55. Analysis of the dephosphorylation kinetics of PRC1 and TPX2 proteins with pT481S and pT369S substitutions, respectively, confirmed this preference for phosphothreonine. PP2A-B55 dephosphorylated the phosphoserine mutant proteins more slowly than their wild-type phosphothreonine-containing counterparts. Thus, an additional feature different to the polybasic regions must allow recognition of phosphoserine by PP2A-B55. Interestingly, phosphoserine substrates frequently contain aromatic or bulky residues just upstream of the dephosphorylation site. Amazingly, introducing an aromatic residue upstream of the engineered PRC1^{pS470} or wild-type PRC1^{pT470} resulted in equal dephosphorylation of phosphoserine and phosphothreonine, suggesting that the absence of this amino acid determines specificity for phosphothreonine.

For successful cell division, it is important that mitotic spindle proteins are preferentially dephosphorylated over nuclear envelope proteins, so that nuclear pores only form and import proteins once chromosomes are successfully segregated (Fig. 1 B). Cundell et al. (2016) asked if the dephosphorylation rate of a protein substrate determines the timing of B55-dependent processes in cells in mitotic exit. Interestingly, the timing of a mitotic exit event (e.g., nuclear envelope reformation), which is known from microscopic analyses, correlates with the rate constants of the PP2A-B55–dependent dephosphorylation of a protein substrate (k_{b55}). Late substrates (NUP153, FG-repeat nucleoporins, and RanBP2) were found to have lower k_{b55} values (0.1 to 0.04 min^{-1}) than earlier PP2A-B55 substrates (e.g., PRC1, TPX2; 0.3 to 0.1 min^{-1}). Considering that k_{b55} is determined by the nature of the polybasic motif, introduction of additional basic residues into the B55 substrate binding site should convert a late substrate into an early substrate, whereas a reduction in the basic nature of the B55 binding site should delay dephosphorylation. Indeed, Cundell et al. (2016) show that mutations in NUP153 that reduce the polybasic region surrounding the dephosphorylation site delayed and diminished recruitment of NUP153 to chromatin in mitotic exit.

Thus, the work by Cundell et al. (2016) deciphered the PP2A-B55 dephosphorylation program by identifying the basic substrate recognition motif that explains the timing of substrate dephosphorylation. It also delineated the PP2A-B55 substrate specificity toward phosphothreonine as determined by the absence of an aromatic residue upstream of the phosphothreonine and determined how ENSA functions as a PP2A-B55 inhibitor. Although we now understand how PP2A-B55 recognizes its substrate, it is important to ask whether other forms of PP2A with different regulatory B subunits follow similar principles. Interestingly, Hertz et al. (2016) just identified the conserved LxxIxE motif as a substrate recognition motif for PP2A-B56. It is noteworthy that the consensus for the B56 substrate recognition motif is quite distinct from that of PP2A-B55. In addition, although the B55 recognition sequences border the dephosphorylation site of the substrate, this is not the case for B56. Rather, the LxxIxE motif is embedded in an unstructured region of the target protein that is distinct from the dephosphorylation site. However, common to B55, variations of the substrate recognition site determine the affinity of PP2A. For B56, acidic amino acids inside or immediately adjacent to the C terminus of the site increase B56 affinity, a finding that is consistent with previous work about the dephosphorylation of tyrosine hydroxylase by PP2A-B56 (Saraf et al., 2010). Binding affinity to B56 is also increased when negative charges are introduced by phosphorylation of residues in or downstream of the LxxIxE motif. By deciphering the B55 and B56 binding codes, Cundell et al. (2016) and Hertz et al. (2016) moved the study of protein dephosphorylation during mitotic exit from one of passive observation to a realm in which direct modulation and engineering of PP2A programs becomes an option.

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