Minireview

Yeast Polo-like kinase substrates are nailed with the right tools

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Abstract

A platform has been built combining chemical genetics and bioinformatics to screen the proteome for physiological substrates of the Polo-like kinase in budding yeast. A novel role for this kinase in regulating the mitotic spindle is revealed.

Protein kinases are important regulators of almost every cellular function in eukaryotes. They catalyze the transfer of a phosphate group onto serine, threonine or tyrosine amino acid residues, resulting in a change of activity of the substrate protein. The identification of the physiological substrates of a kinase constitutes an important but intimidating challenge for many biologists. A recent paper by Jennifer Snead and colleagues in *Chemistry and Biology* [1] reports the development of a multidisciplinary approach to the identification of protein kinase substrates that has led to the discovery of a novel mitotic role for the Polo-like kinase in the budding yeast *Saccharomyces cerevisiae* (where it is called Cdc5), implicating novel molecular substrates.

Progression and regulation of the cell-division cycle relies strongly on kinases, including the cyclin-dependent kinases (Cdks), Aurora kinases and Polo-like kinases [2]. Each of these families has been conserved in eukaryotes, from yeasts to humans. Originally discovered in *Drosophila* [3], Polo-like kinases are known to promote and coordinate several events of mitosis and cytokinesis in a multitude of models [4,5]. Among their most crucial functions, Polo-like kinases promote centrosome maturation and separation (in animal cells), sister-chromatid separation in prophase, activation of the equally important Cdks, establishment of a bipolar spindle and cytokinesis. To facilitate their targeting to the appropriate subcellular locations, Polo-like kinases possess a carboxy-terminal Polo-box domain (PBD), which enables these enzymes to dock to proteins that have been pre-phosphorylated (primed) at a specific motif by Polo-like kinases themselves or by other kinases. Humans have four Polo-like kinases (Plk1-4). Plk1-3 are most closely related to *Drosophila* Polo and these have partially diverging functions, with Plk1 fulfilling most of the functions in cell division. Plk4 has a separate function in centriole duplication in humans and flies. The yeasts have single Plks: Cdc5 in budding yeast and Plo1 in fission yeast. Plk1 is often deregulated in many cancers, which has stimulated the development of several chemical inhibitors with therapeutic potential [6-8]. Nevertheless, surprisingly few phosphorylation substrates of the Polo kinases are known, and it is suspected that many more remain to be identified before we have a full picture of how these kinases impact on cell division at the molecular level.

Existing approaches for identifying kinase substrates

Searching the protein sequence complement for particular sequences can often aid the identification of kinase substrates. Kinases differ in substrate specificity, preferring different sequence motifs around the residue to be phosphorylated. For some of the better-characterized kinases, their substrate-motif preference is known, making it possible to predict whether a given protein is likely to be phosphorylated. The primary sequence of a potential sequence does not, however, allow prediction with high confidence of whether a particular segment of the protein will be accessible to phosophorylation by a kinase (for example, it may be buried in the protein core or sterically hindered by an interacting protein). Moreover, kinase and substrates need to meet in space and time in the cell for the

reaction to occur. For these reasons, genomic analysis alone has a very limited capacity to identify physiological substrates of protein kinases.

Various strategies have been used to attempt the systematic identification of kinase substrates experimentally. *In vitro* screens exploiting the change in electrophoretic mobility of proteins after phosphorylation have been carried out, starting with pools of radiolabeled proteins transcribed and translated *in vitro*, with some success [9,10]. Substrates identified in such *in vitro* reactions must, however, then be validated *in vivo*. Another approach is to purify the kinase by affinity-based methods and identify co-purifying proteins using mass spectrometry [11]. Proteins co-purifying with a kinase are often physiological phosphorylation substrates, but this strategy is most effective for kinase-substrate complexes of relatively high binding affinity and abundance. A technique that allows the identification of the physiological substrates of a kinase *in vivo* and with no bias for affinity or abundance is needed.

In a previous study from the laboratories of Kevan Shokat and David Morgan, Ubersax *et al.* [12] reported a screen using chemical biology to identify substrates of Cdk1, a master cell-cycle regulator. The authors designed a modified Cdk1 (Cdk1-as, analog sensitive) with an enlarged ATP-binding site, capable of accommodating and using a bulkier radiolabeled ATP analog in phosphorylation reactions. The ATP analog was designed to be unable to bind any cellular, unmodified kinases. Because the ATP analog was cell-impermeable, however, Ubersax *et al.* [12] could not assay for Cdk1 substrates *in vivo* and instead carried out the reactions in cell extracts (as close to *in vivo* as possible). The reactions combined recombinant cyclin B-Cdk1-as and multiple cell extracts from yeast strains overexpressing single epitope-tagged candidate substrates that were then purified and assayed for their level of radioisotope incorporation. The study tested a selected group of candidate proteins chosen for their known involvement in the cell cycle and for the presence of Cdk1 phosphorylation motifs, as well as a random group. The result was the identification of some 200 potential substrates, and constitutes a milestone in cell-cycle research. However, their strategy fell short of directly identifying *in vivo* substrates.

A new strategy that goes *in vivo*

Now, they have done it. The same groups now report a strategy [1] that allows the systematic identification of *in vivo* kinase substrates. Again, the trick is to combine elements of chemical biology and substrate prediction using bioinformatics. As in the previous work, Snead *et al.* [1] began by generating a modified but functional Cdc5 (Polo) kinase with an enlarged binding site (L158G-substituted) capable of accepting a cell-permeable inhibitor that has a low affinity for the unmodified, wild-type Cdc5 (Figure 1a) and is not predicted to be accommodated in the binding sites of any other kinases of the genome. The resulting kinase, Cdc5-as1, is still named 'as' for 'analog-sensitive' [13]. This general approach has been used previously to selectively inhibit several kinases [14]. However, the particularities of the Cdc5 ATP-binding site make it resistant to the usual pyrazolopyrimidine (PP1) analog inhibitors and forced the use of a pyrrolopyrimidine inhibitor containing a chloromethylketone (referred to as CMK).

With the new kinase-inhibitor pair in hand, Snead *et al.* [1] constructed a strain of yeast in which the endogenous *CDC5* gene was replaced with *cdc5-as1* at the natural locus, enabling the *in vivo* inhibition of Cdc5-as1, the only source of Cdc5 in the cell. This was achieved without any significant risk of simultaneously inhibiting other cellular kinases, which is usually a problem when working with inhibitors developed to target natural kinases.

Treatment of *cdc5-as1* cells with CMK led to inhibition of proliferation (Figure 1b). Cytological examination revealed that cells arrested at or after anaphase. This on its own was not surprising, as Cdc5 is known to be required for exit from mitosis and cytokinesis [15]. But on close examination, an increased percentage of cells had an elongated spindle that was completely enclosed in the mother cell, instead of normally spanning both the mother cell and the bud (although this defect was eventually corrected). Moreover, the early, short spindle was often misaligned and microtubules were often misoriented or sometimes detached from the spindle pole bodies (SPBs). Therefore, Cdc5 is required for normal positioning and function of the mitotic spindle. Inhibition of Cdc5 also led to premature sister-chromatid separation, as expected. Altogether, these experiments revealed both known and new roles for Cdc5 in the cell cycle. This novel role for Cdc5 at the spindle level was not identified in previous studies using temperature-sensitive (*ts*) alleles of *CDC5*: this may be because such alleles often retain some activity even at the restrictive temperature or because the increase in temperature required to inactivate a *ts* allele can alter cell-cycle progression.

Snead *et al.* [1] then set out to screen for substrates of Cdc5 *in silico* and *in vivo*. Using a sequence-profile-scanning algorithm [16,17], they searched all yeast predicted proteins (over 6,000 proteins) for Plk phosphorylation motifs and for PBD-binding motifs (defined using published information available for human and yeast Plks). As an output, each protein was assigned a 'Cdc5 substrate likelihood score'. From among the highest-scoring proteins, functional criteria (for example, known involvement in mitosis or cell cycle) were used to choose a list of 192 candidates to be tested (Figure 1c).

The experimental part of the screen made use of a library of strains in which genes are fused at their natural loci with a tag that can be used for tandem affinity purification (TAP),

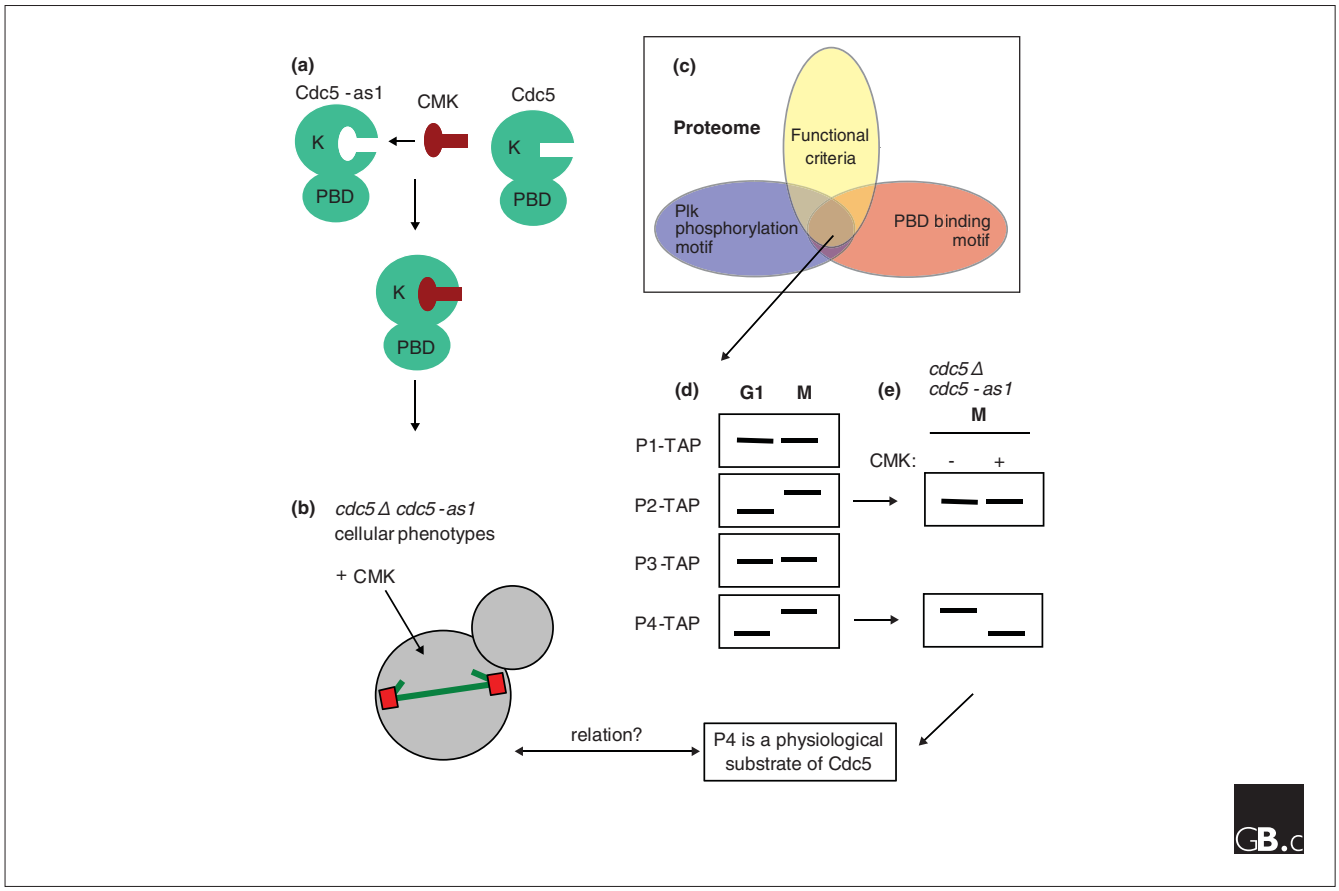

Figure 1
Strategy used by Snead *et al.* [1] to identify substrates of yeast Polo-like kinase (Cdc5). **(a)** A modified Cdc5 (Cdc5-as1) is generated to be selectively inhibited by a small-molecule inhibitor (CMK) that does not affect wild-type Cdc5. K, kinase domain; PBD, Polo-box domain. **(b)** Treatment of *cdc5Δ cdc5-as1* cells with CMK reveals the cellular phenotypes associated with Cdc5 inhibition. **(c)** A sequence-scanning algorithm identifies proteins containing potential Plk phosphorylation motifs (blue), potential PBD-binding motifs (red) and satisfying functional criteria in database annotations (yellow). **(d)** Individual strains expressing candidate substrates (P1, P2, P3, and so on) from their normal loci in fusion with the TAP tag are screened for electrophoretic mobility shifts of the fusion protein between a G1 arrest (Cdc5 inactive) and an M-phase arrest (Cdc5 and other kinases active). **(e)** For fusion proteins showing a shift between G1 and M, strains combining the TAP-tagged gene with the *cdc5Δ cdc5-as1* allele are generated. Those are then screened for a CMK-dependent (Cdc5-dependent) mobility shift in M-phase-arrested cells. Hits from that final step can be considered to be physiological Cdc5 substrates. The cellular phenotypes observed following Cdc5 inhibition in (b) may be hypothesized to result from a failure of phosphorylation of one or more of the Cdc5 substrates identified.

providing easy detection in western blotting. Tagged proteins were then assayed for their phosphorylation state as detected by a shift in electrophoretic mobility. A first screen retained only those proteins from the candidate list that changed in mobility (shifted) between G1 (Cdc5 inactive) and mitosis (Cdc5 and other kinases active) (Figure 1d). Hits from that stage (74) were finally assayed for a shift that was dependent on treatment with CMK in mitotically arrested *cdc5-as1* cells (Figure 1e). Only five proteins passed this final selection step; two of them are known Cdc5 substrates, while three are novel. One of the novel substrates is Spc72, a SPB component with known roles in microtubule nucleation and spindle positioning [18-21]. Since it was known to interact with Cdc5 [22,23], Spc72 was an obvious candidate for a molecular target of Cdc5 in the regulation of the mitotic spindle. Moreover, Cdc5 is known to localize to the SPB. Snead *et al.* [1] showed that Cdc5 requires its PBD to interact with Spc72 and can phosphorylate Spc72 *in vitro*. Finally, they also assayed the binding of recombinant Cdc5 to a set of 90 TAP-tagged proteins annotated (or reported) as SPB-localized in databases. Several of these proteins associated with Cdc5, and Spc72 was among the most efficient interactors, further suggesting a direct interaction.

A significant new advance

Further experiments will be required to clarify how Cdc5 regulates Spc72 and how this affects SPB and spindle function. It is likely that disruption of the Cdc5-Spc72 pathway is at least partly responsible for the cellular phenotypes obtained

when Cdc5 is inhibited *in vivo*. In animal cells, Plk1 is known to promote centrosome maturation and microtubule nucleation activity by various mechanisms [4,24]. Despite marked differences between budding yeasts and animal cells in how the spindle is assembled and regulated, it is becoming clear in the light of the paper by Snead *et al*. that the yeast Polo-like kinase Cdc5 is a major regulator of the mitotic spindle.

Because many phosphoproteins do not produce a marked shift in electrophoretic mobility when phosphorylated at a given site, several Cdc5 substrates are likely to have been missed in this screen. This may be even more problematic for Cdc5 substrates that are also phosphorylated by other mitotic kinases. Nonetheless, the experimental approach presented by Snead *et al*. provides a powerful means of systematically identifying physiological substrates of a kinase in its natural environment - without overexpressing the kinase or the candidate substrates to be screened. This platform should be readily amenable to similar screens for substrates of any kinase of choice in *S. cerevisiae* and therefore constitutes a powerful handle on many signaling pathways in cell biology. The technique is not yet easily transferable to other organisms where exact gene replacement and genomic tagging at the natural loci are, at present, impracticable or challenging, including *Drosophila*, *Caenorhabditis elegans* or mammalian cells. Nonetheless, a recent paper reported the use of a Plk1-as allele (and a specific inhibitor) to analyze Plk1 functions in cytokinesis in human cells where endogenous Plk1 has been knocked out by homologous recombination [25]. The viral expression of Plk1-as in these experiments may not, however, follow the normal levels or cell-cycle profile of endogenous Plk1 expression. In mammalian cells a screen for substrates such as the one presented by Snead *et al*. would almost certainly require the tagged candidates to be artificially expressed. Again, the little budding yeast is first to stick its neck out and one has to hope that technological advances will soon allow other organisms to follow its path.

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