Advances in Enzyme Regulation 50 (2010) 375–399

Contents lists available at ScienceDirect

Advances in Enzyme Regulation

journal homepage: www.elsevier.com/locate/advenzreg

Identification of substrates for cyclin dependent kinases

Alessia Errico [a,1], Krupa Deshmukh [b,1], Yoshimi Tanaka [c], Andrei Pozniakovsky [d], Tim Hunt [a,*]

[a] Cancer Research UK, Clare Hall Laboratories, South Mimms, Herts, EN6 3LD, United Kingdom
[b] E.I Dupont India Pvt. Ltd., Dupont Knowledge Center, ICICI Knowledge Park, Hyderabad 500078, India
[c] National Institute of Genetics, 411-8540 Yata 1111, Mishima, Shizuoka 411-8540, Japan
[d] Max Planck Institute of Molecular Cell Biology and Genetics, Pfotenhauerstr. 108, 01307 Dresden, Germany

Introduction

Protein phosphorylation is a key regulatory mechanism for cell cycle control in eukaryotes. From yeast to humans, cell cycle progression and cell division require the activation of a group of serine–threonine protein kinases called cyclin dependent kinases (CDKs) (Morgan, 1997), which initiate and coordinate these processes by orderly phosphorylation of their targets. CDKs require association with a cyclin subunit to activate them, which provides substrate specificity (Russo, 1997; Solomon et al., 1992), and phosphorylation by an activating protein kinase (CAK) at a conserved threonine residue (Krek and Nigg, 1992; Lorca et al., 1992; Solomon et al., 1992). The concentration of cyclins oscillate during the cell cycle and their abundance is regulated at several levels: transcriptional, translational and at the level of protein stability. In particular, at the end of mitosis, ubiquitin-mediated proteolysis results in the disappearance of mitotic cyclins, which results in inactivation of CDKs (Zachariae and Nasmyth, 1999).

The key cell cycle transitions involving the activity of CDKs occur at the initiation of S-phase and entry into mitosis. Loss of CDK activity at the end of mitosis permits the return to interphase, and is presumably accompanied by dephosphorylation of the majority of target proteins that were modified when the cells entered mitosis. There is rather specific control of the different phases of the cell cycle, so that cells do not enter mitosis with unreplicated or damaged DNA, and do not exit mitosis until all the chromosomes are correctly aligned on the metaphase plate. There is a theoretical conundrum, however, in explaining how it is that activation of S-phase CDKs, such as cyclin E/CDK2 and cyclin A/CDK2 in vertebrate cells promotes entry into S-phase and not into mitosis, or why activation of the M-phase CDKs does not promote a further round of DNA replication. One hypothesis to explain why cells enter S-phase, rather than mitosis, when the S-phase CDK (CDK2-cyclin E in higher eukaryotes) is activated would be a restricted substrate specificity for this particular CDK, yet there is very little information on this point. Another possibility is that CDK2-cyclin E resides in the nucleus, and entry

* Corresponding author.
E-mail address: tim.hunt@cancer.org.uk (T. Hunt).
[1] These authors made equal contributions to the work described here.

0065-2571/$ – see front matter
doi:10.1016/j.advenzreg.2009.12.001

into mitosis requires CDK activity in both nucleus and cytoplasm. Quite possibly, both mechanisms are at work, because we found that, although (normally cytoplasmic) cyclin B-CDK1 cannot trigger entry into S-phase in *Xenopus* egg extracts, it can do so if it is modified so as to be able to enter the nucleus (Moore et al., 2003). Thus, a combination of substrate specificity and substrate availability combine to ensure that the right events occur in the right order. The well known 'block to re-replication' (whereby it is normally impossible to initiate a new round of DNA replication after completion of S-phase) depends on a different kind of mechanism: initiation of replication requires assembly of the origin recognition complex (ORC), which can only occur when CDK activity is low, whereas firing of origins of replication requires high CDK activity (among many other things) (Blow and Dutta, 2005; Remus and Diffley, 2009). Therefore, cells must pass through mitosis into interphase before new origins can be licensed, and re-replication cannot occur upon entry into mitosis.

Two classes of cyclins operate during the G_1 phase in mammalian cells: D type cyclins (D1, D2, D3 in mammals) and E type cyclins (E1 and E2) (Sherr and Roberts, 1999). The D type cyclins bind to CDK4 and CDK6 and determine the cell fate towards cell division rather than cell differentiation. Their primary function is to phosphorylate the retinoblastoma (Rb) family of transcription repressors during G1 (Adams, 2001). Cyclin E associates with CDK2 and this complex regulates the initiation of DNA replication and the progression through the S-phase (Ohtsubo et al., 1995), a process in which cyclin A/CDK2 complex is also involved (Pagano et al., 1992). In this context, cyclin E/CDK2 completes the phosphorylation of pRb, which is an inhibitor of cell cycle progression that represses the transcription of S-phase genes by binding the E2F transcription factor. Phosphorylation of pRb by cyclin E/CDK2 reduces the affinity of pRb for E2F; therefore E2F is able to promote the transcription of genes important for S-phase progression.

The onset of mitosis requires increased activity of CDK1 associated with cyclin A and cyclin B (although Kalaszczynska et al. (2009) recently found that fibroblasts can proliferate without cyclin A). The CDK1/cyclin B complex has maximal activity during metaphase and is the major regulator of the G2/M transition, *i.e.* entry into mitosis and maintenance of the mitotic state. The CDK1/cyclin B complex also acts by priming certain substrates (including INCENP and BubR1) for phosphorylation by another mitotic kinase, Polo kinase (Plk1) (Goto et al., 2006; Wong and Fang, 2007). Entry into mitosis also requires cyclin A, which besides forming a complex with CDK2 in S-phase, forms a complex with CDK1 during the transition from G2 to mitosis (Koff et al., 1991; Lew et al., 1991; Tsai et al., 1991). It is still not entirely clear why two separate forms of CDK1 are needed for entry into mitosis, although a simple explanation may be found in the sub-cellular locations of cyclins A and B, in nucleus and cytoplasm respectively (Pines and Hunter, 1994).

Although the importance of activities of the different CDKs in regulating and driving the different phases of the cell cycle is well established, and a large number of substrates have been identified in budding yeast, we do not really know which substrates require phosphorylation to catalyse cell cycle transitions in vertebrates. The identification of substrates of specific CDK/cyclin complexes is extremely important to understand their involvement in processes central to cell cycle regulation. Several studies based on large scale screening methods and complementary computational approaches have provided lists of potential CDK targets in *S. cerevisiae* and to a limited extent in higher organisms (Chang et al., 2007; Chi et al., 2008; Diederichs et al., 2004; Georgi et al., 2002; Moses et al., 2007; Stukenberg et al., 1997; Ubersax et al., 2003). However, the targets of CDK that have been reported in the literature are limited to less than one hundred (see Table 1).

In this review we describe screening approaches we have undertaken to identify substrates of various purified cyclin/CDK complexes. They include screening of *Xenopus laevis* cDNA libraries and computational methods based on substrate feature recognition. In order to assess the efficiency of our approach to identify physiologically relevant targets of CDK we have compared substrates identified from our studies with available experimental evidence for phosphorylation of these proteins derived from high throughput mass-spectrometric studies (Chi et al., 2008; Molina et al., 2007; Nousiainen et al., 2006). Finally, we enumerate the list of CDK targets identified from our screen that overlap with phosphoproteins containing pSP/pTP peptides as identified from recently reported experimental studies (Chi et al., 2008; Molina et al., 2007; Nousiainen et al., 2006). This list of substrates, derived from multiple independent studies, make up a list of strong candidates. We describe the range of cellular processes linked to cell cycle control based on their potential for phosphorylation by CDKs. Further detailed studies on the

Table 1
List of CDK substrates reported in the literature.

	Substrate	Kinase	Reference
1	Actopaxin	CDK1/Cyclin B	(Curtis et al., 2002)
2	Adenomatous Polyposis Coli (APC)	CDK1/Cyclin B	(Trzepacz et al., 1997)
3	AML/RUNX1 transcription factor	CDK1/Cyclin B CDK2/Cyclin A	(Biggs et al., 2006)
4	Amphiphysin	CDK1 CDK5	(Floyd et al., 2001)
5	Androgen Receptor	CDK1	(Chen et al., 2006)
6	B-Myb	CDK2-Cyclin A	(Saville and Watson, 1998)
7	BAD (BH3 only protein)	CDK1	(Konishi et al., 2002)
8	BARD1	CDK2/Cyclin A CDK2/Cyclin E CDK2/Cyclin B	(Hayami et al., 2005)
9	Beta-tubulin	CDK1	(Fourest-Lieuvin et al., 2006)
10	BRCA2	CDK	(Esashi et al., 2005)
11	C/EBP beta	CDK1 CDK2	(Shuman et al., 2004)
12	Caldesmon	CDK1	(Mak et al., 1991)
13	Cdc14/Clp1	Cdc2	(Wolfe et al., 2006)
14	Cdc20	CDK1 Polo kinase	(Yudkovsky et al., 2000)
15	Cdc25	CDK1/Cyclin B	(Margolis et al., 2003)
16	Cdc6	CDK2	(Petersen et al., 1999)
17	CDK7	CDK1 CDK2	(Garrett et al., 2001)
18	CK2 (Casein kinase 2)	CDK1	(Bosc et al., 1995)
19	Connexin-43	CDK1/Cyclin B	(Kanemitsu et al., 1998)
20	Disabled 2 (Dab2) aka p96 or DOC-2	CDK1	(He et al., 2003)
21	DNA polymerase Lambda	CDK2/Cyclin A	(Frouin et al., 2005)
22	Drc 1	Cdc2	(Noguchi et al., 2002)
23	Dynein light intermediate chain	CDK1/Cyclin B	(Addinall et al., 2001)
24	Dystrophin	CDK1	(Milner et al., 1993)
25	ECT2	CDK1 Polo kinase	(Hara et al., 2006; Yuce et al., 2005)
26	Elongation factor 1, CK2	CDK1 CK2	(Belle et al., 1990)
27	FANCG	CDK1	(Mi et al., 2004)
28	FOXM1c	CDK2-Cyclin E/Cyclin A	(Luscher-Firzlaff et al., 2006)
29	FOXO1	CDK2	(Huang et al., 2006)
30	GM130 (Golgi protein)	CDK1	(Lowe et al., 1998)
31	GRASP-65	CDK1 Polo-kinase	(Lin et al., 2000)
32	HCN1 (APC/C component)	CDK1	(Yoon et al., 2006)
33	hHR6A (homolog of RAD6 ubiquitin ligase)	CDK1 CDK2	(Sarcevic et al., 2002)
34	HIRA (regulator of histone gene expression)	CDK2/Cyclin A	(Hall et al., 2001)
35	Histone H1	CDK2 CDK1	(Meijer et al., 1989)
36	HMG-1 (High mobility group-1)	cdc2	(Nissen et al., 1991)
37	hRad9	CDK1	(St Onge et al., 2003)
38	huCDC7	CDK2-Cyclin E CDK2-Cyclin A CDK1-Cyclin B	(Masai et al., 2000)
39	Human papilloma virus E1 protein	CDK	(Deng et al., 2004)
40	Kar9	Cdc28	(Liakopoulos et al., 2003)
41	KID (chromokinesin)	CDK1	(Ohsugi et al., 2003)
42	KIF11 (Eg5)	CDK1	(Blangy et al., 1995)
43	Kinesin family member23/Zen-4	CDK1/Cyclin B	(Mishima et al., 2004)
44	Lamins A and B	CDK1	(Peter et al., 1990)
45	MAP4	CDK1	(Aizawa et al., 1991)
46	MARCKS	CDK2/Cyclin E	(Manenti et al., 1999)

(*continued on next page*)

Table 1 (*continued*)

	Substrate	Kinase	Reference
47	MCM2	CDC7 CDK2 CDK1 CK2	(Montagnoli et al., 2006)
48	MdmX	CDK2/CDK1	(Elias et al., 2005)
49	MEF (ETS-related transcription factor	CDK2-Cyclin A	(Miyazaki et al., 2001)
50	Mps1 p (control of spindle pole body duplication)	Cdc28	(Jaspersen et al., 2004)
51	MyoD	CDK1 CDK2	(Kitzmann et al., 1999)
52	NBP-60/LaminB-receptor	CDK1	(Courvalin et al., 1992)
53	NDEL	CDK1 Aurora A kinase	(Mori et al., 2007)
54	Net1	CDK Polo kinase	(Queralt et al., 2006)
55	NF-Y	CDK2	(Chae et al., 2004; Yun et al., 2003)
56	Nir2 cytokinesis/Golgi control	CDK1	(Litvak et al., 2004)
57	NPAT (Nuclear Protein Ataxia Telangiectasia locus)	CDK2 – Cyclin E	(Ma et al., 2000)
58	NUCKS (Nuclear phosphoprotein)	CDK1	(Ostvold et al., 2001)
59	Nucleophosmin (NPM)/B23/Numatrin	CDK2/Cyclin E	(Okuda et al., 2000)
60	P107	CDK2/Cyclin A	(Peeper et al., 1993)
61	P21-activated kinase	CDK1	(Thiel et al., 2002)
62	P27kip	CDK2 – Cyclin E	(Sheaff et al., 1997)
63	Polo kinase (Plk-1/cdc5)	Cdc28	(Mortensen et al., 2005)
64	Protein Phosphatase 1 Inhibitor 2 (I-2)	CDK1/Cyclin B	(Li et al., 2006)
65	Rab4	CDK1	(van der Sluijs et al., 1992)
66	RCC1	CDK1/Cyclin B	(Hutchins et al., 2004)
67	Retinoblastoma protein pRb	CDK4/Cyclin D CDK2/Cyclin E	(Lees et al., 1991)
68	RI alpha subunit of PKA	CDK2/Cyclin E	(Gupte et al., 2006)
69	Ribonucleotide Reductase R2	CDK1 CDK2	(Chan et al., 1999)
70	Separase	CDK1/Cyclin B	(Gorr et al., 2005)
71	Ski oncoprotein	CDK1	(Marcelain and Hayman, 2005)
72	Spc42 (spindle pole body duplication)	CDC28	(Jaspersen et al., 2004)
73	Srs2 DNA helicase	CDK1	(Chiolo et al., 2005)
74	Stathmin	CDK1 CDK2	(Beretta et al., 1993)
75	Stem-loop binding protein (control of histone mRNA transcription)	CDK2/Cyclin A	(Koseoglu et al., 2008)
76	Survivin	CDK1-Cyclin B	(O'Connor et al., 2000)
77	Swi5	Cdc28	(Moll et al., 1991)
78	TOPK (MAPKK involved in spermatogenesis)	CDK1/Cyclin B	(Fujibuchi et al., 2005)
79	Upstream binding factor	CDK2/Cyclin A CDK2/Cyclin E	(Voit et al., 1999)
80	WARTS tumor suppressor	CDK1/Cyclin B	(Morisaki et al., 2002)
81	Wee1	CDK1 – Cyclin B1 CDK2 Polo kinase CK2	(Watanabe et al., 2005)

consequences of phosphorylation of these substrates for cell cycle control is necessary, however, to get an insight into the associated cellular changes that mediate regulation of cell cycle.

The experimental approaches

Cyclins A and B associated with CDK1 are major mitotic regulators, but it is not known how many proteins need to be phosphorylated to enter and proceed through mitosis (nor is it known what fraction of such proteins need to be phosphorylated to bring about the altered properties associated with the transition from interphase to mitosis). A screen using a mitotic *Xenopus* extract as the source of mitotic protein kinases identified 20 mitotic phosphoproteins, most of which were shown to be

direct substrates of cyclin B/CDK1 (Georgi et al., 2002; Stukenberg et al., 1997). However this screen was far from exhaustive, and did not distinguish between different forms of CDKs, requiring a more comprehensive and comparative screen for targets of CDK1 and CDK2. We adopted the approach introduced by Stukenberg in 1997 (Stukenberg et al., 1997) to identify substrates among the translation products of *Xenopus* oocyte cDNA library clones, using recombinant cyclins and CDKs. The Stukenberg approach is based on the property of many proteins to display a mobility shift on polyacrylamide gels upon phosphorylation. We identified 35 substrates for cyclin B/CDK1, and also investigated the differential specificity of cyclin B/CDK1 *versus* cyclin A/CDK1 and cyclin A/CDK2. Surprisingly, about half the substrates were equally well phosphorylated regardless of the cyclin or the CDK. A few showed 'preference' for cyclin A or cyclin B. Strikingly, many of these bona fide substrates for cyclin B/CDK1 lacked the canonical consensus, S/T-P-X-K/R, although all had several instances of S/T-P. Clearly, this makes identifying CDK substrates by inspection of their amino acid sequences a difficult business. We discuss this problem at some length below.

Cyclin E/CDK2 activity is necessary for entry into S-phase. Relatively few substrates are known, of which the majority are involved in transcriptional regulation, for example pRb (Harbour et al., 1999; Lundberg and Weinberg, 1998), NPAT (Ma et al., 2000; Zhao et al., 1998), and CBP (Ait-Si-Ali et al., 1998). It is significant that cyclin E/CDK2 kinase activity is necessary for entering S-phase in *X. laevis* cell free systems derived from activated eggs, where no transcription takes place. Clearly, therefore, other substrates must be phosphorylated to enable entry into and progression through S-phase. Beyond its role in S-phase, moreover, cyclin E/CDK2 seems to be important for centrosome duplication (Hinchcliffe et al., 1999), and it has been suggested that nucleophosmin is a critical substrate in this process (Okuda et al., 2000). Using mobility shift assays as described above, we searched for substrates of cyclin E/CDK2 in a curated and annotated *Xenopus* cDNA library comprising 7296 clones. We identified 42 distinct substrates, and tested the differential specificity for cyclin E/CDK2 *versus* cyclin A/CDK2 in phosphorylating these targets. Again, the majority proved equally good substrates for either form of the holoenzyme.

The cDNA library screening produced a list of substrates that are phosphorylated by CDKs containing cyclins A, B and E (Table 2). We believe this represents a set of high affinity targets for these cyclin/CDK complexes. The substrates are generated through *in vitro* transcription and translation in reticulocyte lysate and are therefore present at very low concentrations–probably lower or comparable to their physiological levels, yet they undergo significant mobility shifts upon addition of a CDK. Moreover, the substrates identified from the cDNA library screening include a few previously well-characterized CDK targets, thus validating the approach. The majority (but, significantly, not all) of these substrates contain complete consensus motifs (S/T-P-X-K/R) while all of them have at least minimal consensus motifs, S/T-P.

In parallel, we studied the necessity and sufficiency of the consensus motifs for phosphorylation by CDKs. Using computational methods, we identified potential substrates among bacterial proteins and expressed them as recombinant proteins in *E. coli*. The purified proteins were then tested for their ability to serve as substrates for cyclin A/CDK2. Strikingly, we were unable to identify any protein from *E. coli* that would serve as a substrate for this CDK. Features derived from this study were incorporated into an effective computational method for identification of substrates based on Hidden Markov Models [HMM]. The effectiveness of HMM based methods in identifying CDK substrates was tested on the substrates previously identified by mobility shift assays and targets of CDK1 in *S. cerevisiae* (Ubersax et al., 2003). We found that HMM based methods, employing features in addition to the basic consensus motifs, are more effective than consensus motif based search methods. Using this HMM based search, we analyzed human phosphoproteins to identify potential CDK targets. 168 phosphoproteins containing pSP or pTP sequences possess sequence features corresponding to HMM profiles of known CDK substrates. Therefore HMM based CDK profiles proved to be effective in identification of cellular substrates for CDK. This further aids in the integration of phosphoproteomic data to the corresponding kinase as shown for CDKs in the current study.

Experimental details

To identify cyclin/CDK substrates we used four different CDK complexes to screen two different cDNA libraries. We used the small pool expression screening method developed by Stukenberg et al. (1997) for

Table 2
List of the proteins that were experimentally identified as substrates of Cyclin B/CDK1, Cyclin A/CDK2 or Cyclin E/CDK2. Protein name, function/description and ID are indicated in the different columns. Empty cells indicate that the protein has not been tested for that particular kinase. Previously published substrates of CDKs are highlighted in **bold**. Substrates homologous to human proteins that are reported as CDK2 substrates by high throughput screening (Chi et al., 2008) are *italicized*. Substrates also identified as *in vivo* phosphoproteins containing pSP/pTP are underlined (Molina et al., 2007).

Protein	Function/Description	ID	Cyclin B/CDK1	Cyclin A/CDK2	Cyclin E/CDK2
ETS Variant gene-1	Novel Androgen receptor activated transcription factor that mediates prostrate cancer cell invasion	XER81	+	+	
PASCIN	Synaptic vesicular membrane trafficking and regulation of dynamin mediated endocytosis	AAH41726	+	+	
Histone deacetylase 6	Inhibits acetylation of alpha-tubulin	AAH43813	+	+	
Similar to peroxisomal biogenesis factor 5	Targets proteins to peroxisomes		+	+	
Similar to Trigger of mitotic entry 1	Tome-1 associates with Skp1 and is associated for degradation of Wee1	P0C2X8	+	+	
Distal-less homeobox 1 isoform 1	Homeobox protein interacting with Smad4 and inhibiting activin pathway in hematopoietic cells	AAI27555	+	+	
Wee1 kinase	CDK1-inhibitory kinase	AAC59664	+	+	
Palmitoylated membrane protein 1	MAGUK family of cytoskeletal proteins	AAH47257	+	+	
Inner centromere protein antigens 135/155 kDa	Forms the chromosome passenger complex	AAC60120	+	+	
Uracil-DNA glycosylase isoform	*Prevents mutagenesis by removing uracil from DNA*	AAH72313	+	+	
TATA box-binding protein-associated factor 2F	Component of Transcription initiation complex	AAI06255.1	+	−	
Telomeric repeat binding factor 2	*Plays a role in telomere protection and length regulation*	AAZ52556	+	+	
Bromodomain-containing protein 4 isoform short	Brd4 remains attached to the mitotic chromosomes on acetylated histones and help maintain chromatin integrity	AAI23909	+	+	
CCAAT/enhancer binding protein gamma	Transcriptional regulator of key antioxidant and DNA repair genes	AAH93547	+	+	
ADP-ribosylation factor GTPase activating protein 3	Interacts with proteins in the Coat Protein 1 (COP1) complex for vesicle formation	AAH41750	+	+	
Glycogenin	Self glucosylates to form a primer for glycogen biosynthesis	AAM33243	+	−	
Hermes	RNA binding protein and Localised factor; regulating cleavage of blastomeres in *Xenopus laevis*	AAD16971.1	+	+	
RNA Polymerase 1	*RNA Polymerase 1*	AAL56846.1	+	+	
MAP/microtubule affinity-regulating kinase 3	Inhibits CDC25C by phosphorylation; Regulates activity of microtubules	AAO27567	+	+	
Wee1	CDK1-inhibitory kinase	AAB99952	+	+	

Table 2 (*continued*)

Protein	Function/Description	ID	Cyclin B/CDK1	Cyclin A/CDK2	Cyclin E/CDK2
Pre-B-cell leukemia transcription factor 1	Transcriptional activator that gets constitutively activated due to translocation in B-Cell leukemia	AAM18914	–	+	
Low density lipoprotein receptor adaptor protein 1	A protein required for clathrin mediated internalisation of LDL receptor	AAN78447	+	+	
Heat shock transcription factor 1	Transcription factor that specifically binds to heat shock response elements and activates transcription.	AAA99999	+	+	
Diacylglycerol kinase epsilon	Binds to pRb and can mediate cell cycle arrest	AAH80380	+	+	
NFATC2-interacting protein	Involved in the regulation of cytokine genes	sp|Q0P4K8	+	+	
LOC79447	Interacts with Histone H3 methyl transferase	AAI23183	+	+	
(Caprin 1)	Induces proliferation	AH44999	+	-	
Diphthamide biosynthesis protein 2 isoform a	Modification of histidine residue on elongation factor EF2 to dipthamide; tumor supressor	AAH77348	+	+	
v-raf murine sarcoma viral oncogene homolog B1	Involved in transduction of mitogenic signalling	AAH79794	+	+	
Programmed cell death 4 isoform 2	Supresses CDK1/ cdc2*via*induction of p21(Waf/cip1)	AAH48225	+	+	
SEC14 (*S. cerevisiae*)-like 1 isoform a	Regulate phosphoinositide homeostasis and trafficking of protein and lipids	AAH82398	+	+	
SMARCD2/BAF60:SWI/SNF-related matrix-associated actin-dependent regulator of chromatin d2	Chromatin remodelling	AAH81255	+	+	
Serine protease inhibitor, Kunitz type, 2	Inhibits membrane associated serine protease that can lead to embryonic demise and malignant transformation	AAH72344	+	+	
LOC222229 (Leucine Rich and WD repeat containing protein 1; LWRD1 homolog)	*Unknown function*	AAH74207	+	+	
Hypothetical protein (Homolog of human FAM122A)	*Unknown function*	AAH72943	+	+	
Xenopus laevis protein associated with PRK1(A20-like Zinc finger)	CDC34 interacting protein	AAH42359		+	-
Granulin isoform 1 precursor	Pro-epithelin involved in cell migration and invasion	AAH48224		+	+
Stathmin	*destabilises microtubules*	AAH54159		+	+

(*continued on next page*)

Table 2 (*continued*)

Protein	Function/Description	ID	Cyclin B/CDK1	Cyclin A/CDK2	Cyclin E/CDK2
CPEB-associated factor Maskin	transiently interacts with eIF-4E. Belongs to the TACC family (Transforming acidic coiled-coil-containing protein) that contains proteins TACC 1, 2, 3 concentrated in the centrosomes of eukaryotic and may play a conserved role in organising centrosomal microtubules.	AAF19726		+	+
Dynein (Intermediate chain)	Microtubule and cytoskeletal integrity	AAH70833		+	+
APEX nuclease	Repair of DNA damaged by ionising radiation	AAH72056		+	++
ARAF serine/threonine protein kinase	Transduction of mitogenic signalling (v-raf murine sarcoma 3611 homolog)	AAH72170		+	+
Cyclin E1	S-phase entry	AAH77766		+	+
Erp1/emi2	a Plx1 regulated inhibitor of the APC	AAN76807		+	+
Striatin	membrane binding domain and associate with PP2A	AAH77858		-	+
EPLIN (Epitheilial protein lost in neoplasm)	Cytoskeleton-associated, inhibits actin filament de-polymerisation and cross-link filaments in bundles	AAH84208		+	+
Acidic a-syntrophin	Actin and calmodulin binding protein	AAH87465		+	-
ATRIP	S-phase checkpoint (ATR partner)	AAH97710		+	+
POM21	Nuclear pore protein	AAH99036		+	+
Establishment of cohesion-1 homolog 2	Sister chromatid cohesion	AAH99061		+	+
Regulator of G-protein signalling 12 isoform 2	Cytoskeletal regulation and cellular organisation	AAH99068		+	+
Maternal embryonic leucine zipper kinase	Cell cycle regulated kinase associated with spliceosome assembly	AAI06636		+	+
Lamina-associated protein 2-beta isoform	LAP2 proteins function in nuclear assembly and DNA replication. Truncated LAP2beta proteins alter lamina assembly, envelope formation, nuclear AAH97604, and DNA replication efficiency in *Xenopus laevis* extracts	AAC05383		+	-
Histone stem-loop binding protein	Phosphorylation of SLBP in late S-phase leads to degradation	AAI14223		+	+
Tipin	Checkpoint mediator protein	AAI23143		+	+
Cyclin A1	S-phase and mitosis	AAI33225		++	+
PIF1	DNA helicase (telomere formation and elongation)	AAH97805		+	+
GRASP55_65	*Golgi reassembly stacking protein 2*	AAH97604		+	+
Semaphorin 6D	Maintenance and remodelling of neuronal connections	NP_001089133		+	+

Table 2 (*continued*)

Protein	Function/Description	ID	Cyclin B/CDK1	Cyclin A/CDK2	Cyclin E/CDK2
SmcX; Jumonji/ARID domain-containing protein 1C	Demethylases	NP_004178.2		+	+
Similar to bicaudal C homolog 1	RNA binding protein required during embryonic development	AAH84957		+	+
Phosphatidylinositol-4-phosphate 5-kinase type II ba	Controls insulin signalling	AAI29060		+	–
Hypothetical protein LOC9813	Unknown (ATPase domain)	AAH79685		+	+
No homology to known proteins	Unknown	Xl_1939		+	–
C1orf174	Unknown	AAI06028		+	+
Zinc finger protein 46 (KUP)	Unknown	AAH82227		+	–
C12orf48	Unknown (contain uvrD helicase domain)	AAI08808		+	+
Fizzy1	APC regulation	AAH76805		–	+
No homology to known proteins	Unknown				
gi|9728788|gb|BE509013.1		+	+		
cAMP responsive element binding protein 3-like 2	Unknown	AAI29062		+	–
Chromosome 2 open reading frame 30 (hypothetical protein LOC495829)	Unknown	AAI24844		+	+
Similar to Zinc finger protein 516	Unknown	AAH81149		+	+
Potassium channel tetramerisation domain-containing 3(MGC86234 protein)	Unknown	AAH79768		+	–
Minor histocompatibility antigen HA-1	Unknown	AAH77287		+	+
Coiled-coil domain protein 52	Unknown	AAH76847		–	+
AE binding protein 2	Unknown	AAH71104		+	+

screening substrates of mitotic protein kinases in frog egg extracts. Our first screen used a *Xenopus* oocyte cDNA library of 10,000 non-oriented clones to identify substrates for purified cyclin B/CDK1. Pooled clones from the oocyte library were transcribed and translated *in vitro* to generate a mixture of radio-labelled proteins, which where then incubated either with purified kinase or buffer. After the kinase reaction, samples were analyzed by SDS/PAGE and autoradiography to detect proteins that were retarded after incubation with the CDK. Pools containing clones that show gel mobility shifts were subdivided and examined until single clones displaying a shift were identified. From approximately 10000 non-oriented cDNAs, we identified 39 independent cDNAs as targets of cyclin B1/CDK1 (see Table 1). Although cyclin B1/CDK1 is regarded as the major regulator of G2/M transition, there is strong evidence that cyclin A is also necessary for cells to enter mitosis. Cyclin A binds both CDK1 and CDK2 at different stages of the cell cycle. We therefore examined if cyclin A/CDK1 or cyclin A/CDK2 were able to phosphorylate the substrates phosphorylated by cyclin B/CKD2. About half the clones showed the same level of mobility shift regardless of the kinase, 15% of the clones were specifically phosphorylated by cyclin B1/CDK1, 8% shifted better after addition of cyclin A/CDK2 but none were observed to be "preferred" as substrate of cyclin A/CDK1. All the clones were tested by translating them in *Xenopus* egg extracts, and 72% displayed a mobility shift after incubation in the crude M-phase cytoplasm.

Substrates identified include previously characterised targets such as Wee l (Watanabe et al., 2005) and INCENP (Goto et al., 2006; Stukenberg et al., 1997). Human homologs of nine novel *Xenopus*

substrates have been demonstrated as phosphoproteins containing pSP/pTP peptides by mass-spectrometric studies (Chi et al., 2008; Marklund et al., 1994) (Table 2). A number of proteins with well-established cell cycle functions such as Programmed cell death 4, isoform 2 (Pcd4), bromodomain-containing protein 4 short isoform, Trigger for mitotic entry (TOME-1), and Uracil-DNA glycosylase isoform are included in the list of *Xenopus* substrates.

The screen to identify substrates for cyclin E/CDK2 used a *Xenopus* cDNA library comprising 7296 individual clones, arrayed in nineteen 384 well plates (3268 independent sequences). Annotations for these sequences are available in the database at https://intradb.mpi-cbg.de/xenopus/cgi-bin/login.cgi?dispatch=contig_search.cgi. The pooled DNAs were transcribed and translated *in vitro* to generate pool of ^{35}S labelled proteins, which were then incubated with ATP in presence or absence of the purified kinase. Samples were analyzed by SDS/PAGE to detect proteins that were retarded upon phosphorylation (Fig. 1). The clones were pooled by rows and columns, so that once a shifting band was identified in a row (row L, Fig. 1A), the subsequent identification of a band of the same molecular weight with the same shifting property in one of the columns (column 21, Fig. 1A) of the plates directly identified the potential target on the plate, in this case a protein kinase called Eg3 (see Le Guellec et al. (1991)). The positive clones were then grown independently, and the single ^{35}S labelled proteins assayed for their ability to shift after phosphorylation by cyclin E/CDK2 (Fig. 1B). The identity of the genuine positives were then checked by DNA sequencing.

Using this approach, we identified 42 substrates for cyclin E/CDK2. To assess the difference in target specificity conferred by cyclin A and cyclin E on CDK2, we checked if cyclin A/CDK2 was able to phosphorylate the identified targets (Fig. 1B). In general, we found that cyclin E and cyclin A exhibited very similar specificity towards these substrates and only in 10% of the cases did we identify a substrate that was phosphorylated specifically by one of the two kinases. For example, phosphatidylinositol-4-phosphate 5-kinase was better phosphorylated by cyclin A/CDK2, whereas the APEX nuclease was a better substrate for cyclin E/CDK2. This overlapping substrate specificity probably explains how fibroblasts can proliferate without cyclin A, but show elevated levels of cyclin E (Kalaszczynska et al., 2009). All the substrates underwent a mobility shift when added to a *Xenopus* egg extract, and this mobility shift was blocked by the addition of either roscovitine or p21 to inhibit CDK2 activity (data not shown), consistent with the idea that all these proteins are authentic substrates for CDKs. Sequence analysis of the substrates showed that all of them contained multiple S/T-P motifs, although only 35 of

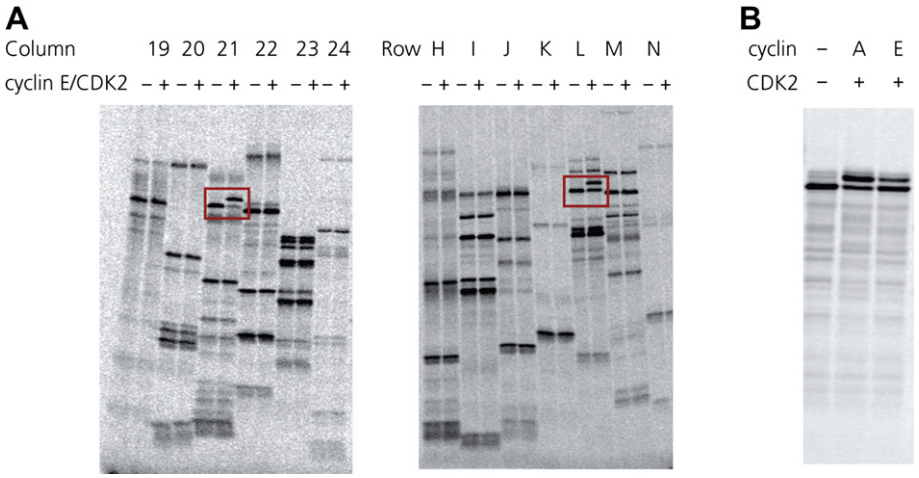

Fig. 1. Identification of Cyclin E/CDK2 substrates using the 'shift' assay. Plates containing single clones cultures are grown at 37° for 24 h. Bacterial cultures are pooled by row (A to P) and column (1–24). The pooled DNAs are prepared and translated *in vitro* and proteins are labeled with ^{35}S. Labeled proteins are used as substrates in a kinase reaction and are supplemented with ATP in presence (+) or absence (−) of the recombinant Cyclin E/CDK2 kinase. After 30 min, reactions are stopped by adding 2x sample buffer and samples are analyzed by SDS/PAGE and autoradiography. Shifting clones are isolated by their position on the plate (L21, red boxes), grown individually and tested again in a kinase assay by adding or not Cyclin E/CDK2 or Cyclin A/CDK2.

the 43 contained at least one complete consensus sequence (S/T-P-X-K/R). In the case of the substrates for cyclin B/CDK1, all contained multiple SP or TP motifs but about 44% of them did not possess any complete consensus motif.

The targets identified include proteins involved in a variety of biological processes. Known CDK targets such as stathmin, dynein and GRASP55 were among the set of clones identified in this way, validating the screening procedure. The majority of the substrates identified are involved in nuclear processes (Table 2). Human homologues of four novel *Xenopus* substrates identified in the screen are also phosphoproteins with pSP/pTP peptides identified from mass-spectrometric studies and are very likely to be conserved targets of CDK. These include EPLIN, thymopoietin, ATRIP, and establishment of cohesion-1 (Eco1) homolog. We further investigated Tipin, another novel substrate, which plays a role in recovery of stalled forks during DNA replication (Errico et al., 2007) although so far, we have been unable to demonstrate any role for the phosphorylation.

Assuming that the library is representative of the entire *Xenopus* repertoire of expressed proteins, the 'shifting' clones represent about 1.2 percent of the total. This is probably an underestimate of the real number of CDK substrates, because it is unlikely that every protein shifts upon phosphorylation. All the same, it suggests that hundreds of proteins are phosphorylated when a cell enters mitosis. And, presumably, dephosphorylated when the cell returns to interphase.

Computational approaches to identify CDK substrates

The consensus sequence motifs for CDK phosphorylation was first well-established by screening oriented peptide libraries (Songyang et al., 1994). They comprise S/T-P and S/T-P-X-R/K, referred to as the minimal and complete motifs respectively. These consensus motifs are shared by the majority of CDK substrates reported to date and in the substrates revealed the current study. However, a few exceptions that lack the adjacent proline residue have been reported for myosin light chain (Satterwhite et al., 1992) and desmin (Kusubata et al., 1993). The requirement of nearby 'Cy' or 'RXL' motifs that bind to the hydrophobic batch on the cyclin A subunit of CDKs is well established (Chen et al., 1996), but only for a few substrates (Brown et al., 1999; Takeda et al., 2001). The consensus motifs are short and extremely common, posing a limitation for their use in methods based on computational identification of novel CDK substrates.

In an attempt of identify novel substrates of CDK we have assessed the sufficiency of the phosphorylation consensus motif, shared by majority of CDK, substrates, by systematically analyzing sequences of various substrates and non-substrates. We focused our attention on features flanking the consensus sequence motifs for phosphorylation by CDKs.

We examined the relative abundance of consensus motifs in proteins, by analyzing the occurrences of minimal and complete consensus motifs in proteins from two bacterial species (*E. coli* and *B. subtilis*) and three eukaryotic species (*S. cerevisiae*, *S. pombe* and *H. sapiens*). The first surprise, considering that bacteria do not contain CDKs, is that the number of proteins containing complete or minimal motifs (Fig. 2) is very similar in prokaryotes and eukaryotes. However, we noted that a larger number of eukaryotic proteins contain *multiple* complete consensus motifs.

We also noticed that when CDKs were expressed in bacteria, the background of phosphorylation of soluble bacterial proteins was essentially undetectable. There are many possible explanations for this, including the possibility that such potential substrates as exist are present at very low concentrations in bacteria. We therefore selected six *E. coli* proteins that contained multiple minimal and/or complete CDK consensus motifs. The choice of the proteins was also based on the availability of the 3-dimensional structures for the *E. coli* proteins or their closely related homologues to check the accessibility of the consensus motifs for CDKs. These six genes were amplified by PCR and cloned in expression vectors (see Methods), induced in *E. coli* and partially purified. These purified proteins were then used as potential substrates for cyclin A/CDK2 in *in vitro* kinase assays. The kinase reaction mixtures were analyzed by SDS-PAGE and autoradiography (Fig. 3). An N-terminal GST-tagged fragment of CDC6 served as a positive control that is efficiently phosphorylated by cyclin A/CDK2 (Fig. 3, lane 9). None of the *E. coli* proteins were detectably phosphorylated by cyclin A/CDK2 (Fig. 3, lane 1–6). This data indicates that the presence of multiple consensus motifs are not sufficient for recognition by CDKs. Further, this implies that features apart from consensus motifs, which are located proximal or distal (such as Cy motifs) to the consensus

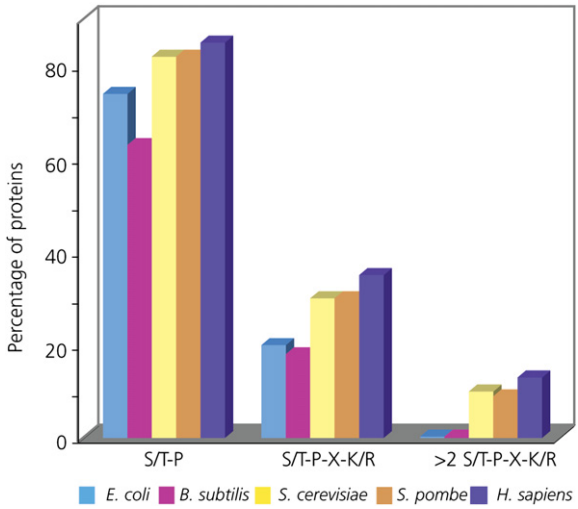

Fig. 2. Occurrence of CDK consensus motifs in proteins from different species. Percentage of proteins containing minimal (S/T-P) and complete (S/T-P–X-K/R) motifs for CDK phosphorylation is shown for various bacterial and eukaryotic genomes.

motifs, are likely to enable recognition of substrates by CDK. As Cy motifs are not shared by all CDK substrates, the nature of other recognition motifs are currently unclear. The *E. coli* proteins described above therefore served as a set of full-length proteins that are non-substrates (negative control). Their sequences were therefore compared with that of known CDK targets to extract the features that distinguish substrates from non-substrates.

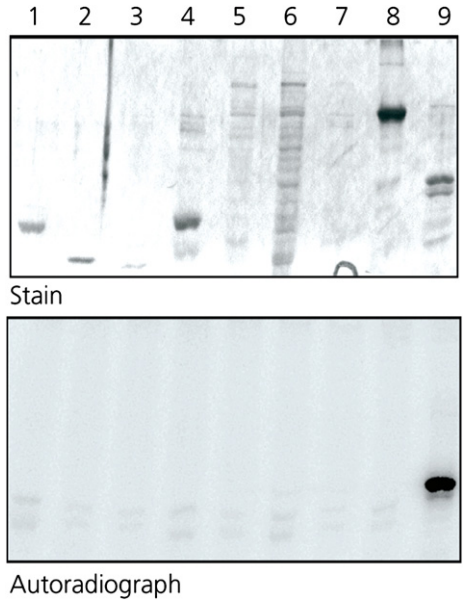

Fig. 3. *In vitro* kinase assay for *E. coli* proteins with Ser/Thr–Pro motifs. Partially purified *E. coli* proteins with consensus motifs (Lane 1–6) were incubated with Cyclin A/CDK2 and $\gamma-[^{32}PO_4]$ATP and analyzed by SDS-PAGE and autoradiography (Coomassie blue staining, top panel; autoradiography, bottom panel). (Lane 1) ERYK, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase; (lane 2) PMSR, peptide methionine sulfoxide reductase; (lane 3) OmpR, catabolic regulation response regulator; (lane 4) LysR, Putative trancription regulator; (lane 5) Gly_Trans, putative glycosyl transferase, colanic acid synthesis; (lane 6) Rha_BAD, positive regulator for rhaBAD operon. (Lane 8), BSA and (lane 9) N-terminal fragment of CDC6 fused to GST (negative and positive controls respectively).

Flanking sequences in substrates and non-substrates are different

We next examined the sequence characteristics of regions flanking the consensus motifs to get an insight into the features that distinguish between substrates and non-substrates. The amino acid preferences of flanking regions in nearly 100 phosphorylation sites (see Methods) and 27 consensus motifs surrounding the non-phosphorylated sites of *E. coli* proteins described above, were examined in this context. We observed no preference for any individual amino acid in the regions excluding the consensus motif (P−12 to P0, P+2, P+4 to P+12), of the substrate and non-substrate sites. On the other hand we observed significant differences (Fig. 4) in the extent of occurrence of specific groups of amino

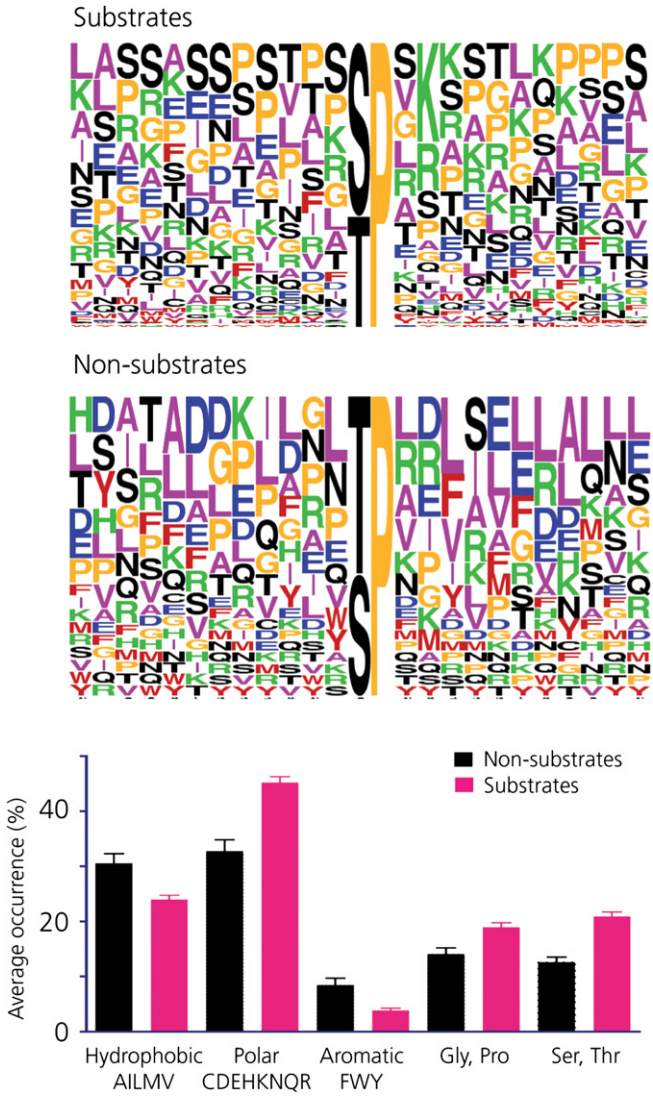

Fig. 4. Comparison of sequences of consensus motifs in substrate and non-substrates. (A) Relative frequency of amino acids in sites flanking S/T-P motifs in substrate and non-substrates (*E. coli* proteins analyzed in this work). This figure was generated using WebLogo (Crooks et al., 2004). Twelve residues flanking the consensus motifs on the N- and C-terminal of S/T of S/T-P motifs are compared. (B) Average frequency of occurrence of different groups of amino acids flanking the consensus motifs for CDK phosphorylation (S/T-P) in substrates (Red) and non-substrates (black) is shown.

acids between the substrate and the non-substrate sites. The substrates sites have higher preference for polar, charged, Pro/Gly and Ser/Thr residues and a reduced occurrence of aromatic and hydrophobic residues. Distinct composition of amino acids were therefore observed in regions flanking substrate and non-substrate sites. The average composition of the substrate and non-substrate sites were significantly different as confirmed by *T*-test on the means of occurrence of individual groups of amino acids with a *p*-value $< 10^{-3}$ (*see* Methods).

A hidden Markov model-based identification of substrates

So, consensus motifs are not specific to substrates and are insufficient for phosphorylation by CDKs. Moreover, the distribution of individual residues flanking the phospho-S/T vary widely among substrates. However there is a bias towards the size and polarity of the residues constituting the site (Fig. 4). We therefore find the substrate phosphorylation sites are more elusive in contrast to the presumed simplistic nature of consensus motifs. Hence we decided to build profiles of known substrate phosphorylation sites using HMMer 2.0 (see Methods) to assess their effectiveness for specificity in identification of substrates. The HMM based method varies from the consensus motif based search procedures in two ways. Firstly, the search patterns of the HMM encompass wider regions – 12 residues on the N- and C-terminal flanks of pS/pTP in the case of optimal profile length. Secondly, each profile is built from multiple phosphorylation site patterns so as to incorporate preferences of diverse substrates. These profiles reflect the preference for a selected set of residues at sites flanking pS/pTP in known CDK substrates for a range of residues, in contrast to single particular residues represented in a linear motif such as those found in consensus sequences.

Substrate HMM profiles were tested on various substrate data-sets derived from independent experimental studies (ruling out any inherent bias in choice of method or organism). The HMM search parameters were optimized for the length of the profile and screening threshold (referred to as *E*-value) by using the published CDK1 targets of *S. cerevisiae* as a positive control data set. We found that a profile length of 25 amino acids and *E*-value of 0.15 were optimal for substrate identification (data not shown). We compared the performance of HMM profiles in identification of substrates with that of a recent method reported by Moses et al. (2007) for identification of CDK substrates, referred to as the 'clustering method'. The identification of substrates by this method is based on the observed higher spatial clustering of multiple consensus motifs in substrates in contrast to non-substrates. The quantitative assessment of the two methods (see Methods) revealed that HMM method has higher sensitivity (76%) and accuracy (70%) compared to the clustering method (15% sensitivity and 55% accuracy) in the identification of CDK1 targets of *S. cerevisiae*. The clustering method displays higher specificity but low sensitivity, indicating that consensus motifs are not often clustered in substrates.

The sensitivity of the two methods has also been tested on the substrates obtained in the *Xenopus* cDNA library screening. 56/77 (72%) of the total targets with available sequence information were identified as CDK targets by HMM based method. Only eight (10%) of these proteins were identified by the clustering method. Thus, the HMM based method proved more effective in all the datasets of available substrates. Neither the HMM based nor the clustering method identified any of the bacterial proteins analyzed in this study as substrates, indicating that clustering method is quite specific and also that HMM based substrate profile is able to distinguish substrate sites from non-substrate sites effectively.

Potential human targets of CDKs

As described in the previous sections, we found that HMM based search methods were quite effective in identification of novel CDK substrates. However, we found that the HMM based method had a limitation of lower specificity ($\sim$65% true hits). Therefore, to increase the specificity of our search, we restricted our search for human CDK targets by analyzing a subset of human proteins, the phosphoproteome data set (Molina et al., 2007; Nousiainen et al., 2006) (see Methods). This set of proteins comprised 513 *in vivo* phosphorylated proteins of which 215 containing pSP/pTP motifs. HMM profiles scanned the data set of 513 proteins and identified 292 proteins as CDK targets (Supplementary Table 1). 155/292 (53%) targets overlap with 215 phosphoproteins containing pSP or pTP motifs. Another 13

proteins identified by HMM have been recently reported to be phosphorylated on S–P or T–P based on mass-spectrometric studies by another group (Chi et al., 2008). A total of 168 proteins identified by HMM from the human phosphoproteome are very strong candidates for CDK phosphorylation (Supplementary Table 1). They include known substrates and homologues of *Xenopus* proteins identified in our analysis from mobility shift-based screening using various cyclin/CDK complexes. These targets are discussed further in detail in the later sections.

The CDK targets obtained from all the methods described in this study can be classified broadly into various functional categories. One third of these proteins are associated with core cell cycle regulatory processes and complexes. Other proteins identified are involved in controlling the dynamics of various aspects of cell cycle machinery, such as chromosome associated factors, microtubules, cytosketelal proteins, the Golgi complex, nuclear pore and membrane complexes, and DNA-binding and repair factors (Tables 2 and 3 and Supplementary Table 1)

Discussion

Cell cycle transitions controlled by various cyclin/CDK complexes are highly specific. However, the number and nature of substrates that each cyclin/CDK complex has to phosphorylate to regulate such cell cycle events are not well-understood in vertebrates. In the current work, we used the combined approaches of cDNA library screening and computational analysis to get a better insight into the spectrum of CDK substrates. Employing the 'shift assay' developed by Stukenberg et al., (1997), we originally screened for substrates of cyclin A/CDK2 (Duncan T., Ph.D. Thesis). Despite the limitations of the method, due to the fact that not all proteins shift after phosphorylation, this assay appears to be extremely reliable. Therefore we decided to use this approach for other CDKs.

We report here a similar screening of *Xenopus* cDNA libraries using different cyclin/CDK complexes. We first identified 35 substrates of cyclin B/CDK1 using a *Xenopus* cDNA library containing 10,000 non-oriented clones, and 42 substrates of cyclin E/CDK2 screening an arrayed *Xenopus* cDNA library containing 3286 independent clones. The rate of detection was around 0.4% for the cyclin B/CDK1 screen and 1.4% for the cyclin E/CDK2 screen, but the 'real' content of the first cDNA library is not known. This screening of substrates using cyclin E/CDK2 has greatly benefited from arrayed and annotated clones analyzed in smaller pools in contrast to the cyclin B screen and the original Stukenberg screen for mitotic phosphoproteins (Stukenberg et al., 1997).

The specificity of cyclin E or cyclin A/CDK2 towards positive clones of the arrayed library was further evaluated. The majority of the substrates (90%) were phosphorylated by both kinases. Moreover, about half the cyclin B/CDK1 targets were phosphorylated by cyclin A/CDK1 or cyclin A/CDK2. Screening with cyclin E/CDK2 identified Stathmin and v-Raf as substrates. Stathmin, a microtubule regulator is well documented as a mitotic substrate of CDK1/cyclin B (Marklund et al., 1994) in addition to other non-CDK kinases. We also identified v-Raf, involved in mitogenic signalling, in both the cyclin E/CDK2 and cyclin B/CDK1 screens. This reiterates the fact that specificity results in part from where in the cell particular substrates and CDKs are found, and in part when they are present and active; the matter of *intrinsic* specificity for a particular cyclin/CDK complex seems to play a relatively minor role in many cases. Screening cDNA libraries for substrate identification suffers from two major limitations, however; firstly, it cannot detect substrates that do not undergo mobility shift upon phosphorylation and second, we risk identifying as substrates proteins that would not be accessible for the kinase *in vivo*. Nevertheless, this approach still represents an efficient and reliable method to identify substrates, which could be explored further. The set of substrates identified are likely to represent high affinity targets, as they are present at very low concentrations in the assay, comparable to or lower than physiological concentrations. The availability of a *Xenopus* arrayed cDNA library represents an excellent resource to pursue the substrate identification further for other kinases, although it suffers from being very incomplete in terms of the genome's potential repertoire.

Sequence analysis of the identified substrates showed that all of them contained multiple Ser/Thr–Pro motifs, and 80% (35/43) contained at least one complete consensus sequence. Previous screening in the lab had also shown that even if the (S/T)-P-X-(K/R) represents a bona fide consensus for CDK phosphorylation, the S/T-P is the minimal motif required for phosphorylation by this family of kinases. Although extensive phosphorylation site mapping on all the screened substrates has not been carried

out, the amino acid sequences of all the substrates identified so far suggests that CDK2 prefers substrates with complete consensus sequence while CDK1 is a less stringent kinase; in fact only 50% of the CDK1/cyclin B substrates identified in our screens have at least one complete consensus sequence.

This means that consensus motifs alone are of very limited use for *in silico* based identification of novel substrates. This is because of the ubiquitous occurrence of Ser/Thr–Pro in both substrates and non-substrates. In addition, the requirement of cyclin docking 'Cy' motif in CDK substrates is not well-understood (and whether there is an equivalent, different motif for cyclin B is unknown). Hence, we explored a computational approach to analyze sequences flanking these consensus motifs. We have effectively used the additional substrate specific features, revealed from the analysis, for identification of novel targets of CDK.

This required a detailed analysis of known substrates in comparison with 'non-substrates' of CDK. As far as we know, no full-length proteins with complete or minimal consensus motifs, have previously been shown *not* to be phosphorylated by cyclin/CDK complexes. We noticed that incubation of $\gamma-[^{32}PO_4]$ATP and cyclin/CDK complexes with *E. coli* cell lysates revealed little or no incorporation of labeled phosphate into bacterial proteins, when such lysates were analyzed by SDS-PAGE and autoradiography. Sequence comparison of *E. coli* and eukaryotic proteins show that the *E. coli* genome encodes a large number of proteins with minimal or complete consensus motifs for phosphorylation by CDKs. We therefore investigated if the lack of observed phosphorylation of the bacterial proteins was due to very low abundance of the relevant proteins in the extract. To test this point, *E. coli* proteins with multiple consensus motifs were over-expressed, partially purified, and tested as substrates of cyclin/CDK complexes. None of the six proteins we tested were phosphorylated by cyclin A/CDK2, showing that consensus motifs alone are not sufficient for recognition as substrates. Analysis of sequences flanking the potential CDK consensus motifs of *E. coli* proteins and substrates revealed distinct preferences of substrate sites for polar and charged amino acids in addition to Pro, Gly, Ser and Thr residues and lower occurrence of aromatic and other hydrophobic amino acids. Most likely, these reflect a requirement for lack of secondary and tertiary structure in substrate sites, or, to put it another way, in bona fide substrates, phosphorylation sites are found in flexible, unstructured regions of proteins.

The phosphorylation sites do not show any particular preference for any individual amino acid in their flanking regions. This makes it impossible to use individual phosphorylation site patterns for '*in silico*' identification of novel substrates, although we can probably rule out proteins lacking S/T-P (such as the core histones) as substrates. We therefore used Hidden Markov Models to build profiles from multiple CDK phosphorylation sites, including additional flanking regions (12 amino acids on N- and C-terminal of phospho-Ser/Thr), to analyze protein sequences for a range of substrate characteristics. We find that HMM based profiles are more sensitive in identification of CDK substrates compared to other consensus motif based methods. We have particularly compared the effectiveness of HMM based method with that of clustering method, which has also been recently used for identification of CDK targets (Moses et al., 2007).

A very small fraction of the known CDK1 targets in *S. cerevisiae* (15%) and *Xenopus* cDNA library screened substrates (10%) are identified by the clustering method. The lower sensitivity of the Clustering method for the identification of a wide range of CDK substrates therefore implies that consensus motifs do not occur in clusters in the majority of CDK substrates. We find that HMM based methods encompassing wider phosphorylation site patterns from diverse substrates, performs better than the Clustering method for substrate identification.

We further scanned the protein sequences of the targets identified from the *Xenopus* cDNA library. Nearly 72% (56 out of 77) of all the substrates identified using cyclin E/CDK2 or cyclin B/CDK1 complexes were picked by HMM based methods as targets of CDKs. Currently the HMM based method is the most sensitive method, although slightly less specific compared to the most specific clustering method. Hence incorporation of more substrate specific features such as amino acid composition of the flanking sites as employed in our search of the human phosphoproteome should improve the specificity of these profiles further. Updating the HMM profiles with phosphorylation sites newly discovered from the experimental methods will also be critical to improving its sensitivity. We find combining such *in silico* approaches to known data-set of experimentally determined phosphoproteins help to identify novel substrates.

Using HMM based method we were able to further associate human phosphoproteome data-sets (Molina et al., 2007; Nousiainen et al., 2006) to CDK profiles in an attempt to identify novel CDK targets. In order to exploit the sensitivity of HMM based methods in identification of substrates from physiologically relevant proteins, we analyzed a large set of human phosphoproteins. Human phosphoproteins with phospho-Ser/Thr–Pro detected from mass-spectrometric studies (Molina et al., 2007; Nousiainen et al., 2006) were therefore analyzed using HMM based CDK substrate profile. Based on this analysis we were able to identify 168 putative human targets which include previously characterized human substrates such as Nucleophosmin (Tokuyama et al., 2001), Nucleolin (Zhu et al., 1999), NDEL1 (Mori et al., 2007), and histone H1 (Arion et al., 1988) and four proteins such as Uracil-DNA glycosylase isoform 1, EPLIN, Dynein light chain and Thymopoietin, identified as novel CDK substrates from our screening of *Xenopus* cDNA library. This further suggests that HMM based profiles are specific to biologically relevant CDK targets.

Cellular functions of potential CDK targets

We identify a total of 244 potential CDK targets from the combination of these approaches, which reveals a diverse spectrum of CDK substrates in many critical cellular processes including nuclear assembly, regulation of CDK activity, cytosketelal organization, vesicular trafficking, cellular migration and invasion. A few representative targets are described in the following sections.

Most multicellular eukaryotes undergo open mitosis, involving breakdown of nuclear envelope and visible condensation of chromosomes. Cyclin dependent kinases facilitate this process by phosphorylating nuclear proteins, such as condensins (Kimura et al., 1998), lamins (Heald and McKeon, 1990; Peter et al., 1991). We identified *Xenopus* thymopoietin, POM121 and tipin as targets of cyclin E/CDK2. Thymopoietin is a nuclear lamina-associated protein also known as lamina-associated polypeptide 2 that is known to have a role in post-mitotic nuclear assembly (Dechat et al., 1998). POM121 is a core nuclear pore protein that is completely dispersed during mitosis (Daigle et al., 2001). Tipin is required for recovery of stalled replication forks during DNA replication (Errico et al., 2007). The HMM based analysis of the *in vivo* human phosphoproteins analyzed in this study suggests that the nuclear pore components nucleoporin 107 KDa and nucleoporin 98 KDa are also potential CDK targets.

Regulation of cyclin dependent kinases is governed by a complex feedback mechanism involving various positive (CDC25) and negative CDK regulators (Wee1, p21Cip/p27Kip) that are themselves targets of CDKs. Cyclin B/CDK1 screening identified Wee1, TOME-1, Brd4, pcd4 (programmed cell death 4 isoform 2), and MARK3 as targets of CDK. TOME-1 is an F-box protein, which is a part of the SCF complex involved in down-regulation of Wee1 that helps to trigger the onset of mitosis (Smith et al., 2007). Brd4 is involved in the transcriptional control of Aurora B kinase that in turn regulates mitosis, by phosphorylation of a variety of substrates (You et al., 2009). Pcd4 is a tumour suppressor that indirectly down-regulates CDK1 function by induction of $p21^{(waf1/cip1)}$ (Goke et al., 2004). MARK-3 (MAP/Microtubule regulating kinase; CTAK1) phosphorylates CDC25C and results in its inhibition (Bachmann et al., 2006). Establishment of cohesion-1 homolog of *Xenopus* was identified as a target for cyclin E/CDK2 in our screening. This protein is a critical acetyl transferease required to establish the cohesin complex that holds sister chromatids together (Ben-Shahar et al., 2008; Milutinovich et al., 2007). Additional cell cycle regulators have been identified as targets of CDK from *in silico* analysis (Supplementary Table 1).

The reorganization of the tubulin cytoskeleton that occurs when cells enter mitosis is critical for chromosome segregation, and intermediate filaments and cell adhesions also are dramatically altered at this time. CDK activity regulates the properties of several cytoskeletal proteins, including vimentin and nestin (Chou et al., 1990, 2003), which leads to changes in their structural and interaction properties. Our screening based on shift assays has identified various cytoskeletal proteins that include Eplin, syntrophin, ETS variant gene-1 and HDAC6. Eplin is a negative regulator of actin filament polymerization (Maul et al., 2003). Syntrophin also interacts with actin and calmodulin to affect their functions (Newbell et al., 1997). ETS variant gene-1 is a transcription factor involved in cell invasion in response to androgen receptor (Cai et al., 2007). Histone deacetylase 6 (HDAC6), despite its name, is a cytoplasmic enzyme that seems to be involved in a variety of cytoskeletal reorganizations (Valenzuela-Fernandez et al., 2008).

Other human cytoskeletal proteins such as Enable Homolog (ENA/VASP family of proteins) and adducin-1 are implied as CDK targetes from our *in silico* analysis of phosphoproteins.

The role of CDK phosphorylation in the control of vesicular trafficking between various cellular organelles and in regulation of proteins localized in various organelles has long been suspected, to account for the reorganization of membranes seen in mitosis (Shorter and Warren, 2002). GRASP55, a protein regulating the reassembly of Golgi stacks is an important target of CDK known to be cell cycle regulated (Duran et al., 2008). Changes in various membranous vesicles during cell cycle transitions is also controlled by various lipid kinases and proteins. Based on shift assays, we identified diacylglycerol kinase and phosphatidyl-inositol-4-phosphate-5-kinase as targets of CDK2. These enzymes are responsible for maintaining phosphoinositide content of the cell for extracellular maxtrix regulation and organization of cytoskeleton (Dunlop and Muggli, 2000; Evangelisti et al., 2006). Epsin 2 and Arf-GAP3 (ADP-ribosylation factor GTPase activating protein 3), involved in endocytosis (Nie et al., 2002) and vesicle formation have also been identified as targets from shift-based assays. Epsin 1 was previously identified as a mitotic substrate of cyclin B/CDK1 by Stukenberg et al., (1997).

Various other proteins involved in regulation of transcription, translation, chromatin remodelling, apoptosis and telomere regulation have been identified from our combined screening approaches. The wide spectrum of substrates reported in this study forms a representative list of CDK targets in vertebrates encompassing different cell cycle phases. While the substrates have been identified using specific cyclin/CDK complexes, they are likely to be phosphorylated by other CDK/cyclin complexes and other mitotic kinases subject to spatio-temporal organisation. Further studies aimed at detailed evaluation of candidate substrates in a biological context is necessary to validate and understand specific roles of these targets in vertebrates. Mere lists are not terribly informative.

Material and methods

Protein expression and purification

A bicistronic vector was prepared in the pGEX6P1 background containing both CDK2 and CIV1. Both CDK2 and CIV1 have an N-terminal GST tag. To have a soluble source of cyclin E, we used a truncated version of human cyclin E1 (residues 81–363) (Honda et al., 2005) with a GST N-terminal tag. Both GST–CDK2 and GST-cyclin E have a prescission protease cleavage site after the GST tag.

All plasmids were transformed in BL21-CodonPlus (DE3)-RIL bacterial strain (Stratagene). GST-CDK2 and GST-cyclin E were expressed separately in bacteria. They were induced at 18 °C with 0.1 mM IPTG (Sigma) for 20 h. Bacteria were lysed in 20 mM Hepes pH 7.8/300 mM NaCl/0.1% MTG (Sigma) using a cell disruptor. The suspension obtained was centrifuged at 38000 rpm in a Ti45 Beckman rotor. Lysates were then mixed for 1 h at 4 °C to allow complex formation; 2 ml of GSH-Sepharose beads (Amersham) were added to the mixed lysate and incubated in agitation at 4C for 1 h. The beads were washed with 100 ml of 20 mM Hepes pH 7.8/300 mM NaCl/0.1% MTG and the cyclin/CDK complexes were eluted with 20 mM glutathione/20 mM Hepes pH 7.8/300 mM NaCl/0.1% MTG. The GST tags were cleaved from the complex using prescission protease (1:50 w/w) overnight at 4 °C, and cyclin E/CDK2 was further purified through a gel filtration column. Cyclin A/CDK2 complex was prepared in the same way, but cyclin A was expressed as a 6-His tagged version with an N-terminal truncation (construct A3) to obtain a soluble protein (Brown et al., 1995).

For cyclin B/CDK1, a polycistronic vector was constructed in the PGEX6P1 backgorund containing a *X. laevis* CDK1 fragment, CIV1 and *X. laevis* cyclin B1. The plasmid was transformed into BL21-CodonPlus (DE3)-RIL bacterial strain (Stratagene). Cyclin B/CDK1 was induced at 25 °C with 0.5 mM IPTG (Sigma) for 20 h. Bacteria were lysed in PBS containing 0.2 mg/mL Lysozyme, 0.1% deoxycholate, 10 mM $MgCl_2$ and 5 μg/mL DNAse I. The lysate was incubated on ice for 1 h, sonicated for 30 s (Heat system Sonicator) and centrifuged at 10000 g for 20 min. The supernatant was dialyzed against PBS with 10 mM $MgCl_2$ and 5% glycerol. For substrate screening, 1 ml of lysate was mixed with 20 μl of GSH-Sepharose beads and rotated for 2 h at 4 °C. Beads were washed in PBS/10 mM $MgCl_2$/5% Glycerol and resuspended in 50 μl of this same buffer. This method provide sufficient bead-bound kinase to perform 20 kinase reactions

The *E. coli* proteins used in the analysis were amplified from *E. coli* genomic DNA and cloned in pET21b vector.The proteins were induced in *E. coli* using 0.1 mM IPTG at 30° and subjected to a single step purification on Ni-NTA beads (Qiagen). These partially purified proteins were used as substrates for the kinase assays.

Preparation of DNA and protein pools from the Xenopus *library and kinase assay*

Plasmid pools form the Xenopus oocyte cDNA library were transcribed and translated *in vitro* using Quick-coupled *in vitro* transcription-translation kit (Promega) in 5 μl reactions. One μl of the translated products were added to 10 μl of kinase buffer (see below) and the bead-bound kinase and incubated at 23° for 30 min.

The arrayed *Xenopus* cDNA library was a gift from Tony Hyman. Bacteria expressing single clones were grown in Luria Broth (with 100 μg/mL Ampicillin) separately in 386 well plates at 37° for 36 h. Single clone cultures were then pooled by row (wells 1–12 and 13–24) and by column (wells A–P) and the DNA from the pooled bacteria was prepared using a miniprep Qiagen kit. Five μl of each miniprep was translated *in vitro* using the Quick-coupled transcription-translation kit (Promega) according to the manufacturer's instruction. H1 kinase assay and other kinase reactions were performed in 80 mM Na β-glycerolphosphate/15 mM $MgCl_2$/20 mM EGTA/100 μM ATP/1 μCi of $\gamma-[^{32}P]$-ATP (Amersham) in 20 μl containing 200 nM of the appropriate kinase and either 1 mg/mL of H1 or 4 μl of *in vitro* translated material as substrates. Kinase reactions were performed for 30 min at 23°.

Kinase assays on the bacterial proteins were essentially performed as described above, using 200 ng of the *E. coli* test proteins or a GST-tagged N-terminal fragment of CDC6 as substrate in the presence of 200 nM cyclin A/CDK2; the samples were incubated for 5 min at 30 °C. Reactions were terminated by adding 2X sample buffer and analyzed on 15% SDS/PAGE.

In silico analysis

Substrates and Non-substrates used in the analysis

Various distinct sets of substrates and non-substrates have been used to systematically analyze the effectiveness of the substrate profiles in identification of substrates.

Data-set 1: List of CDK1 positive targets (333) of *S. cerevisiae* (Ubersax et al., 2003)
Data-set 2: Previously known substrates of CDK1/CDK2 (30) not included for building profile HMM (Supplementary Table 2).
Data-set 3: Substrates of CDK2/cyclin E identified from small pool expression screening of arrayed *X. laevis* cDNA library (reported in current study) (Table 2)
Data-set 4: Substrates identified by screening Non-arrayed *X. laevis* library using CDK1/cyclin B (reported in current study). (Table 2)
Data-set 5: *E. coli* proteins not phosphorylated by CDK2/cyclin A complex constitute the non-substrates. They include 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase; Peptide methionine sulfoxide reductase; putative glycosyl transferase, colanic acid synthesis; positive regulator for rhaBAD operon; Putative transcription regulator LysR and OmpR regulators (Supplementary Table 3).
Data-set 6: List of CDK1 negative targets (362) of *S. cerevisiae* (Ubersax et al., 2003) kindly provided by David Morgan.

HMMbuild module was used to build the substrate profiles matrix (profile HMM; Additional file: HMM_58.hmm) and HMMPfam modules of local version of HMMER 2.3.2 (http://hmmer.janelia.org/) was used to search each of the data-sets described above using substrate profiles for identification of substrates. The performance of HMM method was compared with the substrate detection method based on clustering of phosphorylation sites (Moses et al., 2007). Perl script compute_SLR.pl downloaded from the website (http://www.sanger.ac.uk/Users/am8/protein_motifs.html) was used to identify substrates from all the above data-sets.

Detection of consensus motifs in proteins

All proteins reported in the study were scanned for Ser/Thr–Pro consensus motifs using local version of the Pro-site module PS_SCAN (Moses et al., 2007)(http://expasy.org/prosite/). Bacterial, yeast and human proteins sequences analyzed for occurrence of CDK consensus motifs were downloaded from NCBI (ftp://ftp.ncbi.nih.gov/genomes).

Analysis on composition of substrate and non-substrate sequences

Consensus motifs obtained from *E. coli* proteins analyzed in the study (Supplementary Table 3), which are not phosphorylated by CDK2/cyclin A complex have been considered as the non-substrate data-set. In order to analyze sequences of CDK substrates nearly 70 substrates comprising of a nearly 100 phosphorylation sites were collected by searching through literature and consulting the database PhosphoELM (http://phospho.elm.eu.org/). A representative list of 58 CDK substrates (Supplementary Table 4) to build profile HMMs was further generated, to reduce the bias in the nature of phosphorylation sites, used to build the HMM. The remaining substrates were utilized as another test data set (Methods; Data set 2 (Supplementary Table 2)).

Sequence of 25 amino acids, including Ser/Thr residue of Ser/Thr–Pro motifs in the middle, were extracted using the PS_SCAN from the non-substrate data-sets. Phosphorylation sites of known CDK substrates comprising of 25 amino acids, flanking the N- and C-terminal of phospho-Ser/Thr were considered as the substrate data-set to build substrate profiles (profile HMM). The amino acids toward the N-terminal and C-terminal of the central Ser/Thr (P0) are referred to as 'P − n' and 'P + n' (n stands for number of amino acids away from the P0). Amino acids were categorized into 5 different groups namely, Aromatic (F,Y,W), Hydrophobic (A, I, L, M, V), Polar (C, D, E, H, K, N, Q, R) and P/G group and S/T group. The composition of 27 consensus motifs flanking patterns from 6 Bacterial proteins and ∼100 phosphorylation sites of ∼70 proteins were analyzed using perl scripts.

Two-tailed *T*-tests to validate the differences between the average occurrence of various groups of amino acids in substrate and non-substrate data set were carried out using the statistical package in PRISM.

Hidden Markov Models based detection of substrates

58 representative patterns of phosphorylation sites from the substrate data-set, (Supplementary Table 4) described above were extracted to build profile using HMMBUILD module of HMMER-2.0. HMMPFAM module of HMMER-2.0 was used to search the substrate profile against the query sequence. In order to optimize the HMM search for the best performance, multiple searches over E-value ranging from 0.01 to 1 has been carried out using CDK1 targets (Data set 1 (positive hits) and Data set 7 (negative hits) described earlier). Substrate patterns varying in length (9, 17, 25 amino acids as described in (ii)) have been used for building profile HMMs to define the optimal profile length required for accurate substrate identification.

Evaluation parameters used to compare HMM based method and clustering method

Diagnostic test characteristics described below have been used to evaluate the two methods of substrate identification compared in the study, namely HMM based and clustering methods.

True positives (TP): Number of substrates identified as substrates
False Positive (FP): Number of non-substrates identified as substrates
True Negative (TN): Number of non-substrates identified as non-substrates
False Negative (FN): Number of substrates identified as non-substrates
Sensitivity = TP/TP + FN; Fraction of total substrates identified
Specificity = TN/TN + FP; Fraction of non-substrates excluded
Accuracy = Sensitivity + Specificity/2

Identification of novel human targets of CDK

A set of 513 unique sequences of human phosphoproteins derived from previously reported studies [34] were scanned using substrate profile HMM. Proteins matching HMM profiles were further compared to those phosphoproteins in with pSer-Pro/pThr-Pro peptides.

The list of 292 human CDK targets and 77 Xenopus targets were further compared to the CDK2 phosphorylated phosphopeptides reported in another mass-spectrometric studies (Chi et al., 2008).

Appendix. Supplementary information

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.advenzreg.2009.12.001.

References

Adams PD. Regulation of the retinoblastoma tumor suppressor protein by cyclin/cdks. Biochim Biophys Acta 2001;1471:M123–33.

Addinall SG, Mayr PS, Doyle S, Sheehan JK, Woodman PG, Allan VJ. Phosphorylation by cdc2-CyclinB1 kinase releases cytoplasmic dynein from membranes. J Biol Chem 2001;276:15939–44.

Ait-Si-Ali S, Ramirez S, Barre FX, Dkhissi F, Magnaghi-Jaulin L, Girault JA, et al. Histone acetyltransferase activity of CBP is controlled by cycle-dependent kinases and oncoprotein E1A. Nature 1998;396:184–6.

Aizawa H, Kamijo M, Ohba Y, Mori A, Okuhara K, Kawasaki H, et al. Microtubule destabilization by cdc2/H1 histone kinase: phosphorylation of a "pro-rich region" in the microtubule-binding domain of MAP-4. Biochem Biophys Res Commun 1991;179:1620–6.

Arion D, Meijer L, Brizuela L, Beach D. cdc2 is a component of the M phase-specific histone H1 kinase: evidence for identity with MPF. Cell 1988;55:371–8.

Bachmann M, Kosan C, Xing PX, Montenarh M, Hoffmann I, Moroy T. The oncogenic serine/threonine kinase Pim-1 directly phosphorylates and activates the G2/M specific phosphatase Cdc25C. Int J Biochem Cell Biol 2006;38:430–43.

Belle R, Cormier P, Poulhe R, Morales J, Huchon D, Mulner-Lorillon O. Protein phosphorylation during meiotic maturation of Xenopus oocytes: cdc2 protein kinase targets. Int J Dev Biol 1990;34:111–5.

Ben-Shahar TR, Heeger S, Lehane C, East P, Flynn H, Skehel M, et al. Eco1-dependent cohesin acetylation during establishment of sister chromatid cohesion. Science 2008;321:563–6.

Beretta L, Dobransky T, Sobel A. Multiple phosphorylation of stathmin. Identification of four sites phosphorylated in intact cells and in vitro by cyclic AMP-dependent protein kinase and p34cdc2. J Biol Chem 1993;268:20076–84.

Biggs JR, Peterson LF, Zhang Y, Kraft AS, Zhang DE. AML1/RUNX1 phosphorylation by cyclin-dependent kinases regulates the degradation of AML1/RUNX1 by the anaphase-promoting complex. Mol Cell Biol 2006;26:7420–9.

Blangy A, Lane HA, d'Herin P, Harper M, Kress M, Nigg EA. Phosphorylation by p34cdc2 regulates spindle association of human Eg5, a kinesin-related motor essential for bipolar spindle formation in vivo. Cell 1995;83:1159–69.

Blow JJ, Dutta A. Preventing re-replication of chromosomal DNA. Nat Rev Mol Cell Biol 2005;6:476–86.

Bosc DG, Slominski E, Sichler C, Litchfield DW. Phosphorylation of casein kinase II by p34cdc2. Identification of phosphorylation sites using phosphorylation site mutants in vitro. J Biol Chem 1995;270:25872–8.

Brown NR, Noble ME, Endicott JA, Johnson LN. The structural basis for specificity of substrate and recruitment peptides for cyclin-dependent kinases. Nat Cell Biol 1999;1:438–43.

Brown NR, Noble ME, Endicott JA, Garman EF, Wakatsuki S, Mitchell E, et al. The crystal structure of cyclin A. Structure 1995;3:1235–47.

Cai C, Hsieh CL, Omwancha J, Zheng Z, Chen SY, Baert JL, et al. ETV1 is a novel androgen receptor-regulated gene that mediates prostate cancer cell invasion. Mol Endocrinol; 2007.

Chae HD, Yun J, Bang YJ, Shin DY. Cdk2-dependent phosphorylation of the NF-Y transcription factor is essential for the expression of the cell cycle-regulatory genes and cell cycle G1/S and G2/M transitions. Oncogene 2004;23:4084–8.

Chan AK, Persad S, Litchfield DW, Wright JA. Ribonucleotide reductase R2 protein is phosphorylated at serine-20 by P34cdc2 kinase. Biochim Biophys Acta 1999;1448:363–71.

Chang EJ, Begum R, Chait BT, Gaasterland T. Prediction of cyclin-dependent kinase phosphorylation substrates. PLoS One 2007;2:e656.

Chen J, Saha P, Kornbluth S, Dynlacht BD, Dutta A. Cyclin-binding motifs are essential for the function of p21CIP1. Mol Cell Biol 1996;16:4673–82.

Chen S, Xu Y, Yuan X, Bubley GJ, Balk SP. Androgen receptor phosphorylation and stabilization in prostate cancer by cyclin-dependent kinase 1. Proc Natl Acad Sci U S A 2006;103:15969–74.

Chi Y, Welcker M, Hizli AA, Posakony JJ, Aebersold R, Clurman BE. Identification of CDK2 substrates in human cell lysates. Genome Biol 2008;9:R149.

Chiolo I, Carotenuto W, Maffioletti G, Petrini JH, Foiani M, Liberi G. Srs2 and Sgs1 DNA helicases associate with Mre11 in different subcomplexes following checkpoint activation and CDK1-mediated Srs2 phosphorylation. Mol Cell Biol 2005;25:5738–51.

Chou YH, Bischoff JR, Beach D, Goldman RD. Intermediate filament reorganization during mitosis is mediated by p34cdc2 phosphorylation of vimentin. Cell 1990;62:1063–71.

Chou YH, Khuon S, Herrmann H, Goldman RD. Nestin promotes the phosphorylation-dependent disassembly of vimentin intermediate filaments during mitosis. Mol Biol Cell 2003;14:1468–78.

Courvalin JC, Segil N, Blobel G, Worman HJ. The lamin B receptor of the inner nuclear membrane undergoes mitosis-specific phosphorylation and is a substrate for p34cdc2-type protein kinase. J Biol Chem 1992;267:19035–8.

Crooks GE, Hon G, Chandonia JM, Brenner SE. WebLogo: a sequence logo generator. Genome Res 2004;14:1188–90.

Curtis M, Nikolopoulos SN, Turner CE. Actopaxin is phosphorylated during mitosis and is a substrate for cyclin B1/cdc2 kinase. Biochem J 2002;363:233–42.

Daigle N, Beaudouin J, Hartnell L, Imreh G, Hallberg E, Lippincott-Schwartz J, et al. Nuclear pore complexes form immobile networks and have a very low turnover in live mammalian cells. J Cell Biol 2001;154:71–84.

Dechat T, Gotzmann J, Stockinger A, Harris CA, Talle MA, Siekierka JJ, et al. Detergent-salt resistance of LAP2alpha in interphase nuclei and phosphorylation-dependent association with chromosomes early in nuclear assembly implies functions in nuclear structure dynamics. EMBO J 1998;17:4887–902.
Deng W, Lin BY, Jin G, Wheeler CG, Ma T, Harper JW, et al. Cyclin/CDK regulates the nucleocytoplasmic localization of the human papillomavirus E1 DNA helicase. J Virol 2004;78:13954–65.
Diederichs S, Baumer N, Ji P, Metzelder SK, Idos GE, Cauvet T, et al. Identification of interaction partners and substrates of the cyclin A1-CDK2 complex. J Biol Chem 2004;279:33727–41.
Dunlop ME, Muggli EE. Extracellular matrix components cooperate to activate phosphatidyl inositol-4-phosphate 5-kinase. Biochem Biophys Res Commun 2000;279:931–7.
Duran JM, Kinseth M, Bossard C, Rose DW, Polishchuk R, Wu CC, et al. The role of GRASP55 in Golgi fragmentation and entry of cells into mitosis. Mol Biol Cell 2008;19:2579–87.
Elias B, Laine A, Ronai Z. Phosphorylation of MdmX by CDK2/Cdc2(p34) is required for nuclear export of Mdm2. Oncogene 2005; 24:2574–9.
Errico A, Costanzo V, Hunt T. Tipin is required for stalled replication forks to resume DNA replication after removal of aphidicolin in Xenopus egg extracts. Proc Natl Acad Sci U S A 2007;104:14929–34.
Esashi F, Christ N, Gannon J, Liu Y, Hunt T, Jasin M, et al. CDK-dependent phosphorylation of BRCA2 as a regulatory mechanism for recombinational repair. Nature 2005;434:598–604.
Evangelisti C, Bortul R, Tabellini G, Papa V, Cocco L, Martelli AM. Nuclear expression of diacylglycerol kinases: possible involvement in DNA replication. Eur J Histochem 2006;50:9–13.
Floyd SR, Porro EB, Slepnev VI, Ochoa GC, Tsai LH, De Camilli P. Amphiphysin 1 binds the cyclin-dependent kinase (cdk) 5 regulatory subunit p35 and is phosphorylated by cdk5 and cdc2. J Biol Chem 2001;276:8104–10.
Fourest-Lieuvin A, Peris L, Gache V, Garcia-Saez I, Juillan-Binard C, Lantez V, et al. Microtubule regulation in mitosis: tubulin phosphorylation by the cyclin-dependent kinase Cdk1. Mol Biol Cell 2006;17:1041–50.
Frouin I, Toueille M, Ferrari E, Shevelev I, Hubscher U. Phosphorylation of human DNA polymerase lambda by the cyclin-dependent kinase Cdk2/cyclin A complex is modulated by its association with proliferating cell nuclear antigen. Nucleic Acids Res 2005;33:5354–61.
Fujibuchi T, Abe Y, Takeuchi T, Ueda N, Shigemoto K, Yamamoto H, et al. Expression and phosphorylation of TOPK during spermatogenesis. Dev Growth Differ 2005;47:637–44.
Garrett S, Barton WA, Knights R, Jin P, Morgan DO, Fisher RP. Reciprocal activation by cyclin-dependent kinases 2 and 7 is directed by substrate specificity determinants outside the T loop. Mol Cell Biol 2001;21:88–99.
Georgi AB, Stukenberg PT, Kirschner MW. Timing of events in mitosis. Curr Biol 2002;12:105–14.
Goke R, Barth P, Schmidt A, Samans B, Lankat-Buttgereit B. Programmed cell death protein 4 suppresses CDK1/cdc2 via induction of p21(Waf1/Cip1). Am J Physiol Cell Physiol 2004;287:C1541–6.
Gorr IH, Boos D, Stemmann O. Mutual inhibition of separase and Cdk1 by two-step complex formation. Mol Cell 2005;19: 135–41.
Goto H, Kiyono T, Tomono Y, Kawajiri A, Urano T, Furukawa K, et al. Complex formation of Plk1 and INCENP required for metaphase-anaphase transition. Nat Cell Biol 2006;8:180–7.
Gupte RS, Traganos F, Darzynkiewicz Z, Lee MY. Phosphorylation of RIalpha by cyclin-dependent kinase CDK 2/cyclin E modulates the dissociation of the RIalpha-RFC40 complex. Cell Cycle 2006;5:653–60.
Hall C, Nelson DM, Ye X, Baker K, DeCaprio JA, Seeholzer S, et al. HIRA, the human homologue of yeast Hir1 p and Hir2 p, is a novel cyclin-cdk2 substrate whose expression blocks S-phase progression. Mol Cell Biol 2001;21:1854–65.
Hara T, Abe M, Inoue H, Yu LR, Veenstra TD, Kang YH, et al. Cytokinesis regulator ECT2 changes its conformation through phosphorylation at Thr-341 in G2/M phase. Oncogene 2006;25:566–78.
Harbour JW, Luo RX, Dei Santi A, Postigo AA, Dean DC. Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. Cell 1999;98:859–69.
Hayami R, Sato K, Wu W, Nishikawa T, Hiroi J, Ohtani-Kaneko R, et al. Down-regulation of BRCA1-BARD1 ubiquitin ligase by CDK2. Cancer Res 2005;65:6–10.
He J, Xu J, Xu XX, Hall RA. Cell cycle-dependent phosphorylation of Disabled-2 by cdc2. Oncogene 2003;22:4524–30.
Heald R, McKeon F. Mutations of phosphorylation sites in lamin A that prevent nuclear lamina disassembly in mitosis. Cell 1990; 61:579–89.
Hinchcliffe EH, Li C, Thompson EA, Maller JL, Sluder G. Requirement of Cdk2-cyclin E activity for repeated centrosome reproduction in Xenopus egg extracts. Science 1999;283:851–4.
Honda R, Lowe ED, Dubinina E, Skamnaki V, Cook A, Brown NR, et al. The structure of cyclin E1/CDK2: implications for CDK2 activation and CDK2-independent roles. EMBO J 2005;24:452–63.
Huang H, Regan KM, Lou Z, Chen J, Tindall DJ. CDK2-dependent phosphorylation of FOXO1 as an apoptotic response to DNA damage. Science 2006;314:294–7.
Hutchins JR, Moore WJ, Hood FE, Wilson JS, Andrews PD, Swedlow JR, et al. Phosphorylation regulates the dynamic interaction of RCC1 with chromosomes during mitosis. Curr Biol 2004;14:1099–104.
Jaspersen SL, Huneycutt BJ, Giddings Jr TH, Resing KA, Ahn NG, Winey M. Cdc28/Cdk1 regulates spindle pole body duplication through phosphorylation of Spc42 and Mps1. Dev Cell 2004;7:263–74.
Kalaszczynska I, Geng Y, Iino T, Mizuno S, Choi Y, Kondratiuk I, et al. Cyclin A is redundant in fibroblasts but essential in hematopoietic and embryonic stem cells. Cell 2009;138:352–65.
Kanemitsu MY, Jiang W, Eckhart W. Cdc2-mediated phosphorylation of the gap junction protein, connexin43, during mitosis. Cell Growth Differ 1998;9:13–21.
Kimura K, Hirano M, Kobayashi R, Hirano T. Phosphorylation and activation of 13S condensin by Cdc2 in vitro. Science 1998;282: 487–90.
Kitzmann M, Vandromme M, Schaeffer V, Carnac G, Labbe JC, Lamb N, et al. cdk1- and cdk2-mediated phosphorylation of MyoD Ser200 in growing C2 myoblasts: role in modulating MyoD half-life and myogenic activity. Mol Cell Biol 1999;19:3167–76.
Koff A, Cross F, Fisher A, Schumacher J, Leguellec K, Philippe M, et al. Human cyclin E, a new cyclin that interacts with two members of the CDC2 gene family. Cell 1991;66:1217–28.

Konishi Y, Lehtinen M, Donovan N, Bonni A. Cdc2 phosphorylation of BAD links the cell cycle to the cell death machinery. Mol Cell 2002;9:1005–16.
Koseoglu MM, Graves LM, Marzluff WF. Phosphorylation of threonine 61 by cyclin a/Cdk1 triggers degradation of stem-loop binding protein at the end of S phase. Mol Cell Biol 2008;28:4469–79.
Krek W, Nigg EA. Cell cycle regulation of vertebrate p34cdc2 activity: identification of Thr161 as an essential in vivo phosphorylation site. New Biol 1992;4:323–9.
Kusubata M, Matsuoka Y, Tsujimura K, Ito H, Ando S, Kamijo M, et al. cdc2 kinase phosphorylation of desmin at three serine/threonine residues in the amino-terminal head domain. Biochem Biophys Res Commun 1993;190:927–34.
Le Guellec R, Paris J, Couturier A, Roghi C, Philippe M. Cloning by differential screening of a Xenopus cDNA that encodes a kinesin-related protein. Mol Cell Biol 1991;11:3395–8.
Lees JA, Buchkovich KJ, Marshak DR, Anderson CW, Harlow E. The retinoblastoma protein is phosphorylated on multiple sites by human cdc2. EMBO J 1991;10:4279–90.
Lew DJ, Dulic V, Reed SI. Isolation of three novel human cyclins by rescue of G1 cyclin (Cln) function in yeast. Cell 1991;66:1197–206.
Li M, Stefansson B, Wang W, Schaefer EM, Brautigan DL. Phosphorylation of the Pro-X-Thr-Pro site in phosphatase inhibitor-2 by cyclin-dependent protein kinase during M-phase of the cell cycle. Cell Signal 2006;18:1318–26.
Liakopoulos D, Kusch J, Grava S, Vogel J, Barral Y. Asymmetric loading of Kar9 onto spindle poles and microtubules ensures proper spindle alignment. Cell 2003;112:561–74.
Lin CY, Madsen ML, Yarm FR, Jang YJ, Liu X, Erikson RL. Peripheral Golgi protein GRASP65 is a target of mitotic polo-like kinase (Plk) and Cdc2. Proc Natl Acad Sci U S A 2000;97:12589–94.
Litvak V, Argov R, Dahan N, Ramachandran S, Amarilio R, Shainskaya A, et al. Mitotic phosphorylation of the peripheral Golgi protein Nir2 by Cdk1 provides a docking mechanism for Plk1 and affects cytokinesis completion. Mol Cell 2004;14:319–30.
Lorca T, Labbe JC, Devault A, Fesquet D, Capony JP, Cavadore JC, et al. Dephosphorylation of cdc2 on threonine 161 is required for cdc2 kinase inactivation and normal anaphase. EMBO J 1992;11:2381–90.
Lowe M, Rabouille C, Nakamura N, Watson R, Jackman M, Jamsa E, et al. Cdc2 kinase directly phosphorylates the cis-Golgi matrix protein GM130 and is required for Golgi fragmentation in mitosis. Cell 1998;94:783–93.
Lundberg AS, Weinberg RA. Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. Mol Cell Biol 1998;18:753–61.
Luscher-Firzlaff JM, Lilischkis R, Luscher B. Regulation of the transcription factor FOXM1c by Cyclin E/CDK2. FEBS Lett 2006;580:1716–22.
Ma T, Van Tine BA, Wei Y, Garrett MD, Nelson D, Adams PD, et al. Cell cycle-regulated phosphorylation of p220(NPAT) by cyclin E/Cdk2 in Cajal bodies promotes histone gene transcription. Genes Dev 2000;14:2298–313.
Mak AS, Carpenter M, Smillie LB, Wang JH. Phosphorylation of caldesmon by p34cdc2 kinase. Identification of phosphorylation sites. J Biol Chem 1991;266:19971–5.
Manenti S, Yamauchi E, Sorokine O, Knibiehler M, Van Dorsselaer A, Taniguchi H, et al. Phosphorylation of the myristoylated protein kinase C substrate MARCKS by the cyclin E-cyclin-dependent kinase 2 complex in vitro. Biochem J 1999;340(Pt 3):775–82.
Marcelain K, Hayman MJ. The Ski oncoprotein is upregulated and localized at the centrosomes and mitotic spindle during mitosis. Oncogene 2005;24:4321–9.
Margolis SS, Walsh S, Weiser DC, Yoshida M, Shenolikar S, Kornbluth S. PP1 control of M phase entry exerted through 14-3-3-regulated Cdc25 dephosphorylation. EMBO J 2003;22:5734–45.
Marklund U, Larsson N, Brattsand G, Osterman O, Chatila TA, Gullberg M. Serine 16 of oncoprotein 18 is a major cytosolic target for the Ca2+/calmodulin-dependent kinase-Gr. Eur J Biochem 1994;225:53–60.
Masai H, Matsui E, You Z, Ishimi Y, Tamai K, Arai K. Human Cdc7-related kinase complex. In vitro phosphorylation of MCM by concerted actions of Cdks and Cdc7 and that of a criticial threonine residue of Cdc7 bY Cdks. J Biol Chem 2000;275:29042–52.
Maul RS, Song Y, Amann KJ, Gerbin SC, Pollard TD, Chang DD. EPLIN regulates actin dynamics by cross-linking and stabilizing filaments. J Cell Biol 2003;160:399–407.
Meijer L, Arion D, Golsteyn R, Pines J, Brizuela L, Hunt T, et al. Cyclin is a component of the sea urchin egg M-phase specific histone H1 kinase. EMBO J 1989;8:2275–82.
Mi J, Qiao F, Wilson JB, High AA, Schroeder MJ, Stukenberg PT, et al. FANCG is phosphorylated at serines 383 and 387 during mitosis. Mol Cell Biol 2004;24:8576–85.
Milner RE, Busaan JL, Holmes CF, Wang JH, Michalak M. Phosphorylation of dystrophin. The carboxyl-terminal region of dystrophin is a substrate for in vitro phosphorylation by p34cdc2 protein kinase. J Biol Chem 1993;268:21901–5.
Milutinovich M, Unal E, Ward C, Skibbens RV, Koshland D. A multi-step pathway for the establishment of sister chromatid cohesion. PLoS Genet 2007;3:e12.
Mishima M, Pavicic V, Gruneberg U, Nigg EA, Glotzer M. Cell cycle regulation of central spindle assembly. Nature 2004;430:908–13.
Miyazaki Y, Boccuni P, Mao S, Zhang J, Erdjument-Bromage H, Tempst P, et al. Cyclin A-dependent phosphorylation of the ETS-related protein, MEF, restricts its activity to the G1 phase of the cell cycle. J Biol Chem 2001;276:40528–36.
Molina H, Horn DM, Tang N, Mathivanan S, Pandey A. Global proteomic profiling of phosphopeptides using electron transfer dissociation tandem mass spectrometry. Proc Natl Acad Sci U S A 2007;104:2199–204.
Moll T, Tebb G, Surana U, Robitsch H, Nasmyth K. The role of phosphorylation and the CDC28 protein kinase in cell cycle-regulated nuclear import of the S. cerevisiae transcription factor SWI5. Cell 1991;66:743–58.
Montagnoli A, Valsasina B, Brotherton D, Troiani S, Rainoldi S, Tenca P, et al. Identification of Mcm2 phosphorylation sites by S-phase-regulating kinases. J Biol Chem 2006;281:10281–90.
Moore JD, Kirk JA, Hunt T. Unmasking the S-phase-promoting potential of cyclin B1. Science 2003;300:987–90.
Morgan DO. Cyclin-dependent kinases: engines, clocks, and microprocessors. Annu Rev Cell Dev Biol 1997;13:261–91.
Mori D, Yano Y, Toyo-oka K, Yoshida N, Yamada M, Muramatsu M, et al. NDEL1 phosphorylation by Aurora-A kinase is essential for centrosomal maturation, separation, and TACC3 recruitment. Mol Cell Biol 2007;27:352–67.
Morisaki T, Hirota T, Iida S, Marumoto T, Hara T, Nishiyama Y, et al. WARTS tumor suppressor is phosphorylated by Cdc2/cyclin B at spindle poles during mitosis. FEBS Lett 2002;529:319–24.

Mortensen EM, Haas W, Gygi M, Gygi SP, Kellogg DR. Cdc28-dependent regulation of the Cdc5/Polo kinase. Curr Biol 2005;15:2033–7.
Moses AM, Heriche JK, Durbin R. Clustering of phosphorylation site recognition motifs can be exploited to predict the targets of cyclin-dependent kinase. Genome Biol 2007;8:R23.
Newbell BJ, Anderson JT, Jarrett HW. Ca2+-calmodulin binding to mouse alpha1 syntrophin: syntrophin is also a Ca2+-binding protein. Biochemistry 1997;36:1295–305.
Nie Z, Stanley KT, Stauffer S, Jacques KM, Hirsch DS, Takei J, et al. AGAP1, an endosome-associated, phosphoinositide-dependent ADP-ribosylation factor GTPase-activating protein that affects actin cytoskeleton. J Biol Chem 2002;277:48965–75.
Nissen MS, Langan TA, Reeves R. Phosphorylation by cdc2 kinase modulates DNA binding activity of high mobility group I nonhistone chromatin protein. J Biol Chem 1991;266:19945–52.
Noguchi E, Shanahan P, Noguchi C, Russell P. CDK phosphorylation of Drc1 regulates DNA replication in fission yeast. Curr Biol 2002;12:599–605.
Nousiainen M, Sillje HH, Sauer G, Nigg EA, Korner R. Phosphoproteome analysis of the human mitotic spindle. Proc Natl Acad Sci U S A 2006;103:5391–6.
O'Connor DS, Grossman D, Plescia J, Li F, Zhang H, Villa A, et al. Regulation of apoptosis at cell division by p34cdc2 phosphorylation of survivin. Proc Natl Acad Sci U S A 2000;97:13103–7.
Ohsugi M, Tokai-Nishizumi N, Shiroguchi K, Toyoshima YY, Inoue J, Yamamoto T. Cdc2-mediated phosphorylation of Kid controls its distribution to spindle and chromosomes. EMBO J 2003;22:2091–103.
Ohtsubo M, Theodoras AM, Schumacher J, Roberts JM, Pagano M. Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. Mol Cell Biol 1995;15:2612–24.
Okuda M, Horn HF, Tarapore P, Tokuyama Y, Smulian AG, Chan PK, et al. Nucleophosmin/B23 is a target of CDK2/cyclin E in centrosome duplication. Cell 2000;103:127–40.
Ostvold AC, Norum JH, Mathiesen S, Wanvik B, Sefland I, Grundt K. Molecular cloning of a mammalian nuclear phosphoprotein NUCKS, which serves as a substrate for Cdk1 in vivo. Eur J Biochem 2001;268:2430–40.
Pagano M, Pepperkok R, Verde F, Ansorge W, Draetta G. Cyclin A is required at two points in the human cell cycle. EMBO J 1992;11:961–71.
Peeper DS, Parker LL, Ewen ME, Toebes M, Hall FL, Xu M, et al. A- and B-type cyclins differentially modulate substrate specificity of cyclin-cdk complexes. EMBO J 1993;12:1947–54.
Peter M, Heitlinger E, Haner M, Aebi U, Nigg EA. Disassembly of in vitro formed lamin head-to-tail polymers by CDC2 kinase. EMBO J 1991;10:1535–44.
Peter M, Nakagawa J, Doree M, Labbe JC, Nigg EA. In vitro disassembly of the nuclear lamina and M phase-specific phosphorylation of lamins by cdc2 kinase. Cell 1990;61:591–602.
Petersen BO, Lukas J, Sorensen CS, Bartek J, Helin K. Phosphorylation of mammalian CDC6 by cyclin A/CDK2 regulates its subcellular localization. EMBO J 1999;18:396–410.
Pines J, Hunter T. The differential localization of human cyclins A and B is due to a cytoplasmic retention signal in cyclin B. EMBO J 1994;13:3772–81.
Queralt E, Lehane C, Novak B, Uhlmann F. Downregulation of PP2A(Cdc55) phosphatase by separase initiates mitotic exit in budding yeast. Cell 2006;125:719–32.
Remus D, Diffley JF. Eukaryotic DNA replication control: lock and load, then fire. Curr Opin Cell Biol; 2009.
Russo AA. Purification and reconstitution of cyclin-dependent kinase 2 in four states of activity. Meth Enzymol 1997;283:3–12.
Sarcevic B, Mawson A, Baker RT, Sutherland RL. Regulation of the ubiquitin-conjugating enzyme hHR6A by CDK-mediated phosphorylation. EMBO J 2002;21:2009–18.
Satterwhite LL, Lohka MJ, Wilson KL, Scherson TY, Cisek LJ, Corden JL, et al. Phosphorylation of myosin-II regulatory light chain by cyclin-p34cdc2: a mechanism for the timing of cytokinesis. J Cell Biol 1992;118:595–605.
Saville MK, Watson RJ. The cell-cycle regulated transcription factor B-Myb is phosphorylated by cyclin A/Cdk2 at sites that enhance its transactivation properties. Oncogene 1998;17:2679–89.
Sheaff RJ, Groudine M, Gordon M, Roberts JM, Clurman BE. Cyclin E-CDK2 is a regulator of p27Kip1. Genes Dev 1997;11:1464–78.
Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 1999;13:1501–12.
Shorter J, Warren G. Golgi architecture and inheritance. Annu Rev Cell Dev Biol 2002;18:379–420.
Shuman JD, Sebastian T, Kaldis P, Copeland TD, Zhu S, Smart RC, et al. Cell cycle-dependent phosphorylation of C/EBPbeta mediates oncogenic cooperativity between C/EBPbeta and H-RasV12. Mol Cell Biol 2004;24:7380–91.
Smith A, Simanski S, Fallahi M, Ayad NG. Redundant ubiquitin ligase activities regulate wee1 degradation and mitotic entry. Cell Cycle 2007;6:2795–9.
Solomon MJ, Lee T, Kirschner MW. Role of phosphorylation in p34cdc2 activation: identification of an activating kinase. Mol Biol Cell 1992;3:13–27.
Songyang Z, Blechner S, Hoagland N, Hoekstra MF, Piwnica-Worms H, Cantley LC. Use of an oriented peptide library to determine the optimal substrates of protein kinases. Curr Biol 1994;4:973–82.
St Onge RP, Besley BD, Pelley JL, Davey S. A role for the phosphorylation of hRad9 in checkpoint signaling. J Biol Chem 2003;278:26620–8.
Stukenberg PT, Lustig KD, McGarry TJ, King RW, Kuang J, Kirschner MW. Systematic identification of mitotic phosphoproteins. Curr Biol 1997;7:338–48.
Takeda DY, Wohlschlegel JA, Dutta A. A bipartite substrate recognition motif for cyclin-dependent kinases. J Biol Chem 2001;276:1993–7.
Thiel DA, Reeder MK, Pfaff A, Coleman TR, Sells MA, Chernoff J. Cell cycle-regulated phosphorylation of p21-activated kinase 1. Curr Biol 2002;12:1227–32.
Tokuyama Y, Horn HF, Kawamura K, Tarapore P, Fukasawa K. Specific phosphorylation of nucleophosmin on Thr(199) by cyclin-dependent kinase 2-cyclin E and its role in centrosome duplication. J Biol Chem 2001;276:21529–37.
Trzepacz C, Lowy AM, Kordich JJ, Groden J. Phosphorylation of the tumor suppressor adenomatous polyposis coli (APC) by the cyclin-dependent kinase p34. J Biol Chem 1997;272:21681–4.

Tsai LH, Harlow E, Meyerson M. Isolation of the human cdk2 gene that encodes the cyclin A- and adenovirus E1A-associated p33 kinase. Nature 1991;353:174–7.
Ubersax JA, Woodbury EL, Quang PN, Paraz M, Blethrow JD, Shah K, et al. Targets of the cyclin-dependent kinase Cdk1. Nature 2003;425:859–64.
Valenzuela-Fernandez A, Cabrero JR, Serrador JM, Sanchez-Madrid F. HDAC6: a key regulator of cytoskeleton, cell migration and cell-cell interactions. Trends Cell Biol 2008;18:291–7.
van der Sluijs P, Hull M, Huber LA, Male P, Goud B, Mellman I. Reversible phosphorylation–dephosphorylation determines the localization of rab4 during the cell cycle. EMBO J 1992;11:4379–89.
Voit R, Hoffmann M, Grummt I. Phosphorylation by G1-specific cdk-cyclin complexes activates the nucleolar transcription factor UBF. EMBO J 1999;18:1891–9.
Watanabe N, Arai H, Iwasaki J, Shiina M, Ogata K, Hunter T, et al. Cyclin-dependent kinase (CDK) phosphorylation destabilizes somatic Wee1 via multiple pathways. Proc Natl Acad Sci U S A 2005;102:11663–8.
Wolfe BA, McDonald WH, Yates 3rd JR, Gould KL. Phospho-regulation of the Cdc14/Clp1 phosphatase delays late mitotic events in S. pombe. Dev Cell 2006;11:423–30.
Wong OK, Fang G. Cdk1 phosphorylation of BubR1 controls spindle checkpoint arrest and Plk1-mediated formation of the 3F3/2 epitope. J Cell Biol 2007;179:611–7.
Yoon HJ, Feoktistova A, Chen JS, Jennings JL, Link AJ, Gould KL. Role of Hcn1 and its phosphorylation in fission yeast anaphase-promoting complex/cyclosome function. J Biol Chem 2006;281:32284–93.
You J, Li Q, Wu C, Kim J, Ottinger M, Howley PM. Regulation of aurora B expression by the bromodomain protein Brd4. Mol Cell Biol 2009;29:5094–103.
Yuce O, Piekny A, Glotzer M. An ECT2-centralspindlin complex regulates the localization and function of RhoA. J Cell Biol 2005; 170:571–82.
Yudkovsky Y, Shteinberg M, Listovsky T, Brandeis M, Hershko A. Phosphorylation of Cdc20/fizzy negatively regulates the mammalian cyclosome/APC in the mitotic checkpoint. Biochem Biophys Res Commun 2000;271:299–304.
Yun J, Chae HD, Choi TS, Kim EH, Bang YJ, Chung J, et al. Cdk2-dependent phosphorylation of the NF-Y transcription factor and its involvement in the p53-p21 signaling pathway. J Biol Chem 2003;278:36966–72.
Zachariae W, Nasmyth K. Whose end is destruction: cell division and the anaphase-promoting complex. Genes Dev 1999;13: 2039–58.
Zhao J, Dynlacht B, Imai T, Hori T, Harlow E. Expression of NPAT, a novel substrate of cyclin E-CDK2, promotes S-phase entry. Genes Dev 1998;12:456–61.
Zhu Y, Lu D, DiMario P. Nucleolin, defective for MPF phosphorylation, localizes normally during mitosis and nucleologenesis. Histochem Cell Biol 1999;111:477–87.