Chapter 14

Phosphorylation Mediated Regulation of Cdc25 Activity, Localization and Stability

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1. Introduction

Dual specificity phosphatases of the Cdc25 family are critically important regulators of the cell cycle. They activate cyclin-dependent kinases (CDKs) at key cell cycle transitions such as the initiation of DNA synthesis and mitosis. They also represent key points of regulation for pathways monitoring DNA integrity, DNA replication, growth factor signaling and extracellular stress. Since their mis-regulation allows cells to function in a genetically unstable state, it is not surprising that these phosphatases are involved in transformation to a cancerous state. Cdc25 phosphatases are heavily regulated by phosphorylation. Many regulatory phosphorylation sites on Cdc25 influence catalytic activity, substrate specificity, subcellular localization and stability. This chapter summarizes the current literature on the phospho-regulation of these proteins.

2. Yeast genetics and Xenopus oocyte maturation – Setting the stage

The study of cell division in eukaryotes was dramatically changed with the isolation of temperature sensitive "cell division cycle" (*cdc*$^-$) mutants of the yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. These mutants arrested uniformly at a particular cell cycle stage and uncoupled cell growth from cell cycle progression.[1-3] *S. pombe* cells are cylindrical cells growing from the tips while keeping a constant diameter.[3] At the restrictive temperature, fission yeast *cdc*$^-$ mutants arrest at their restriction point and become abnormally elongated. Within the fission yeast *cdc*$^-$ collection was the *cdc25-22* mutation which arrests at mitotic entry when incubated at its restrictive temperature.[3] The collection also included *cdc9-50* which divided at approximately half the length of a wildtype cell; it was later renamed *wee1-50 i*n reference to its small size.[4] The *wee1-50* mutation suppresses the cell cycle phenotype of *cdc25-22* demonstrating that these two proteins act in opposition during the G2/M transition.[5] Analysis of these mutations showed that Cdc2 is rate limiting

for mitotic entry and is inhibited by Wee1 and activated by Cdc25.[6,7] Regulation of Cdc2 by Wee1 and Cdc25 in fission yeast was one of the first connected pathways in cell cycle research in any organism. The gene names Cdc25 and Wee1 are used in almost all organisms today.

Concurrently, Maturation Promoting Factor (MPF) was discovered through a series of elegant cytoplasmic transfer experiments conducted on frog, starfish and sea urchin oocytes [8-10]. Immature *Xenopus* oocytes arrest at prophase I. Progesterone induced MPF activation induces maturation whereby oocytes complete meiosis I and arrest at meiotic metaphase II.[9,11] Microinjection of cytoplasm from a mature oocyte into an immature oocyte also induces maturation.[8,9] This bioassay for maturation promoting factor (MPF) activity allowed it to be tracked through the meiotic and later mitotic cycles. Activity falls after anaphase I, rising again as cells enter prophase II. Although MPF activity is high, mature oocytes arrest due to the presence of a second soluble factor called Cyto-Static Factor (CSF).[9] Subsequent entry of the sperm nucleus causes an influx of extracellular calcium, ultimately removing CSF inhibition and serving as the trigger for completion of meiosis and the start of mitotic divisions. Extracts from prophase II arrested mature oocytes can be induced to undergo alternating DNA synthesis and mitotic phases by the addition of calcium, providing an excellent cell free system for studying MPF regulation.[12] A peak of MPF activity accompanies each round of mitosis, reaching maximal activity during meiotic prophase, followed by a catastrophic drop as chromosomes segregate at anaphase. Fertilization is followed by a rapid succession of 12 rounds of DNA synthesis and cell division with no intervening gap phases, driven solely by maternally derived mRNA.[13] Gap phases are re-established at the mid-blastula transition followed by typical somatic cell cycles.[14] MPF consists of three proteins: Cdk1 kinase, cyclin B (Cdc2 [19-21] and Cdc13 [22,23] respectively in fission yeast) and the small regulatory protein Suc1.[15-19] The activator of both MPF in *Xenopus* and the Cdc2-Cdc13 complex in *S. pombe* is Cdc25 phosphatase.[24,25]

The cytoplasmic transfer experiments showed that MPF activity correlates with increased protein phosphorylation in donor oocytes [26], targeting a large number of nuclear proteins.[27,28] MPF is a histone H1 kinase, an activity which is still used today to measure CDK function.[29] MPF induces many of the cytological changes associated with mitosis such as nuclear envelope breakdown, chromosome condensation and mitotic spindle formation.[30-32] Mass spectrometry has shown that the single Cdk1 homologue in budding yeast (CDC28) phosphorylates 547 sites on 308 proteins *in vivo*.[33] The regulation of only about 75 of these CDC28 substrates has been examined in any detail.[34] Undoubtedly, the network of CDK mediated phosphorylation events in vertebrate cells will turn out to be far more complex.

3. Taxonomic distribution, duplication and divergence of Cdc25 homologues

Cdc25 is present in all eukaryotic cells. The yeasts, *S. cerevisiae* and *S. pombe*, possess a single Cdc25 protein (referred to as MIH1 in the former).[35] Duplication and divergence

led to three Cdc25 paralogues in vertebrates, Cdc25A, Cdc25B, and Cdc25C.[36,36-40] Cdc25A acts early in the cell cycle, regulating the G1/S transition, whereas Cdc25B and Cdc25C act at G2/M. Human Cdc25A, Cdc25B and Cdc25C have several isoforms through alternative splicing.[41-44] Cdc25C was discovered based on sequence similarity to fission yeast Cdc25 by using degenerate PCR primers to conserved residues in the catalytic region.[38] Human Cdc25A and Cdc25B were cloned by complementation of the temperature sensitive *cdc25-22^{ts}* allele of *S. pombe,* demonstrating the strong conservation of function in this protein family.[37] *Xenopus* Cdc25A and Cdc25C were initially discovered based on sequence similarity to human Cdc25C.[40,45] Injection of recombinant *Xenopus* Cdc25A or Cdc25C into oocytes induces their maturation, the first direct indication that this protein was a positive regulator of MPF.[40,45,46] *Xenopus* Cdc25C also complements the *cdc25-22^{ts}* mutation in fission yeast.[45] Four Cdc25 homologues are present in *Caenorhabditis* [47], whereas *Drosophila* contains two, *string* and *twine.*[48,49] The conserved C-terminal domain of *string* contains the intrinsic phosphatase activity against a Cdk1-derived peptide containing phosphorylated Y15 and against the synthetic substrate *p*-nitrophenyl phosphate.[50] Few plant Cdc25 homologues can be found by sequence comparison in the NCBI database and those only in unicellular algae, eg *Ostreococcus tauri*.[51] In *Arabidopsis thaliana* [52] the full length protein is only 146 residues long representing only the phosphatase domain and it shows marginal similarity to vertebrate and fission yeast Cdc25. Overexpression of *A. thaliana* Cdc25 causes premature mitotic entry in fission yeast.[53]

4. Regulation of cell cycle transitions by Cdc25

4.1. The fission yeast G2/M transition – The prototype Cdc25/cyclin-CDK circuit

Regulation of the transition from G2 to mitosis in *S. pombe* is typical of the positive feedback loops seen between Cdc25 and CDKs in all systems. Cdc2 (Cdk1) drives all cell cycle transitions in fission yeast.[20] During G2 and early mitosis Cdc2 complexes with the B-type cyclin Cdc13 [54,55], the only essential cyclin in fission yeast.[56] Whereas Cdc2 is constitutively expressed throughout the cell cycle [57], Cdc25 and Cdc13 begin to accumulate in G2 and are degraded during mitotic entry.[58-60] Prior to M-phase, the Cdc2-Cdc13 complex is kept inactive by phosphorylation of threonine 14 (T14) and tyrosine 15 (Y15).[61,62] Y15 is phosphorylated by the S-phase specific kinase Mik1 [63,64] and T14 and Y15 are modified by Wee1.[63,65-67] In G2 Cdc25 translocates to the nucleus via the importin-β homologue Sal3.[68] After cells grow to a critical size for mitotic entry, Cdc25 becomes active and dephosphorylates Cdc2 Y15.[69] Cdc25 is activated and hyperphosphorylated by Cdc2 as the cell enters mitosis.[58, 70] CDKs phosphorylate a serine or threonine residue in the context of a consensus site (S/T)PX(K/R).[71,72] The positions of the Cdc2 phosphorylation sites on Cdc25 have not been determined, but mutagenesis of 15 potential CDK consensus sites abrogates phosphorylation by Cdc2-Cdc13.[73] In *S. pombe*, the feedback loop between Cdc25 and Cdc2-Cdc13 is strengthened by the involvement of the *S. pombe* Polo kinase homologue Plo1 which phosphorylates Cdc25 downstream of Cdc2 activation.[74] After the metaphase-anaphase transition, Polo

kinase activation encourages cyclin degradation through activation of the anaphase promoting complex (APC).[75] Thus, this kinase simultaneously ensures that Cdc2 activation will be robust, and brief, as its cyclin partner Cdc13 is degraded by the APC immediately following anaphase initiation.

4.2 Vertebrate cell cycle

While fission yeast Cdc25 is solely involved in the G2/M transition, Cdc25 orthologues in vertebrates also play a role in G1 and S-phase progression. Vertebrates have several CDK-cyclin complexes which participate in these transitions through positive feedback loops with Cdc25. In addition to associating with cyclin B, Cdk1 associates with cyclin A during late S-phase and G2.[76] A second CDK, Cdk2, was discovered as a cDNA which could complement the loss of the budding yeast Cdk1 homologue, CDC28.[77] Cdk2 functions early in the cell cycle and is likewise negatively regulated by phosphorylation of Y15.[78,79] It forms a complex with Cyclin A and Cyclin E.[80,81] Cdk4 and Cdk6 operate early in G1 in association with D-type cyclins.[82] In many cases a particular cyclin class (ie. A, B, D, E) has multiple members. For the sake of clarity, cyclins will be referred to by their subtype only. Furthermore, only the CDKs and cyclins directly responsible for cell cycle transitions in concert with Cdc25 orthologues will be discussed. For instance, Cdk1/cyclin B is activated by a CDK-Activating Kinase (CAK), a complex of Cdk7 and cyclin H [83], but as CAKs are not activated by Cdc25 they are outside the scope of this review.

4.2.1. Cell cycle re-entry from G_o

Most somatic cells spend their time in G_o. Cells in G_o may commit to entry into the cell cycle when they receive stimuli in the form of growth factors The cells then deactivate the cell cycle repressors which have kept them in G_o and transcribe the positive regulators of the next cell cycle transition, G1/S.

In non-dividing cells, the Retinoblastoma protein (Rb) binds to E2F thus preventing transcription of genes required for cell cycle progression including cyclin D, cyclin A and Cdc25A.[84-87] (Figure 1) After exposure to growth factors, cells in G_o re-enter the cell cycle through activation of the Ras pathway via the Raf/MAP (Mitogen Activated Protein) kinase pathway.[88] This leads to the degradation of the Cdk4 inhibitors $p15^{INK4B}$ and $p16^{INK4A}$ and the Cdk2 inhibitors $p27^{KIP}$, $p21^{CIP}$ and induction of cyclin D. Cdk4 and Cdk6 bound to cyclin D inhibit the Rb protein [89] allowing transcription of cyclin E and cyclin A.[89,90] Cdc25A activates Cdk4-cyclin D but not Cdk6-cyclin D *in vitro*.[91] Cyclin E associates with Cdk2 in late G1 and helps complete the inhibition of Rb.[89,92]

4.2.2. The G1/S transition

Cdc25A is transcribed following relief of Rb-mediated transcriptional repression, reaching its maximal level at the end of G1 and dephosphorylating Y15 on Cdk2.[87,91] Cdk2 is phosphorylated on Y15 by Wee1.[93] Dephosphorylation of Cdk2 takes place via both

Cdc25A and Cdc25B.[94-97] Cdk2 immunoprecipitated from cell lysates where Cdc25A has been overexpressed has high histone H1 kinase activity and low levels of Y15 phosphorylation.[91] Such overexpression accelerates entry into S-phase through activation of Cdk2-cyclin E.[91,98] DNA-synthesis can be blocked in these cells by injecting them with anti-Cdc25A antibodies.[99] Cdk2-cyclin E and Cdc25A are mutually activated by a positive feedback loop allowing passage of the G1/S boundary.[95] Depletion of Cdk2 or cyclin E prevents phosphorylation of Cdc25A and recombinant Cdc25A can be activated by Cdk2-cyclin E *in vitro*.[95] In addition phosphorylation of Cdc25A by Cdk2 destabilizes the phosphatase. Conversely, exposure of cells to CDK inhibitors roscovitine and olomoucine causes stabilization of Cdc25A.[99]

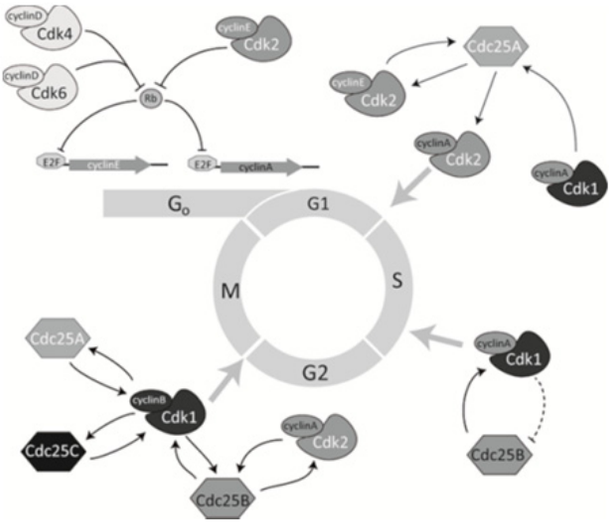

Figure 1. Positive feedback loops between Cdk-cyclin complexes and Cdc25 family members drive cell cycle transitions.

S-phase initiation requires activation of DNA replication proteins by Cdk2-cyclin A. Injecting G1 cells with anti-cyclin A antibodies stops entry into S-phase.[100] Phosphorylation of the essential DNA replication initiator Cdc6 by Cdk2-cyclin A leads to its nuclear import.[101,102] Cdk2 is recruited to chromatin by the replication initiation factor Cdc45 where it phosphorylates histone H1 and induces chromosome de-condensation.[103] As S-phase progresses high Cdk2-cyclin A activity induces degradation of Cdc6, preventing re-initiation of DNA synthesis at origins which have already fired.[102]

Cdc25B has a role late in S-phase as Cdc25B immunoprecipitated from late S-phase HeLa cell extracts is phosphorylated, activated, and able to dephosphorylate Cdk2-cyclin A.[97] The murine homologue of Cdc25B purified from S-phase extracts promotes cyclin A and cyclin E associated histone H1 kinase activity *in vitro*.[94] In addition, siRNA knockdown of human Cdc25B causes a delay in initiation of DNA synthesis.[104] The Cdk1-cyclin A complex regulates late origin firing in S-phase. A constitutively activated Cdk1 allele increases firing of late S-phase origins, whereas a loss of function temperature sensitive allele of Cdk1 has a defect in late origin firing at the restrictive temperature.[76]

4.2.3. The G2/M transition

Unlike the simple circuit of Cdc25-Cdc2 activation in fission yeast, the vertebrate G2/M transition involves a series of interconnected loops with positive and negative inputs from a variety of pathways. This led to what could be considered the "traditional model" of G2/M transition in vertebrates with respect to Cdc25 regulation by CDK-cyclin complexes. In reality things may be more complex. Cdc25C is not explicitly required for mitotic entry; siRNA knockdown of Cdc25C does not prevent the G2/M transition.[105] In addition, mouse lines which lack Cdc25B and/or Cdc25C are viable with the only obvious phenotype being a defect in oocyte maturation observed in *cdc25B*$^{-/-}$ mice.[106-108] In addition, the creation of CDK and cyclin knockout mice has revealed a network of compensatory mechanisms which cloud the traditional model. Cdk2$^{-/-}$, Cdk4$^{-/-}$, or Cdk6$^{-/-}$ mice show some abnormalities as adults, but are not embryonic lethal indicating that these Cdks are not individually essential for cell division.[92] There is considerable redundancy. Mitotic entry is best viewed as a three step process. First, Cdc25B and CDK-cyclin A are activated followed by basal activation of Cdk1-cyclin B at the centrosome.[109] Second, Cdk1-cyclinB, Cdc25B and Cdc25C localize to the nucleus. Third, Cdk1-cyclin B and Cdc25C mutually activate. Cdk1-cyclin B then induces overt mitotic events such as breakdown of the nuclear envelope and spindle formation. [81,110,111]

4.2.4. Activation of Cdc25B and Cdk1/2-cyclinA

Phosphorylation of human Cdc25B by Cdk1-cyclin A during G2 causes Cdc25B activation but also destabilizes the protein.[112,113] Cdc25B activates Cdk2-cyclinA in a positive feedback loop.[114] Cdk2-cyclin A mediated destabilization of Cdc25B has not been reported, although the Cdk1 and Cdk2 kinase complexes modify Cdc25B to approximately the same degree and have a similar set of substrates.[113,115] However, Cdk2 is the preferred binding partner of cyclin A and is more active than Cdk1-cyclin A during G2.[80,100] Cdk2 cyclin A has two peaks of activation, one during S-phase and one prior to G2/M. [80,116] Inhibition of Cdk2-cyclin A delays mitotic entry.[109] Depletion of Cdk1-cyclin A, activation of the CDK inhibitor p21^{cip} , or addition of an inhibitory ATP analogue destabilizes Cdc25B in *Sf9* cell extracts.[112] Similarly, in cycloheximide treated cells Cdc25B, but not Cdc25A or Cdc25C, is unstable and uniquely labile during the cell cycle.[117]

Cyclin B accumulates throughout G2 but Cdc25A and Cdc25B are required to induce formation of the Cdk1-cyclin B complex at G2/M.[118] Cdk1-cyclin B interaction occurs earlier in G2 when Cdc25A or Cdc25B are overexpressed. Cdk1 and cyclin B are almost exclusively cytoplasmic during interphase with a small portion of the complex associating with the centrosome at G2/M.[119-121] A population of Cdk2-cyclinA is likewise localized to the centrosomes prior to prophase.[116] In *Xenopus*, centrosomal localization of Cdk1-cyclin B requires Aurora kinase.[122] Aurora is involved in a diverse set of mitotic events such as spindle assembly, centrosome maturation, and cytokinesis.[123] Aurora can also phosphorylate Cdc25B at S353 *in vivo*, activating the phosphatase.[124] (Table 1) Although

some Cdk1-cyclin B is activated by Cdc25B [120,125], this does not represent a full mitotic activation and is only sufficient for progression as far as prophase. Injection of a dominant-negative mutant of Cdc25B into HeLa cells causes arrest in prophase with condensed chromosomes and disassembled nucleoli, but without nuclear envelope breakdown [109]. Microinjection of Cdk1-cyclin B lets cells pass this block and complete mitosis.

Species	Member	Kinase	Site(s)	References
S. pombe	Cdc25	Cds1/Chk1/Srk1	S99, S148, S178, S192, S204, S206, T226, S234, S359, T561, S567, T569	[60, 156, 157, 162]
Human	Cdc25A	Cdk1-cyclin B	S18, S40, S88, S116, S261, S283, S321	[149, 151, 167]
		Chk1	S76, S124, S178, T507	[168-173, 200]
		Chk2	S124, S278	[173, 175]
		Casein Kinase 1ε	S82	[199]
		Casein Kinase 1α	S79, S82	[198]
		p38	S76, S124	[168, 175]
		GSK-3β	S76	[193, 194]
		NEK11	S82, S88	[187]
		Plk3	S80	[193, 194]
	Cdc25B*	Cdk1-cyclinB	S50, S160, S321	[135, 136, 246]
		Chk1	S151, S230, S323, S563	[236, 238, 239]
		Aurora	S353	[124]
		Casein Kinase 2	S186, S187	[235]
		JNK	S101, S103	[280]
		MEK/ERK	S249	[259]
		p38	S323	[208]
		MK2	S323	[210]
		Plk1	T167, S209, T404, S465	[136]
	Cdc25C	Cdk1/cyclin B	T48, T67, S122, T130, S168, S214	[42, 143, 145, 246]
		Chk1	S216, S247, S263	[219-221, 225, 226]
		Chk2	S216	[209, 242]
		Casein Kinase 2	T236	[234]
		MK2	S216	[210]
		C-TAK1	S216	[276]
		JNK	S168	[281, 282]
		MEK/ERK	S216	[278]
		Plk1	S198	[140]
		Plk3	S191, S198	[141]
Xenopus	Cdc25A	Chk1	S73, T504	[201, 207]
	Cdc25C	Cdk1/cylinB Cdk1-cyclinA	T48, T67, T138, S205, S285;	[193, 247, 249]
		Chk1	S287, T533	[207, 229-231, 241]
		p42	S48, T138, S205	[272]
		$p90^{rsk}$	S287	[269, 270]
		Rsk2	S317, S318, S319	[271]

Table 1. Summary of known phosphorylation sites on Cdc25 family members and the kinases responsible

4.2.5. Everybody into the nucleus

Cyclin B has a cytoplasmic retention sequence which is sufficient to induce cytoplasmic localization of the normally nuclear protein and contains a nuclear export signal (NES).[126]

Nuclear export is blocked by phosphorylation of S126 at the end of prophase.[127-129] Cyclin B is phosphorylated by Cdk1-Cyclin B as starfish oocytes pass the prophase II to metaphase II arrest.[130] Human cyclin B S126 is followed by a proline residue suggesting Cdk1-cyclin B autophosphorylation. Cyclin B is phosphorylated by the *Xenopus* Polo kinase homologue Plx1 on S133 and S147 (homologous to human cyclin B S177 and S181) enhancing its nuclear import.[125,131] Initial Cdc25B mediated activation of Cdk1-cyclin B may result in a priming autophosphorylation of S126 followed by docking of Polo kinase and inhibition of nuclear export. Such a Cdk1 mediated priming phosphorylation has been shown to induce Plx1 phosphorylation of Cdc25B (see below). Human Polo kinase Plk1 also deactivates Cdk1-cyclin B inhibitory kinases. Plk1 inhibits the cytoplasmic Cdk1 inhibitory kinase Myt1.[132,133] Wee1 is phosphorylated on S53 and S123 by Plk1 and Cdk1-cyclin B, respectively, resulting in its degradation by β-TrCP.[134]

The activated cytoplasmic pool of Cdk1-cyclin B phosphorylates Cdc25B on S160 inducing its nuclear import. [135] (All phosphorylated residues on human Cdc25B are numbered according to the sequence of the longest splice variant Cdc25B3). Overexpression of human Cdc25B causes an increase in cells with condensed chromatin, whereas overexpression of Cdc25B-S160G does not induce mitotic entry. S160 phosphorylation does not affect the *in vitro* activity of human Cdc25B against a fluorescein diphosphate substrate, but instead positively regulates its nuclear import. *In vitro* phosphorylation assays show Cdk1-cyclin B can phosphorylate residues on Cdc25B which are not targeted by Cdk1-cyclin A or Cdk2-cyclin A.[113] Interestingly, Cdk1-cyclin A cannot phosphorylate Cdc25B that has previously been phosphorylated by Cdk1-cyclin B.[112]

Plk1 is involved in human Cdc25B nuclear import following the initial activation of Cdk1-cyclin B. After addition of the Plk1 inhibitor thiophene benzimidazole, nuclear accumulation of GFP-Cdc25B is reduced. Conversely, expression of a constitutively active Plk1 mutant enhances Cdc25 nuclear localization.[136] Co-overexpression of Plk1 and Cdc25B in U2OS osteosarcoma cells induces chromosome condensation to a greater degree compared to cells expressing Plk1 or Cdc25B alone. This is partially dependent on the presence of a functional nuclear localization signal (NLS) in Cdc25B. Plk1 docking to Cdc25B requires prior phosphorylation of S50 by Cdk1/cyclin B.[137] Mass spectrometry identified thirteen phosphorylated Plk1 sites on Cdc25B *in vitro* and showed that T167, S209, T404, S465 and S513 appear to be particularly strong targets.[137] Plk1 is required for Cdk1-cyclin B activation, at least in part by negatively regulating Cdc25C nuclear export. Depletion of Plx1 from oocyte extracts prevents the activation of Cdc25C and Cdk1-cyclin B.[138] Unlike Cdc25B, phosphorylation of Cdc25C by Cdk1 is not a prerequisite for Plk1 targeting the phosphatase; Plk1 affects Cdc25C phosphatase activity and its localization. *In vitro* phosphorylation of Cdc25C enhances its ability to dephosphorylate kinase dead Cdc2 on Y15.[139] Plk1 phosphorylates human Cdc25C on S198 which resides within the nuclear export signal (NES) of the phosphatase, promoting its nuclear localization.[140] Polo kinase family member Plk3 also interacts with human Cdc25C and phosphorylates it on S191, and S198 to a lesser degree.[141] Substitutions S191D, S198D or Plk3 overexpression result in constitutive nuclear localization, while siRNA knockdown of Plk3 or substitutions S191A

and S198A leads to nuclear exclusion.[141] Another polo family member, Plk4, phosphorylates human Cdc25C on undetermined sites.[142]

4.2.6. Full activation of Cdk1-CyclinB and Cdc25C

Activated Cdk1-cyclin B phosphorylates human Cdc25C on T48, T67, S122, T130, S168 and S214 *in vitro* and *in vivo*, driving a positive feedback loop which culminates in the phosphorylation of mitotic Cdk1 substrates.[42,143] Cdk1 phosphorylated Cdc25C has an increased Cdk1 Y15 phosphatase activity.[143] Recombinant Cdc25C can activate Cdk1 thereby increasing its histone H1 kinase activity.[144] Use of phospho-specific antibodies recently showed that phosphorylation of T48, T67 and T130 occur on spatially separate pools of human Cdc25C.[145] T67-phosphorylated Cdc25C is chromatin associated from prophase until telophase while T130-phosphorylated Cdc25C localizes to the centrosomes. T130 phosphorylation creates a Plk1 binding site on Cdc25C. Three distinct pools of T48, T67 and T130 mono-phosphorylated Cdc25C protein can be detected by immunoprecipitation with each individual phospho-specific antibody; Cdc25C pulled down with one antibody is not bound by the other two. In *Xenopus* oocyte extracts Cdc25C is heavily phosphorylated while cells undergo germinal vesicle breakdown (meiosis I).[45] *Xenopus* Cdc25C residues T48, T67, T138, S205 and S285 are phosphorylated by Cdk1-cyclin A and Cdk1-cyclin B *in vitro*.[146] Mutation of the major targets, T48, T67 and T138, to alanine prevents *Xenopus* Cdc25C activation *in vitro*.[147] Although G2/M is not normally associated with Cdc25A function, it also contributes to Cdk1-cyclin B activation. Depleting cells of Cdc25A reduces Cdk1-cyclin B activation by approximately fifty percent.[148] In cells arrested in mitosis by addition of nocodazole, a microtubule polymerization inhibitor and spindle poison, Cdc25A is phosphorylated by Cdk1-cyclin B on S18 and S116. Substitution of these residues with alanine leads to Cdc25A instability. Cdc25A S40, S88, S261 and S283 are also Cdk1-cyclin B phosphorylated *in vitro*.[149]

4.2.7. Mitotic exit

Following chromosome alignment on the metaphase plate a cascade of APC mediated degradation events occurs to reset conditions for the start of the next cell cycle.[150] The APC regulates two important processes required for completion of mitosis. First, it targets Securin, the inhibitory subunit of Separase, which is responsible for Cohesin cleavage and chromosome separation. Second, it targets cyclin A and cyclin B for destruction, inactivating Cdk1. Cyclin B is degraded after the metaphase-anaphase transition while cyclin A is degraded during metaphase. [121] Cdk1 mediated phosphorylation of Cdc25 paralogues is reversed by Cdc14 family phosphatases. Cdk1-cyclin B phosphorylates human Cdc25A S18, S40, S88, S116, S261 and S283 *in vitro*.[149] Of these sites, human Cdc14A dephosphorylates S116, Cdc14B targets S88 and S261, while both phosphatases can dephosphorylate S40. Cdc14A was independently identified as also dephosphorylating Cdc25A S321.[151] siRNA knockdown of Cdc14B leads to accumulation of phosphorylated Cdc25B and Cdc25C.[149] In *S. pombe* Cdc14 homologue Clp1 dephosphorylates Cdc25 and this is required for its ubiquitination and APC mediated proteolysis at the end of mitosis.[73] Similarly, in vertebrates Cdc25A and Cdc25B are targeted by the APC at mitotic exit.[152,153]

5. Cdc25 phosphorylation by the DNA damage and replication checkpoint

DNA damage causes activation of checkpoints which delay cell cycle transitions to allow sufficient time for repair. Stalling of replication forks causes a similar cell cycle arrest, with additional need for stabilizing replication forks and/or modulating replication origin firing until the cause of the stalling is eliminated. Checkpoint effector kinases impinge on the central cell cycle machinery by phosphorylating Cdc25. This modification variously inhibits Cdc25 phosphatase activity, induces degradation or creates binding sites for 14-3-3 proteins which modify localization of the protein.

5.1. Fission yeast

Cell cycle arrest following DNA damage requires that Cdc2 is kept in a Y15-phosphorylated, inhibited state.[154] Cdc25 is inhibited through phosphorylation by Chk1 and Cds1 kinases in response to DNA damage and replication fork arrest, respectively.[155-157] Cells over-expressing Cdc25 or expressing a Y15F phospho-mimetic mutation of Cdc2 fail to arrest cell cycle progression after exposure to ionizing radiation.[154] In the absence of Cds1, Chk1 can cause cell cycle arrest following stalling of replication forks by hydroxyurea (HU) exposure. Cells lacking both kinases are unable to arrest.[155] Cds1 also phosphorylates multiple substrates to stabilize stalled replication forks and prevent the occurrence of inappropriate recombination events.[158] Upstream regulation of the DNA damage and replication checkpoint pathway occurs through activation of the ATM (Ataxia-telangiectasia mutated) homologue Rad3 through a well conserved signaling cascade.[159]

Phosphorylation of Cdc25 by Chk1 and Cds1 creates binding sites for the 14-3-3 homologues Rad24 and Rad25.[160,161] Phosphorylation and 14-3-3 binding stabilizes Cdc25, a phenomena referred to as “stockpiling”, thought to allow the cell to rapidly re-enter the cell cycle once replication or DNA damage arrest has been lifted.[70] The first Chk1/Cds1 phosphorylated Cdc25 residue identified, S99, partially impairs the replication and DNA damage checkpoint when mutated to alanine.[157] S99 modified Cdc25 is also phosphorylated on S192 and S359 by Cds1 and Chk1 *in vivo* and *in vitro*.[156] By phosphorylating Cdc25 *in vitro* with Cds1, nine additional sites were identified by mass spectrometry (S148, S178, S204, S206, T226, S234, T561, S567, T569) as well as the three sites previously known.[162] Nine sites between S99 and S359 are distributed through the poorly conserved N-terminal two thirds of the protein, while three sites reside in the extreme C-terminus. Alanine substitutions of all nine sites in the amino two thirds of Cdc25, *cdc25(9A)*, overrides the DNA replication checkpoint when expressed under control of a relatively weak heterologous promoter.[162] However, when *cdc25(9A)* is expressed from the native *cdc25*$^{+}$ locus under the control of its own promoter it does not cause a cell cycle phenotype, and the cell is checkpoint competent.[60] In addition, Cdc25(9A) is unstable following replication arrest suggesting redundancy in checkpoint control, such that Cdc25 which cannot be inhibited by Cds1 is eliminated from the cell. The S-phase specific Cdc2-Y15 kinase Mik1 is sufficient to prevent mitotic entry in these cells. Alanine substitutions of only

the three C-terminal Cdc25 sites (T561, S567, T569) have a clear replication checkpoint defect in a *mik1*$^{-}$ background and appear to be involved in maintenance, but not establishment, of the DNA damage checkpoint. [305]

5.2. Vertebrate Cdc25 regulation by DNA damage and replication checkpoints

In vertebrate cells detection of DNA damage is relayed through ATM and ATR (ATM-Related) to two checkpoint effectors, Chk1 and Chk2. While the *S. pombe* ATM homologue Rad3 is involved in activation of both Chk1 and Cds1; DNA damage signaling in vertebrates shows separation of ATR-Chk1 and ATM-Chk2 axes.[163,164] The target of these effector proteins is determined by the cell cycle stage at which the damage occurs and the nature of the damage itself. ATM-Chk2 signaling is initiated by double strand breaks, while ATR-Chk1 is activated by stalled replication forks and single stranded breaks. The p38 MAP kinase pathway is critical for cell cycle arrest following UV induced DNA damage. (Figure 2)

5.2.1. G1/S and Intra-S checkpoints

The G1/S checkpoint prevents the start of DNA synthesis in the presence of DNA damage while the Intra-S checkpoint protects replication forks, prevents activation of late replication origins, and keeps the cell from entering mitosis until S-phase is completed. G1-S checkpoint arrest is manifested through inhibition of Cdc25A, thus preventing activation of Cdk4-cyclin D and Cdk2-cyclin E. The checkpoint also activates p53 resulting in the induction of the Cdk2 inhibitor $p21^{CIP}$ and the targeting of Cyclin E to the SCF complex to reinforce Cdk2 inhibition.[165] In rat fibroblasts UV induced DNA damage during G1 results in cell cycle arrest at the G1/S transition requiring inhibition of Cdc25A and phosphorylation of Cdk4-Y14.[166] In U2OS osteosarcoma cells Cdk2-cyclin E kinase activity decreases and Y15 phosphorylation increases coincident with Cdc25A degradation following UV exposure.[167] Conversely, Cdc25A overexpression in UV exposed U2OS osteosarcoma cells results in bypass of the checkpoint and dephosphorylation of Cdk2-cyclin E. Cdc25A inhibition involves a combination of destabilization and inhibition of phosphatase activity by Chk1.[167] Treatment with caffeine (an ATM/ATR inhibitor) or the Chk1 inhibitor UNC-01, or depletion of Chk1, stabilizes the phosphatase.[167,168] In humans, Chk1 phosphorylates Cdc25A S76, S124 , S178, and T507.[168-170] Cdc25A catalytic activity is reduced three-fold when it is phosphorylated by hChk1 *in vitro*.[169] S76 and S124 are phosphorylated following ionizing radiation resulting in Cdc25A instability.[168,169,171-173] Mutation of S76 to alanine stabilizes human Cdc25A [171,172]; however, neither S76A nor S124A overrides checkpoint arrest following ionizing radiation or UV.[168] Mouse cells homozygous for the Cdc25A S124A mutation display Cdc25A stabilization following ionizing radiation;[174] however, there are no changes in proportion of cells in S-phase, or radiation resistant DNA synthesis indicating their S-phase checkpoint is intact.

Human Cdc25A S76, S124 and/or S178 are identified in several publications as “S75, S123 and S177,” respectively.[168,169,174-176] In the original cloning of human Cdc25A.[37],

there are several substitutions in the N-terminus (Accession: AAA58415.1) and a one residue gap corresponding to residue R12 in all other full length human Cdc25A sequences in the NCBI database (Accession: P30304). Residues have been re-numbered as per "P30304" for the sake of consistency.

Cdc25A S76, S124, S178 and T507 match the consensus site for 14-3-3 binding, RXX_pS/T [177] However, only the Chk1 dependent phosphorylation of S178 and T507 results in association with 14-3-3.[170] Substitution of these residues to alanine results in a complete loss of 14-3-3 interaction *in vivo*. Phosphorylation of T507 in particular, and subsequent 14-3-3 binding, interferes with Cdk1-cyclin B association by blocking a cyclin B docking site. A recent study suggests that a ternary complex between Cdc25A, 14-3-3γ and Chk1 is formed following ionizing radiation.[178] The Chk1/14-3-3γ interaction requires auto-phosphorylation of Chk1 S296. Substituting Chk1 S296 for alanine precludes 14-3-3 binding and Cdc25A S76 phosphorylation and deactivates the DNA damage checkpoint.[178] 14-3-3 proteins preferably exist as thermostable homo- and heterodimers. Each isoform in a heterodimer binding a different protein provides a common mechanism for bringing enzymes and their substrate proteins into close proximity.[179,180]

Defects in the Intra-S checkpoint allow replication of damaged DNA.[173,176,181] In mammalian cells, DNA damage results in destabilization of Cdc25A and inhibition of Cdk2.[173] Cdk2 is involved in loading the Cdc45 origin binding factor. Inhibition of Cdc25A stops further origin firing once DNA damage is detected.[182] Cdc25A is also unstable after HU induced replication fork stalling, which unlike in fission yeast, is controlled by activation of Chk1 in mammalian cells.[183]

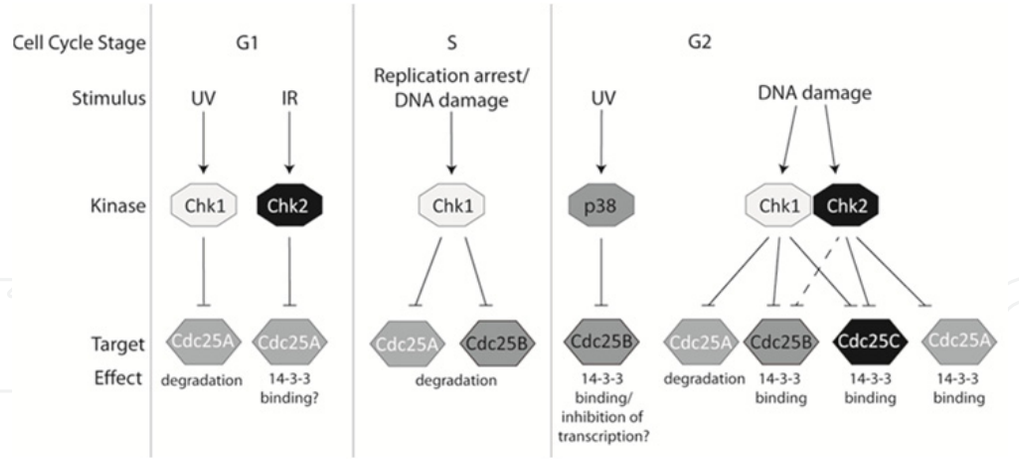

Figure 2. Inhibitory phosphorylation of Cdc25 family members following DNA damage and replication arrest

5.2.1.1. Phosphorylation mediated degradation of Cdc25A by the SCF- βTrCP complex

The mechanism by which Cdc25A is destabilized following DNA structure checkpoint activation is well understood (Figure 3). Human Cdc25A phosphorylation by Chk1 following S-phase DNA damage causes degradation by F-box protein β-TrCP associated with the Skp1-cullin-Fbox (SCF) complex.[171,184] Mutating the destruction box or KEN

box (APC interaction motifs) of Cdc25A does not stabilize the protein following exposure to ionizing radiation.[152] This indicates that SCF-mediated degradation of Cdc25A after DNA damage is independent of the cell cycle regulated APC-mediated degradation which occurs at mitotic exit. β-TrCP recognizes a degron motif of $DSG(X)_4S$ where both serine residues are phosphorylated.[185] siRNA knockdown of β-TrCP causes stabilization of Cdc25A, and radiation-resistant DNA synthesis in cells exposed to ionizing radiation.[171,184] Human Cdc25A contains such a motif: $DS_{82}GFCLDS_{88}$. The S88A substitution does not stabilize the phosphatase, suggesting that S88 phosphorylation is not explicitly required for β-TrCP binding to Cdc25A.[171] Following ionizing radiation, Chk1 primes Cdc25A for destruction through phosphorylation of S76.[171,186] The NimA-related NEK11 kinase targets Cdc25A S82 and S88, within the DSG degron sequence.[187] Depletion of NEK11 causes a marked decrease in S82 and S88 phosphorylation *in vivo*, and prevents Cdc25A degradation following IR. NEK11, itself thought to be a Chk1 substrate, can directly phosphorylate S82 and S88 *in vitro.*

Following ATM activation, Chk2 phosphorylates and activates the oncogene p53, and inhibits its negative regulator MDM2 after ionizing radiation.[188-191] p53 then induces the Cdk2-cyclin E inhibitor $p21^{WAF}$.[192] p53 also activates Glycogen Synthase Kinase (GSK-3β), which phosphorylates human Cdc25A S76 following a priming phosphorylation of S80 which can be targeted *in vitro* by Plk3.[193,194] Plk3 is unique among Polo kinase family members in causing Cdc25A stabilization when knocked down with siRNA.[194] In Plk3 null mice Cdc25A is stabilized following DNA damage.[195] Plk3 also phosphorylates and activates p53 following DNA damage and contributes to Chk2 activation.[196,197] In HeLa cells, Casein Kinase 1α (CK1α) sequentially phosphorylates both S79 and S82 following priming phosphorylation of S76 by Chk1 or GSK-3β.[198] The CK1ε isoform negatively regulates Cdc25A by S82 phosphorylation in HEK293 cells.[199] S82 phosphorylation *in vitro* and *in vivo* takes place in response to DNA damage, dependent on CK1α first phosphorylating S79.[198] S79A substitution prevents S82 phosphorylation. S76 phosphorylation appears to be the first target in the cascade, since it does not require prior phosphorylation of S79, T80, S82 or S88. Only alanine substitutions of S76, S79 and S82 impair β-TrCP interaction with Cdc25A and stabilize the phosphatase suggesting that T80 and S88 are not critical for Cdc25A degradation. Additional sites (S107, S156, S192, S279 S293) are phosphorylated by Chk1 or Cds1 *in vitro* but mutating any of these sites does not eliminate β-TrCP binding.[200] Although an interaction between phosphorylated *Xenopus* Cdc25A and β-TrCP has not been demonstrated, Chk1 is required for Cdc25A degradation at the mid-blastula transition through phosphorylation of S73.[201] Interestingly, *Xenopus* Cdc25A lacks the first serine in the $DSG(X)_4S$ degron motif found in human Cdc25A, instead possessing DDG. *Xenopus* Cdc25A S73 lies just upstream of the mutated degron and is analogous to S76 in human Cdc25A. The DAG motif of *Xenopus* Cdc25A lies within a larger PEST motif which regulates stability of the phosphatase through β-TrCP binding, independent of Chk1.[202] In mouse embryonic stem cells, which lack a G1/S DNA damage response, Cdk2 kinase activity is not affected by Cdc25A degradation following exposure to ionizing radiation.[203] Cdc25A degradation is independent of Chk1 and Chk2 but instead dependent on GSK-3β. Like Cdc25A, mammalian Cdc25B is unstable following HU induced

arrest.[117] Cdc25B binds β-TrCP strongly and contains the residues DAG rather than the DSG degron motif.[202,204] In contrast, Cdc25B accumulates following G2 DNA damage checkpoint arrest induced by a variety of agents.[205] This is reminiscent of the "stockpiling" phenomena noted earlier in *S. pombe*.[70] β-TrCP interaction with Cdc25B may also be required for mitotic exit.[204] A Cdc25B-DDA degron mutant which cannot bind β-TrCP accelerates mitotic entry slightly, but has a significant delay completing mitosis and progressing to G1. This mitotic delay is due to an extended metaphase in which Cdc25B-DDA shows a high proportion of lagging, misaligned and bridged chromosomes as well as mis-oriented spindles.[204]

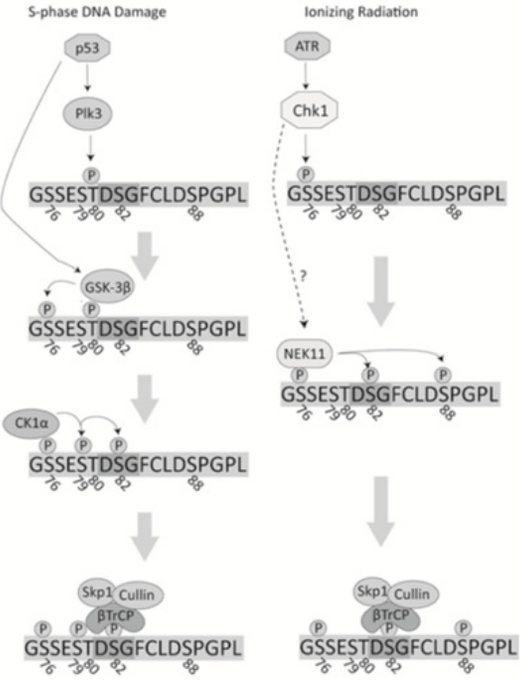

Figure 3. Multi-step phosphorylation cascades involved in targeting human Cdc25A for degradation by the β-TrCP SCF complex following DNA damage in S-phase and G2.

5.2.1.2. Cdc25A inhibition by 14-3-3 binding

Cdc25A S76, S124, S178 and T507 match the consensus site for 14-3-3 binding RXX_pS/T [177] However, only the Chk1 dependent phosphorylation of S178 and T507 results in association with 14-3-3.[170] Substitution of these residues to alanine results in a complete loss of 14-3-3 interaction *in vivo*. Phosphorylation of T507 in particular, and subsequent 14-3-3 binding, interferes with Cdk1-cyclin B association by blocking a cyclin B docking site. A recent study suggests that a ternary complex between Cdc25A, 14-3-3γ and Chk1 is formed following ionizing radiation.[178] The Chk1/14-3-3γ interaction requires auto-phosphorylation of Chk1 S296. Substituting Chk1 S296 to alanine precludes 14-3-3 binding and Cdc25A S76 phosphorylation and deactivates the DNA damage checkpoint.[178] 14-3-3 proteins preferably exist as thermostable homo- and heterodimers. Each isoform in a heterodimer binding a different protein provides a common mechanism for bringing enzymes and their substrate proteins into close proximity.[179,180]

Following exposure to ionizing radiation during G1 phosphorylation of Cdc25A on S124 by Chk2 prevents entry to S-phase .[173] Chk2 cannot efficiently phosphorylate Cdc25A on S76 and so cannot induce Cdc25A degradation by the β-TrCP route.[206] The mechanism by which S124 phosphorylation induces Cdc25A degradation is not clear because it is not required for degradation via β-TrCP.[173] S124 conforms to a 14-3-3 phospho-serine binding site, but doesn't bind 14-3-3.[170]In contrast to the effects of Cdc25A phosphorylation sites discussed thus far, modification of *Xenopus* Cdc25A T504 by Chk1 negatively regulates interaction with Cdk1-cyclin A, Cdk1-cyclin B and Cdk2-cyclin E but in a 14-3-3 independent manner.[207]

5.2.2. G2/M DNA damage checkpoint

The response to damage in G2 is dependent on the nature of the damage signal. Exposure to UV activates p38 MAP kinase and checkpoint arrest is independent of ATM and ATR since the arrest is not caffeine sensitive.[208] The primary target following UV irradiation is Cdc25B and although p38 can phosphorylate Cdc25C *in vitro*, UV exposure does not affect the Cdc25C/14-3-3 interaction.[208] Ionizing radiation activates ATM and ATR and results in Cdc25C phosphorylation by Chk1 and Chk2.[209]

5.2.2.1. Cdc25B inhibition following UV induced DNA damage

A number of conflicting reports have appeared relating to Cdc25B regulation following UV exposure. Some groups have reported that UV has no effect on Cdc25B protein levels [208,210], but others have shown that UV causes either MAP kinase mediated Cdc25B degradation or Cdc25B accumulation.[205,208,211] Cell line specific effects have no doubt contributed to these inconsistencies as human molecular biology relies heavily on transformed cell lines and mis-regulation of Cdc25B is a common phenomenon in tumors.[212] Cdc25B isolated from UV irradiated A2058 melanoma cells still retains a substantial portion of its Y15 phosphatase activity and is localized to the nucleus.[213] In HeLa cells Cdc25B is localized almost exclusively to the cytoplasm as detected by cell fractionation and immunofluorescence.[120] Variation in the apparatus used for UV irradiation could also have contributed to contradictory accounts of Cdc25B regulation. A recent re-examination of the effect of UV on Cdc25B showed that after 10 J/m^2 exposure, Cdc25B levels did not decrease, although following 60 J/m^2 Cdc25B was clearly downregulated.[214] Exposure of U2OS osteosarcoma cells to 10 J/m^2 UV leads to Cdc25B nuclear export. Based on chemical inhibitor experiments Cdc25B downregulation is not mediated by ATM/ATR, p38 MAPK or JNK, but rather following 60 J/m^2, by inhibiting Cdc25 translation. The eukaryotic initiation factor regulating Cdc25B expression, eIF2α, is phosphorylated and inhibited following UV exposure.[215] UV mediated DNA damage during G2 involves human Cdc25B S323 phosphorylation through the p38 kinase during interphase.[208] ATM/ATR inhibitor caffeine and the Chk1 inhibitor UNC-01 have no effect on UV mediated checkpoint arrest. Isoforms 14-3-3β and 14-3-3ε bind preferentially to Cdc25B phosphorylated S323, allowing its nuclear export.[216] Nuclear export of Cdc25B is abolished in cells expressing the Cdc25B S323A substitution, regardless of which 14-3-3

isoform is co-expressed.[216] Two amino-truncated Cdc25B isoforms localize to the nucleus *in vivo* and regulate recovery from G2/M checkpoint arrest but neither is required for mitotic entry.[44] Although one of these isoforms contains the DDG degron described above, both are more stable than the full length Cdc25B.

5.2.2.2. Inhibition of Cdc25C following DNA damage

Cdk1 activation is prevented by UV induced checkpoint activity coincident with the appearance of a phosphorylated form of Cdc25C.[213] In contrast with fission yeast Cdc25, which gradually accumulates in the nucleus during G2, human Cdc25C is primarily localized to the cytoplasm during interphase and only enters the nucleus at mitotic entry.[217] Thus, nuclear export of Cdc25C is not a requirement for G2 DNA damage response, since the phosphatase is already cytoplasmic at this time. However, exposure to ionizing radiation decreases the enzymatic activity of human Cdc25C.[218] Several research groups showed relatively early in the Cdc25 phosphorylation story that residue S216 is phosphorylated by Chk1 [219-221] and Chk2 *in vitro*.[209,222] Phosphorylation of S216 results in 14-3-3 binding and nuclear export.[219] Residues surrounding S216, $RSPS_{216}MP$, correspond to the canonical 14-3-3 consensus binding site RSX_PSXP.[177] Cdc25C S216A mutants are unable to bind 14-3-3.[219] This is likely due to close proximity of S216 to the NLS, leading to the obstruction of the import signal and trapping of the phosphatase in the cytoplasm.[221,223] A higher proportion of cells expressing Cdc25 S216A have nuclear abnormalities indicative of progression to mitosis prior to completion of DNA synthesis; this effect is exacerbated by addition of HU.[217,219] Such cells also have reduced ability to delay entry to mitosis following ionizing radiation exposure.[224]

Although Cdc25C S216A is a relatively poor substrate for Chk1 compared to the wildtype protein, Chk1 can still execute some degree of phosphorylation on the mutant phosphatase.[220] This observation suggests the possibility that additional Cdc25C phosphorylation negatively regulates its enzymatic activity. Recent bioinformatics approaches to generate profiles from peptide binding arrays based on three diverse 14-3-3 binding sites have generated an improved 14-3-3 binding motif consensus.[225] This helped to identify two additional phosphorylated Cdc25C residues, Ser247 and Ser263, which interact with 14-3-3. Mutation of either residue to alanine reduces 14-3-3 binding, but neither of these mutant peptides was affected by Cdc25 S216 phosphorylation when expressed in cells. S263 was previously identified in an isolated report which showed phosphorylation of this residue induces Cdc25B nuclear export.[226] Cdc25C purified from cells treated with the topoisomerase II inhibitor etoposide is phosphorylated on S263, but S263A substitution results in enhanced nuclear localization. The kinase targeting this residue has yet to be determined experimentally. However, the residues surrounding S263 ($KKTV_PSLCD$) conform to a Chk1 consensus site as Chk1 can tolerate a lysine (K) at the -3 position relative to the phosphorylated serine or threonine *in vitro*.[227]

Regulation of *Xenopus* Cdc25C localization is similar to its human counterpart. In cultured *Xenopus* tissue Cdc25C is primarily in the cytoplasm while the Cdc25C S287A mutant, corresponding to S216 in human Cdc25C, is almost exclusively nuclear.[228] Cdc25C is

phosphorylated on multiple sites by Chk1 but only S287 phosphorylation is required for 14-3-3 binding.[229-231] Although *Xenopus* Cdc25C can be made exclusively cytoplasmic by co-expression with 14-3-3ε, Cdc25C S287A is not affected. Nuclear export depends on the intrinsic 14-3-3ε nuclear export signal and re-import is prevented by blocking Cdc25C association with importin-α.[228] In egg extracts depleted of endogenous Cdc25C, expressing Cdc25C S287A accelerates mitotic entry relative to overexpression of the wildtype protein.[229] However, Cdc25C S287A has a less than two fold increase of *in vitro* Cdk1 Y15 dephosphorylation activity over wildtype Cdc25C when pre-incubated with 14-3-3ε. Thus, it seems likely that *Xenopus* Cdc25C phosphorylation and 14-3-3 binding regulates the phosphatase at the level of cellular localization, rather than inhibiting its activity *per se*.

Cdc25A is considered to regulate the G1/S transition in the "Traditional Model" of the human cell cycle but it also has a significant role in mitotic entry. As such, Cdc25A is an important target of the DNA damage checkpoint. In fact, mice lacking Cdc25B and Cdc25C do not have a G2/M checkpoint defect.[106,107] Phosphorylation by Chk1 causes degradation of Cdc25A following DNA damage during G2 ionizing radiation and exposure to the DNA intercalating agent adriamycin.[172,181]

5.3. Chk1 regulation of Cdc25 orthologues in unperturbed cell cycles

Cdc25A, B and C are all phosphorylated by Chk1 in the absence of externally induced DNA damage. As Cdc25 phosphatases are such potent positive regulators of cell cycle transitions it is perhaps not surprising that the cell maintains their activity at a low level until their precise point of activation. Chk1 regulates human Cdc25A stability during unperturbed cell cycles. Phosphorylation of S82 and S88 can be detected using phospho-specific antibodies in unperturbed cells.[184] Depletion of the S82/S88 kinase NEK1 and S76 kinase CK1ε by siRNA, results in Cdc25A stabilization in the absence of DNA damage.[187,199] Cdc25A S124 phosphorylation by Chk1 also occurs in the absence of damage and destabilizes the phosphatase.[168,169,200] Inhibiting ATM/ATR, or a variety of upstream checkpoint components, also stabilizes in the absence of externally induced DNA damage which may indicate there is some basal level of spontaneous damage checkpoint signaling.[232] Cdc25A is also phosphorylated by Casein kinase 2β (CK2β) in a damage independent manner.[233] CK2 phosphorylates human Cdc25C T236 adjacent to the NLS *in vitro*.[234] A T236D mutation reduces β-importin binding, thus excluding Cdc25 from the nucleus.[234] CK2 phosphorylates Cdc25B on residues S186 and S187, just downstream from the KEN box, modestly increases its phosphatase activity, and potentially blocking APC mediated degradation.[235]

Human Cdc25B is phosphorylated *in vitro* by Chk1 at S230 and S563 in the absence of DNA damage.[236] S230 phosphospecific antibodies show that Cdc25B modified on this residue is centrosome associated from S-phase until mitosis.[236] A population of Chk1 is localized to the centrosome during G2 and can prevent promiscuous Cdk1-cyclin B activation by Cdc25B.[237] S323 was previously identified as the major 14-3-3 binding site.[238] S151 and S230 account for the remainder of the interaction, but both need to be dephosphorylated

before interaction with 14-3-3 is lost.[239] Human Cdc25B S563 resides in the extreme C-terminus of Cdc25B and is analogous to Cdc25A S504 and *S. pombe* Cdc25 T569. Cdc25B lacking the S323 phosphorylation site is almost exclusively nuclear. It is interesting that this residue is targeted by Chk1, but does not appear to bind 14-3-3 when phosphorylated. If the function of this phosphorylation is conserved between Cdc25A S504 and Cdc25B S563, phosphorylation may affect interaction with Cdk1-cyclin B.[240]

Chk1 phosphorylation of human Cdc25C and nuclear export by 14-3-3 binding keeps the phosphatase cytoplasmic during unperturbed cell cycles.[217] Nuclear localization of Cdc25C(S216A) is enhanced, suggesting that part of the function of 14-3-3 binding is to obscure the NLS located adjacent to this residue.[224] Overexpression of Cdc25C(S216A) induces a higher degree of premature mitotic entry.[217] *Xenopus* Cdc25C is likewise phosphorylated on S287 by Chk1 during interphase.[228,241] Phospho-S287 is bound by 14-3-3ε and 14-3-3ζ obscuring the NLS and preventing nuclear import.[228] *Xenopus* Cdc25C is phosphorylated on T533 by Chk1, but not Chk2.[207] Injecting Cdc25C T533A mRNA into *Xenopus* oocytes results in more rapid dephosphorylation of Cdk1 Y15.[207] Again, this suggests that regulation of CDK-cyclin interaction with Cdc25 orthologues by C-terminal phosphorylation is a common mechanism for inhibiting cell cycle progression.

Although Cdc25A and Cdc25B are dispensable for embryonic development, Chk1 and ATR kinases are essential.[242,243] The Cdc25B/14-3-3 interaction is important for maintaining G2 arrest, and inhibiting germinal vesicle breakdown in *Xenopus* oocytes prior to progesterone exposure.[244]

In *S. pombe*, Chk1 does not appear to negatively regulate Cdc25 in the absence of DNA damage. Loss of a negative regulator of Cdc25 is expected to cause the cell to divide at a reduced length. However, deletion of Chk1 does not cause a cell cycle phenotype.[245] In addition, expressing Cdc25 where all twelve putative Cds1/Chk1 phosphorylation sites are mutated to alanine does not cause acceleration of the cell cycle.[60]

5.4. Cdk1 phosphorylation of Cdc25 precludes checkpoint mediated inhibition during mitosis

If Cdc25C is phosphorylated and inactivated during interphase via 14-3-3 binding, how is it then activated at mitotic entry? Cdk1-cyclin B phosphorylation of Cdc25C causes 14-3-3 dissociation and allows removal of interphase phosphorylations. Re-phosphorylation of these residues is simultaneously blocked. Cdk1-cyclin B thus potentiates Cdc25C for its pro-mitotic function and ensures that it remains active. The region surrounding S216 in human Cdc25C and S287 in *Xenopus* Cdc25C is a well conserved stretch in the N-terminal region of the two proteins; LYRSPS$_{216}$MPE is identical between Human and *Xenopus* and contains S216 and S287, respectively.(Figure 4) In both organisms, the two serine residues upstream of the major phosphorylated 14-3-3 binding residue, S214 in human and S285 in *Xenopus*, is targeted by Cdk1-cyclin B.[42,246-248] In human cells phosphorylation of S214 precludes phosphorylation of S216 by Chk1 and 14-3-3 binding.[42,246] Substituting S214D prevents phosphorylation of S216.[246] S216 is not phosphorylated during M-phase *in vivo*, and

ionizing radiation in M-phase cells cannot induce its phosphorylation.[246] 14-3-3 is unable to bind Cdc25C immunoprecipitated from cells arrested in mitosis with nocodazole.[219] In *Xenopus*, Cdc25C S285 phosphorylation prevents the phosphorylation of S287.[247,249] S285 can be phosphorylated by both Cdk1-cyclin B and Cdk1-cyclin A *in vitro*.[146] Until the mid-blastula transition, *Xenopus* Cdc25C is phosphorylated on S285 which precludes Chk1 mediated phosphorylation of S287 and subsequent 14-3-3 binding.[247] Human Cdc25C S214 is also phosphorylated in maturing human oocytes.[250] Removal of phospho-S287 bound 14-3-3 and phosphorylation of *Xenopus* Cdc25C S285 requires prior phosphorylation of Cdc25C T138.[249] T138 is a substrate of Cdk1-cyclin B.[146] However, selective depletion of Cdk2 from *Xenopus* egg extracts using the N-terminus of the Cdk2 inhibitor p21, completely prevents removal of 14-3-3 from Cdc25C.[251] In *Xenopus* egg extracts Cdk2 is required for mitotic entry.[81] Other CDK phosphorylation sites on *Xenopus* Cdc25C such as T48 and T67 negatively regulate its activity in an S287 independent manner.[249] Phosphorylation of T138 is not sufficient to cause 14-3-3 dissociation.[248] How is 14-3-3 physically removed from phospho-S287? The pelleted fraction from ultracentrifuged interphase egg extracts contains a 14-3-3 dissociating activity, which was determined to be the intermediate filament component Keratin 8/18[248]. Keratin is phosphorylated during mitosis, binds 14-3-3, and has a role in mitotic progression in hepatocytes.[252] Keratin may act as a "14-3-3 sink" during mitosis, stripping 14-3-3 from S287.[248] *Xenopus* Cdc25C T138 corresponds with T130 in human Cdc25C. A phospho-T130 specific antibody shows that Cdc25C phosphorylated on this site localizes to the centrosome.[145] A localization for the de-inhibition of Cdc25C fits well with the putative centrosomal localization of Cdc25C activation.

PP1 phosphatase removes S287 phosphorylation once 14-3-3 has dissociated.[251] Binding of PP1 to *Xenopus* Cdc25C requires a docking motif "VXF", amino acids 105-107, the loss of which prevents S287 dephosphorylation.[251] Phosphorylation of *Xenopus* Cdc25C S285 by Cdk1-cyclin B enhances recruitment of phosphatase PP1 to Cdc25C, inducing the dephosphorylation of S287.[249] PP2A/B56δ dephosphorylates *Xenopus* Cdc25C T138 during interphase, mitotic exit and following replication arrest.[248] This maintains the phosphatase in a state where it can be inhibited by S287 phosphorylation. Inhibition of PP2A by okadaic acid prematurely induces mitotic entry in *Xenopus* egg extracts.[253] T138 is also dephosphorylated during replication arrest where B56δ is itself phosphorylated by Chk1, enhancing complex formation with PP2A.[248] PP2A mediated dephosphorylation of Cdc25 is assisted by the action of the Pin1 prolyl isomerase. Pin1 isomerizes the peptide bond between phospho-serine/threonine and proline placing their R-groups in a *trans* orientation.[254] This isomerization makes the phosphorylated residue a better substrate for the PP2A phosphatase.[255] *In vitro*, Pin1 decreases the catalytic activity of Cdc25C that has previously been phosphorylated by Cdk1.[256] Pin1 is also involved in maintenance of replication checkpoint arrest in *Xenopus* encouraging the reversal of Cdk1 mediated Cdc25C phosphorylation.[257] In Humans, Cdc25C is deactivated in a similar manner. T130 on Cdc25C is dephosphorylated by PP2A.[258] Mitotic exit is delayed when PP2A is knocked down, suggesting that dephosphorylation of Cdc25C T130 in human cells is also important for the transition from M to G1 of the next cycle. A similar situation may exist where human Cdc25B1

S321 phosphorylation blocks phosphorylation of the major 14-3-3 binding residue S323 by p38.[246] Expression of the phosphorylation-mimicking Cdc25B1 S321D prevents p38 mediated phosphorylation of S323 and abolishes binding by 14-3-3β and 14-3-3ε.[246,259] 14-3-3σ binding is unaffected by S321 phosphorylation.[259] 14-3-3σ preferentially interacts with Cdc25B3 S230 [216] and is induced by p53 following activation by ATM/Chk2.[260]

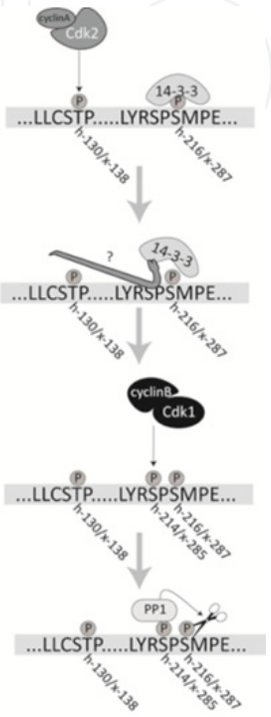

Figure 4. Cdk-cyclin mediated removal of inhibitory S216/S287 phosphorylation and 14-3-3 binding in human and *Xenopus* Cdc25C, respectively.

There are no Cdc2 phosphorylation motifs (S/TP) directly upstream of any of the twelve Cds1 *in vitro* phosphorylation sites in *S. pombe*. Large scale phosphoproteome analysis has detected S99, S178 and S359 phosphorylation of fission yeast Cdc25 in M-phase arrested cells.[261] Thus it appears that the vertebrate mechanism for reversing and preventing Chk1 phosphorylation of Cdc25C evolved relatively recently. However, parallels exist between some aspects of Cdc25 dephosphorylation between fission yeast and vertebrates. Treatment with okadaic acid or deletion of PP2A homologue Ppa2 causes premature mitotic entry in fission yeast.[262,263] Loss of *ppa2* suppresses the *cdc25-22*^{ts} mutation, a genetic interaction indicative of a negative regulator.[263] Loss of PP1 homologue Dis2 results in cell elongation, suggesting its role as a positive regulator of Cdc25 is conserved in *S. pombe*.[264] Temperature sensitive *dis2* mutants have a defect in exit from mitosis, similar to the effect of siRNA inhibition of human PP2A.[258,264]

6. Cdc25 phosphorylation by MAP kinase cascades

In addition to regulation by DNA damage and DNA replication checkpoints, Cdc25 is the target of several MAPK cascades responding to stress and mitogenic signals. A detailed

description of the variety of MAPK pathways is outside of the scope of this manuscript, but excellent reviews are available.[265]. In general, MAP kinase cascades involve the sequential activation of three kinases; a MAP kinase kinase kinase (MAPKKK) phosphorylates a MAP kinase kinase (MAPKK), which phosphorylates a MAP kinase (MAPK). There are three such cascades which are salient to our discussion of Cdc25 regulation.

6.1. Raf/MEK/ERK

The ERK1/ERK2 MAPKs are activated by Raf MAPKKKs working on MEK1/MEK2 MAPKKs. This cascade is primarily activated by extracellular signaling through receptor tyrosine kinases in response to mitogenic signals.[266] During Cyto-Static Factor arrest in mature *Xenopus* oocytes metaphase II arrest is enforced by the combined activity of the MEK/ERK pathway upregulating ribosomal subunit S6 kinase/CamKII homologue $p90^{rsk}$, and APC inhibition by the Emi2 kinase.[267] Calcium influx as a result of fertilization activates $p90^{rsk}$ (a Ca^{2+}/calmodulin dependent kinase II homologue) which phosphorylates Emi2.[268] This allows APC activation, cyclin B degradation, and progression through meiosis II. Cdc25C is phosphorylated on S287 by $p90^{Rsk}$ *in vitro* resulting in its inhibition via 14-3-3 binding.[269,270] Conversely, *Xenopus* Cdc25C S317, T318 and S319 are phosphorylated by $p90^{rsk}$ orthologue Rsk2 which serves to activate the phosphatase in mature oocyte extracts.[271] The *Xenopus* ERK homologue, p42, phosphorylates Cdc25C T48, T138 and S205 *in vitro.*[272] T138 and S205 are also Cdk1-cyclinB targets [146], but T48 is uniquely phosphorylated by p42. In HeLa cells after activation of MEK/ERK signaling by addition of 12-O-tetradecanoylphorbol-13- acetate (TPA), G2/M transition is inhibited by degradation of Cdc25B.[273] This is mediated through MEK dependent phosphorylation of Cdc25B S249 by CamKII, an activator of human Cdc25C.[274] Inhibition of CamKII results in a G2 arrest. Overexpression of CamKII can also arrest cells in G2, but with a high level of Cdk1/Cyclin B activity.[275] Another member of the CamKII family, C-TAK1, phosphorylates human Cdc25C on S216 resulting in inhibition via 14-3-3 binding and nuclear export.[276] *Xenopus* Cdc25C can be phosphorylated by CamKII *in vitro.*[277] Lastly, $p14^{arf}$ upregulation in response to anti-proliferative signals results in human Cdc25C downregulation through MEK/ERK MAPK signaling.[278] Human Cdc25C becomes phosphorylated on S216 and is subsequently ubiquitinated and degraded.[278]

6.2. p38 and JNK MAPKs

The p38 and JNK kinases activate in response to extracellular stimuli such as heatshock, oxidative stress, ionizing radiation, UV and growth factor deprivation. Both are activated by MAPKKs of the MKK family which are themselves activated by a large variety of MAPKKKs. We have already discussed the function of p38 in response to UV irradiation. p38 also phosphorylates human Cdc25A on S124 and S76 in response to osmotic stress, destabilizing the protein [168] and a 42 C heatshock causes p38 and Chk2 to phosphorylate S76 and S178 of human Cdc25A, respectively.[175] MAPKAP kinase 2 (MK2) functions downstream of p38 and regulates G1 and S-phase cell cycle progression in response to UV.[210] Downstream of p38, it phosphorylates RxxS/T motifs and activation of MK2

correlates with increased binding of 14-3-3 to Cdc25B. p38 and MK2 kinase form a tight complex and are imported into the nucleus together, so previous work showing that p38 directly phosphorylates S216 on Cdc25C and S323 on Cdc25B may in fact have been inadvertently monitoring MK2 activity.[279] Cdc25A may also be an MK2 substrate as MK2 knockdowns ablate the G1 and S-phase checkpoints. JNK activity targets two serines within the region DAGLCMDS$_{101}$PS$_{103}$P of the DSG degron on human Cdc25B.[280] Simultaneous S101A and S103A substitution prevents β-TrCP binding and Cdc25B ubiquitination. JNK also phosphorylates Cdc25C on S168 inhibiting its phosphatase activity.[281,282] This residue is transiently phosphorylated *in vivo* on nuclear Cdc25C prior to and after mitotic entry.[281] S168 is phosphorylated following UV irradiation and osmotic shock.[281,282]

6.3. The *S. pombe* stress activated MAP kinase pathway

In fission yeast, Cdc25 is phosphorylated by the CamKII homologue Srk1 in response to extracellular stress.[283] Srk1 is activated downstream of the Spc1 MAPK, Wis1 MAPKK, and Win1 or Wak1MAPKKKs.[284] This phosphorylation occurs on residues also targeted by Cds1 as Cdc25(9A) is not sensitive to Srk1 mediated inhibition.[283] Srk1 phosphorylation of Cdc25 results in its nuclear export, similar to the response to DNA damage and replication arrest.

7. Other kinases which phosphorylate Cdc25s

PKA prevents oocyte maturation by inhibition of Polo kinase mediated Cdc25 activation, and deactivating the Mos/MEK/ERK MAP kinase cascade which inhibits Myt1.[285] Progesterone exposure in *Xenopus* oocytes down regulates adenylate cyclase, lowering cyclicAMP levels and consequently deactivating PKA. Prior to maturation, *Xenopus* Cdc25C is also inhibited by PKA phosphorylation of S287 and T318 *in vitro*.[286] Murine PKA phosphorylates Cdc25B S321, negatively regulating the phosphatase; S321A mutants cause enhanced germinal vesicle breakdown when injected into mouse oocytes.[287]

Pim1 is a serine/threonine kinase induced by the SAT3 and SAT5 transcription factors following cytokine exposure thus linking pro-proliferative signals to the cell cycle control machinery.[288] Pim1 phosphorylates and activates Cdc25A and represses the Cdk4/6 inhibitor p21^{CIP} to encourage the G1/S transition.[289,290] Pim1 is also able to phosphorylate and inhibit the CamKII homologue c-TAK and accelerate Cdc25C mediated mitotic entry.[291]PAR-1/MARK (partitioning-defective 1/Microtubule affinity-Regulating Kinase) protein homologue pEG3 phosphorylates human Cdc25A S263, Cdc25B S169 and Cdc25C S216.[292,293] Overexpressing pEg3 results in G2 arrest which can be reversed by co-expressing Cdc25B.[292] Anti-phospho PAR1 S169 antibodies stain spindle pole and centrosome in immunofluorescence experiments.[293] In *C. elegans* the first cell division is unequal which produces a larger anterior cell and a smaller posterior one. The next cell division is asynchronous with the anterior cell dividing prior to the posterior one.[294] Rapid anterior cell cycle timing is due to enrichment of Polo kinase and Cdc25.1 in the anterior cell. This is dependent on a network of polarity proteins, including Par1.[295]

8. Cdc25 and disease

Cdc25 orthologues are the subject of much attention as they are commonly upregulated in human tumors.[296] This is perhaps not surprising considering the role of Cdc25 inhibition in maintaining genomic stability and the regulation of these phosphatases by Rb, p53 and a number of other oncogenes. Cdc25A and Cdc25B themselves are oncogenes in humans.[212] Cdc25B is overexpressed in 32% of breast cancer tissue samples, and high Cdc25B levels correlate with high incidences of recurrence and decreased 10 year survival.[212] Overexpression of Cdc25A is similarly linked to poor clinical outcome.[296,297] Cells bearing oncogenic mutations of myc have elevated Cdc25A and Cdc25B levels.[298] Anti-Cdc25B autoantibody has been shown to be a predictor of poor prognosis in esophageal cancer patients.[299] Overexpression of Cdc25B has recently been shown to cause a variety of S-phase effects including increased Cdc45 recruitment to chromatin, impairment of replication fork progression DNA damage and chromosome instability.[300]

An interesting link between Cdc25 and disease comes from the finding that the HIV-1 protein vpr causes G2/M arrest.[301] When expressed in *S. pombe*, vpr activates Srk1 kinase, resulting in Cdc25 phosphorylation and 14-3-3 mediated nuclear export.[302,303] The vpr protein also acts through upregulation of PP2A phosphatase acting on both Wee1 kinase and Cdc25, reversing activating Cdc2 phosphorylation.[304]

9. Conclusion

It has been more than thirty five years since Cdc25 was first isolated as an elongated temperature-sensitive fission yeast mutant and twenty one years since its biochemical function was determined. The field of cell cycle research and the study of Cdc25 in particular are extremely active with numerous new manuscripts appearing each year. This research has revealed that Cdc25 is one of the most intricately regulated proteins in the cell. Cdc25 accepts input from numerous pathways and checkpoints monitoring whether conditions inside and outside the cell are permissive for cell cycle progression. When conditions warrant caution, Cdc25 is inhibited by phosphorylation leading to alterations in its catalytic activity, cellular localization, substrate recognition and stability. When the green light is given Cdc25 participates in an intricate series of interconnected positive feedback loops with the beating heart of cell cycle regulation, the Cyclin-CDK complex. When the cell loses control of Cdc25 regulation, the results are deadly.

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