Dephosphorylation of the inhibitory phosphorylation site S287 in *Xenopus* Cdc25C by protein phosphatase-2A is inhibited by 14-3-3 binding

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Abstract **Cdc25C phosphatase induces mitosis by dephosphorylating and activating Cdc2/cyclin B protein kinase. Phosphorylation of *Xenopus* Cdc25C at serine 287 creates a binding site for a 14-3-3 protein and restrains activation during interphase. Here, we show that dephosphorylation of S287 is catalysed by protein phosphatase-2A in *Xenopus* egg extracts. 14-3-3 protein binding to Cdc25C inhibits dephosphorylation of S287, providing a mechanism to maintain phosphorylation of that site during interphase. The rate of dephosphorylation of S287 is not increased in mitotic extracts, indicating that the phosphorylation status of the site is likely to be controlled through modulation of kinases or 14-3-3 binding activity.**

Key words: Cdc25; Cell cycle checkpoint; Protein phosphatase; 14-3-3 protein; Chk1

1. Introduction

The initiation of mitosis in eukaryotic cells is coordinated by the activation of the cyclin-dependent protein kinase Cdc2/cyclin B. In addition to association with cyclin B, the Cdc2 catalytic subunit requires correct phosphorylation for activity. Phosphorylation of a threonine residue in the T-loop (T161 in vertebrate Cdc2) is essential for activity, but Cdc2 is kept inactive during interphase by dominant inhibitory phosphorylation at T14 and Y15. The final activation step of Cdc2 is the removal of these inhibitory phosphates by the dual-specificity protein phosphatase Cdc25 [1].

In vertebrates, Cdc25C is activated at the G2/M transition by phosphorylation on multiple Ser/Thr residues [2,3] catalysed by the polo-like kinase Plk1/Plx1 [4] and Cdc2/cyclin B [5], creating a feedback activation loop that causes the rapid activation of Cdc2 upon entry into mitosis [6]. Studies using specific inhibitors have shown the enzyme responsible for dephosphorylating these activating sites of Cdc25C is a type-2A protein phosphatase (PP2A), which is specifically repressed in mitosis [2,7]. Conversely, Cdc25C is phosphorylated during interphase at an inhibitory site (S287 in *Xenopus*, S216 in human), which is critical for determining the length of G2 and for cell cycle arrest induced by checkpoint signals [8–10]. S287 is the target of Chk1 and Cds1/Chk2 protein kinases, which are activated in response to damaged or unreplicated DNA. Phosphorylation of S287 of Cdc25C directly inhibits its catalytic activity [11,12] and creates a mode 1 binding site for 14-3-3 proteins [8,9,13,14] (Fig. 1A), sequestering Cdc25C in the cytoplasm [15–19]. In *Xenopus* egg extracts, Cdc25C is stoichiometrically bound via phospho-S287 to either the ε or ζ isoform of 14-3-3 during interphase, but 14-3-3 binding is lost in mitosis suggesting that S287 becomes dephosphorylated [9]. Although not previously described, the protein phosphatase that acts on S287 may play a role in the control of phosphorylation at this site and thereby determine the activity of Cdc25C.

Here, we show that S287 is dephosphorylated in *Xenopus* egg extracts by PP2A, the same activity that removes phosphate from activating phosphorylation sites on Cdc25C. We show that binding of a 14-3-3 protein to Cdc25C inhibits the dephosphorylation of S287 in interphase, indicating that 14-3-3 may play a role in the maintenance of inhibitory phosphorylation of Cdc25C in interphase and under checkpoint-induced conditions.

2. Materials and methods

2.1. Production of glutathione S-transferase (GST) fusion proteins

A 135 bp section of a *Xenopus* Cdc25C cDNA [3], encoding amino acids 271–316, and the 744 bp coding sequence of *Xenopus* 14-3-3ζ [20] were amplified by PCR and cloned into the pGEX-4T-1 vector (Amersham Biosciences). Site-directed mutagenesis was carried out using the QuikChange kit (Stratagene) and confirmed by automated DNA sequencing. GST fusion proteins were expressed in *Escherichia coli* BLR (DE3) cells, purified by affinity chromatography on glutathione–Sepharose 4B (GS4B, Amersham Biosciences) and stored in 10 mM HEPES–KOH, pH 7.5, 1 mM DTT at −70°C.

2.2. Xenopus egg extracts

Interphase extracts were prepared from *Xenopus* eggs by the method of Hutchison [21]. Cystostatic factor (CSF)-arrested, M-phase egg extracts were prepared by the method of Murray [22]. CSF release into interphase induced by the addition of 0.8 mM $CaCl_2$ was confirmed by loss of mitotic spindle morphology [22] and reduction in Cdc2/cyclin B kinase activity [23]. Cdc2 was activated in interphase *Xenopus* egg extract by addition of 2 μM *Arbacia punctulata* cyclin BΔ90 [24] and incubation at room temperature for 120 min [23].

2.3. Protein kinases, protein phosphatases, inhibitors

GST-tagged human Chk1 (GST–hChk1) was expressed and purified as described [25]. The GST moiety was removed using thrombin protease. Purified rabbit PP1 catalytic subunit, human PP2A (AC heterodimer), human PP2Cα and human inhibitor-2 (I-2) were from Up-

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state. Stock solutions of 500 μM microcystin-LR (MC) and okadaic acid (OA; Calbiochem) were prepared in DMSO.

2.4. Preparation of ^{32}P-phosphorylated GST–Cdc25C(271–316)

GST–Cdc25C(271–316) protein (200 μg) was phosphorylated in 100 μl buffer A (20 mM HEPES–KOH, pH 7.5, 150 mM NaCl, 2 mM DTT) plus 10 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP (approx. 37 kBq/nmol), 50 ng Chk1 and 20 μl (bed volume) GS4B, at room temperature with rotation for 30 min. The beads were washed in buffer A, then GST–Cdc25C(271–316) protein was eluted in buffer A containing 50 mM glutathione. The eluate was buffer-exchanged (for buffer A) and concentrated in an Ultrafree-0.5 centrifugal filter device (Millipore).

2.5. S287 dephosphorylation assay

Dephosphorylation of [^{32}P-S287]GST–Cdc25C(271–316) protein (0.1 mg/ml) in interphase *Xenopus* egg extract was carried out at room temperature for 30 min, unless indicated otherwise. Diluted extract (1:100) was prepared in buffer A plus 1 mg/ml bovine serum albumin. For purified protein phosphatases, 1 U is defined as that activity which releases 1 nmol phosphate from a phosphoprotein substrate per min. The reaction with PP2C also contained 10 mM $MgCl_2$. To analyse the loss of radiolabel, samples were separated on a SDS–12% PAGE gel and analysed by autoradiography.

2.6. Binding of 14-3-3 proteins to Cdc25C

GST–Cdc25C(271–316) protein (50 μg) was first phosphorylated in buffer AM (buffer A plus 10 mM $MgCl_2$) plus 100 μM ATP and 60 ng Chk1 for 2 h at 37°C. 20 μl of GS4B (50% slurry, in buffer AM) was then added and incubated for 1 h at room temperature with rotation to bind the GST–Cdc25C(271–316) to the beads. 100 μl interphase *Xenopus* egg extract (diluted 1:10 in buffer AM) was added, with 30 ng Chk1 and 0.1 μM OA where indicated, and incubated for 1 h at room temperature with agitation. The beads were washed five times with 200 μl buffer AM plus 1% (v/v) detergent: Nonidet P-40 (Roche), Triton X-100 (Sigma) or Empigen-BB (a gift from Ellis and Everard Ltd.), eluted in SDS–PAGE sample buffer and analysed by Western blotting with a rabbit polyclonal anti-14-3-3 antibody that recognises all isotypes (sc-629, Santa Cruz Biotechnology).

3. Results

3.1. Dephosphorylation of S287 of Cdc25C in Xenopus egg extracts

To generate a probe to investigate the dephosphorylation of the S287 residue of Cdc25C, a fragment derived from residues 271–316 of the N-terminal regulatory domain of *Xenopus* Cdc25C (Fig. 1A) was expressed as a GST-fusion protein in *E. coli* and purified to near homogeneity. Variant forms of GST–Cdc25C(271–316) were produced by site-directed mutagenesis, named according to the amino acid sequence of residues 285–287. When incubated with Mg^{2+}[γ-^{32}P]ATP and Chk1 in vitro, the SPS and APS proteins became ^{32}P-phosphorylated to a very similar extent, while the SPA or SPD proteins (which have non-phosphorylatable residues in the 287 position) were not phosphorylated, nor was GST (Fig. 1B), demonstrating that S287 is the exclusive target of Chk1 within GST–Cdc25C(271–316).

When incubated with interphase *Xenopus* egg extract, [^{32}P]phosphate was rapidly lost from [^{32}P-S287]SPS protein (Fig. 1C). The loss of [^{32}P]phosphate was completely blocked by 1 μM OA, an inhibitor of type-1 or type-2A serine/threonine protein phosphatases [26], demonstrating that interphase egg extract contains a protein phosphatase which targets S287-phosphatase (hereafter referred to as the S287-phosphatase). Scintillation counting of excised bands from the SDS–PAGE gel showed that the loss of radiolabel was approximately linear with respect to time, enabling it to be used as an assay for S287-phosphatase activity.

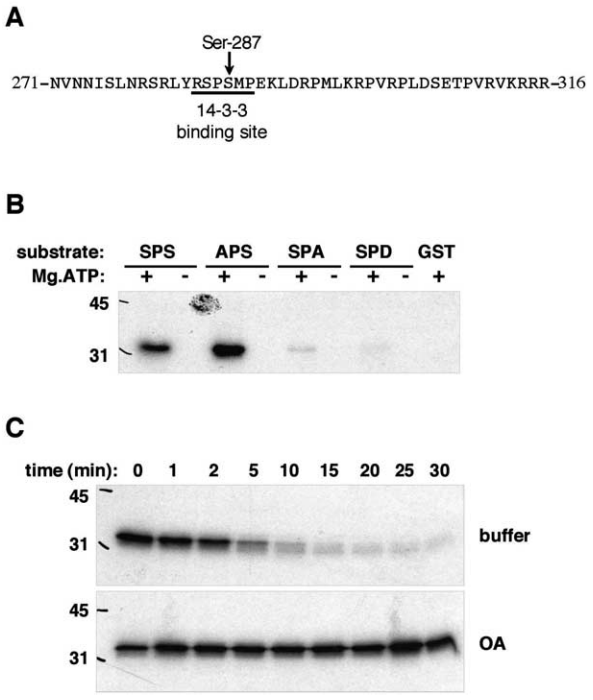

Fig. 1. Phosphorylation and dephosphorylation of the Ser-287 residue of *Xenopus* Cdc25C. A: Amino acid sequence and features of Cdc25C(271–316). B: Phosphorylation of GST–Cdc25C(271–316) SPS and variants by Chk1, analysed by SDS–PAGE and autoradiography. GST provided an additional control. C: Dephosphorylation of [^{32}P-S287]SPS protein in dilute interphase *Xenopus* egg extract with (OA) or without (buffer) addition of 1 μM OA, analysed by SDS–PAGE and autoradiography.

3.2. Characterisation of the S287-phosphatase in Xenopus egg extracts

To characterise biochemically the type of protein phosphatase acting on S287, the dephosphorylation of [^{32}P-S287]SPS protein by dilute egg extract was assayed in the presence of inhibitors and activators of protein Ser/Thr phosphatases [27] (Fig. 2). Similar to the effect of 1 μM OA, dephosphorylation was completely inhibited by 1 μM MC [28] (Fig. 2A), confirming the involvement of PP1 or PP2A. However, I-2, which specifically inhibits PP1 [27], had no inhibitory effect on S287 dephosphorylation (Fig. 2B), showing that the predominant S287-phosphatase activity in *Xenopus* egg extract is type-2A. Neither EGTA nor EDTA (which chelate Ca^{2+} and Mg^{2+} required by PP2B and PP2C, respectively) inhibited the S287-phosphatase, nor were Ca^{2+} or Mg^{2+} ions (at 10 mM) able to stimulate dephosphorylation of S287 in the presence of 1 μM OA (Fig. 2A), confirming that PP2B and PP2C do not make a significant contribution towards S287-phosphatase activity.

Half-maximal inhibition of S287-phosphatase activity in dilute interphase egg extract occurred at 4.8 nM OA (Fig. 2C), consistent with the classification of this phosphatase as type-2A [7,27,29]. Indeed, when incubated with 0.1 U of purified PP1 (catalytic subunit), PP2A (AC dimer) or PP2C, [^{32}P-S287]SPS protein was most efficiently dephosphorylated by PP2A (Fig. 2D).

3.3. Dephosphorylation of S287 by interphase and mitotic egg extracts

S287 in *Xenopus* Cdc25C is phosphorylated and bound to a

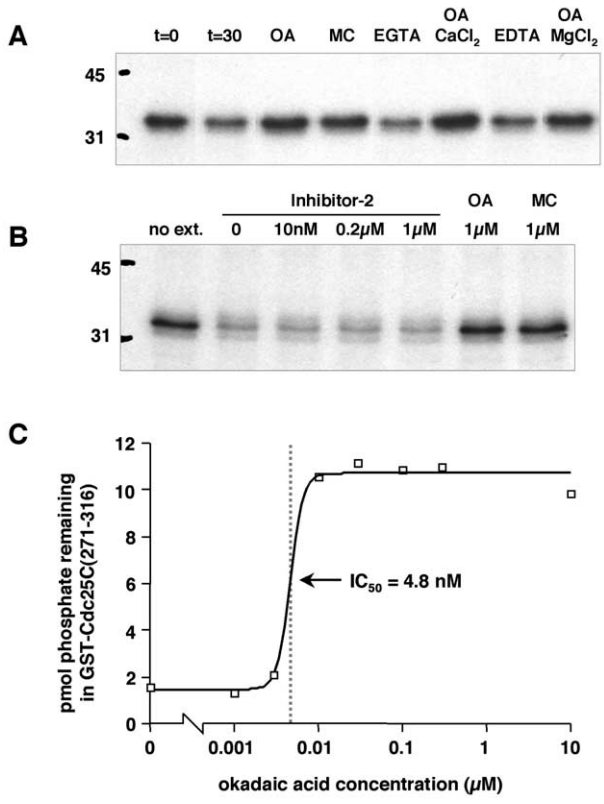

Fig. 2. Characterisation of the phosphatase activity targeting the Ser-287 site of Cdc25C. A,B: Dephosphorylation of [^{32}P-S287]SPS protein in dilute egg extract plus the stated compounds (OA or MC at 1 μM; EGTA, EDTA, $CaCl_2$ or $MgCl_2$ at 10 mM) or I-2. C: Inhibition of [^{32}P-S287]SPS dephosphorylation in dilute egg extract by OA. Following SDS–PAGE and autoradiography, bands were scintillation counted to determine the amount of phosphate remaining in the substrate. D: Dephosphorylation of [^{32}P-S287]SPS protein by dilute egg extract or 0.1 U of purified protein phosphatase.

14-3-3 protein during interphase in egg extract [9], but release of 14-3-3 in M-phase extract [8,9] suggests that S287 is dephosphorylated in mitosis, although cell cycle-dependent changes in the activities of protein kinases or phosphatases targeting this site have not been reported. One possibility is that the activity of the S287-phosphatase changes during the cell cycle, being elevated during mitosis to keep the inhibitory S287 residue of Cdc25C dephosphorylated. However, we found that [^{32}P-S287]SPS and -APS proteins were dephosphorylated faster in interphase than in the M-phase egg extract (Fig. 3A), similar to the activating sites on Cdc25C phosphorylated by Cdc2/cyclin B [7]. Similarly, the rate of S287 dephosphorylation in an interphase extract was reduced by addition of cyclin BΔ90, which stably activates Cdc2 protein kinase (Fig. 3B). Thus, a change in S287-phosphatase activity is unlikely to account for specific dephosphorylation of S287 in mitosis. Interestingly, the APS protein was consistently dephosphorylated in egg extract at a faster rate than the SPS protein (Fig. 3A).

3.4. Effect of 14-3-3 protein binding on S287 dephosphorylation

GST–Cdc25C(271–316) SPS protein bound and precipitated 14-3-3 proteins from interphase *Xenopus* egg extract incubated with Chk1 and OA (Fig. 4A). However, the APS, SPA or SPD proteins did not interact with 14-3-3 proteins, demonstrating the requirement for phosphoserine at residue 287 and serine at residue 285, consistent with other 14-3-3 binding sites that require either serine or an aromatic residue at the −2 position [13]. Interaction of 14-3-3 with the SPS protein depended on the detergent present during washing: binding was detected when the non-ionic detergents Nonidet P-40 and Triton X-100 were used, but was completely absent with the zwitterionic detergent Empigen-BB, which disrupts 14-3-3 binding to certain phosphorylation sites [30].

The inability of 14-3-3 to bind the APS protein suggested an explanation for the more rapid dephosphorylation of this protein in concentrated egg extracts (Fig. 3A). We tested the possibility that 14-3-3 binding inhibited S287 dephosphorylation by adding GST–14-3-3ζ to extract diluted 1:100 where the endogenous 14-3-3 concentration (27 nM; [9]) would be less than that of the exogenous phosphatase substrate. An excess of GST–14-3-3ζ (10 or 20 μM) inhibited the dephosphorylation of 3 μM [^{32}P-S287]SPS in diluted interphase egg

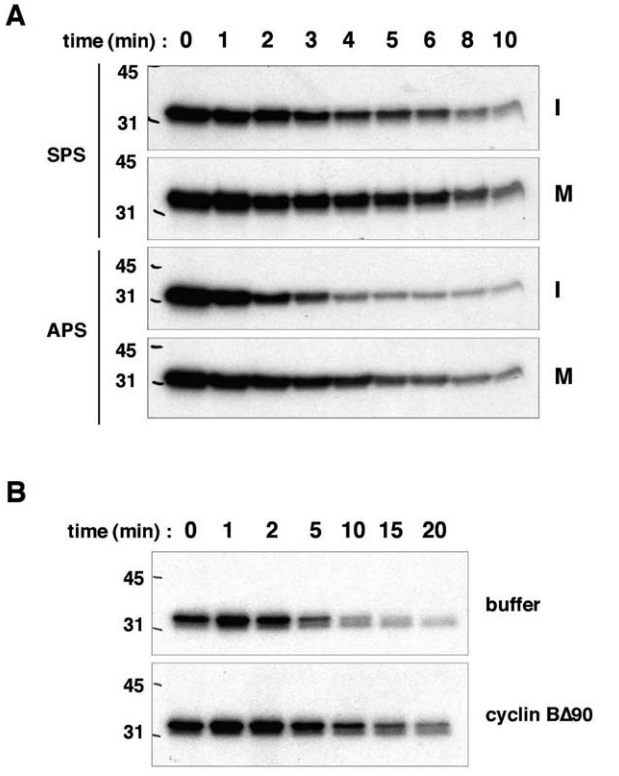

Fig. 3. Rate of S287 dephosphorylation in interphase and mitotic *Xenopus* egg extracts. A: Dephosphorylation of [^{32}P-S287]SPS or -APS protein (400 μg/ml) in a concentrated CSF-arrested egg extract which had previously been incubated for 40 min with either 0.8 mM $CaCl_2$ (interphase, I) or buffer (M-phase, M). B: Dephosphorylation of [^{32}P-S287]SPS protein (400 μg/ml) in a concentrated interphase egg extract which had previously been incubated with buffer or cyclin BΔ90 to activate Cdc2 protein kinase.

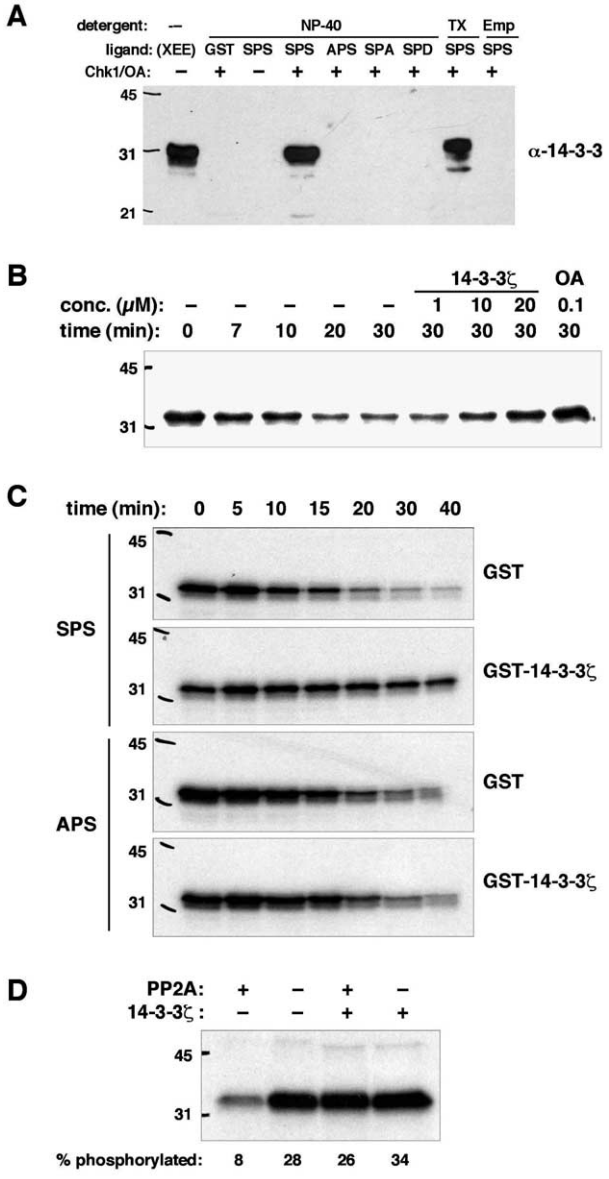

Fig. 4. Effect of 14-3-3 protein binding on S287 dephosphorylation. A: Binding of 14-3-3 from interphase *Xenopus* egg extract to immobilised GST–Cdc25C(271–316) proteins (SPS or mutants, as indicated) in the presence or absence of Chk1 and OA, and the indicated detergent (TX, Triton X-100; Emp, Empigen BB). The leftmost lane (XEE) represents 1 μl of egg extract. B: Effect of 14-3-3ζ on dephosphorylation of [^{32}P-S287]SPS protein (3 μM) incubated for the times indicated with dilute egg extract. C: Dephosphorylation of [^{32}P-S287]SPS or -APS protein (5 μM) incubated with dilute egg extract in the presence of GST or GST–14-3-3ζ (50 μM). D: Dephosphorylation of [^{32}P-S287]SPS protein (50 μg/ml) incubated with 0.02 U purified PP2A and 14-3-3ζ (500 μg/ml) as indicated.

extract (Fig. 4B). However, under conditions where SPS dephosphorylation was inhibited by GST–14-3-3ζ, the APS protein was dephosphorylated (Fig. 4C). Removal of phosphate from S287 catalysed by purified PP2A was also completely inhibited by GST–14-3-3ζ (Fig. 4D). Taken together, these data indicate that binding of 14-3-3 to Cdc25C via the S287-phosphatase motif inhibits the dephosphorylation of this residue.

4. Discussion

In this study we have investigated the dephosphorylation of the inhibitory S287 residue of Cdc25C. The protein phosphatase that acts on this site in *Xenopus* egg extracts is classified as type-2A, the same as the phosphatase that dephosphorylates activating phosphorylation sites on Cdc25C. This creates a paradox in understanding how the phosphorylation of these sites may be differentially regulated. However, dephosphorylation of S287 is inhibited by 14-3-3, a protein which forms a stable interaction with the phosphorylation site during interphase, maintaining the stable phosphorylation of this site during interphase when PP2A activity towards Cdc25C is high and the activating sites are kept dephosphorylated.

Phosphatase activity towards S287 on Cdc25C may play an important role in overcoming G2 timing controls and checkpoint restraint of mitosis in response to DNA damage or replication arrest. A reduction in S287-phosphatase and loss of 14-3-3 binding to Cdc25C in mitosis [8,9] indicates that the balance between kinase and phosphatase activities acting on this site switches at the G2/M transition. However, we found that the rate of S287 dephosphorylation is not increased in mitotic extracts compared to interphase extracts, indicating that net dephosphorylation and loss of 14-3-3 binding to Cdc25C in mitosis are not a consequence of elevated PP2A activity towards that site. Rather it seems that PP2A activity towards both inhibitory (this report) and activating sites [7] on Cdc25C is decreased during mitosis, either due to competition from the increased abundance of phosphoproteins during mitosis [31] or a more specific down-regulation of PP2A activity towards Cdc25C [7]. Net dephosphorylation of S287 in mitosis may therefore be a consequence of inactivation of the relevant kinase activities, assuming that the interaction between 14-3-3 and the phosphorylated binding site is dynamic. Another possibility is that 14-3-3 itself becomes modified, e.g. by phosphorylation [32,33], reducing its binding activity and causing dissociation from Cdc25C.

14-3-3 proteins are members of a functional group of signalling molecules which bind proteins containing phosphorylated residues, usually within a particular sequence context [34]; these include Src-homology 2 domains [35], forkhead-associated (FHA) domains [36], and certain WW domains [37]. Like the effect on Cdc25C S287 dephosphorylation, 14-3-3 proteins can inhibit dephosphorylation of binding partners such as histones [38], tyrosine and tryptophan hydroxylases [39,40], and the Ser/Thr kinase Raf-1 [30,41,42]. Similarly, the FHA1 domain of Rad53p inhibits dephosphorylation of the pThr18 residue of human p53 to which it binds [36]. Crystal structures of a 14-3-3 dimer [13,43], FHA domains and WW domains bound to phosphorylated peptides [44–46] provide an explanation for these effects: the side-chains of the phosphoserine or phosphothreonine residues are buried within specificity pockets in these binding domains and protein phosphatases would be unable to gain access. In the physiological context, the dynamics of the interaction between the phosphoprotein and its binding partner are likely to determine the effect on phosphorylation status. One function of proteins that bind to phosphorylated epitopes may be to control the persistence of phosphorylation at particular sites after the temporal decline of kinase activity or spatial separation of a phosphoprotein from the kinase within a cell. In the case of Cdc25C, 14-3-3 binding also determines cellular local-

isation of the phosphoprotein, since a nuclear localisation signal on Cdc25C is masked by 14-3-3 binding and Cdc25C is excluded from the nucleus by active export [18].

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