



Differential phosphorylation of Cdc25C phosphatase in mitosis

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ABSTRACT

Cdc25 dual-specificity phosphatases coordinate entry into mitosis through activating dephosphorylation of the Mitosis-Promoting Factor, Cdk1-cyclin B1. Activation of Cdc25C at the G2/M transition, involves its dissociation from 14-3-3, together with its hyperphosphorylation on several sites within its regulatory N-terminal domain, mediated by cyclin-dependent kinases and Plk1. Growing evidence suggests that phosphorylation intermediates are likely to precede complete hyperphosphorylation of Cdc25C. To address whether such variants occur in mitotic cells, we raised antibodies directed against different mitotic phosphorylation sites of human Cdc25C, and characterized the phosphorylated species detectable in HeLa cells. In the present study, we provide first-time evidence for the existence of multiple species of Cdc25C in mitotic cell extracts, including full-length and splice variants with different phosphorylation patterns, thereby revealing an intricate network of Cdc25C phosphatases, likely to have distinct biological functions.

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Transitions between the different phases of the mammalian cell cycle are driven by the sequential activation of cyclin-dependent kinases, whose activity is dictated by the periodic expression of cyclin subunits, and a number of posttranslational modifications [1]. In particular, Cdc25 dual-specificity phosphatases coordinate entry into mitosis through activating dephosphorylation of the Mitosis-Promoting Factor, Cdk1-cyclin B1 [2]. In turn, active Cdk1-cyclin B1 establishes a positive feedback loop with Cdc25 phosphatases, contributing to their hyperactivation through phosphorylation of several S/T-P sites [3–7].

In human, three isoforms of Cdc25, A, B, and C, cooperate in regulating cell cycle progression [8]. It is widely accepted that Cdc25B is responsible for initial activation of Cdk1-cyclin B1 at centrosomes, while Cdc25A and Cdc25C would be involved in further amplification of its activity [8–12]. Since its initial discovery as the first Cdc25 phosphatase, Cdc25C has been extensively characterized in terms of function and regulation [13,14]. During interphase, phosphorylation of Cdc25C on Ser216 prevents its activation, by promoting its sequestration in the cytoplasm through association with 14-3-3 proteins [15–17]. At the G2/M transition, dissociation of Cdc25C from 14-3-3, together with its hyperphosphorylation on several sites within its regulatory N-terminal domain, Thr48, Thr67, Ser 122, Thr130, and Ser214, coincides with mitotic activation of Cdc25C [18–22]. Recent studies in *Xenopus* have revealed that Thr130 phosphorylation is a prerequisite to 14-3-3 dissociation [23–25]. Moreover, phosphor-

ylation of human Cdc25C on Ser214 in mitosis has been shown to prevent re-phosphorylation of Ser216 and subsequent inactivation of Cdc25C through re-binding of 14-3-3 [18].

Hyperphosphorylation of Cdc25C is readily detected in mitotic extracts due to a significant reduction in its electrophoretic mobility, first described in the literature for *Xenopus* Cdc25C [26]. However, it remains unclear whether phosphorylated intermediates of fully active Cdc25C occur in mitotic cells. Moreover, several studies report the identification of alternatively spliced variants of Cdc25C, namely Cdc25C1 (full-length protein, 53 kDa) Cdc25C2 (48 kDa), C3 (33 kDa) C4 (49 kDa), and C5 (42 kDa), some of which are upregulated in cancer cell types [27–31]. These splice variants diverge by some phosphorylation sites and regulatory interaction patches, suggesting that they are likely to integrate and respond to different regulatory inputs. However, there is no evidence relating to phosphorylation of these splice variants. In this study, we have raised and purified antibodies that recognize specific phosphorylation sites of human Cdc25C, and have used these to probe for phosphorylated species of Cdc25C in HeLa cell extracts. We have identified several phosphorylated forms of this phosphatase in mitotic HeLa extracts, providing first-time evidence for the occurrence of multiple phosphorylated Cdc25C species, including phosphorylated splice variants, thereby revealing the existence of a complex network of Cdc25C isoforms.

Materials and methods

Cell culture, synchronization, and siRNA transfection. HeLa cells were cultured in DMEM supplemented with 10% FCS, at 37 °C in an atmosphere containing 5% CO₂. Cell culture media, serum, and antibiotics were purchased from Invitrogen. Cells were synchronized in mitosis for 12 h with 250 µg/ml Nocodazole, and collected

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by mitotic shakeoff. siRNA smart-pools™ targeting Cdc25C and control siRNA (non-targeting) were obtained from Dharmacon. 50 nM siRNA were transfected twice at a 24 h interval with Oligofectamine™ (Invitrogen) according to the manufacturer's instructions. Cells were collected by trypsinization 72 h after the first transfection, frozen in liquid nitrogen and stored at -80°C .

Immunization and purification of Cdc25C phosphospecific antibodies. All peptides derived from Cdc25C phosphorylation sites were synthesized by GL Biochem Shanghai Ltd. (China). Rabbits were immunized with thyroglobulin-coupled pThr130, pThr48, and pSer214 peptides (CAQLLCSpTPNGLDRG, CVPRpTPVGKFLG, and CGLYRpSPSMPELN, respectively) by the Animal Core Facility. Sera were first purified against phosphopeptides and then against the corresponding unphosphorylated peptides to eliminate all remaining non phospho-specific antibodies, on Sulf-olink™ resin (Pierce, USA) as recommended by the manufacturer. Purified antibodies were concentrated by ammonium sulfate precipitation, dialyzed into PBS and stored at -20°C in 50% glycerol.

Western blotting and antibodies. Cells were lysed in 50 mM Tris, 150 mM NaCl, 0.1% NP40, 0.1% deoxycholate, 2 mM EDTA, 1 mM PMSF, Complete™ protease inhibitors (Roche), 50 mM NaF, 40 mM β -Glycero-phosphate, 1 mM NaVO₄, on ice and vortexed every 5 min for 30 min, sonicated twice for 2 s at low intensity, then centrifuged at 17,000g at 4°C . The soluble fraction was dosed by BCA assay (Pierce), and boiled in Laemmli buffer. For Lambda-phosphatase assays, cells synchronized with nocodazole were processed in the same fashion, treated with 400 U Lambda phosphatase for 30 min at 30°C in buffer deprived of phosphatase inhibitors, and then boiled in Laemmli buffer. Thirty micrograms of total protein were loaded by lane and separated on 10% SDS-PAGE, then transferred onto a PVDF membrane (Perkin-Elmer). Protein loading was controlled by amido-black staining of the membrane (Sigma). Membranes were blocked in TBS 0.1% Tween 20, 5% non-fat dry milk for 1 h. Antibodies against the C-terminus of Cdc25C (C-20, sc-327) were purchased from Tebu-Bio (Santa-Cruz). For Western blotting, all primary antibodies were used at a 1:500 dilution, incubated overnight at 4°C in TBS 0.1% Tween 20, 5% non-fat dry milk except for phospho-specific antibodies which were incubated in TBS 0.1% Tween 20, 3% BSA. Membranes were rinsed three times for 5 min in TBS-Tween 0.1%, then incubated with secondary donkey anti-rabbit IgG, coupled to horseradish peroxidase (GE Healthcare) at a 1:10,000 dilution in TBS 0.1% Tween 20, 5% non-fat dry milk for 1 h at room temperature. Western blots were revealed with the Western-Lighting ECL (Perkin-Elmer).

Results

Characterization of human Cdc25C variants in interphasic and mitotic cells

In order to characterize the different species of Cdc25C present in interphasic and mitotic HeLa cells, we probed cell extracts by Western blotting with a generic antibody directed against the C-terminus of the phosphatase (C-20). We identified several forms

of Cdc25C, which we classified into three categories, according to their electrophoretic mobility (Fig. 1A). In asynchronous HeLa cell extracts, two major species of Cdc25C were detected: a doublet in the 60 kDa range (molecular weight range 2), corresponding to full-length “interphasic” forms of Cdc25C, unphosphorylated and phosphorylated on Ser216, as well as bands with an apparent molecular weight of 45 kDa (range 1), which are most likely Cdc25C splice variants Cdc25C5 (42 kDa), Cdc25C2 or Cdc25C4 (around 48 kDa). In mitotic cell extracts, although these forms are also detectable, the most abundant species are found around 80 kDa (molecular weight range 3), inferring that these are hyperphosphorylated forms of Cdc25C. The specific identity of bands detected with the C-20 antibody, was verified by probing cell extracts from interphase and mitotic cells treated with siRNA targeting Cdc25C (or with non-targeting siRNA, as a control). Most of the bands detected by the C-20 antibody disappeared upon treatment with Cdc25C-specific siRNA, with the exception of a prominent band at 45 kDa, between the splice variants described above. Moreover, to verify whether the high molecular weight forms detected in mitotic cell extracts were indeed phosphorylated, cell extracts were treated with Lambda phosphatase, which lead to their complete disappearance, to the benefit of the 60 kDa form (Fig. 1B).

Cdc25C phospho-specific antibodies specifically recognize phosphoproteins with the same electrophoretic mobility as Cdc25C in mitotic cell extracts

To further characterize mitotic phosphorylation variants of Cdc25C, we raised and purified several antibodies against three different phosphopeptides derived from phosphorylation sites Thr48, Thr130, and Ser214, and used these antibodies to probe extracts from asynchronous (and therefore mostly interphasic) or mitotic HeLa cells (Fig. 2). Anti-Thr130 and anti-Ser214 antibodies only recognized proteins present in mitotic extracts. Moreover these bands completely disappeared after Lambda phosphatase treatment of mitotic extracts, indicating that these antibodies were truly phosphospecific (Fig. 2A and B). Anti-pThr48 was somewhat less specific and detected several proteins in asynchronous cell extracts, but otherwise recognized phospho-specific bands in the mitotic extract, which were depleted upon Lambda phosphatase

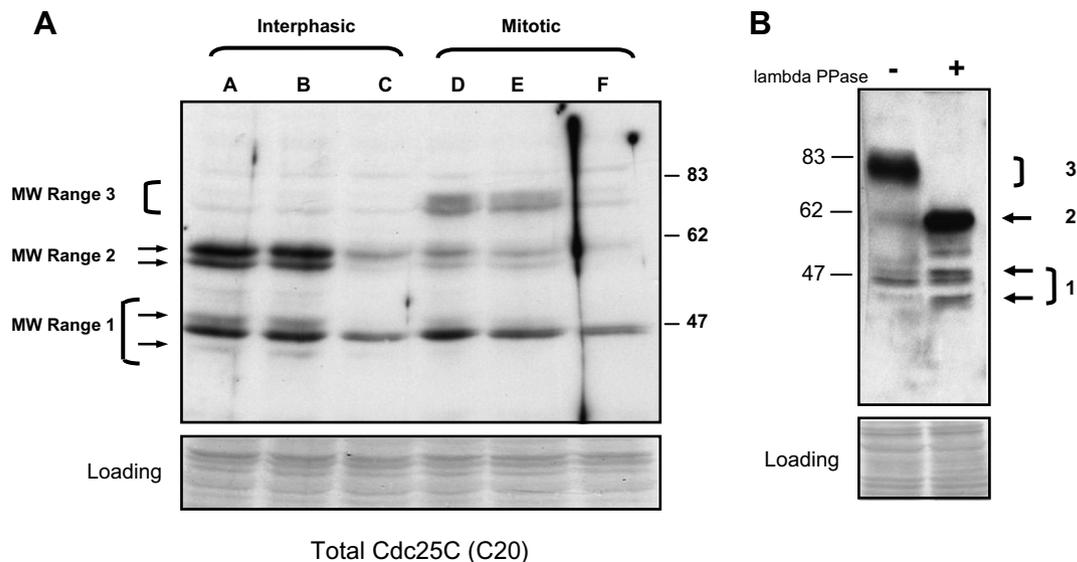


Fig. 1. Identification of multiple Cdc25C variants in interphasic and mitotic HeLa cells. (A) Interphasic (lanes A–C) or Mitotic (lanes D–F) HeLa cells either untreated (A, D), or treated with control siRNA (B, E) or with siRNA targeting Cdc25C (C, F). (B) Mitotic extracts were either untreated (–) or treated with Lambda phosphatase (+). Loading control under each panel: amido-black staining.

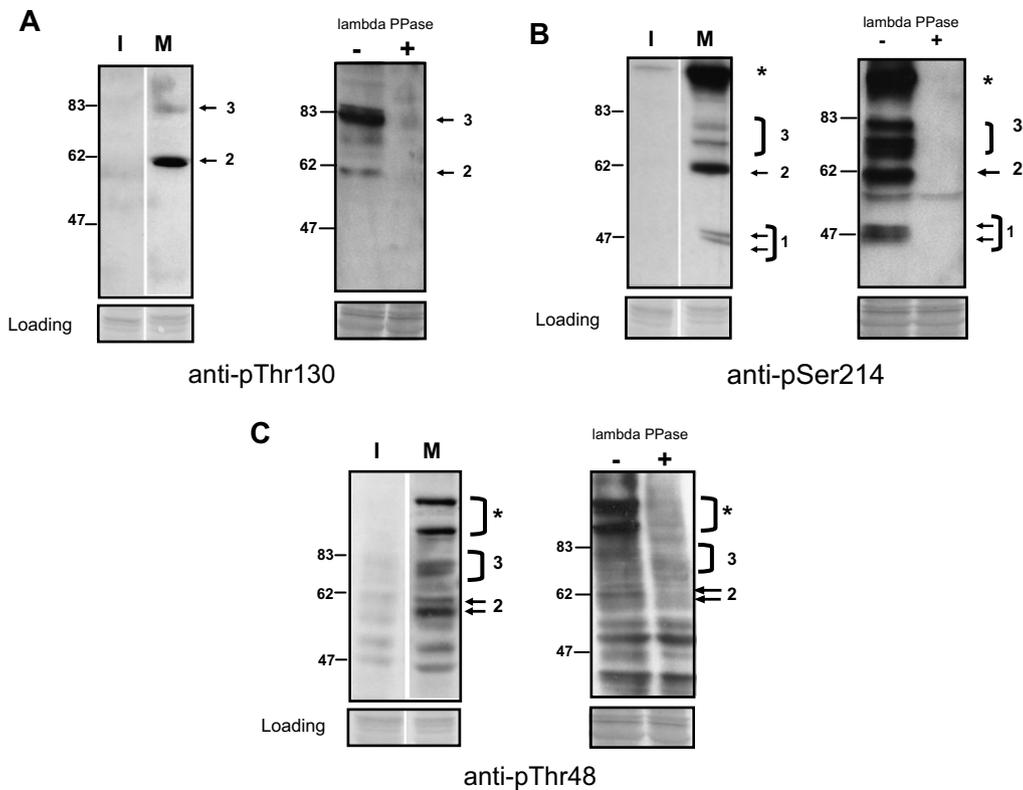


Fig. 2. Antibodies raised against phospho-epitopes of Cdc25C specifically recognize phosphoproteins in mitotic extracts. (A) Anti-pThr130 Cdc25C; (B) anti-pSer214; (C) anti-pThr48. For each panel: Left: Western blots of interphasic (I) and mitotic (M) HeLa cell extracts. Right: Mitotic extracts either untreated (–) or treated with Lambda phosphatase (+). Loading control under each panel: amido-black staining.

treatment (Fig. 2C). Interestingly, the phosphoproteins detected with our phosphospecific antibodies exhibited similar electrophoretic mobilities to those detected by the C-20 antibody, therefore suggesting they were Cdc25C variants.

Anti-phosphoCdc25C antibodies reveal the coexistence of multiple differentially phosphorylated forms of Cdc25C in mitosis, including splice variants

To rule out the possibility that our antibodies recognized phosphoproteins other than Cdc25C with the same electrophoretic mobility, they were used to probe mitotic cell extracts treated with siRNA targeting Cdc25C, or control siRNA. All three antibodies revealed a net reduction in the intensity or a complete disappearance of the phosphoprotein bands upon siRNA treatment, thereby confirming that these antibodies specifically recognize Cdc25C phosphovariants in mitotic extracts (Fig. 3). Anti-pThr130 detects two species: one in molecular weight range 2 and one in range 3 (Fig. 3A). Anti-pSer214 recognizes five species depleted by siRNA: two in range 3, one in range 2, and two in range 1 (Fig. 3B). Anti-pThr 48 recognizes 4–6 variants of Cdc25C: two in range 2, at least two in range 3 appearing as a smeared band, and two in range 1, although the identity of the latter is questionable, as they are not significantly depleted upon siRNA treatment. In addition, anti-pSer214 and anti-pThr48 detect high molecular weight species, which are only quite partially depleted by siRNA treatment, and which most likely correspond to unrelated phosphoproteins which are non-specifically recognized by these antibodies, although we cannot completely exclude that they are hyperphosphorylated species of Cdc25C (Fig. 3B and C, asterisk). However, it seems unlikely that Cdc25C should undergo a change in electrophoretic mobility greater than 25 kDa upon phosphorylation of five residues, and

the extraction conditions used in this experiment do not preserve posttranslational modifications which might occur in concert with phosphorylation, such as ubiquitinylation or sumoylation. The species detected by all three antibodies with an apparent molecular weight of 60 kDa probably correspond to monophosphorylated forms of Cdc25C, whereas species with reduced electrophoretic mobility are most likely phosphorylated on multiple sites. The forms detected by pSer214 and pThr48 with an apparent molecular weight of 45 kDa are most likely Cdc25C splice variants detected with the C-20 antibody. Indeed, an alignment of the primary sequences of the different Cdc25C splice variants shows that they all harbour Thr48, Ser214, and Ser216, but neither Ser122 nor Thr130; moreover only C1, C2, and C3 splice variants retain Thr67, whereas C4 and C5 harbour a Ser instead (Fig. 3D). These findings infer an additional level of complexity in the network of Cdc25 phosphatases. A schematic model illustrating the variety of Cdc25C species which occur in mammalian cells, including multiple phosphorylation intermediates and splice variants, is represented in Fig. 4.

Discussion

Mitotic hyperphosphorylation of Cdc25C is associated with an increase in its phosphatase activity and is readily detected through a significant shift in its electrophoretic mobility [3,15–20,26]. Although it is commonly assumed that Cdc25C oscillates between an hypophosphorylated (Ser216 phosphorylated) inactive state, and an hyperphosphorylated active mitotic form, growing evidence suggests that intermediate phosphorylation variants are likely to occur [18,23–25]. To address this issue, we raised antibodies against three different mitotic phosphorylation sites of human Cdc25C (Thr48, Thr130, and Ser214), and characterized the

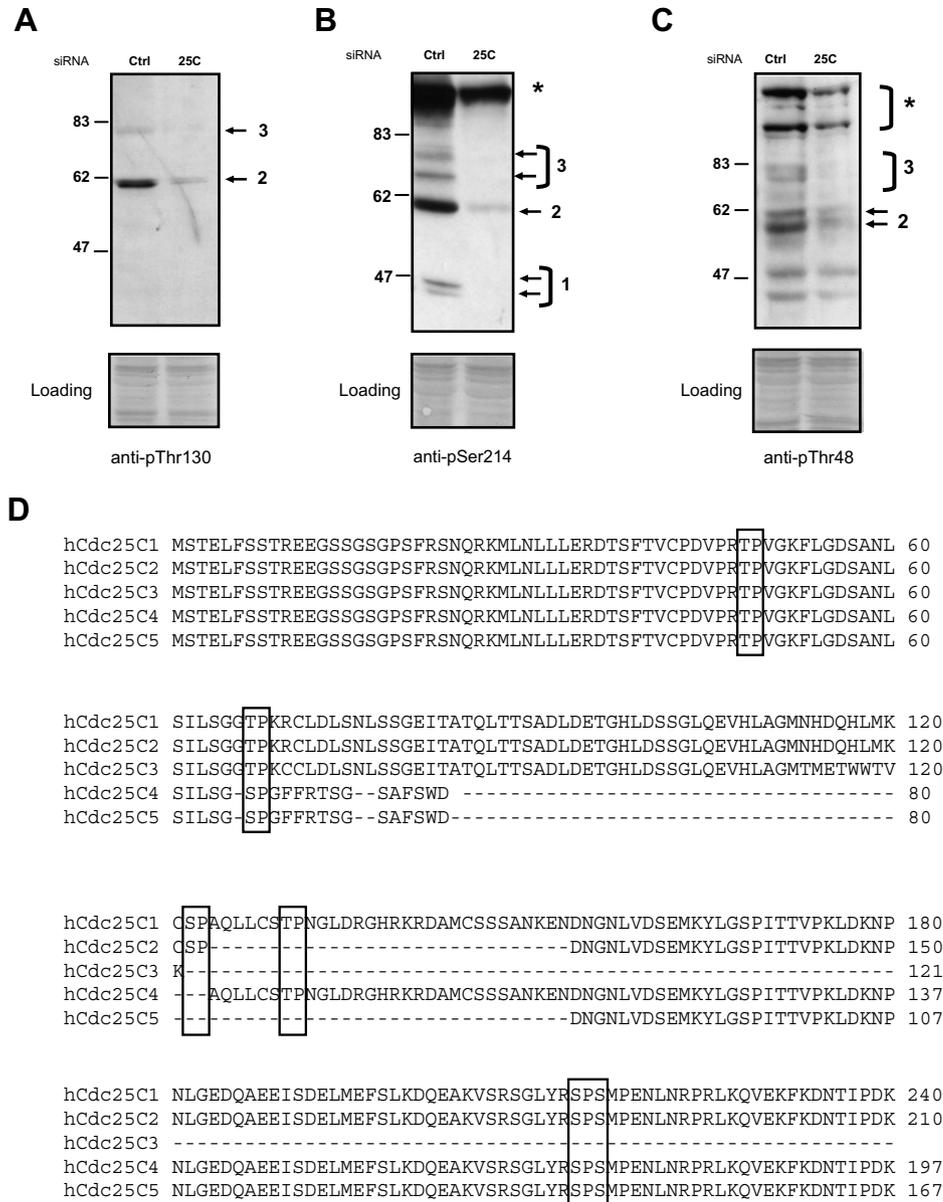


Fig. 3. Coexistence of differentially phosphorylated species of Cdc25C in mitotic extracts. HeLa cells were treated with control siRNA (Ctrl) or with siRNA targeting Cdc25C (25C) for 48 h, then synchronized in mitosis with nocodazole, and cell extracts were probed with the phosphospecific Cdc25C antibodies. (A) Anti-pThr130; (B) anti-pSer214; (C) anti-pThr 48. Anti-pSer214 and anti-pThr48 recognize non-specific high molecular weight phosphoproteins (marked by an asterisk). Loading control under each panel: amido-black staining. (D) Alignment of the N-terminal domains of human Cdc25C splice variants highlighting mitotic phosphorylation sites (boxed residues).

different phosphorylated species detectable in HeLa cell extracts. Whilst each of our antibodies reveals a different spectrum of Cdc25C variants, together they identify a complex network of phosphorylation intermediates, harbouring at least two forms of Cdc25C phosphorylated on Thr130, five on Ser214 and 4–6 forms phosphorylated on Thr48. Based on their electrophoretic mobility, we infer that these include intermediates bearing different combinations of multiple phosphorylated residues, as well as mono- or bis-phosphorylated species, and phosphorylated splice variants. Hence, while mitotic phosphorylations of Cdc25C are commonly believed to occur simultaneously, our data instead imply that intermediate phosphorylated forms occur independently, further inferring a specific biological function for these intermediates. Phosphorylation may regulate subcellular localization, interactions with different partners/substrates, or even influence the catalytic activity of Cdc25C per se. Further studies will be required to gain

insight into the biological function of the phosphorylated species identified in this study.

An additional level of complexity in the biology of Cdc25 phosphatases was uncovered with the identification of alternatively spliced variants of all three Cdc25 isoforms [27–34]. However, unlike Cdc25B splice variants, which have been extensively studied [32–34], the function and regulation of Cdc25C splice variants remains largely unknown. The present study reveals that splice variants of Cdc25C are phosphorylated on Ser214 and possibly on Thr48 in mitosis, suggesting that they are subject to the same regulatory mechanisms as full-length Cdc25C. Phosphorylation of Ser214 has been shown to promote mitotic activation of Cdc25C, by preventing 14-3-3 binding [18], therefore implying that the phosphorylated Cdc25C5 and C2/C4 splice variants identified in this study are also active during mitosis. Future studies will be required to investigate their function.

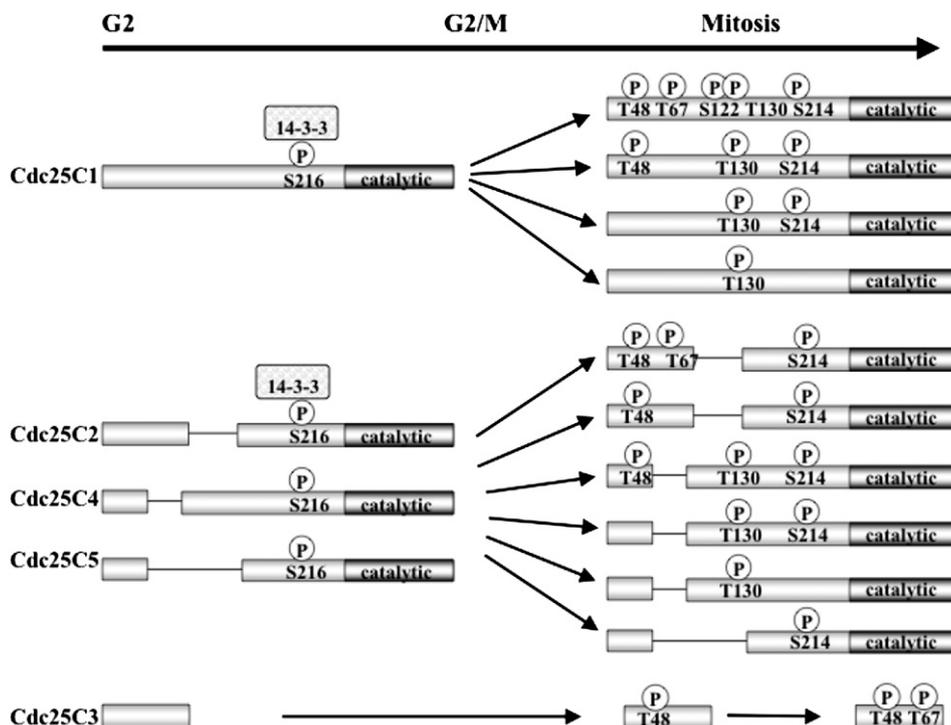


Fig. 4. Schematic representation of the different species of Cdc25C. Mammalian cells harbour several forms of Cdc25C phosphatase, including a full-length form known as Cdc25C1, and several splice variants, Cdc25C2, C3, C4, and C5, which may undergo differential phosphorylation on several phosphorylation sites, in particular Ser216 during interphase, Thr48, 67, 130, and Ser130, 214 in mitosis.

Taken together our data provide first-time evidence for the coexistence of a mosaic of phosphorylated Cdc25C species. The combination between splicing and differential phosphorylation adds to the complexity of the Cdc25 network. It is tempting to speculate that this additional level of complexity applies to all members of the Cdc25 family.

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