Identification of N-Terminally Truncated Stable Nuclear Isoforms of CDC25B That Are Specifically Involved in G2/M Checkpoint Recovery

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Abstract

CDC25B phosphatases must activate cyclin B-CDK1 complexes to restart the cell cycle after an arrest in G2 phase caused by DNA damage. However, little is known about the precise mechanisms involved in this process, which may exert considerable impact on cancer susceptibility and therapeutic responses. Here we report the discovery of novel N-terminally truncated CDC25B isoforms, referred to as ΔN-CDC25B, with an exclusively nuclear and nonredundant function in cell cycle re-initiation after DNA damage. ΔN-CDC25B isoforms are expressed from a distinct promoter not involved in expression of canonical full-length isoforms. Remarkably, in contrast to the high lability and spatial dynamism of the full-length isoforms, ΔN-CDC25B isoforms are highly stable and exclusively nuclear, strongly suggesting the existence of two pools of CDC25B phosphatases in the cell that have functionally distinct properties. Using isoform-specific siRNA, we found that depleting full-length isoforms, but not ΔN-CDC25B isoforms, delays entry into mitosis. Thus, in an unperturbed cell cycle, the full-length isoforms are exclusively responsible for activating cyclin B-CDK1. Strikingly, in the late response to DNA damage, we found a CHK1-dependent shift in accumulation of CDC25B isoforms toward the ΔN-CDC25B species. Under this physiological stress condition, the ΔN-CDC25B isoform was found to play a crucial, nonredundant function in restarting the cell cycle after DNA damage-induced G2 phase arrest. Our findings reveal the existence of a previously unrecognized CDC25B isoform that operates specifically in the nucleus to reinitiate G2/M transition after DNA damage. Cancer Res; 71(5); 1968–77. ©2011 AACR.

Introduction

In mammals, there are 3 CDC25 phosphatases—CDC25A, CDC25B, and CDC25C—(1, 2) which are believed to be partially functionally redundant in controlling mitotic entry (3, 4). In the prevailing model, CDC25B has an exclusive role in the initiation of cyclin B-CDK1 activation at the centrosome (5), although not essential as its RNAi-mediated depletion only results in a short delay in the entry into mitosis (5, 6). In special physiological conditions in which the cell cycle has to be restarted from an extended block, the functional redundancy between the 3 CDC25s is lost, and CDC25B is the only member of the CDC25 family whose function becomes essential for mitotic entry. This notion stems in part from the fact that in oocyte of female CDC25B-null mice, resumption of the meiotic progression does not occur owing to a defect of activation of cyclin B-CDK1 (7), a phenotype not observed in a CDC25C-null background (8). Moreover, Medema and colleagues have shown that CDC25B, but not CDC25A or CDC25C, was critically required for the recovery from a prolonged arrest in G2 induced by the activation of the DNA damage checkpoint (9). What makes CDC25B so unique among the CDC25s in activating cyclin B-CDK1 in the context of a resumption of the cell cycle is currently unknown. CDC25B is a very dynamic molecule that has been shown to be subjected to a complex nucleo-cytoplasmic shuttling dependent on the combined effects of a nuclear localization signal and a nuclear export sequence (10–12). There are several lines of evidence showing that CDC25B is sequestered in the cytoplasm in response to various cellular stresses (11, 13), especially those activating the p38-MK2 signaling (14). The reason why the cell concentrates CDC25B in the cytoplasm in response to some stress, and the relationship between cytoplasmic or nuclear compartmentalization and the control of the phosphatase activity remain poorly understood. CDC25B has been described as a very unstable protein whose degradation is mediated by the ubiquitin-proteasome machinery (15, 16). The mechanism by which CDC25B is directed to proteolysis involves a DDG motif, located in the N-terminal regulatory domain, which plays a critical role in the recruitment of the E3 ubiquitin ligase SCFβTrCP to the...
phosphatase (17, 18). The degradation of CDC25B, which determines the cellular level of the protein, seems to be controlled by an intricate array of phosphorylation events emanating from various signaling pathways (13, 19) still sparsely characterized. The description of the regulation of the level of CDC25B in response to genotoxic stress has led to conflicting data (13, 20). These discrepancies stem from the fact that CDC25B expression appears to be particularly sensitive to the nature and magnitude of the stress applied to the cell, and most importantly from the lack of well characterized CDC25B antibodies. In addition, because of the latter reason, an accurate biochemical characterization of the CDC25B isoforms present in the cell is still missing. To date, although 5 mRNA splice variants (CDC25B1 to B5) have been described (21, 22), only CDC25B3 and CDC25B2 have been biochemically detected in human cells (22).

Here, we have investigated the molecular diversity generated by CDC25B gene and identified a novel class of CDC25B isoforms, nuclear, and functionally dedicated to the recovery from the G2/M checkpoint. Besides raising the concept of the existence of 2 molecular pools of CDC25B phosphatases with opposite stability and nucleo-cytoplasmic trafficking properties, our results point to novel regulatory modes of CDC25B, and function in the nucleus critical for the resumption of the cell cycle.

Materials and Methods

Cell culture, transfection, synchronization, and checkpoint recovery

U2OS and HCT116 cells were grown in DMEM (Invitrogen) supplemented with 10% FCS. Plasmid DNA was transfected using Jet PEI reagent (Poly Plus transfection). Thymidine-synchronization was carried out by treating the cells with 2.5 mmol/L thymidine (Sigma) either for 24 hours (simple block), or 16 hours followed by a release of 15 hours, and readdition of thymidine for 17 hours again (double block). Etoposide (Sigma) was applied to the cells for one hour at a concentration of 40 μmol/L, before being washed extensively. Checkpoint recovery experiment was carried out essentially as described (6). For protein stability studies, cycloheximide point recovery experiment was carried out essentially as described (13, 19) still sparsely characterized. The description of the regulation of the level of CDC25B in response to genotoxic stress has led to conflicting data (13, 20). These discrepancies stem from the fact that CDC25B expression appears to be particularly sensitive to the nature and magnitude of the stress applied to the cell, and most importantly from the lack of well characterized CDC25B antibodies. In addition, because of the latter reason, an accurate biochemical characterization of the CDC25B isoforms present in the cell is still missing. To date, although 5 mRNA splice variants (CDC25B1 to B5) have been described (21, 22), only CDC25B3 and CDC25B2 have been biochemically detected in human cells (22).

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Western blotting, and Immunofluorescence staining

Whole cell extracts for immunoprecipitation were prepared by lysis of the cell in a buffer containing 50 mmol/L Tris HCl pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40, and supplemented with proteases inhibitors. Clarified extracts were incubated with anti-HA affinity matrix (Roche) overnight at 4°C, and pulled down material was separated from the beads by addition of SDS loading buffer. Western blots were conducted using whole cell extracts obtained by lysing the cells either in SDS PAGE loading buffer or in NP40 lysis buffer. Proteins were separated using NuPAGE Bis Tris gradient precasted gels (Invitrogen), and transferred to Nitrocellulose. U2OS tet-off expressing CDC25B3-HA or CDC25B55-HA were grown on coverslips, fixed with 4% paraformaldehyde, and permeabilized with 0.5% Triton X100, and immunostained using anti-HA antibody.

Plasmids

Plasmid vectors allowing the expression of C-terminally HA-tagged CDC25B3, B6, and B7 were obtained by PCR-amplifying their respective coding sequences using the forward primers B3-Bam 5′-attgtatccattcgagggtcgcggcagccg-3′ or B6-Bam 5′-tagatccatcatgtcgcggcagccg-3′ and B6-Bam 5′-tagatccatcatgtcgcggcagccg-3′ associated with 25B-HA XhoI 5′-tacctgtagtgatcatcgtcgcg-3′ and cloning the resulting PCR fragments to the BamH1-XhoI sites of the vector pCDNA3.1+.

Flow cytometry

Cells were harvested and fixed with cold 70% ethanol. They were subsequently stained with either anti-phospho H3 Ser-10 antibodies or the monoclonal 3.12.I.22 (23) that were revealed using anti-HA antibody. DNA was stained with Propidium Iodide (Sigma). DNA content and mitotic index were determined using a FACScalibur flow cytometer (BD).

siRNA-mediated knockdown

siRNA were transfected using Interferin reagent (Poly Plus transfection) at a final concentration of 1 to 3 nmol/L. The mRNA sequences targeted by the siRNAs used in this study are: siRNA CDC2B: agagccagauaccccuau, siRNA 55: ggcaguaugacgu, siRNA 65: aggggauagagau, siRNA CHK1 (24). Nontargeting control siRNA and Smart Pool siRNA to Plk1 were purchased from Dharmacon.

Sequence database search

The visualization of the cDNA sequences aligning to the CDC25B gene locus was achieved using Blat search from UCSC genome browser (25).

Results

Biochemical characterization of CDC25B polypeptides diversity identifies novel short isoforms

In order to investigate the diversity of CDC25B polypeptides expressed in human cells, Western blots were carried out on HCT116 whole cell lysates using 2 independent antibodies.
(C20 and 19.29) directed against 2 different domains of CDC25B (see Fig. 1C). Both antibodies produced a similar signal pattern depicted in Figure 1A consisting in 2 band doublets migrating at an apparent molecular weight of 65 and 61 kDa for the slowest migrating pair (Fig. 1A, band 65 and 61), and 55 and 52 kDa for the fastest (Fig. 1A, band 55 and 52). The fact that those electrophoretic species were specifically depleted in cells transfected with a siRNA targeting CDC25B mRNA (Fig. 1B) excluded the possibility of nonspecific signals and hence showed that they corresponded to bona fide CDC25B molecules. We next used 2 antibodies directed against either the A- or B-domain of CDC25B, both localized in the N-terminal regulatory domain (Fig. 1C; see the schematic representation of the primary structure of CDC25B), and absent in CDC25B1 and CDC25B2 isoforms, respectively (21). Remarkably, the antibodies to the most N-terminal A-domain failed to recognize the 55 kDa species whereas those against the more C-terminal B-domain did bind to it showing that this molecular species corresponds to a N-terminal truncation of CDC25B whose amino-terminal extremity maps between the A- and B-domains (Fig. 1C). We were not able to characterize the 52 kDa species primary structure using this approach because it co-migrated with the IgG heavy chains. The fact that the 65 kDa signal displayed the epitopes of the 4 antibodies to CDC25B used in our study, and the 61 kDa CDC25B signal was specifically ignored by the anti-B domain antibodies (although recognized by the anti-A domain) (Fig. 1C), identified these 2 molecular species, collectively referred hereafter to as full-length CDC25B (Fl-CDC25B), as CDC25B3 and CDC25B2, respectively. We next assessed to which extent the repertory of isoforms identified above was present in various tumoral and nontumoral human cell lines (described in Table S1). As shown in Figure 1D, although each cell line expressed a unique pattern of CDC25B molecules, the 65 kDa CDC25B3 isoform seemed to constitute the major isoform present in every cell lines. Most importantly, the 55 kDa and to a lower extent the 52 kDa species were detected in most of, but not all, the cell lines analyzed, excluding the possibility of a narrow range of expression restrained to the HCT116. Thus, using classical immunoblotting approach, we have been able to detect 4 distinct CDC25B isoforms in human cells. Whereas our data identified the doublet of higher molecular weight as CDC25B3 and B2, the 2 molecular species of lower apparent molecular weight did not exhibit the molecular weight and reactivity to antibodies features of the known CDC25B molecules suggesting that they correspond to uncharacterized isoforms. They were called CDC25B6 (55 kDa) and B7 (52 kDa) according to the current CDC25B isoforms nomenclature (see Table 1 for details).

**Identification of mRNA sequences coding for N-terminally truncated CDC25B isoforms**

In order to characterize the primary structures of CDC25B6 and B7 biochemical species, the sequences of the CDC25B transcriptome present in the human expressed sequence tags (ESTs) sequence-databases were analyzed in silico. We identified 2 classes of mRNA sequences structurally distinct in their 5’ sequence that initiate from 2 distinct positions sepa-
rated of about 570 bp in the genomic locus, suggesting the existence of 2 promoters referred to as P1, and P2 (see Fig. 2A). Although P2 transcripts encoded the canonical CDC25B isoforms (Fig. 2A), P1 promoted mRNAs were characterized by the fact that the conventional ATG codon was removed by the splicing of the first intron, shifting the initiation of translation to the third exon (Fig. 2A). The open reading frames thus created encoded N-terminally truncated versions of CDC25B initiating from the methionine-113 (CDC25B3 numbering). RT-PCR experiments confirmed the existence of these P1 mRNAs in various human cell lines including U2OS and HCT116 (Fig. 2B). Interestingly, PCR produced 2 major amplicons (488 and 365 bp) whose sequence analysis revealed that P1 mRNAs occur as 2 major splice variants (Fig. 2A) encoding for Met113-truncated versions of CDC25B3 and B2 (Fig. 2D). We next aimed to investigate whether

Table 1. Record of all the identified CDC25B isoforms, detailing their accession numbers, protein length, and whether or not they have been biochemically characterized

<table>
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<tr>
<th>Name</th>
<th>Nucleotide accession number</th>
<th>Protein accession number</th>
<th>Protein length</th>
<th>Experimental biochemical confirmation</th>
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<td>601</td>
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Figure 2. Identification of the mRNA sequences of CDC25B6 and B7. A, schematic representation of an alignment of the exon-intron structure of the 2 classes of CDC25B mRNAs initiated from P1 and P2 identified from the ESTs databases. Untranslated transcribed region and coding sequences are depicted in gray and white, respectively. The box A and B represent the exon spliced in CDC25B1 and CDC25B2 splice variants, respectively. B, P1 mRNAs occur as 2 major splice variants in U2OS and HCT116. RT-PCR conducted on RNA extracted from U2OS or HCT116 cells using primers in the exon 1 and 7 of P1 mRNA (arrows in A) results in 2 DNA fragments whose sizes correspond to P1 mRNAs with (488 bp predicted) or without the exon 6 (365 bp predicted). C, CDC25B6 and B7 isoforms are the products of P1 mRNAs. Western blot analysis of cell lysates prepared from U2OS or HCT116 cells transfected with the mentioned siRNA. The localization of the mRNA sequences targeted by the siRNA 55 and siRNA 65 are shown in A. The sequence of siRNA 25B falls in the coding sequence of the catalytic domain shared by all the CDC25B mRNAs (not depicted). D, schematic representation of the primary structures deduced from translation of the CDC25B mRNA species depicted in A, and their relationship with the CDC25B electrophoretic species shown in C. NLS: nuclear localization sequence. DDG: SCFßTrCp E3 ubiquitin ligase interacting motif.
CDC25B and B7 isoforms were generated from these P1 mRNAs (Fig. 2A). We designed a siRNA specifically affecting the transcripts generated from P1 (Fig. 2A; siRNA 55), and ignoring P2 mRNAs, using a sequence within the first exon of the P1 mRNAs. When transfected to the cells, CDC25B6 and B7 were depleted whereas Fl-CDC25B isoforms level remained unaffected (Fig. 2C, lane 55). Conversely, transfection of a siRNA designed to specifically target the P2 mRNAs (see Fig. 2A; siRNA 65) resulted in a strong reduction of Fl-CDC25B level whereas CDC25B6 and B7 were left almost unaffected (Fig. 2C, lanes 65), suggesting that P2 mRNAs exclusively produce Fl-CDC25B molecules. We concluded from these results that the B6 and B7 isoforms originate from P1 mRNAs. Taken with the characterization of the sequences of the P1 mRNAs, we deduced the primary structures of CDC25B6 and B7 as those of CDC25B3 and CDC25B2 translated from the Met113, respectively. This class of truncated isoforms will be collectively referred hereafter to as ΔN-CDC25B by opposition to full-length (Fl) variants (see Table 1).

**ΔN-CDC25B isoforms are nuclear and stable**

CDC25B phosphatases are viewed as very dynamic molecules subjected to a constant nucleocytoplasmic shuttling (10, 26), associated with a high turnover (16). To our surprise, although ΔN-CDC25B contains the DDG degron described by Kanemori and collaborators (17), treatment for one hour with the proteasome inhibitor MG132 did not affect significantly the amount of ΔN-CDC25B whereas under the same treatment, the level of Fl-CDC25B, as well as that of CDC25A (used here as a highly labile protein control) dramatically increased (Fig. 3A). These results suggested that ΔN-CDC25B was much less prone to degradation by the proteasome than its full-length counterpart. In order to confirm this observation, and visualize the kinetics of degradation, cycloheximide chase experiments were conducted in U2OS and HCT116 cells. Inhibition of protein synthesis resulted in a rapid disappearance of Fl-CDC25B whereas CDC25B6 and B7 level remained remarkably stable even 3 hours after addition of cycloheximide (Fig. 3B and C). We next sought to determine CDC25B6 and B7 subcellular localization. Because of the lack of

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![Image](image_url)
antibodies to CDC25B capable of detecting the endogenous isoforms in immunostaining, the localization of ectopically expressed HA-tagged CDC25B6, B7, and B3 was determined using an anti-HA antibody. In agreement with the lack of nuclear export sequence (NES), we found that both CDC25B6 and CDC25B7 localized to the nucleus (Fig. 3D). In contrast, and in line with our previous results (10), CDC25B3-HA exhibited a pan-cellular localization (Fig. 3D, left panels). Taken together, our data revealed that the ΔN-CDC25B isoforms are characterized by a behavior opposite to that of the Fl-CDC25B molecules as they exhibit a particularly low turnover in addition to be compartmentalized in the nucleus.

Specific depletion of ΔN-CDC25B does not delay entry into mitosis

It is established that, in an otherwise unperturbed cell cycle, the knockdown of CDC25B results in a delay of about a couple of hours in the entry into mitosis (5, 6). We thus sought to address the specific contribution of each class of CDC25B molecules in this phenotype using our isoform-specific siRNAs (see Fig. 2B). U2OS cells were transfected with the relevant siRNAs, and subsequently synchronized with thymidine (Fig. 4A). The efficacy of the siRNA-mediated depletion of Fl-CDC25B and/or ΔN-CDC25B 10 hours after release from synchronization was validated by Western blot analysis.
(Fig. 4D). The analysis of the refilling of the 2N DNA content as a function of the time after thymidine release showed that, in agreement with published data (5), cells treated with siRNA leading to the full depletion of CDC25B remained in a 4N DNA content about one hour and half longer than cells transfected with control siRNA (Fig 4B, siRNA CDC25B versus siRNA control). Remarkably, although transfection of the siRNA 65 resulted in a similar delay in the reduction of the 4N DNA content, interfering with the expression of ∆N-CDC25B did not produce any significant inhibition of the evolution of the DNA content when compared to the control siRNA (Fig 4B; siRNA 65 versus siRNA 55). We reached identical conclusion using a similar experimental set-up in which nocodazole was added after release from thymidine block in order to follow the accumulation of mitotic cells. In this experimental approach, again, the siRNA 55 was the sole siRNA to CDC25B not to delay the kinetic of accumulation of mitotic cells versus the control. We concluded from these results that the full-length species is the class of CDC25B isoforms functioning in the mitotic entry network. In addition, our results support the idea that they cannot be replaced by the ∆N-CDC25B species in this function. Moreover, if ∆N-CDC25B plays any role in the entry into mitosis, this function can be fulfilled by Fl-CDC25B.

∆N-CDC25B and Fl-CDC25B isoforms are both required for the G2/M checkpoint recovery

The incapacity to recover from a prolonged checkpoint-induced G2 block is the most prominent phenotype of cells in which CDC25B function has been abrogated (6). We thus sought to characterize the specific contribution of Fl-CDC25B and ∆N-CDC25B in the G2/M checkpoint recovery process using an experimental set-up (Fig. 6A) initially developed by Medema and collaborators (6). U2OS cells transfected with the isoform-specific or control siRNAs, were treated with etoposide while progressing through G2. After 18 hours of block in G2, a synchronous resumption of the cell cycle was simulated by the addition of caffeine, an inhibitor of the ATR and ATM kinases. We validated the efficiency of the siRNA-mediated depletion of each isoforms of CDC25B by Western blotting (Fig. 6B). In line with published data (6), 6 hours after addition of caffeine, whereas a substantial part of the cells transfected with the control siRNA had entered into mitosis, the depletion of all the CDC25B isoforms, and PLK1 knockdown, both resulted in a strong abrogation of the recovery (Fig 6C; compare control with CDC25B and PLK1). Remarkably, a similar inhibition of caffeine-induced entry into mitosis was observed after specific knockdown of ∆N-CDC25B, or Fl-CDC25B (Fig. 6C; siRNA 55 and 65). Importantly, the recovery phenotype associated with the siRNA 65 and 55 was rescued by ectopically expressing nontargetable versions of CDC25B3 and CDC25B6, respectively (supplementary Fig. S2), excluding the possibility of off target effects. We therefore concluded that the expression of both Fl-CDC25B and ∆N-CDC25B was required for the recovery from the G2/M checkpoint. Moreover, these results identified a function for ∆N-CDC25B, exerted when the cell cycle is restarted after a prolonged establishment of the G2/M checkpoint.
To date, little effort has been done to characterize the CDC25B polypeptides diversity expressed in human cells. Yet this knowledge is essential for our full understanding of how CDC25B functions, and the regulation of its activity. We have discovered the existence of a N-terminally truncated class of CDC25B isoforms, co-expressed along with the canonical full-length variants in the cell. Although the human CDC25B promoter has so far never been characterized, our data reveal the existence of 2 CDC25B promoters, P1 and P2, which seem to control separately the expression of ΔN- and Fl-CDC25B isoforms, respectively. This notion is consistent with the apparent independent dynamics of expression-level of each class of isoforms observed during the progression of the cell cycle and the response to genotoxic stress, although...
mechanisms other than transcriptional regulation, like protein stability, are likely to contribute to this differential control. Most importantly, we show that CHK1 is involved in the upregulation of ΔN-CDC25B level during the response to genotoxic stress, a result consistent with the checkpoint-dependent induction of CDC25B described by Banzal and Lazo (20). The nature of the CHK1-dependent mechanisms that stimulate transcription from P1 promoter, and whose effect take place hours after DNA damage remain to be established. Conceptually, our results reveal a unique regulation mode of CDC25B in response to DNA damage in which CHK1 in one hand negatively regulates CDC25B activity to establish the checkpoint (24, 27), and on the other hand participates in the upregulation of the expression of special isoforms of the same positive regulator of the cell cycle, presumably as an anticipatory event toward the resumption of the cell cycle.

The fact that ΔN-CDC25B escapes degradation while containing the DDG degron (17) reveals that the latter, yet necessary, is not sufficient for targeting CDC25B to the proteasome, and shows that the first 113 residues domain of the full length isoforms contains essential structural and/or regulatory determinants of CDC25B degradation.

Our finding that specific knockdown of the Fl-CDC25B isoforms, but not that of ΔN-CDC25B, results in the same delayed entry into mitosis phenotype observed after full depletion of CDC25B (5, 6) identifies the canonical full length species as the isoforms in charge of initiating the activation of cyclin B-CDK1 at the centrosome. The fact that this function cannot be fulfilled by the ΔN-CDC25B isoforms alone may results from their inability to access to the cytoplasmic centrosomal pool of cyclin B-CDK1 owing to their nuclear compartmentalization. The absence of cell cycle progression phenotype associated with the knockdown of the ΔN-CDC25B isoforms raises the question of their function, if any, during an unperturbed cell division cycle, and may explain why their level of expression was found below the detection threshold in some cell lines.

Remarkably, our results reveal that ΔN-CDC25B isoforms become essential for the G2/M transition when the cell cycle is restarted after a prolonged G2/M block induced by the DNA damage checkpoint (6), a function consistent with the dramatic increase of ΔN-CDC25B level observed under this physiological state. This accumulation is not a matter of simply maintaining or increasing the level of CDC25B phosphatase in the cell. Indeed, the 2 classes of isoforms do not seem to be functionally redundant (at least at physiological level of expression; see supplementary Fig. S2) as ΔN-CDC25B, which constitutes approximately 95% of the total pool of CDC25B 18 hours after DNA damage, fails to support the recovery in cells depleted for Fl-CDC25B.

The consideration that, in cells blocked G2, cyclin B is mainly cytoplasmic whereas cyclin A resides in the nucleus (28), suggests that the substrate of ΔN-CDC25B might be cyclin A-CDK complexes. This idea is in line with a recent report (29) revealing that cyclin A-CDK activity has to be maintained to a certain level during the establishment of the checkpoint to allow the activation of FoxM1, a transcription factor whose activity is critical for the recovery (30). Our findings suggest a model in which ΔN-CDC25B would be dedicated variants of CDC25B that would keep the cyclin A-CDK activity above a critical threshold required for the resumption of the cell cycle.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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