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APC/C^{Cdh1}-dependent degradation of Cdc20 requires a phosphorylation on CRY-box by Polo-like kinase-1 during somatic cell cycle

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ABSTRACT

Cdc20 is an activator of the anaphase-promoting complex (APC/C), and APC/C^{Cdc20} is essential for metaphase–anaphase transition. To allow progression beyond mitosis, Cdc20 is degraded through KEN-box-dependent APC/C^{Cdh1} activity. Mammalian Cdc20 contains the CRY box, a second APC/C^{Cdh1}-dependent degron, but the molecular mechanism in degradation process remains undefined. Polo-like kinase-1 (Plk1) is an essential mitotic kinase regulating various targets in kinetochore, centrosome, and midbody for proper mitotic progression. Plk1 directly bound to Cdc20 and phosphorylates it on serine-170 located in CRY-box. Whereas wild-type Cdc20 was degraded according to progress cell cycle beyond mitosis, the phosphorylation-defective mutant, which serine-170 was changed into alanine, was not destroyed in early G1 phase. The phosphorylation on serine-170 by Plk1 was important for ubiquitination and Cdh1-dependent proteolysis. However, this modification by Plk1 on CRY box had no effect on the subcellular localization of Cdc20 and the formation of APC/C-inhibitory checkpoint complexes under spindle assembly checkpoint. This mechanism will be the first finding of inhibitory phosphorylation related to Cdc20 instability.

1. Introduction

The Polo-like kinase-1 (Plk1) is a serine/threonine protein kinase that plays numerous roles during M-phase progression. Plk1 is required for the initiation of G2/M by phosphorylation of Cdc25C and mitotic cyclins, centrosome maturation, and the establishment of a bipolar spindle [1–3]. For initiation of anaphase and exit from mitosis, activation of the anaphase-promoting complex (APC/C) by Plk1 is essential [4,5]. Localization of Plk1 to centrosome persists from early mitosis until late anaphase [6,7], and Plk1 moves to kinetochore and may regulate chromosome and chromatin separation during anaphase [8]. The non-catalytic region in C-terminus of Plk1 has a conserved motif, named Polo-box and this motif is important for Plk localization. Previously, through yeast two-hybrid with Polo-box motif as bait, centrosome/kinetochore components or regulators were characterized as positive clones [9]. Cdc20 is an activator of APC/C (anaphase promoting complex/cyclosome) ubiquitin ligase, and is essential for initiation of the key mitotic regulators to facilitate mitosis [10,11]. APC/C^{Cdc20} degrades mitotic cyclins to regulate cdk1 activity before anaphase onset. One of the key target proteins of APC/C^{Cdc20} is securin, whose degradation is pivotal for releasing separase to cleave cohesin allowing sister chromatid separation at the metaphase–anaphase transition.

During normal cell division, the Cdc20 protein level is strictly regulated. It starts to accumulate in S phase, peaks in mitosis, and drops as cells exit from mitosis [12]. Stability of mammalian Cdc20 is regulated by a single degron, the KEN box, which is recognized by APC/C^{Cdh1} and required for the efficient ubiquitination by APC/C [13]. Cdc20 is also degraded through a newly described CRY box (CRY × PS) in an independent manner of KEN box by studying the degradation of fluorescent protein chimaeras in mammalian oocytes and embryo [14]. Although the regulation of APC/C^{Cdc20} catalytic activity in normal cell division or SAC by hyperphosphorylation has been relatively well-known, less is known about the regulation of Cdc20 degradation in late mitosis by APC/C^{Cdh1} through degrons. Furthermore, it is unclear whether modification on degron regions of Cdc20 regulates its degradation. Here we show that phosphorylation on CRY box, one of the degrons of Cdc20, by Plk1 is essential for Cdc20 degradation.

2. Materials and methods

2.1. Cell culture and cell synchronization, transfection, and gene silencing

Cell culture and cell cycle arrest were performed as previous report [15]. Transient transfection with plasmids was performed by

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standard calcium chloride method except that HEPES-buffered saline was used [16].

To deplete the endogenous Plk1 and Cdh1 in HeLa cells, a plasmid-based small interference RNA constructs were used (SuperArray Bioscience Corporation, USA). The gene specific-sequences for knock-down of Plk1 and Cdh1 are 5′-GCTGCTTAATGACGAGTT CTT-3′ and 5′-AGAAGGGTCTGTTCACGTATT-3′, respectively.

2.2. Generation and expression of Cdc20 constructs

Mutations at the indicated sites in the Cdc20 gene were generated by PCR method. All the PCR products and deletion constructs were cloned into pMAL-CRI (New England Biolabs) and pGEX vectors (Promega) for *Escherichia coli* expression and into pCMV-Tag2 vector (Stratagene) for expression in mammalian cells.

2.3. Protein purification, kinase assay, immunoprecipitation, and in vitro binding assay

For expression in *E. coli* system, isopropyl-β-D-thiogalactoside (IPTG) induction was performed for 5 h (0.3 mM final concentration). MBP- and GST-fusion proteins were recovered by binding to amylase (New England Biolabs) and glutathione resins (Peptron, Korea), respectively. For Plk1 kinase assay was performed as previously described [17]. 1–2 μg of His-Plk1 purified from Hi5 cells was used as a kinase source [15]. For immunoprecipitation, the HeLa cell extract was incubated with anti-FLAG monoclonal antibody (Sigma) for Cdc20s expressed ectopically. Bound proteins in the immunocomplex were analyzed by Western blot with various antibodies [15].

2.4. Phosphoamino acid analysis

The radio-labeled proteins were separated by SDS–PAGE and eletrotransferred onto PVDF membrane (Millipore). The phosphoproteins on membrane hydrolyzed in 6 N HCl were spotted and analyzed by electrophoresis at pH 1.9 in the first dimension (1.5 μV for 20 min) and pH 3.5 in the second dimension (1.3 V for 16 min) with the HTLE-7000 system as previously described [15].

2.5. Confocal microscopy

For confocal microscopy, Cells were fixed with 3% paraformaldehyde/methanol [16], and. DNA and proteins in cells were detected by DAPI and primary antibodies, followed by treatment with Cy3 conjugated secondary antibody using Zeiss LSM510 microscopy.

3. Results

3.1. CRY box in Cdc20 protein is phosphorylated by Plk1

We previously showed that one of the kinetochore regulators, Cdc20 was characterized as positive clones through yeast two-hybrid with Polo-box motif of Plk1 as bait [9]. We conducted a binding experiment between Cdc20 and Plk1 in mammalian cell system *in vivo* and *in vitro*. Full-length Cdc20 was separated into three parts, based on phosphorylations: N-terminal phosphor-part (residue 1–164, D1 in Fig. 1A), middle part (residue 164–348, D2 in Fig. 1A), and C-terminal part (residue 348–499, D3 in Fig. 1A). After co-transfection of full length Cdc20 and Plk1 constructs in mitotic HeLa cells, the lysates of these cells were subjected to immunoprecipitation with anti-FLAG antibody and blotted with anti-GFP. Full-length Cdc20 was co-precipitated with both wild-type Plk1 and a kinase-dead mutant Plk1 (K82M) (Fig. 1B, lanes 2 and 3 in panel a). Indeed, C-terminal domain of Plk1 was sufficient to associate with Cdc20 (Fig. 1B, lane 4 in panel a). In GST full down assay, the N-terminal domain (D1) including KEN box interacted with full-length Plk1 (Fig. 1C, lane 2 in a) and the C-terminal domain (D3) including WD40 repeat domain interacts with both full-length and C-terminal of Plk1 (Fig. 1C, lane 4 and 8 in a). To investigate a question of whether Cdc20 is one of the substrates of Plk1

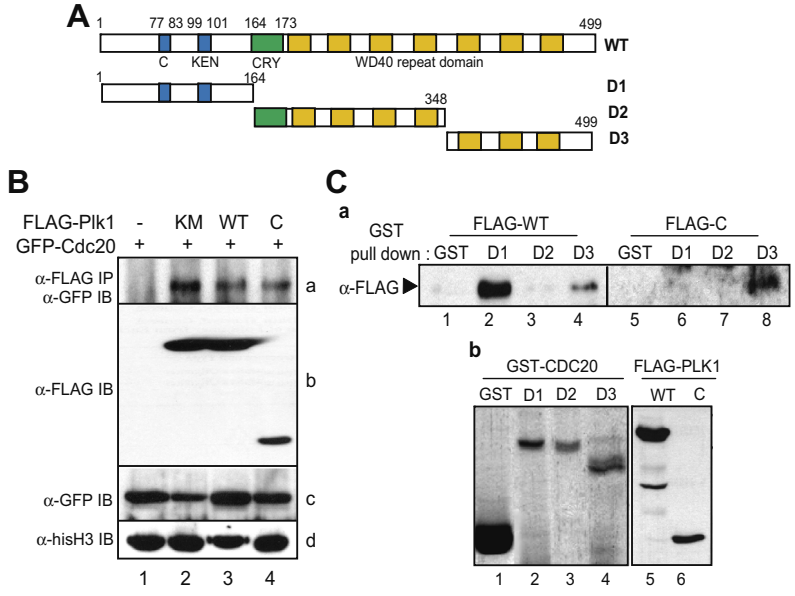

Fig. 1. Interaction between Cdc20 and Plk1 requires the C-terminal domain of both proteins. (A) Schematic domain structure of Cdc20. The full length of Cdc20 (WT) consists of 499 amino acids. (B and C) Interaction between Cdc20 and Plk1 in HeLa cells. Plk1s were tagged with FLAG motif for detection by using anti-FLAG antibody. (B) GFP-Cdc20s were expressed in HeLa cells, immunoprecipitated, and subjected to immunoblot for detection of Plk1 binding (a). Phosphor-Histone H3 was analyzed as a control for confirmation of mitotic condition (d). (C) Three domains of Cdc20 were purified and subjected to *in vitro* protein binding assay incubating with HeLa cell lysates. (a) *in vitro* protein binding between domain constructs of Cdc20 and Plk1. b, expression level of GST-Cdc20s and FLAG-Plk1s. 1, GST only; 2∼4, domain constructs of Cdc20; 5, full-length Plk1; 6, C-terminal construct of Plk1.

or not, we performed *in vitro* kinase assay using a recombinant Plk1 purified from insect cells [15]. Both full-length Cdc20 and D2 domain was phosphorylated by recombinant Plk1 (Fig. 2, filled circle in lane 4 and 7, panel ^{32}P). Phosphoamino acid analysis revealed that the site(s) phosphorylated by Plk1 is mainly serine (Fig. 2B). There are three Plk1 consensus regions matched partially in the primary structure of Cdc20, and one of them is located in CRY-box, which is conserved in mammalian species (Fig. 2C). To validate the phosphorylation sites, we replaced each of the serine (S170, S193, and S205) with an alanine residue. When serine-170 was replaced with alanine, the phosphorylation signal was disappeared in *in vitro* kinase assay (Fig. 2, lane 2 in panel b). Neither serine-193 nor serine-205 was involved in phosphorylation by Plk1 (Fig. 2, lanes 3–5 in panel b). These data suggest that serine-170 located in CRY-box, one of the degrons of Cdc20, was phosphorylated by Plk1.

3.2. The phosphorylation of S170 is related to Cdc20 instability during mitotic exit

Cdc20 protein normally plays a role in the metaphase–anaphase transition and is degraded gradually according to enter the G1 phase. For detection of Cdc20 degradation, after transfections with S170A mutant and wild type Cdc20, mitotic HeLa cells were released into fresh media for 3 h. According to progression from mitosis to next G1, endogenous Cyclin B1 decrease (Fig. 3A, middle panel). As expected, wild type Cdc20 was decreased gradually during releasing (Fig. 3A, lanes 1–5 in upper panel in a). However, S170A mutant of Cdc20 did not disappear under the same condition (Fig. 3A, lanes 1–5 in upper panel in b). To validate the effect of KEN box in the CRY box-dependent degradation, lysine, glutamate, and aspargine in KEN box were displaced with alanines [14]. The degradation of this KEN mutant protein was detected gradually in lower level than the of wild-type Cdc20 (Fig. 3A, lanes 1–5 in upper panel in c). As expected, when both KEN and CRY boxes were mutated, Cdc20 remained according to the cell cycle progression (Fig. 3A, lanes 1–5 in upper panel in d). These results suggested that the phosphorylation in CRY box is more important and dominant for Cdc20 degradation than the function of KEN box. Indeed, the wild-type Cdc20 was highly ubiquitinated and degraded completely in further incubation (Fig. 3B, lanes 2, 4 and 6 in upper and middle panels), whereas S170A mutant remained (lanes 1, 3 and 5).

To further explain that Plk1 participates in the phosphorylation and degradation of Cdc20, we examined the Cdc20 degradation under Plk1 depletion. When Plk1 was depleted, Cdc20 were remained in higher levels (Fig. 3C, lanes 3 and 4 in upper panel). Moreover, the overexpression of wild-type and a constitutive activated Plk1 reduced Cdc20 level in comparison with that in control (Fig. 3D, lanes 3 and 4 in middle panel). Expression of a kinase-dead mutant of Plk1 (K82M) and treatment with proteasome inhibitor did not decrease Cdc20 levels (lanes 2 and 5 in middle panel). Overexpression of Plk1 had no effect on stability of phosphorylation-defective mutant of Cdc20 (S170A) (lanes 6 and 7 in middle panel). These data suggested that the kinase activity of Plk1 is required for the phosphorylation on Cdc20 and degradation of Cdc20 through APC/C.

Next, we investigated whether phosphorylation on S170 residue was affected on binding affinity with APC/C components, such as Cdc27. In mitotic cells, the Cdc27 interaction with wild-type

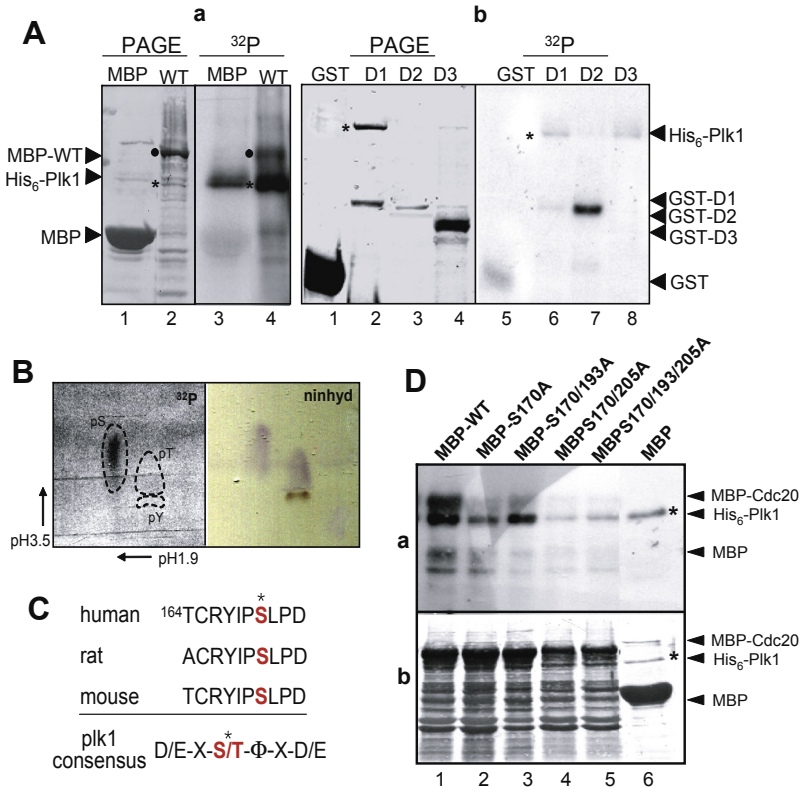

Fig. 2. Polo-like kinase-1 phosphorylates Cdc20. (A) *In vitro* phosphorylation of full length (a) and domain of Cdc20 (b) by Plk1. The asterisks in panels indicated His-Plk1 auto-phosphorylated. Cdc20 labeled with ^{32}P from PAGE gel was excised and subjected into further analysis (filled circle in a). (B) Phosphoamino acid analysis of Cdc20 phosphorylated by Plk1. The amino acids are separated by 2D-electrophoresis, detected by ninhydrin staining (ninhyd), and exposed on film (^{32}P). S, phosphor-serine; T, phosphor-threonine; Y, phosphor-tyrosin. (C) Comparison of CRY box in human, rat, and mouse. Asterisk denotes the identical serine residue, which can be phosphorylated in Plk1 consensus region. (D) *In vitro* Plk1 assay of various Cdc20 constructs. ^{32}P signals and protein constructs of Cdc20 are shown in panel a and b, respectively. Asterisks in panels indicate the autophoshporylation signal of Plk1. All constructs were fused with MBP.

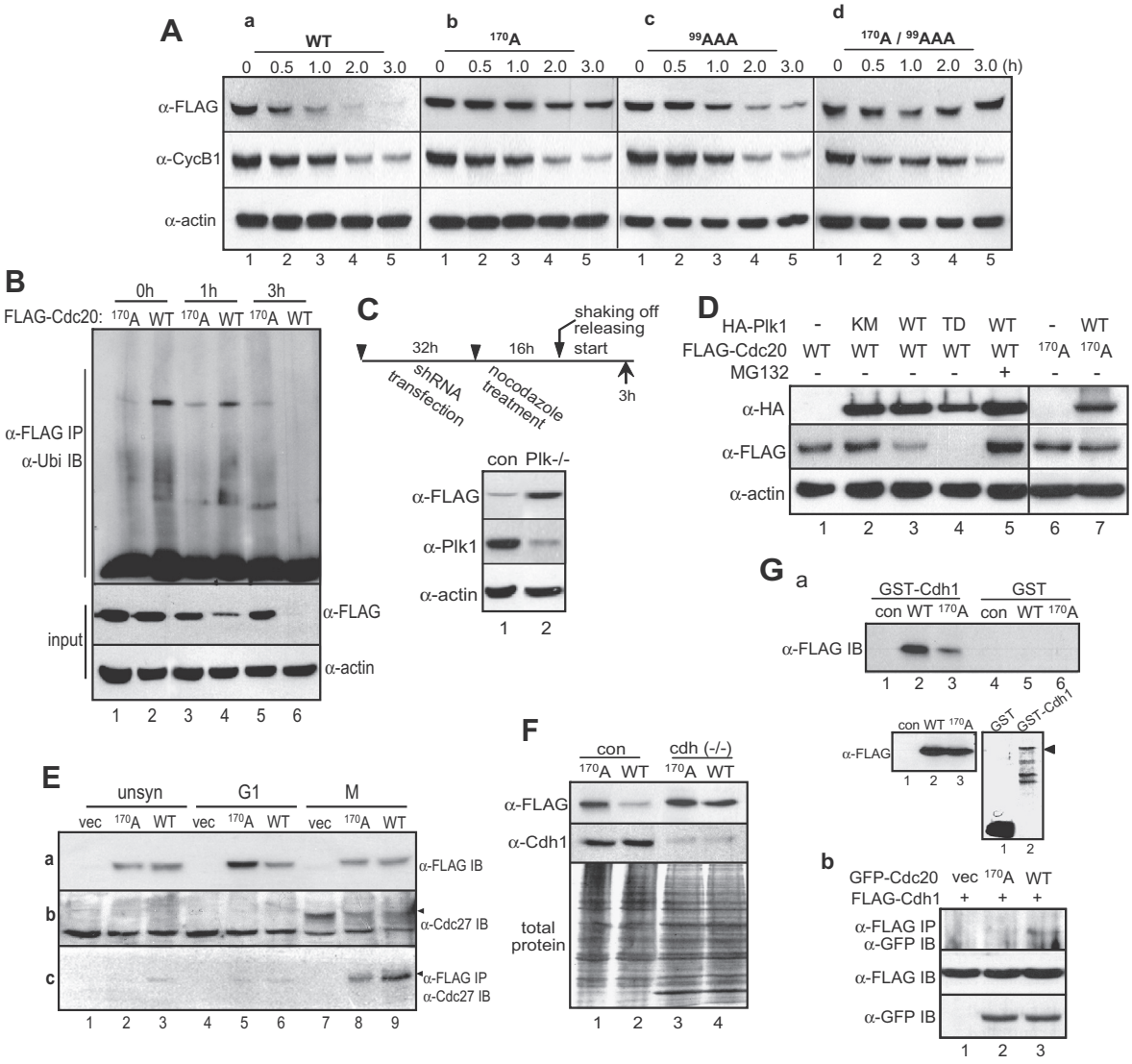

Fig. 3. Phosphorylation on serine-170 by Plk1 is essential for APC^{Cdh1}-dependent degradation of Cdc20. (A) Stability of Cdc20 during cell division. After transfection, cells are treated with nocodazole and collected by shaking-off. Mitotic cells are released into fresh media for indicated time. a, wild type Cdc20 (WT); b, S170A mutant construct (^{170}A); c, KEN mutant construct (^{99}AAA); d, double mutant of S170A and KEN degron (^{170}A^{99}AAA). (B) Ubiquitination of Cdc20. Under the same condition in (A), GFP-Cdc20s were expressed in HeLa cells, immunoprecipitated, and subjected to immunoblot with anti-ubiquitin antibody (α-FLAG IP/α-Ubi IB). Lane 1, 3, and 5, cells transfected with S170A mutant; lane 2, 4, and 6, cells transfected with wild-type Cdc20. (C) Inactivation of Plk1 by using shRNA stabilities Cdc20. Co-transfection of shRNA and FLAG-Cdc20 constructs, treatment with nocodazole, and release for the indicated times are performed as indicated in the schematic. Cells are analyzed by immunoblot for levels of Cdc20 transfected, Plk1, and actin. Lane 1 and 2, transfected with control vector; lane 3 and 4, transfected with Plk1 shRNA. (D) Overexpression of Plk1 decreased Cdc20 amounts in a APC-dependent manner. HeLa cells are co-transfected with Cdc20s and/or Plk1s. For inhibition of APC, cells were treated with MG132. After 24 h, cells are harvested and subjected to immunoblot analysis. (E) Interaction between Cdc20 and Cdc27. HeLa cells in G1 or prometaphase were transfected with FLAG-Cdc20 constructs, and Cdc20 was immunoprecipitated and immnublotted for Cdc27. (F) Phosphorylation on serine-170 facilitates degradation of Cdc20 in Cdh1-dependent manner. Co-transfection with Cdh1-shRNA and Cdc20s, treatment with nocodazole, and release for the indicated times are shown as indicated in the schematic. Lane 1, co-transfected with control vector and S170A; lane 2, control vector and wild type Cdc20; lane 3, Cdh1-shRNA and S170A; lane 4, Cdh1-shRNA and wild type Cdc20. (E) Interaction between Cdc20 and Cdh1 *in vitro* (pane a) and *in vivo* (panel b). For *in vitro* interaction, GST-Cdh1 was incubated with HeLa cell lysates containing FLAG-Cdc20s. Bound FLAG-Cdc20 proteins are detected by using anti-FLAG antibody (panel a). For *in vivo* interaction, both Cdc20s and Cdh1 are co-transfected in HeLa cells, and immunoprecipitates of FLAG-Cdh1 from the cell lysates are subjected for immunoblot of Cdc20 by using anti-GFP antibody (α-FLAG IP/α-GFP IB).

Cdc20 was stronger than that with S170A mutant (Fig. 3E, lanes 8 and 9 in lower panel), indicated that the phosphorylation of CRY-box might be important for ubiquitination and degradation of Cdc20 in early G1 phase by interaction with APC/C component.

3.3. The phosphorylation of S170 and APC/C^{Cdh1}-dependent degradation

To investigate whether the phosphorylation on S170 is essential for Cdh1-dependent degradation in HeLa cells, we compared the Cdc20 amounts under Cdh1-depleted conditions. Under normal conditions, wild-type Cdc20 was unstable than S170A mutant (Fig. 3F, lanes 1 and 2). When endogenous Cdh1 was depleted in cells, wild type Cdc20 remained in the same level with S170A mutant (Fig. 3F, lanes 3 and 4). The expression levels of S170A mutant protein did not change under the condition of knockdown in cells (Fig. 3F, lanes 1 and 3), suggested that the phosphorylation on S170 is essential for APC/C^{Cdh1}-dependent degradation in HeLa cells.

To investigate the interaction between Cdc20 and Cdh1 is dependent on the CRY box as is usually the case with KEN box

(reviewed in [11,18]), we performed the protein binding experiments. As expected, *in vitro* interaction between wild-type Cdc20 and Cdh1 was stronger than that with S170A mutant, and S170A mutation reduced its binding efficiency to Cdh1 in immunoprecipitation experiment (Fig. 3G, lanes 2 and 3, upper panel in a and b), indicated that the phosphorylation of serine-170 in CRY box contributes to the interaction with Cdh1.

3.4. The mutation of Cdc20 did not affect on the subcellular localization and interaction with SAC components of Cdc20

Both wild-type and S170A mutant of Cdc20 were localized into kinetochore region in prometaphasic cells, and were colocalized with Aurora B and Plk1 (Fig. 4A, a and b), suggested that the phosphorylation on Ser-170 is not involved in the sub-cellular localization of Cdc20, but in regulation of Cdc20 stability. Upon activation of the spindle assembly checkpoint, Cdc20 is highly phosphorylated by MAP kinase, Cdk1 and Bub1, and forms the mitotic checkpoint complex (MCC) containing BubR1, Bub3, and Mad2, which associates with and inhibits APC/C [19,20]. To investigate whether phosphorylation of Ser-170 is involved in the function of Cdc20 under the spindle assembly checkpoint, we performed an *in vivo* protein binding experiment between Cdc20s and components of checkpoint complex. During activation of the checkpoint, both Cdc20 wild-type and S170A mutant proteins interacted with endogenous Mad2, BubR1 and Bub3 in the spindle assembly checkpoint (Fig. 4B, lanes 2 and 3 in upper panel in a–c). These data indicated that phosphorylation on Ser-170 by Plk1 is not essential for the formation of mitotic checkpoint complex under spindle assembly checkpoint.

4. Discussion

During the somatic cell cycle, Cdc20 is an essential activator of anaphase-promoting complex (APC/C), which is involved in the metaphase-anaphase transition [21]. APC/C^{Cdh1}-mediated degradation of Cdc20 during interphase is regulated by two degradation signals (degrons), KEN box and CRY box in Cdc20 [13,14]. Most APC/C substrates contain APC/C degron, which is required for their ubiquitination by APC/C. The KEN box is recognized by APC/C^{Cdh1} [22], and it has been found in other APC/C^{Cdh1} substrate

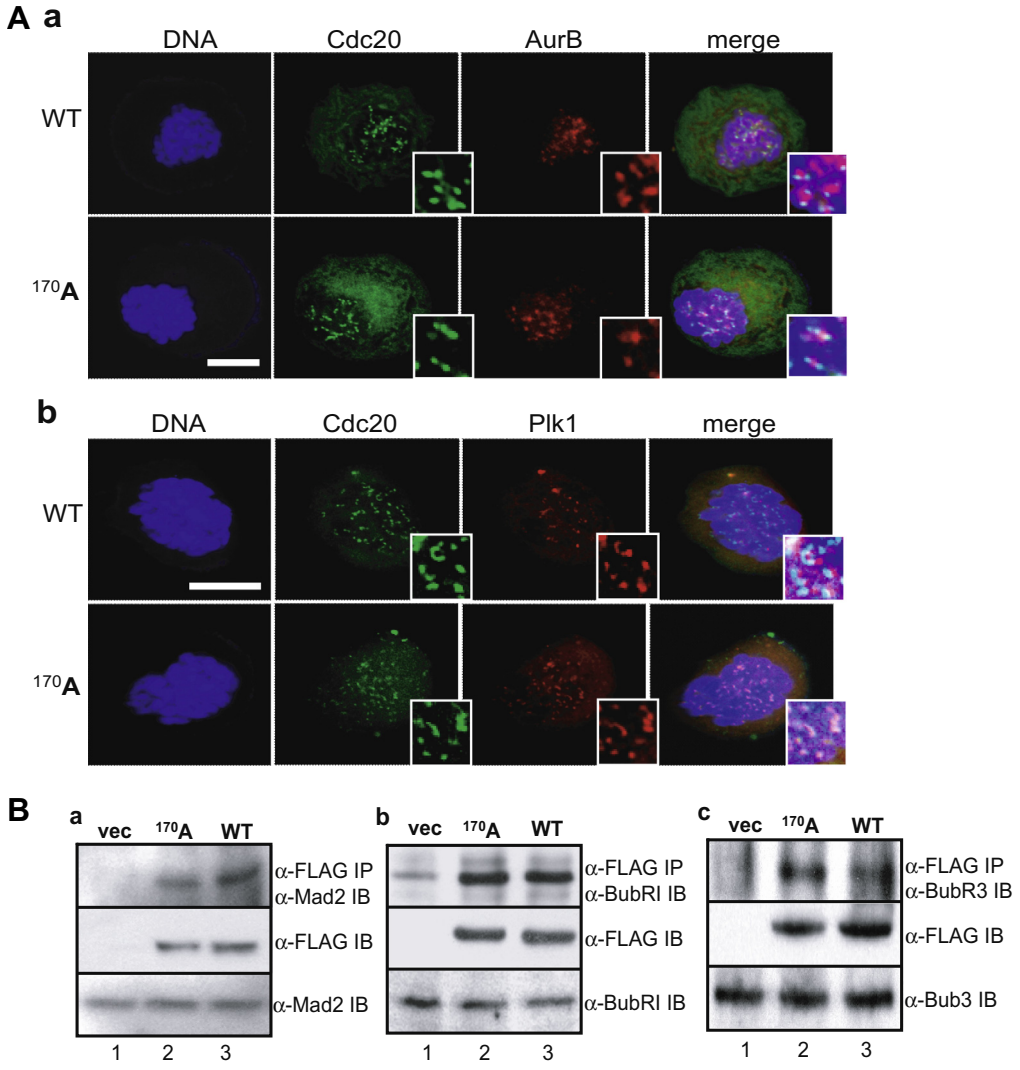

Fig. 4. Subcellular localization of wild type and S170A mutant Cdc20. (A) Kinetochore localization of wild-type and S170A mutant Cdc20. After transfection with FLAG-Cdc20s, cells were treated with nocodazole and synchronized in prometaphase. To detect kinetochore region, anti-aurora B (a) and anti-Plk1 (b) antibodies are used as primary antibodies. Cdc20 proteins are detected by using anti-FLAG antibody. (B) Interaction between Cdc20s and the spindle assembly checkpoint components, Mad2, BubR1, and Bub3. HeLa cells expressed Cdc20s constructs were induced spindle assembly checkpoint by treatment with nocodazole for 24 h. Cdc20s were immunoprecipitated with anti-FLAG antibody followed immunoblot analysis for endogenous Mad2 (α-FLAG IP/α-Mad2 IB in a), BubR1 (α-FLAG IP/α-BubR1 IB in b), and Bub3 (α-FLAG IP/α-Bub3 IB in c).

proteins, such as securin for degradation during late mitosis and early G1 phase [23]. The CRY box was known as a second degron in mammalian Cdc20, and contributed to Cdc20 degradation in germinal vesicle (GV)-stage mouse oocytes and embryo in APC/C^{Cdh1}-dependent manner [14]. In this report, we investigated the role of CRY box in Cdh1-dependent Cdc20 degradation by APC/C. Interestingly, the serine-170 of Cdc20, which is in CRY box, was phosphorylated strongly by Plk1 *in vitro* (Fig. 2A) Although some Plk1 substrates use the defined optimal D/E-X-S/T-Φ-X-D/E sequence [24], several reported Plk1 phosphorylation sites don't [25,26]. In this study, Plk1 could phosphorylate Cdc20 on serine-170, which lacked an acidic residue in the −2 position (Fig. 2C), and the mutation of aspartate-173 in the +3 position caused only a modest decrease in the rate of Cdc20 degradation [14]. These data suggested that the information of consensus sequences is helpful, but not restrictive. In spite of this limitation, Plk1-dependent phosphorylation and degradation of Cdc20 were proven by a knock-down and a constitutive activation of Plk1 (Fig. 3C and D). During progression from mitosis to G1 phase, a mutant S170A was not degraded in comparison with wild-type Cdc20, and ubiquitination in S170A mutant was occurred in weaker levels than that of wild type (Fig. 3A and B). In addition to ubiquitination and degradation, the phosphorylation on serine-170 by Plk1 was involved in the interaction with Cdh1 and was essential for the dependency on Cdh1 (Fig. 3F).

So far, an example for degron phosphorylation involved in protein degradation has been reported. In prophase, phosphorylation of APC/C inhibitor Emi1 by Plk1 generates a phosphor-degron to recruit the $SCF^{\beta TrCP}$ ubiquitin ligase, causing Emi degradation and allowing progression into prometaphase [27]. These data suggested that Plk1 activates the APC/C by directing the SCF-dependent degradation of Emi1 in prophase. Similarly, we suggested in this report that Plk1 phosphorylate the CRY box to generate a phosphor-degron in Cdc20 to induce the interaction with APC/C^{Cdh1}, facilitating Cdc20 degradation to progress mitotic exit and cytokinesis in normal somatic cell cycle. Another inhibitory regulation of APC/C^{Cdc20} under spindle assembly checkpoint (SAC) has occurred by multi-phosphorylations of Cdc20 by the Cdk1, MAP Kinase, Bub1, and PKA [14,15,19,20,26,28,29]. These phosphorylations are essential for formation of mitotic checkpoint complex. However, whether these inhibitory phosphorylations have an influence on Cdc20 stability as a phosphorylation on CRY box or not is still ambiguous. Although the amino acid residues phosphorylated during SAC are concentrated in N-terminal region of Cdc20, they are not overlapped with the serine-170 in CRY box. Although Ser-170 phosphorylation in CRY box seems to be essential for Cdc20 degradation in APC/C^{Cdh1}-dependent way, further studies are needed to determine what, if any, functional relationship exists in between KEN box and CRY box modification, or in between APC/C binding region and CRY box modification for Cdc20 degradation by Cdh1. Base on a structure model of Cdc20 [11], CRY box locates in a junction region between N-terminal region containing C box and KEN box and huge WD40 repeats domain. Under this situation, the phosphorylation of CRY box can induce global structural change by increasing negative charge, and can ease the shift for Cdh1 or core APC/C component into both degrons and C box.

Because Plk1 starts to be activated from the early mitotic stage, Cdc20 can be phosphorylated in all mitotic periods. However, until the late anaphase, as Cdk1 activity remains high enough to prevent Cdh1 from binding to APC/C, Cdc20 can escape from Cdh1-dependent degradation. APC/C^{Cdc20}-mediated degradation of mitotic cyclins and direct inhibition of Cdk1 by separase that is free from securin degradation result in the activation of APC/C^{Cdh1} in late mitosis and G1 phase. Finally, both Cdc20 and Plk1 are degraded by the activating APC/C^{Cdh1}, and cells enter into G1 phase. In this report, we suggested a novel mechanism of phosphor-degron of CRY box on Cdc20 degradation in APC/C^{Cdh1}-dependent way. Moreover, this will be the first finding that the inhibitory phosphorylation may have an influence on Cdc20 stability during the somatic cell cycle.

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