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Stress-activated MAP kinases JNK and p38 target Cdc25B for degradation

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Abstract

Cdc25 dual-specificity phosphatases positively regulate the cell cycle by activating CDK/cyclin complexes. Of the three mammalian Cdc25 isoforms, Cdc25A is phosphorylated by genotoxic stress-activated Chk1 or Chk2, which triggers its $SCF^{\beta TrCP}$-mediated degradation. However, the roles of Cdc25B and Cdc25C in cell stress checkpoints remain inconclusive. We herein report that c-Jun NH_2-terminal kinase (JNK) induces the degradation of Cdc25B. Treatment of cells with anisomycin, which induces non-genotoxic stress, caused rapid degradation of Cdc25B as well as Cdc25A. Cdc25B degradation was dependent mainly on JNK and partially on p38 MAPK (p38). Accordingly, co-transfection with JNK1, JNK2, or p38 destabilized Cdc25B. *In vitro* kinase assays and site-directed mutagenesis experiments revealed that the critical JNK and p38 phosphorylation site in Cdc25B was Ser101. Cdc25B with Ser101 mutated to alanine was refractory to anisomycin-induced degradation, and cells expressing such mutant Cdc25B proteins were able to override the anisomycin-induced G2 arrest. These results highlight the importance of a novel JNK/p38-Cdc25B axis for a non-genotoxic stress-induced cell cycle checkpoint.

Introduction

Cell cycle progression in eukaryotic cells requires the successive activation and inactivation of CDK–cyclin complexes. Activation of CDK–cyclin complexes depends on members of the Cdc25 dual-specificity phosphatases. In mammalian cells, the Cdc25 family consists of Cdc25A, Cdc25B, and Cdc25C. Although Cdc25C was the first member to be identified, Cdc25A is now regarded as one of the major players in multiple phases of cell cycle progression in mammalian somatic cells (1, 2). Besides its well-known role in G1–S transition, depletion of Cdc25A delays the G2–M transition and chromosome condensation (1, 3, 4). Cdc25A is also a well-known target of the DNA damage checkpoint (2, 5). Upon genotoxic insults, Cdc25A is rapidly phosphorylated by the checkpoint kinases Chk1 and Chk2 followed by $SCF^{\beta\text{-}TrCP}$-mediated ubiquitinylation and degradation (2, 6).

In contrast to Cdc25A, the roles of Cdc25B and Cdc25C remain elusive. Experiments with Cdc25B- or Cdc25C-knockout mice have indicated that these genes are dispensable not only for normal cell cycle control but also for the DNA damage checkpoint (7, 8). The only prominent phenotype is a defect in oocyte maturation in Cdc25B-depleted female mice (9). However, several lines of evidence suggest that Cdc25B is important for controlling CDK1-cyclin B activity at the centrosome, where it contributes to centrosome separation at prophase (4, 10). Chk1 is believed to down-regulate the activity of Cdc25B until prophase (11, 12). The

phosphorylation of Cdc25B (Ser309 of Cdc25B1 or Ser323 in Cdc25B3) has also been implicated in the function of Cdc25B in conjunction with 14-3-3 binding (13, 14). A mutation that abolishes the specific phosphorylation site causes Cdc25B to the nuclear localization and enhances its ability to abrogate DNA damage-induced G2 arrest (15, 16). These results suggest that despite its non-essentiality in mouse models, Cdc25B does play some role in the G2–M transition (14).

Stress stimuli that do not target genome DNA lead to the activation of the so-called stress activated MAP kinases, including p38 MAP kinase (p38) and c-Jun NH_2-terminal kinase (JNK; 17). Recently, MAPKAP kinase 2 (MK2) has received attention for its ability to phosphorylate Cdc25B, leading to 14-3-3 binding (18). MK2 is usually complexed with and activated by p38 (18). Therefore, a fraction of p38 can be found to co-immunoprecipitate with MK2, which could mislead one to believe that p38 phosphorylates substrates that are normally phosphorylated exclusively by MK2. The p38-MK2 cascade is, therefore, believed to regulate cell cycle progression by controlling the stability and subcellular localization of Cdc25A and Cdc25B, respectively, when cells are exposed to genotoxic or non-genotoxic stress (14, 18, 19, 20). JNK also phosphorylates and inactivates Cdc25C (21). In addition, nuclear Cdc25B is exported to the cytoplasm upon UV or non-genotoxic stress by an unknown mechanism (22).

We previously reported that overexpression of 14-3-3 causes the relocation of Cdc25B from the nucleus to the cytoplasm (13, 23, 24). To delineate the role of the cytoplasmic export of

Cdc25B, we established HeLa cell lines that constitutively express Cdc25B and investigated conditions that induce nuclear export of the phosphatase. We found that non-genotoxic stress, but not genotoxic cell stress induced loss of Cdc25B nuclear localization, and that non-genotoxic stress was also an effective inducer of Cdc25B degradation. Moreover, we found that the stability of Cdc25B could be controlled by JNK and p38. These data reveal a novel pathway linking the stress response kinases to the G2-M cell cycle engine.

Materials and methods

Reagents, plasmids, and antibodies: Reagents of the highest grade were obtained from Wako or Sigma. Restriction enzymes were obtained from New England Biolabs, and MKK7-activated recombinant JNK1 and MKK6-activated p38α (both isolated from baculovirus-infected Sf21 cells) were obtained from Upstate. Oligonucleotides were synthesized by Invitrogen. The cDNAs and antibodies used in the experiments were described in *Supplementary Materials and methods*.

Cell culture and plasmid transfection: HeLa cells were grown in DMEM as previously described (13). Plasmids were transiently transfected with LipofectAMINE 2000 (Invitrogen). Cell cycle synchronization was performed using the double thymidine block protocol. To obtain stable Flag-tagged Cdc25B-expressing HeLa cells, we used 10 μg/ml blasticidin S (Invitrogen) for the selection of transformed cells. Established Flag-Cdc25B-expressing HeLa cells were maintained

with 2 μg/ml blasticidin S.

Preparation of crude extracts, immunoblotting, immunoprecipitation, and kinase assay: Crude extracts for the analysis of proteins followed by immunoblotting or immunoprecipitation were performed as described previously (13). To detect endogenous Cdc25B, we subjected proteins that had been immunoprecipitated with rabbit anti-Cdc25B antibodies to SDS-PAGE, followed by immunoblotting. The Cdc25B protein was detected with mouse monoclonal anti-Cdc25B antibody. The immunoprecipitates used for the kinase assay were washed twice with buffer containing 100 mM Tris-HCl (pH 8.0), 5 mM EGTA, and 20 mM $MgCl_2$. The samples were then incubated with the same buffer that was supplemented with appropriate GST-fusion Cdc25B proteins, 1 mM DTT, 200 μM ATP, and γ-^{32}P-ATP (1 μCi = 37 KBq; Perkin Elmer). The reaction mixtures were incubated at 30°C for 60 min and subjected to SDS-PAGE, and radioactivity was detected using the FUJI BAS system (FUJI FILM).

Indirect immunofluorescence and flow cytometry: Indirect immunofluorescence of cells grown on glass coverslips was conducted as described previously (13). Cells treated for fluorescence-activated cell sorting (FACS) were analyzed on a FACSCalibur (BD Biosciences) using ModiFit software. The Ser10-phosphorylated histone H3 (phospho-S10-H3)-positive cells were analyzed using CellQuest software.

Results

Nuclear localization of Cdc25B is lost following treatment of cells with anisomycin but not with DNA-damaging agents

Phosphorylation of Ser309 of Cdc25B1 followed by binding of 14-3-3 disrupts nuclear localization of Cdc25B (13). Ser309 can be phosphorylated by Chk1/2 and MK2 (18, 25, 26). To learn the types of cellular stresses that induce nuclear export, we treated cells with various chemicals and analyzed the subcellular localization of Cdc25B. Given that an antibody that displayed high specificity toward endogenous Cdc25B was not available commercially or in house, we first established a HeLa cell-based cell line that constitutively expressed Flag-tagged Cdc25B (F-Cdc25B). No gross abnormality of cell growth or morphology was discerned for F-Cdc25B-expressing cells, which are hereafter called HeLa-W40 cells.

We treated HeLa-W40 cells with hydroxyurea, aphidicolin, etoposide, or camptothecin, which activate either Chk1 or Chk2, and DNA damage induced by the chemicals was confirmed by assaying for phosphorylated histone H2AX (γ-H2AX; 27). Cdc25B localized to the nucleus in cells with or without DNA damage or replication arrest (Fig. 1A). We next investigated the possible involvement of the p38/MK2 pathways in this process. To activate the p38/MK2 pathway, we treated the cells with anisomycin, which activates p38 and JNK (28, 29). Treatment with anisomycin disrupted the nuclear localization of Cdc25B and promoted the redistribution of

Cdc25B to the cytoplasm (Fig. 1B). These results suggest that Cdc25B nuclear localization can be disrupted by stress pathways other than those initiated by DNA damage.-

To assess the possibility that the p38 pathway regulates Cdc25B, we added the p38 inhibitor SB202190 to HeLa-W40 cells. Of interest is that SB202190 exerted only a small effect on the anisomycin-induced diffusion of Cdc25B (Fig. 1C). In contrast, the JNK inhibitor SP600125 induced strong nuclear staining of Cdc25B under conditions of anisomycin stress. Furthermore, stronger nuclear signals for Cdc25B were generated by simultaneous treatment with both SB202190 and SP600125 than by treatment with SP600125 alone. Inhibition of MEK1 with U0126, thereby inhibiting the activation of Erk1/2, did not prevent cytoplasmic diffusion of Cdc25B (data not shown). Collectively, these data indicate that anisomycin-mediated stress can disrupt nuclear localization of Cdc25B in a JNK-dependent manner.

Cdc25B is degraded in cells treated with anisomycin or sodium chloride but not with DNA-damaging agents

The signals for F-Cdc25B faded in cells after long-term exposure to anisomycin but not after treatment with DNA-damaging agents (data not shown). These observations led us to analyze the level of F-Cdc25B protein in HeLa-W40 cells treated with anisomycin and another non-genotoxic reagent, sodium chloride (NaCl). We also examined the levels of Cdc25A and Cdc25C to

determine whether different cellular stresses affect members of this phosphatase family. Genotoxic stress caused by exposure to hydroxyurea, aphidicolin, etoposide, or camptothecin effectively induced Cdc25A degradation (Fig. 2A). In contrast, expression of Cdc25B or Cdc25C was unaffected under the same conditions. Although neither anisomycin nor NaCl activated Chk1 or Chk2, both Cdc25B and Cdc25A were degraded (Fig. 2A). Cdc25A and Cdc25B decreased in a time-dependent fashion after exposure to anisomycin (Fig. 2B). The endogenous Cdc25B protein in parental HeLa cells was also degraded by anisomycin treatment but not by DNA-damaging agents (Supplemental Fig. S1). The abundance of Cdc25C was not affected by any of the stimuli examined here. UV irradiation that is known to activate not only Chk1/2, but also p38/JNK abrogated nuclear localization of Cdc25B as reported (22) and induced Cdc25B degradation (Supplemental Fig. S2A and S2B).

Although anisomycin inhibits protein synthesis (29), the more rapid decline of Cdc25B in anisomycin-treated cells compared to cycloheximide-treated cells suggested that degradation of Cdc25B was specifically caused by anisomycin-induced stress and not by a general inhibition of protein synthesis (Fig. 2C). More convincing evidence was obtained when we examined the stability of Cdc25B that contained mutations at the constitutive β-TrCP binding site of ^{254}DDGFVD^{259} (amino acid numbering is based on human Cdc25B1; 30). The site is responsible for the steady state-degradation of Cdc25B by a constitutive SCF$^{\beta\text{-TrCP}}$–mediated

ubiquitin–proteasome pathway. We established a HeLa cells line expressing a mutant Cdc25B that contained mutations at the β-TrCP binding site ($Cdc25B^{DAA}$, of which D255 and G256 were replaced by Ala). Fig. 2D shows that $Cdc25B^{DAA}$ was unstable after anisomycin treatment. This indicates that the Cdc25B degradation was specific to anisomycin treatment. Collectively, these data indicate that anisomycin-, but not DNA damage–induced stress, triggers a loss of nuclear localization and stability of Cdc25B.

Inhibition of JNK attenuates the degradation of Cdc25B by anisomycin

To determine the role of p38 or JNK on the stability of Cdc25A and Cdc25B, we treated HeLa cells with inhibitors of p38 or JNK and analyzed the cellular proteins. The activation of p38α/β was monitored as the slower migrating form of MK2, corresponding to phosphorylated MK2 (Fig. 3A); JNK activation was monitored using antibodies recognizing phospho-JNK1 or phospho-c-Jun. We found that the JNK inhibitor SP600125 protected Cdc25B from anisomycin-induced degradation (Fig. 3A, lanes 5–8). A relatively small but definite contribution of the p38 pathway to the degradation of Cdc25B was revealed by these experiments (Fig. 3A, lanes 3–8). The MEK1 inhibitor U0126 again had no effect on the stability of either Cdc25A or Cdc25B (data not shown). Time-course experiments further validated the critical role of JNK in regulating the degradation of Cdc25B (Fig. 3B). The stability of Cdc25C was essentially

unaffected by these inhibitors (Fig. 3A). Exactly the same results were obtained with HeLa-W40 cells that express F-Cdc25B (Supplemental Fig. S3A). JNK inhibitor also attenuated the NaCl-induced F-Cdc25B degradation (Supplemental Fig. S3B). Results shown in Fig. 3C indicate the correlation between the degradation of Cdc25A/Cdc25B and activation of p38/JNK. Anisomycin-, NaCl-, or UV-induced degradation of Cdc25B was inhibited by the proteasome inhibitor MG132, suggesting that the anisomycin-induced degradation of Cdc25B is mediated by the ubiquitin–proteasome pathway (Supplemental Fig. S3C-1, -2, and -3). Results of indirect immunofluorescence also supported that the instability of Cdc25B in anisomycin-treated cells was due to its proteasome-dependent degradation (Supplemental Fig. S3D).

The co-expression of either JNK1 or JNK2 with MKK7 triggered Cdc25B degradation (Fig. 4A). In addition, p38α was able to induce Cdc25B degradation. Furthermore, the expression of kinase-dead JNK1 and JNK2 stabilized Cdc25B (Fig. 4B), indicating dominant negative effects. We further examined the contribution of MK2 to Cdc25B degradation. In this experiment, we used the D316N mutant of p38α, which is unable to activate MK2 (31). As shown in Fig. 4C, expression of $p38\alpha^{D316N}$ induced the degradation of Cdc25B. Taken together, these results suggest that JNK and p38 are integrally involved in Cdc25B degradation.

JNK phosphorylates Cdc25B

We found that bacterially expressed Cdc25B was phosphorylated by kinase-active JNK1 but not by its kinase-dead form, indication that JNK1 can directly phosphorylate Cdc25B (Supplemental Fig. S4A). Likewise, recombinant JNK1 could also phosphorylate Cdc25B (Supplemental Fig. S4B). To identify the phosphorylation site(s) on Cdc25B, GST-fusion constructs of different fragments of Cdc25B were produced in *Escherichia coli* and used as substrates for kinase assays. As shown in Fig. 5A, JNK phosphorylation sites were present in the N-terminal 175 amino acids. The same fragment was also phosphorylated by recombinant p38α (Supplemental Fig. S4C). Furthermore, JNK and Cdc25B were able to form complex, which supports the idea of Cdc25B being a JNK substrate (Supplemental Fig. S5A; the loss of the complex formation in wild type JNK with MKK7 suggests that JNK ran away after phosphorylation event).The importance of the N-terminal region was also supported by the JNK-induced degradation of the construct containing GFP fused to the N-terminal 175 amino acid fragment of Cdc25B (Supplemental Fig. S5B).

Cdc25B/S101 is a candidate JNK phosphorylation site

To identify JNK phosphorylation site(s), we first mutated all six candidate serine residues to alanine in six potential JNK substrate SP sequences (and no TP) in N175-Cdc25B. The 6SA mutant was refractory to JNK- or p38α-induced degradation (Supplemental Fig. S6A). The

co-expression of the individual SA mutant Cdc25B with JNK indicated the importance of S101, followed by S103 (Fig. 5B). The importance of S101 and S103 was further supported by the slower degradation rate of the Cdc25B protein with a double mutation (S101/103A) compared to the mutants with a single mutation (Fig. 5C, lanes 4, 6, and 8). Essentially the same results were obtained when p38α was used as a kinase for the Cdc25B mutants (Supplemental Fig. S6B). Taken together, these data indicate that phosphorylation of Cdc25B at S101 and S103 by JNK and p38 is important for degradation.

HeLa cells expressing the Cdc25B S101A mutant ignore anisomycin-induced G2 arrest

We next examined the effects of anisomycin on cell cycle progression, especially during G2/M phase, when Cdc25B peaks during the cell cycle. HeLa cells were synchronized at G1/S and released into S phase. At 6 h after release, the cells were treated with anisomycin followed by monitoring of M phase entry by detection of phospho-S10-H3. The Cdc25B protein and phospho-S10-H3 increased progressively in DMSO-treated cells (Fig. 6A), but no phospho-S10-H3 was detected in anisomycin-treated cells, indicating that anisomycin treatment inhibited mitotic entry. In such cells, Cdc25B disappeared completely by 30 min after addition of anisomycin (Fig. 6A, lanes 7 and 8). FACS analysis revealed the induction of a G2 delay by anisomycin treatment in synchronously or asynchronously growing HeLa cells (Supplemental

Fig. S7A).

To delineate the significance of Cdc25B S101 phosphorylation in anisomycin-induced G2 arrest, we added anisomycin to asynchronously growing HeLa cells that constitutively express the unphosphorylatable Cdc25B S101A mutant (HeLa-101-1). The results shown in Fig. 6B indicate that the half-life of the S101A mutant Cdc25B was twice as long as that of the wild type after anisomycin treatment (~35 min in S101A and ~15 min in wild type), indicating that phosphorylation of S101 is essential for proper degradation. Treatment of HeLa-101-1 with either NaCl or UV also supported the idea that S101 is important for stress-induced Cdc25B degradation (Supplemental Fig. S8A).

We next investigated whether the S101A mutant would exhibit abrogation of anisomycin-induced G2 arrest. We used cell lines expressing wild-type Cdc25B (W40) or S101A (101-1). The amount of Cdc25B protein in W40 cells and that of 101-1 is shown in Supplemental Fig. S7B. Asynchronously growing HeLa cells, W40 cells, and 101-1 cells were treated with 100 ng/ml anisomycin, and the number of cells entering M phase during a 3-h treatment with anisomycin was determined by detecting phospho-S10-H3. As shown in Fig. 6C, HeLa-101-1 cells exhibited resistance to anisomycin-induced G2 retardation. Thus, cells expressing S101A-mutated Cdc25B seem to resist anisomycin-induced degradation or to recover more rapidly than wild-type Cdc25B-expressing cells.

Collectively, anisomycin induced G2 retardation in HeLa cells, and HeLa cells expressing Cdc25B with an unphosphorylatable mutation at the possible JNK target S101 residue were more refractory to anisomycin-induced cell cycle stress.

Discussion

We report here for the first time that Cdc25B is targeted for degradation in cells that are challenged with anisomycin or NaCl, and that the degradation of Cdc25B is mediated mainly by JNK. We uncovered this phenomenon by using HeLa cells that constitutively express recombinant Cdc25B. In our hands, commercially available antibodies did not properly recognize endogenous Cdc25B in crude extracts by immunoblotting. Immunoprecipitation followed by immunoblotting was necessary to detect endogenous Cdc25B.

Our experiments highlight the critical role of JNK in controlling Cdc25B stability. A level of DNA damage that is sufficient for activating Chk1/2 and Cdc25A degradation did not exert any effects on the stability of Cdc25B. Export of Cdc25B from the nucleus to the cytoplasm is thought to be a mechanism of checkpoint response (18). However, Cdc25B did not follow this expected pattern after DNA damage (Fig. 1A). We therefore suspect that Cdc25B is not a primary target of the DNA damage checkpoint, although we cannot exclude the possibility that its phosphatase activity is directly repressed by a Chk1-dependent mechanism (32).

We demonstrated that M phase entry is delayed in HeLa cells treated with anisomycin (Fig. 6). The G2 retardation observed in this situation is caused in part by the degradation of Cdc25A and Cdc25B. Depletion of either Cdc25A or Cdc25B is insufficient for G2 phase arrest, but the depletion of both Cdc25A and Cdc25B is necessary for more robust G2 arrest (4). Therefore, anisomycin-induced G2 retardation can be explained at least in part by the simultaneous degradation of Cdc25A and Cdc25B (Fig. 2). Our results strongly suggest the presence of a non-genotoxic stress-dependent cell cycle checkpoint wherein Cdc25 phosphatases are degraded to down-regulate CDK activity. This checkpoint is mediated by stress-activated MAP kinases, including p38 and JNK, as depicted in Fig. 6D. The proteasome inhibitor MG132 attenuated anisomycin-induced Cdc25B degradation, which strongly suggests that Cdc25B is degraded by the ubiquitin–proteasome pathway. Cdc25B is degraded in the steady state by the $SCF^{\beta TrCP}$-mediated ubiquitinylation mechanism. It may be less plausible to assume that phosphorylation of S101 stimulates binding of $SCF^{\beta TrCP}$ to the constitutive binding site, which is located more than 100 amino acids downstream of S101. It is necessary to identify the responsible ubiquitinylation system to understand JNK-mediated Cdc25B degradation more precisely.

Several reports have indicated a correlation between the malignancy of tumors and the overexpression of Cdc25A and Cdc25B (33, 34). Cdc25A and Cdc25B are oncogenic (35), and Cdc25A is a main target of the DNA damage checkpoint (1, 2, 34). Assuming that Cdc25B is a

target of a non-genotoxic stress checkpoint, overexpression of Cdc25B may allow cells to become less sensitive to intrinsically harmful cell stresses that do not directly compromise genome integrity. Ignoring the detrimental stress signal, such as NaCl-induced distortion of the cytoskeleton by hyperosmolarity or anisomycin-induced inhibition of protein synthesis, may disrupt the proper regulation of chromosome segregation and cytokinesis during mitosis, which are well-known causative events of genome instability (36).

In conclusion, we have demonstrated that Cdc25B is targeted for JNK-mediated degradation by cellular stress. The stress-induced checkpoint is initiated by the activation of JNK and p38, which phosphorylates Ser101 of Cdc25B. This results in the rapid degradation of Cdc25B and in cell cycle arrest.

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Figure Legends

Figure 1: Nuclear localization of Cdc25B is maintained after DNA damage but is disturbed by anisomycin treatment. (A) HeLa-W40 cells were treated with the indicated chemicals and Flag-Cdc25B and γ-H2AX were detected by indirect immunofluorescence. The nuclei were identified using DAPI. The conditions for chemical treatment were described in *Supplemental Materials and methods*. **(B)** HeLa-W40 cells were treated with 50 ng/ml anisomycin for 15 min and processed for indirect immunofluorescence. **(C)** HeLa-W40 cells were pre-treated with the indicated MAP kinase inhibitors for 1 h followed by anisomycin challenge. The cells were fixed and Flag-Cdc25B, and nuclei were analyzed. The concentration for inhibitors were as follows: SB202190, 20 μM; SP600125, 20 μM.

Figure 2: Degradation of Cdc25A and Cdc25B after treatment with DNA-damaging or non-DNA-damaging agents. (A) HeLa-W40 cells were treated with genotoxic chemicals as described in *Supplemental Materials and methods*. The cells were incubated with anisomycin or 300 mM NaCl for 30 min or 1 h, respectively. Cell extracts were prepared, and indicated proteins were detected by immunoblotting. β-Actin analysis is a loading control. **(B)** Parental HeLa cells were treated with anisomycin, and cell extracts were prepared at the indicated time to detect endogenous Cdc25A, Cdc25B, and Cdc25C. **(C)** HeLa-W40 cells were treated with

anisomycin or 50 μg/ml cycloheximide. Crude extracts were prepared at the indicated time, and the expression of Flag-Cdc25B was analyzed. **(D)** HeLa cells constitutively expressing the $Cdc25B^{DAA}$ mutant were treated with anisomycin or cycloheximide and the expression of Flag-Cdc25B proteins was determined by immunoblotting.

Figure 3: JNK inhibitor SP600125 attenuates the anisomycin-induced Cdc25B degradation. **(A)** HeLa cells were treated with the indicated MAP kinase inhibitors for 1 h followed by anisomycin treatment. Cell extracts were prepared at 20 min after anisomycin addition. SB and SP represent SB202190 and SP600125, respectively. **(B)** HeLa cells were treated with MAP kinase inhibitors, and the expression of proteins was determined at the indicated time. **(C)** HeLa-W40 cells were treated with anisomycin, and the expression of protein was determined at the indicated time. Slower migrating bands of c-Jun and MK2 represent phosphorylated forms of the proteins.

Figure 4: Involvement of JNK in the destabilization of Cdc25B. (A) HeLa cells were transfected with Flag-Cdc25B in combination with MKK6 and p38α, or MKK7 and JNK1 or JNK2. The expression of the indicated proteins was determined by immunoblotting. **(B)** Expression of Flag-Cdc25B was determined after co-transfection with MKK7 and either the wild-type or kinase-dead form of JNK1 or JNK2. **(C)** Expression of Flag-Cdc25B was examined

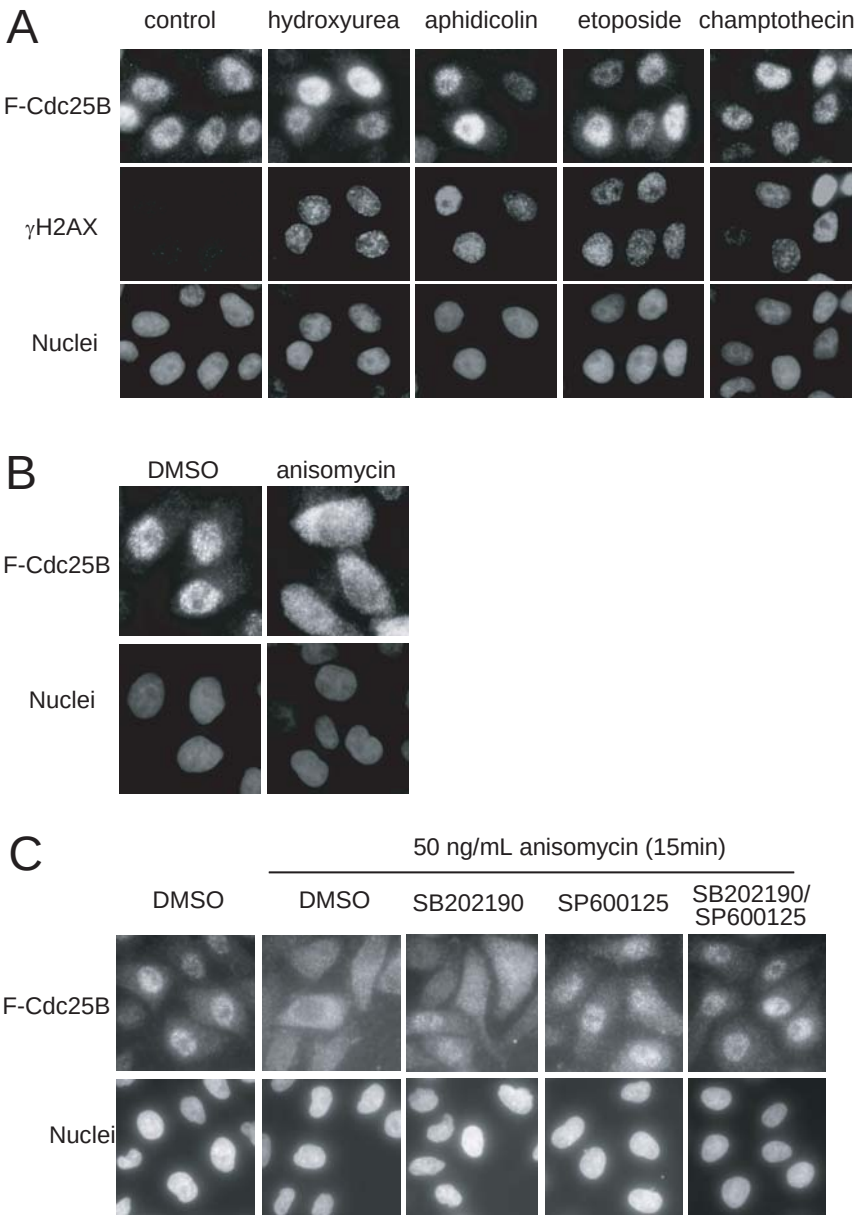
after co-transfection with MKK6 and wild type (WT) or the D316N mutant (MT) of p38α. Arrowhead indicates phospho-MK2.

Figure 5: Phosphorylation of Cdc25B by JNK at possible phosphorylation sites. (A) GST-Cdc25B fragments were incubated with a recombinant JNK1 in the presence of γ-^{32}P-ATP followed by autoradiography (left panel). The right panel indicates the substrate GST-Cdc25B fragment as detected by anti-GST antibody. **(B)** Cdc25B of the wild type or mutants that have SA mutations at candidate JNK phosphorylation sites were co-transfected into HeLa cells with or without MKK7 and JNK1, and expression of Cdc25B was determined by immunoblotting. **(C)** The wild type or SA mutant at S101, S103, or S101/103 was co-transfected into HeLa cells with or without MKK7 and JNK1, and the expression of Cdc25B was determined.

Figure 6: The Cdc25B mutant is refractory to anisomycin-induced G2 arrest. (A) HeLa cells synchronized at G1/S were released into the cell cycle for 6 h, followed by the addition of anisomycin. At the indicated times, the expression of proteins was determined. **(B)** HeLa-W40 or HeLa-101-1 cells were treated with anisomycin, and the expression of the proteins was determined at the indicated time points after treatment. The lower panel indicates quantitative results. Typical results of three independent experiments are shown. **(C)** Asynchronously growing

HeLa, HeLa-W40, or HeLa-101-1 cells were treated with 100 ng/ml anisomycin and 100 ng/ml nocodazole for 3 h. Cells were collected, and the cells in M-phase were determined by detecting phospho-histone H3-Ser10 on FACS. The bars indicate mean ± SD of three independent experiments. The percentage of mitotic cells in asynchronous cells was as follows: HeLa, 2.76 ± 0.27; W40, 3.57 ± 0.13; 101-1, 3.61 ± 0.14. **(D)** A model of the induction of Cdc25A and Cdc25B degradation after non-genotoxic stress followed by G2 arrest.

Uchida *et al*., Figure 1



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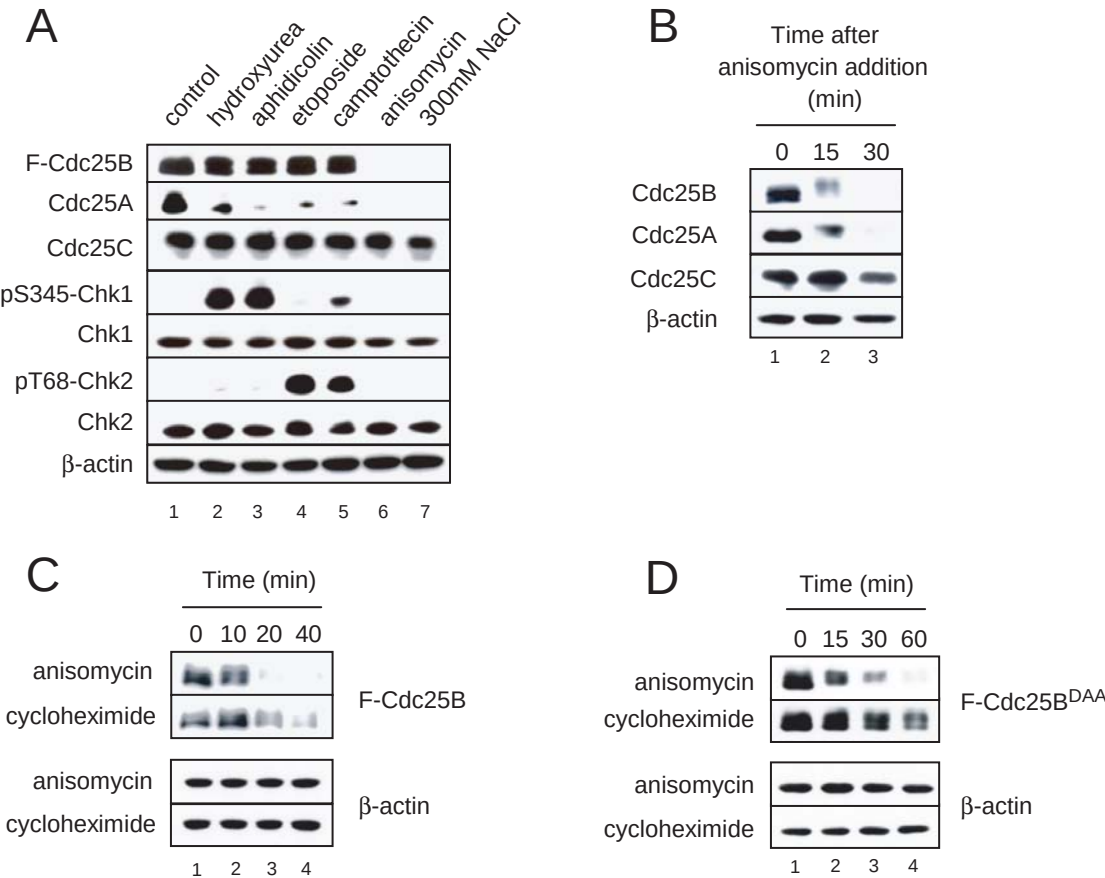

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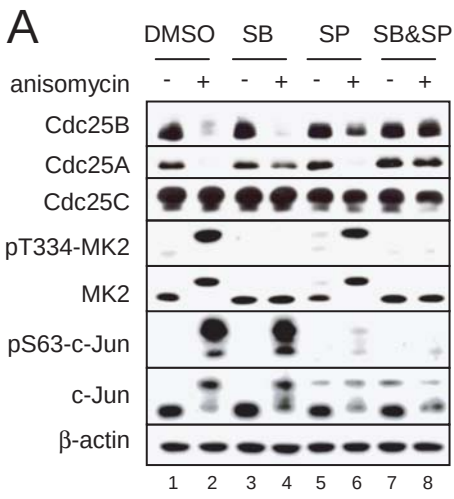
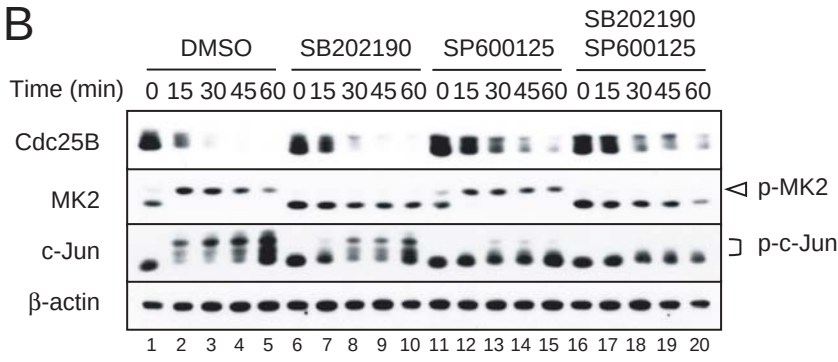
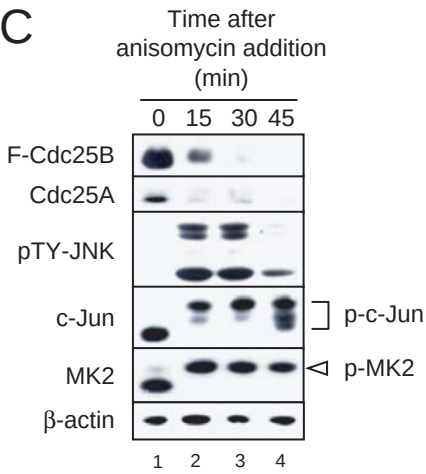
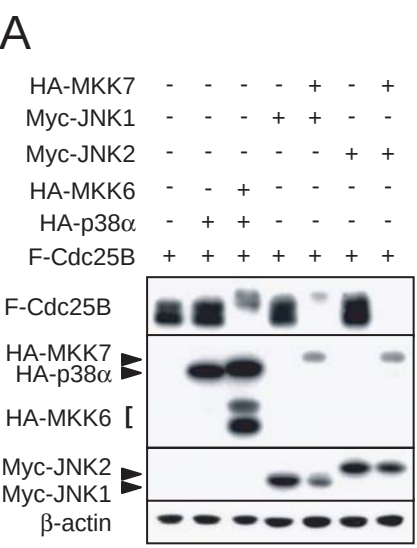
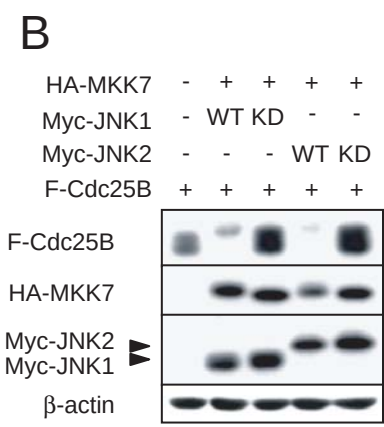


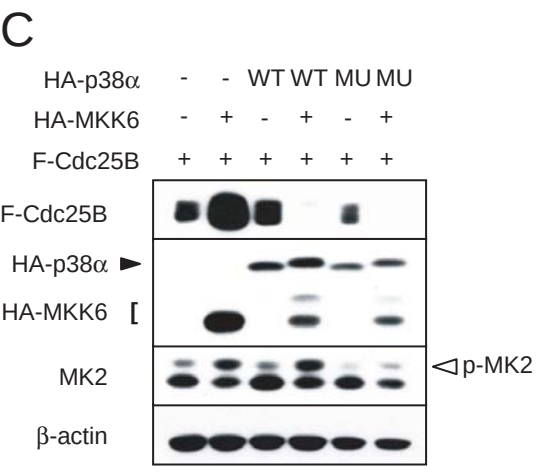




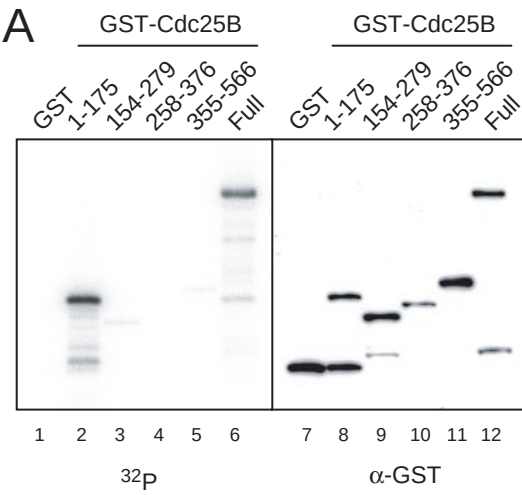
Uchida *et al.*,Figure 4

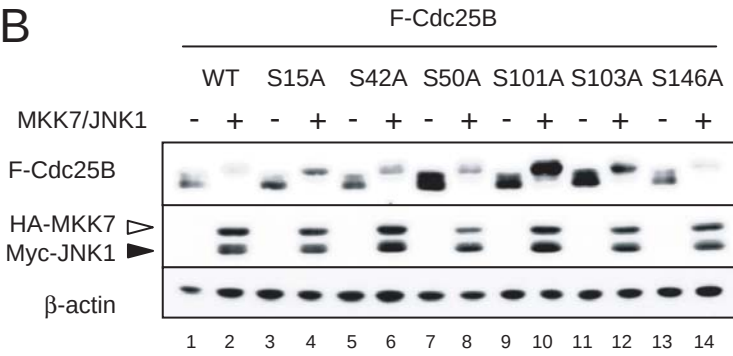


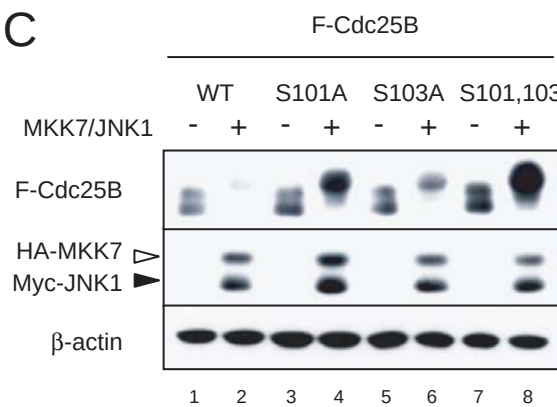




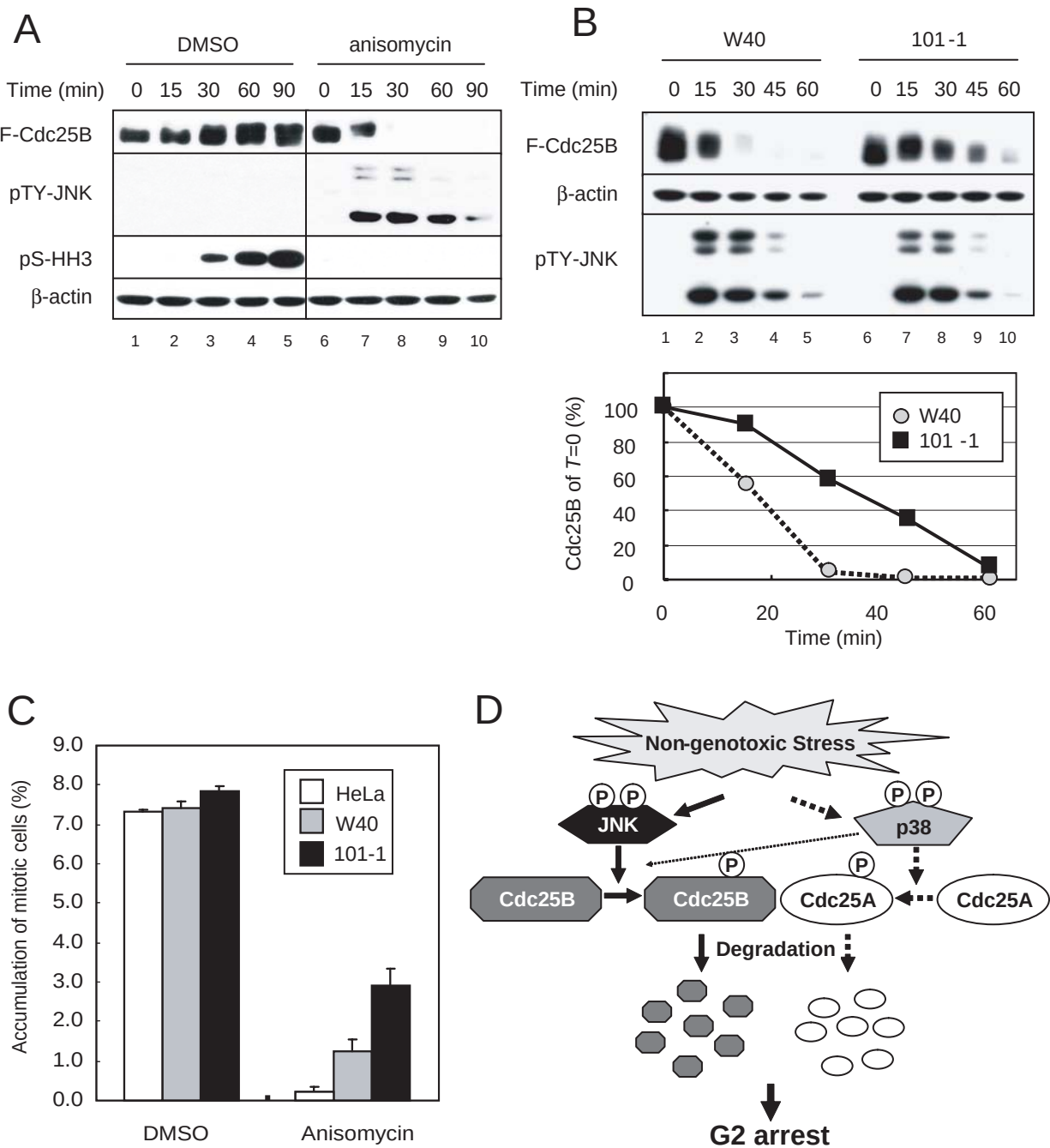
Uchida *et al*, Figure 5







Uchida *et al*., Figure 6



Supplemental Materials and methods

Reagents, plasmids and antibodies: The following cDNAs were used: human Cdc25A, human Cdc25B1, mouse p38α, mouse MKK6, mouse JNK1, mouse JNK2, and mouse MKK7. Flag, HA, and Myc-tagged expression plasmids were constructed using the pEF6/Myc-His vector (Invitrogen) as previously described (13). Mutant versions of the above cDNAs were generated using PCR-based mutagenesis (13), and their nucleotide sequences were confirmed by sequencing. Hydroxyurea, aphidicolin, etoposide, and camptothecin were obtained from Wako; and anisomycin, SB202190, SP600125, U0126, and MG132 were obtained from Calbiochem. The rabbit anti-Flag antibody was raised in house, and the rabbit anti-γ-H2AX and goat anti-GST antibodies were obtained from Upstate and GE Healthcare, respectively. The mouse monoclonal anti-Cdc25B (Ab-1) was purchased from Calbiochem. The following antibodies were obtained from Santa Cruz Biotechnology: Cdc25A (F-6), Cdc25B (C-20), Cdc25C (C-20), and JNK1 (C-17). The following were obtained from Cell Signaling: β-actin, Myc-tag (9B11), HA-tag 262K, Chk1 and phospho-Chk1-S345, Chk2 and phospho-Chk2-T68, p38 (5F11) and phospho-p38-T180/T182 (28B10), MK2 and phospho-MK2-T334, phospho-JNK-T183/Y185 (G9), c-Jun and phospho-c-Jun-S63II, p44/p42 ERK and phospho-p44/p42 ERK-T202/Y204 (E10), phospho-ATF2-T71, and phospho-histone H3-S10 (unconjugated and Alexa Fluor 488-conjugated). Secondary antibodies labeled with horseradish

peroxidase or with Alexa Fluor were purchased from DAKO and Invitrogen, respectively.

Anti-FLAG-M2-agarose beads were purchased from Sigma.

Chemicals and their treatment: Following chemicals were purchased from WAKO; hydroxyurea, aphidicolin, etoposide, camptothecin, and cycloheximide. Anisomycin and MAP kinase inhibitors such as SB202190, SP600125, and U0126 were obtained from Calbiochem. MG132 was also obtained from Calbiochem.

Treatment of cells with chemicals; The conditions for chemical treatment were as follows: hydroxyurea, 1.5 mM for 1 h; aphidicolin, 2.5 μg/ml for 1 h; etoposide, 10 μM for 1 h; camptothecin, 2 μM for 1 h. Cells were treated with anisomycin either at 50 ng/ml or 100 ng/ml. No apoptosis were observed in anisomycin treated cells at the conditions used.

Figure S1: Degradation of endogenous Cdc25B by anisomycin treatment. Cdc25B protein was immunoprecipitated with mouse monoclonal anti-Cdc25B antibodies from crude cell extracts from HeLa cells that were treated with the indicated chemicals as follows: hydroxyurea, 1.5 mM for 1 h; etoposide, 10 mM for 1 h; anisomycin, 50 ng/ml for 30 min. Rabbit anti-Cdc25B antibody was used for the detection of Cdc25B expression. Other proteins were detected by immunoblotting of crude cell extracts with indicated antibodies.

Figure S2: UV-induced cytoplasmic distribution of Cdc25B and degradation. (A) HeLa-W40 cells were treated with 20 J/m^2 of UV and incubated for 15 min. Cells were fixed for the detection of Flag-Cdc25B by indirect immunofluorescence. Nuclei were stained with DAPI. **(B)** After irradiation of UV (20 J/m^2), HeLa-W40 cells were collected at indicated time and expression of proteins were determined by immunoblotting.

Figure S3: Inhibition of anisomycin-induced degradation of Cdc25A and Cdc25B by MAP kinase inhibitors or a proteasome inhibitor. (A) HeLa-W40 cells were treated with the indicated MAP kinase inhibitors for 1 h, and cells were challenged with anisomycin for 20 min. The expression of indicated proteins in crude cell extracts was determined by immunoblotting. The following abbreviations are used: SB, p38 inhibitor SB202190 (20 μM); SP, JNK inhibitor SP600125 (20 μM). **(B)** HeLa-W40 cells were treated with the indicated MAP kinase inhibitors as in (A) and cells were exposed with 300 mM of NaCl for 1 h, followed by determination of indicated proteins in crude cell extracts by immunoblotting. **(C)** HeLa-W40 cells were treated with 5 μM of the proteasome inhibitor MG132 for 1 h, and cells were challenged with anisomycin (50 ng/ml) for 30 min **(C-1)**, NaCl (300 mM) for 1 h **(C-2)**, or UV (20 J/m^2) followed by incubation for 1 h **(C-3)**. The expression of indicated proteins in crude cell extracts was determined by immunoblotting. **(D)** HeLa-W40 cells were treated with 5 μM of the proteasome inhibitor MG132 for 1 h, and cells were challenged with or without anisomycin (50 ng/ml; 30 min). The expression of Flag-Cdc25B was determined by indirect immunofluorescence. Nuclei were stained with DAPI.

Figure S4: Phosphorylation of GST-Cdc25B by JNK and p38 *in vitro*. (A) An *in vitro* kinase assay was conducted with bacterially expressed GST-Cdc25B with 6xHis at the C-terminus as a substrate. Either the wild-type or kinase-dead JNK was recovered from Cos7cells that were transfected with wild-type JNK1 or kinase-dead JNK1 in

combination with MKK7. After the kinase reaction, proteins were separated using SDS-PAGE, and radioactivity was detected using image analysis. The asterisk indicates an unknown cross-reacting protein that was present in the purified *Escherichia coli*–produced GST-Cdc25B-His6 sample. The expression of Myc-tagged JNK1 proteins and the activator MKK7 are shown in right panel. Conditions for the kinase assay were the same as described in *Materials and methods*. **(B)** Recombinant JNK1 isolated from baculovirus-infected insect cells was used as a kinase for *in vitro* phosphorylation assay as described in panel A. **(C)** *In vitro* kinase assays were conducted with p38α as a kinase isolated from baculovirus-infected insect cells. Substrates were bacterially produced GST-fused Cdc25B proteins (full length or fragments).

Figure S5: Involvement of JNK in Cdc25B degradation. (A) Wild type JNK or its kinase-dead form was co-expressed with Flag-Cdc25B in Cos7 cells. Crude cell extracts were prepared 24 h after transfection, Flag-Cdc25B was recovered by immunoprecipitation in the presence or absence of Flag peptide (5 μg/ml), and Cdc25B or JNK1 in the immunoprecipitates were detected by immunoblotting. The expression of proteins in crude extracts is also indicated. **(B)** N-terminally Flag-tagged and C-terminally GFP-tagged Cdc25B fragments with the N-terminal 175 amino acids were co-transfected with JNK1 and MKK7. Crude cell extracts were prepared 24 h after transfection, and the expression of Flag-Cdc25B/N175-GFP was monitored using the anti-GFP antibody. The expression of other proteins is also indicated.

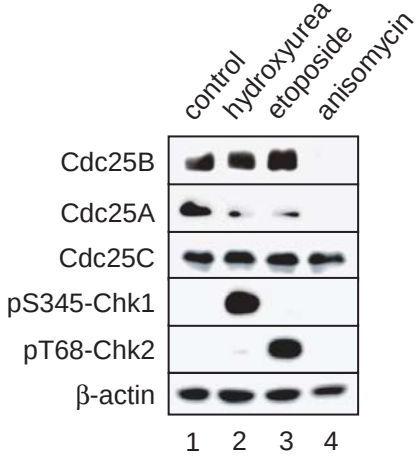
Figure S6: Identification of critical phosphorylation sites for JNK/p38-induced Cdc25B degradation. (A) Cdc25B of wild type (WT) or a JNK phosphorylation mutant that has six candidate serine residues mutated to alanine (6SA) was co-transfected with either JNK1 and MKK7, or p38α and MKK6 into HeLa cells. Expression of proteins was determined 24 h after transfection. **(B)** Cdc25B of wild type, the single mutants S101A or S103A, or the S101A/S103A double mutant was co-transfected with p38α and MKK6 into HeLa cells. Their expression was determined 24 h after transfection.

Figure S7: G2 retardation of HeLa cells treated with anisomycin. (A) Asynchronously growing HeLa cells were treated with either 100 ng/ml anisomycin or 100 ng/ml nocodazole or both. At the indicated time points, cells were collected, and cells in M phase with phospho-S10-H3 were determined by FACS. **(B)** The level of protein expressed in HeLa cells that stably express Flag-tagged Cdc25B of the wild type

or the S101A mutant that were used in **Fig. 6C**. W40, wild type; 101-1, S101A.

Figure S8: The Cdc25B mutant is refractory to NaCl- or UV-induced degradation. HeLa-W40 or HeLa-101-1 cells were treated with 300 mM of NaCl **(A)** or 20 J/m^2 of UV **(B)**, and the expression of the proteins was determined at the indicated time points after treatment.

Uchida *et al*., Supplemental Figure S1



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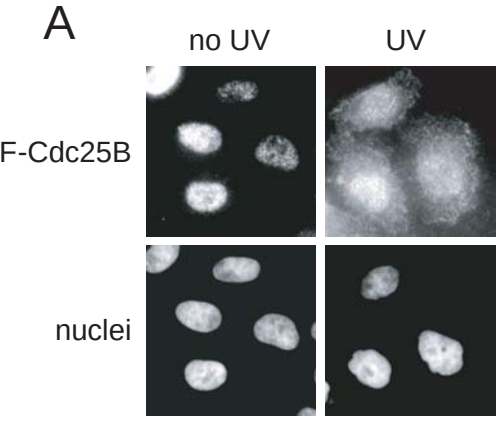

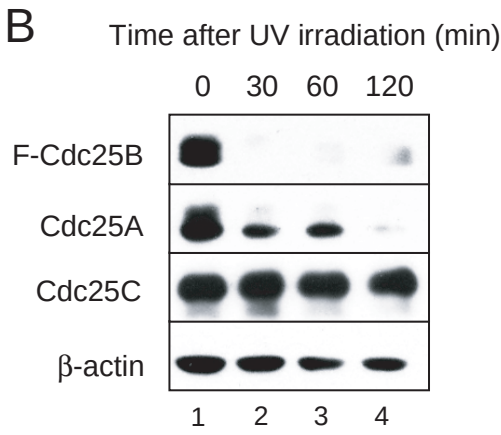

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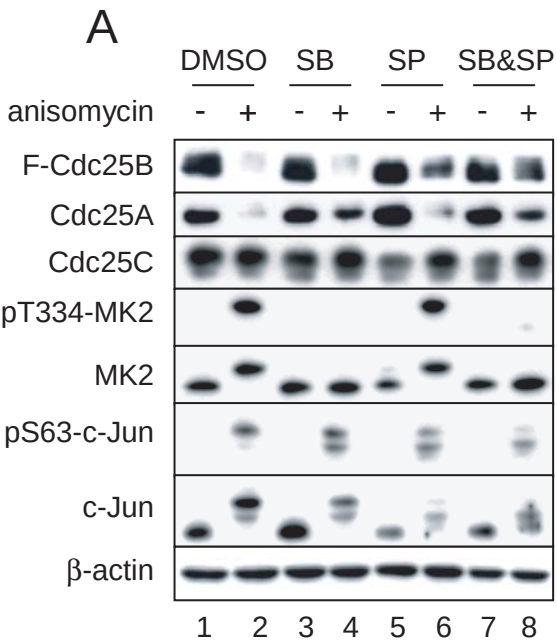

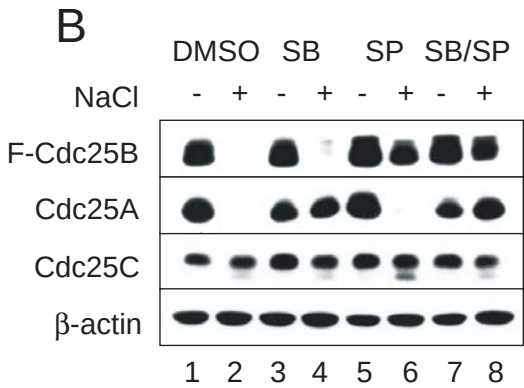

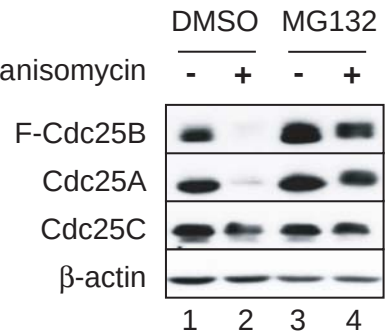

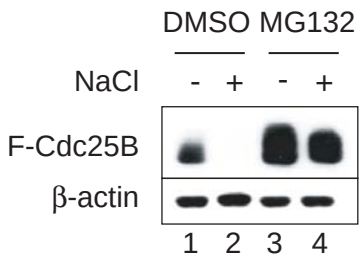

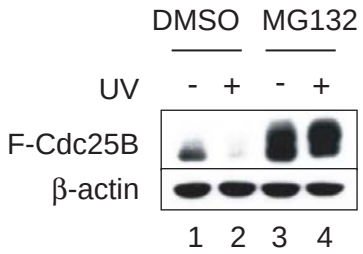

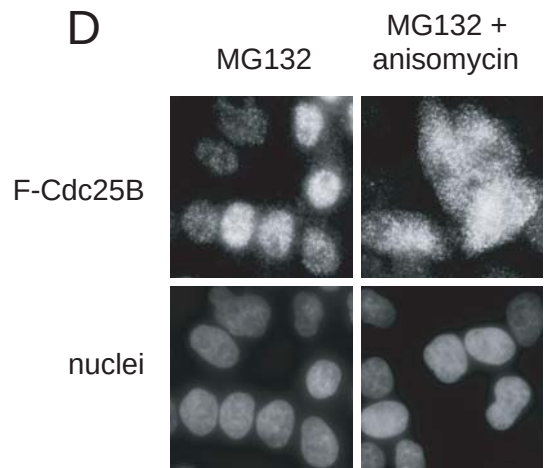

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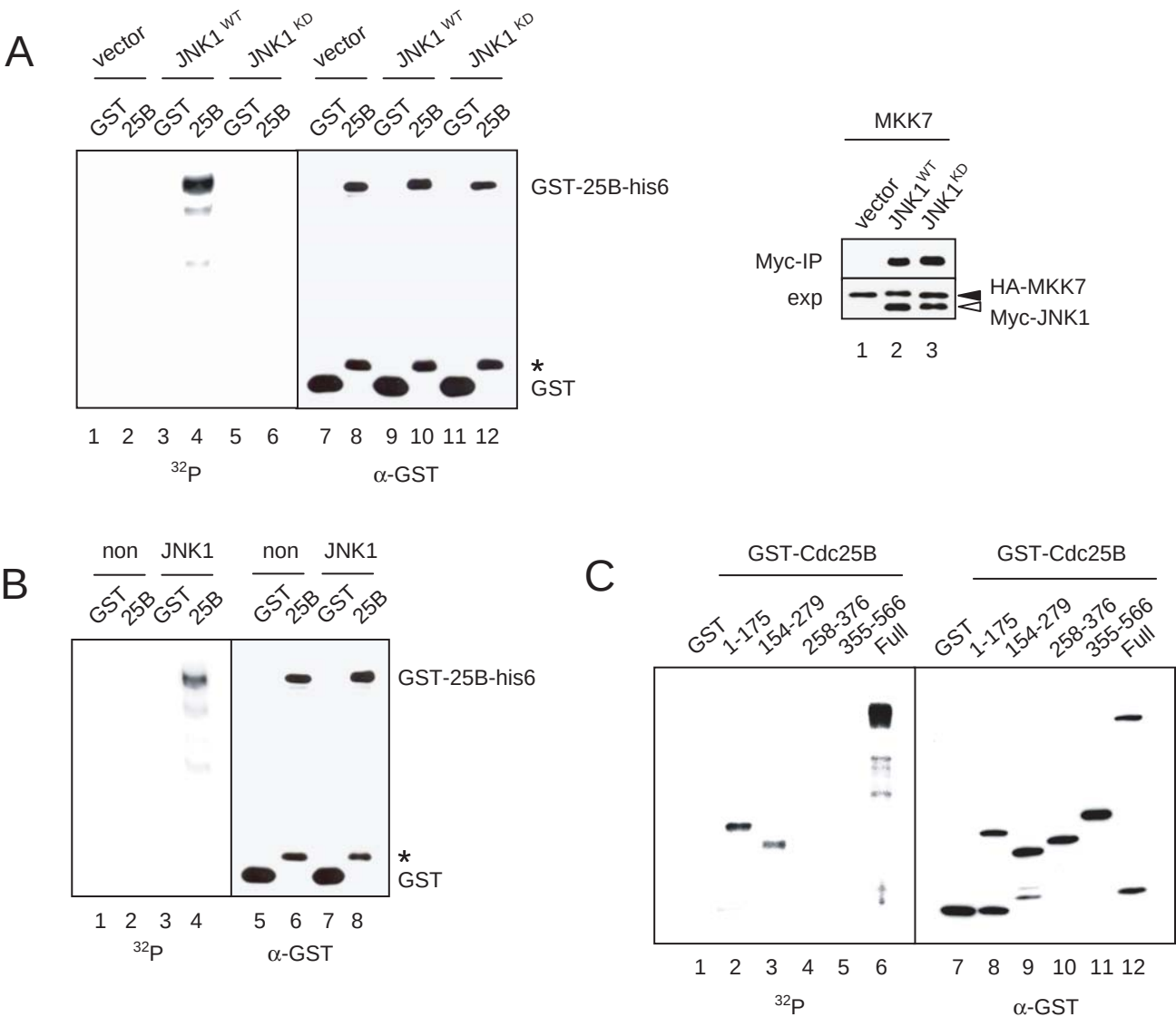

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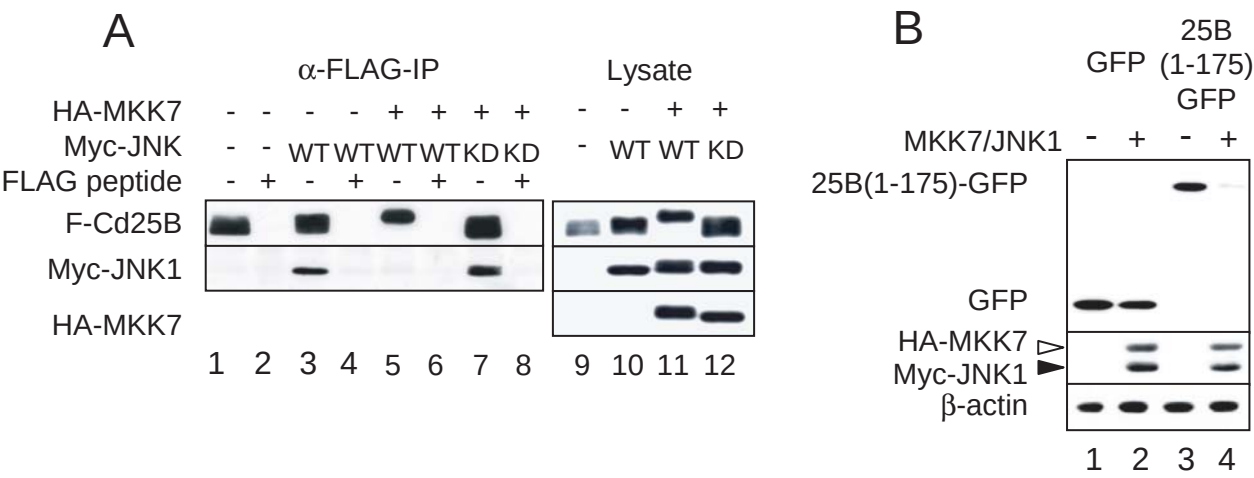

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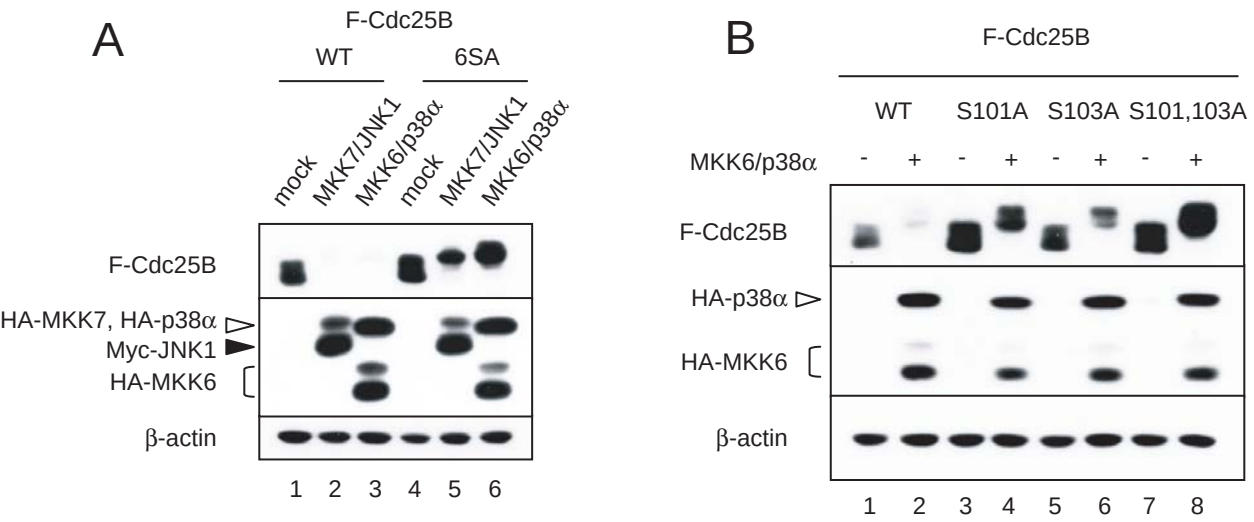

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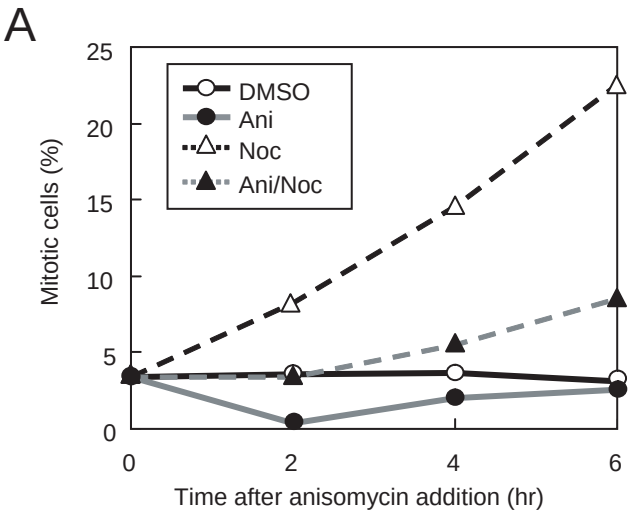

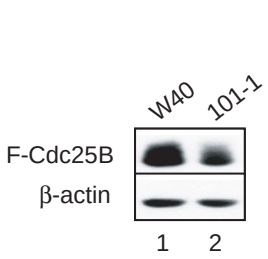

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