for AML1, MOZ–CBP might prevent AML1 from forming active AML1/MOZ/p300/CBP transcription factor complex. Interestingly, both the HAT- and bromo-domain of MOZ–CBP is required for inhibitory action. MOZ–CBP undergoes autoacetylation and can acetylate AML1 as well as histones [20]. Similar to the observation that p300/CBP acetylates and dissociates the coactivator ACTR from the estrogen receptor [2], MOZ–CBP-mediated aberrant acetylation may disrupt some transcription factor and cofactor(s) complexes. Alternatively, MOZ–CBP may inhibit transcription by aberrantly binding to acetylated proteins, then antagonize the function of the AML1 complex (Fig. 3a). Analysis of MOZ–CBP-interacting proteins and MOZ–CBP-acetylating proteins should lead to a better understanding of the mechanism through which MOZ–CBP induces leukemia.

3.3 MOZ–TIF2-induced the aberrant histone code insensitive to physiological stimuli

MOZ is also fused to nuclear receptor coactivator TIF2 by inv(8) (Fig. 2). MOZ–TIF2 has transforming properties in vitro and causes AML in a murine bone marrow transplant assay [3]. The PHD-finger motif of MOZ is essential for transformation, whereas MOZ HAT activity is dispensable. However, MOZ–TIF2 interaction with CBP through the TIF2 CBP interaction domain (CID) is essential for transformation. These results indicate that nucleosomal targeting by MOZ and recruitment of CBP by TIF2 are critical requirements for MOZ–TIF2 transformation and indicate that MOZ gain of function contributes to leukemogenesis. MOZ–TIF2 inhibits transcription by CREB-binding protein (CBP)/p300-dependent activators such as nuclear receptors and p53 [17]. On the retinoic acid (RA) receptor promoter, MOZ–TIF2 modestly increases ligand-independent acetylation of H3K9 or H3K14, but inhibits further acetylation induced by RA. These effects are

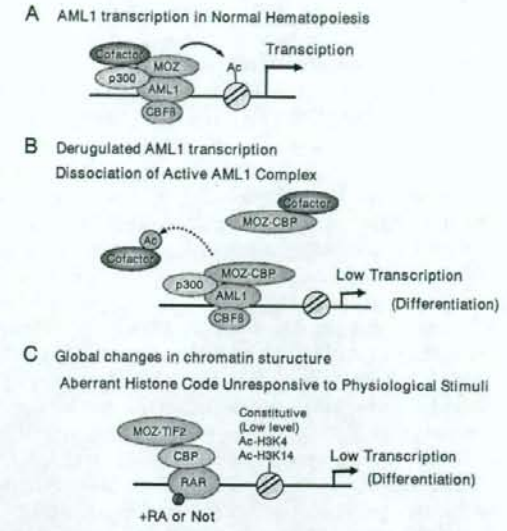

Fig. 3 A model for MOZ fusion products on MOZ-dependent transcription by altering chromatin structures. **a** AML1 transcription in normal hematopoiesis. The transcriptional activity of AML1 depends on MOZ. **b** Deregulation of AML1-mediated transcription by MOZ-related chimeras. MOZ–CBP dissociates AML1/MOZ/p300 active transcription factor complexes, and then inhibits the expression of genes regulated by AML1 (B-1). MOZ–TIF2 associates with AML1. MOZ–TIF2 also forms complex with CBP via AD1 domain of the fused TIF2, and enhances AML1-mediated transcription by increasing histone acetylation (B-2). **c** Alteration of global chromatin structures by a MOZ-related chimera. MOZ–TIF2 alters the histone code of genes those are regulated by p300/CBP, their response to normal physiological stimuli, and then deregulates their expression

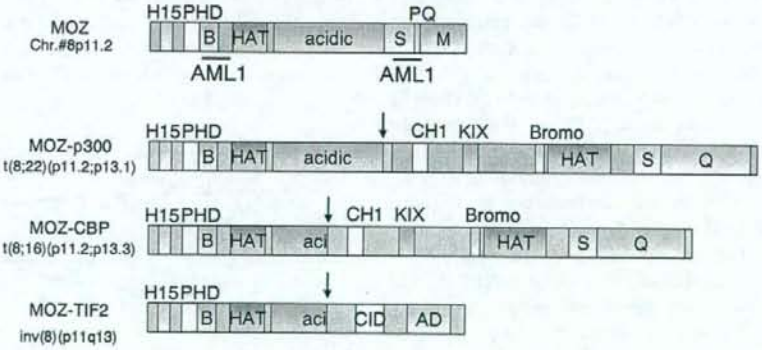

Fig. 2 Schematics for MOZ and its related chimeras. Amino acid numbers of each fusion points are given. *H1/5* histone H1/H5 homology region; *PHD* plant homeodomein; *HAT* histone acetyltransferase; *S*, *PQ*, and *M*, serine-, proline and glutamine-, and methionine-rich region; *CH* cystein/histidine rich region; *KIX* kinase inducible domain; *Br* bromodomain; *SID* nuclear hormone receptor interacting domain; *HLH* helix–loop–helix motif; *NR* nuclear receptor box; *AD* activation domain

site-specific, because H4K8 is unaffected by MOZ–TIF2 and remains RA dependent (Fig. 3c). Aberrant dimethylation of H3R17 and H4R3 is also induced by MOZ–TIF2 by recruiting the arginine methyltransferases such as CARM1 and PRMT1. Thus MOZ fusion proteins have differential effects on the activities of CBP-dependent and MOZ-dependent activators because of their ability to alter cofactor recruitment and chromatin modification at target promoters.

4 Conclusion remarks

Essential components of AML1 including CBF heterodimer as well as transcriptional cofators MOZ, p300, and CBP are indispensable for hematopoiesis, and frequent targets of chromosomal rearrangements in human leukemias. We think that molecular pathogenesis of almost all leukemias can be explained in view of deregulated function of AML1 complex. Therefore, AML1 complex would be a common molecular target of therapy for a wide variety of leukemia.

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Identification of Novel Human Cdt1-binding Proteins by a Proteomics Approach: Proteolytic Regulation by APC/C^{Cdh1}

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In mammalian cells, Cdt1 activity is strictly controlled by multiple independent mechanisms, implying that it is central to the regulation of DNA replication during the cell cycle. In fact, unscheduled Cdt1 hyperfunction results in rereplication and/or chromosomal damage. Thus, it is important to understand its function and regulations precisely. We sought to comprehensively identify human Cdt1-binding proteins by a combination of Cdt1 affinity chromatography and liquid chromatography and tandem mass spectrometry analysis. Through this approach, we could newly identify 11 proteins, including subunits of anaphase-promoting complex/cyclosome (APC/C), SNF2H and WSTF, topoisomerase I and IIα, GRWD1/WDR28, nucleophosmin/nucleoplasmin, and importins. In vivo interactions of Cdt1 with APC/C^{Cdh1}, SNF2H, topoisomerase I and IIα, and GRWD1/WDR28 were confirmed by coimmunoprecipitation assays. A further focus on APC/C^{Cdh1} indicated that this ubiquitin ligase controls the levels of Cdt1 during the cell cycle via three destruction boxes in the Cdt1 N-terminus. Notably, elimination of these destruction boxes resulted in induction of strong rereplication and chromosomal damage. Thus, in addition to SCF^{Skp2} and cullin4-based ubiquitin ligases, APC/C^{Cdh1} is a third ubiquitin ligase that plays a crucial role in proteolytic regulation of Cdt1 in mammalian cells.

INTRODUCTION

Recent research progress has uncovered molecular mechanisms by which DNA replication is cell cycle-controlled in eukaryotic cells (reviewed by Bell and Dutta, 2002; Diffley, 2004; Fujita, 2006). The current paradigm is that a multiprotein complex, termed the prereplication complex (pre-RC), is constructed from late mitosis through G1 phase based on origin recognition complex (ORC) binding to chromosomal DNA. CDC6 and Cdt1 (Maiorano *et al.*, 2000; Nishitani *et al.*, 2000) proteins are recruited to chromatin by interaction with ORC, and the resultant machinery may act as a loader for the minichromosome maintenance (MCM) heterohexameric complex, which could function as a replicative helicase (Ishimi, 1997). Once cyclin-dependent kinases (Cdks) become active at the onset of S phase, pre-RC initiates replication, accompanied by further assembly of multiple other proteins or protein complexes (Bell and Dutta, 2002). The mechanism seems basically conserved from yeast to metazoan cells, although more complicated in the latter (Bell and Dutta, 2002; Diffley, 2004; Fujita, 2006).

To maintain the genome integrity, chromosomal DNA should replicate only once during a single cell cycle. Therefore, the reestablishment of pre-RC, in other words rebinding of MCM, needs to be suppressed during the S, G2, and M phases of the cell cycle. Recent studies have suggested that Cdks play a central role also in this context. Thus, Cdk activity has a bipartite function in the regulation of DNA replication. Cdks prevent reestablishment of pre-RC through multiple redundant mechanisms (Bell and Dutta, 2002; Diffley, 2004; Fujita, 2006). One is phosphorylation of CDC6, leading to degradation in yeast or nuclear export in mammalian cells. In human cells, ORC1 is degraded after S phase, presumably depending on phosphorylation by Cdks. In budding yeast, the function of ORC2 is suppressed through Cdk phosphorylation (Nguyen *et al.*, 2001). It has also been shown that the MCM complex is phosphorylated by Cdks (Bell and Dutta, 2002; Diffley, 2004; Fujita, 2006). In budding yeast, it is necessary to block all three pathways for induction of rereplication without inhibiting Cdk activity (Nguyen *et al.*, 2001). At least in nontransformed mammalian somatic cells, deregulation of an individual replication initiation component alone also does not lead to overt rereplication. For example, several studies have shown that overexpression of ORC1 or CDC6 does not affect normal cell cycle progression (Petersen *et al.*, 1999; Tatsumi *et al.*, 2006). Although Cdt1 overexpression results in overt rereplication in several cancer-derived cell lines (Vaziri *et al.*, 2003), it does not in nontransformed somatic cell lines (Tatsumi *et al.*, 2006).

In metazoans, it has generally been considered that suppression of Cdt1 function after S phase is mainly executed by binding to an inhibitory protein, geminin (McGarry and Kirschner, 1998; Wohlschlegel *et al.*, 2000; Tada *et al.*, 2001; Mihaylov *et al.*, 2002). However, we and others have demonstrated that Cdt1 function is also negatively regulated

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Abbreviations used: pre-RC, prereplication complex; APC/C, anaphase-promoting complex/cyclosome.

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through phosphorylation by Cdks (Li *et al.*, 2003; Liu *et al.*, 2004; Sugimoto *et al.*, 2004). Phosphorylated Cdt1 is recognized by SCF^{Skp2} and targeted for degradation. However, phosphorylation-deficient mutant Cdt1 proteins show only partial resistance to S phase degradation (Sugimoto *et al.*, 2004; Takeda *et al.*, 2005; Senga *et al.*, 2006), implying the existence of another proteolytic mechanism. Cdk phosphorylation also negatively regulates Cdt1 chromatin binding (Sugimoto *et al.*, 2004). On the other hand, the cullin4-based ubiquitin ligase system also has been suggested to be involved in proteolytic regulation of Cdt1. In *Caenorhabditis elegans*, this system appears to contribute to cell cycle regulation of Cdt1 to ensure proper replication of the genome (Zhong *et al.*, 2003), whereas in mammals it was originally implicated in DNA damage–induced Cdt1 degradation (Higa *et al.*, 2003; Hu *et al.*, 2004). It is now known that in addition to the Cdk-SCF^{Skp2} pathway, PCNA (proliferating cell nuclear antigen)-dependent, cullin4-$DDB1^{Cdt2}$-mediated ubiquitination also operates in S phase degradation of Cdt1 (Arias and Walter, 2006; Higa *et al.*, 2006; Hu and Xiong, 2006; Jin *et al.*, 2006; Lovejoy *et al.*, 2006; Nishitani *et al.*, 2006; Sansam *et al.*, 2006; Senga *et al.*, 2006). Nevertheless, details of cell cycle regulation of Cdt1 remain to be fully elucidated (Fujita, 2006).

Inhibition of Cdt1 activity after S phase is carried out by multiple independent mechanisms, implying that deregulation of Cdt1 is a more deleterious insult than deregulation of other initiation proteins. In fact, recent studies have shown that unscheduled Cdt1 hyperfunction results in rereplication and/or chromosomal damage, leading to chromosomal instability and, presumably, eventual carcinogenesis (Arentson *et al.*, 2002; Vaziri *et al.*, 2003; Fujita, 2006; Tatsumi *et al.*, 2006). However, molecular mechanisms by which deregulated Cdt1 damages chromatin without rereplication are so far unclear (Tatsumi *et al.*, 2006). As components of the MCM complex-loading machinery, ORC and CDC6 proteins act using their ATPase activity, probably like replication factor C, a loader for PCNA (Bell and Dutta, 2002). How does Cdt1, lacking an ATPase motif, act during MCM loading? It could be that interactions with unidentified proteins contribute to the physiological and/or pathological functions of Cdt1.

It is clearly important to generate a better understanding of the functions and regulation of Cdt1. As a way to address the issue, we have tried to comprehensively identify human Cdt1-binding proteins by a combination of Cdt1 affinity chromatography and liquid chromatography and tandem mass spectrometry (LC/MS/MS) analysis. We adopted this approach rather than commonly used coimmunopurification methods to identify proteins not only stably binding but also dynamically interacting with Cdt1. Through this approach, we identified 11 novel Cdt1-binding proteins. Possible contributions of these proteins to Cdt1 function and regulation are discussed, with a particular focus on anaphase-promoting complex/cyclosome (APC/C). Our data indicate that, in addition to SCF^{Skp2} and Cullin4-based ubiquitin ligases, APC/C^{Cdh1} ubiquitin ligase also plays a crucial role in proteolytic regulation of Cdt1 in mammalian cells.

MATERIALS AND METHODS

Cells

HeLa, T98G, and 293T cells were grown in DMEM with 8% fetal calf serum.

Cdt1 Affinity Chromatography

Glutathione *S*-transferase (GST)-Cdt1 and GST were bacterially produced and purified as described previously (Sugimoto *et al.*, 2004). Fifteen milligrams of GST-Cdt1 or 20 mg of GST in coupling buffer (0.1 M $NaHCO_3$, pH 8.3, 500 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride [PMSF]) were cross-linked to 2 ml of cyanogenbromide-activated Sepharose 4B (Amersham, Piscataway, NJ). HeLa cell nuclear extracts were prepared as follows. HeLa cells (5 l; $\sim 3 \times 10^9$ cells) were first extracted with 50 ml ice-cold mCSK buffer (10 mM PIPES, pH 6.8, 100 mM NaCl, 300 mM sucrose, 1 mM EGTA, 1 mM $MgCl_2$) containing 0.1% Triton X-100 and a multiple protease inhibitor cocktail (Fujita *et al.*, 1998), and the remnant nuclear pellet was resuspended in 50 ml mCSK buffer containing 0.1% Triton X-100, 0.5M NaCl, and protease inhibitors. After centrifugation (3500 rpm, 10 min), the soluble nuclear fraction was filtrated. The nuclear extracts (30 ml) were diluted with 90 ml buffer A (25 mM Tris-HCl, pH 7.4, 0.01% Nonidet P-40 [NP-40], 1 mM dithiothreitol [DTT], 0.5 mM PMSF, 10% glycerol) and loaded onto tandemly joined GST and GST-Cdt1 columns pre-equilibrated with buffer A containing 0.15 M NaCl. After washing with 200 ml buffer A containing 0.15 M NaCl, the bound proteins were eluted with 8 ml buffer A containing 0.5 M NaCl. The fractions containing eluted proteins were determined by SDS-PAGE followed by Coomassie brilliant blue (CBB) staining.

Mass Spectrometry

The bound fractions were collected, concentrated with Centricon YM-30 (Millipore, Bedford, MA), and separated by 4–20% gradient SDS-PAGE (9-cm length) followed by CBB staining. The area from the top to bottom of the separation gel corresponding to molecular weights from ~250 to 10 kDa was cut into ~40 slices of 2-mm thickness on average, numbered from the bottom to top. Proteins in each gel slice were digested with trypsin (Promega, Madison, WI) and introduced into an electrospray mass spectrometer (LCQ-DECA, ThermoQuest Co., San Jose, CA) coupled on-line to a capillary HPLC column (Magic 2002, Michrom BioResources, Auburn, CA), as described previously (Kitabayashi *et al.*, 2001). The mass spectrometer was operated in the autodetection mode to acquire MS/MS spectra of the peptide. Then sequence databases were searched with the Mascot program (Matrix Science, Boston, MA) to identify proteins showing high hits with the obtained ion spectra.

GST-Cdt1 Pulldown Assay

GST-Cdt1 or GST was bound to glutathione beads, and the beads were mixed with the diluted nuclear extracts prepared from HeLa cells. After washing three times with 10 ml buffer A containing 0.15 M NaCl, the bound proteins were eluted and analyzed by immunoblotting.

Plasmids

Mammalian expression vectors, pcDEBdelta-T7-Cdt1, pCLMSCVhyg-T7-Cdt1, pcDNA3/myc-Cdh1, pcDNA3/myc-Cdc20, and pcDNA3-HA-Skp2, and the bacterial expression vector pGEX-6P-1-Cdt1 were described previously (Taguchi *et al.*, 2002; Sugimoto *et al.*, 2004; Tatsumi *et al.*, 2006). To express the Cdt1 N-terminal 2–101 amino acids, a fragment was excised from pCMV-Cdt1[2–101]9myc3NLSmyc (Nishitani *et al.*, 2006) and inserted into pcDNA-3xHA (Nishitani *et al.*, 2004). To introduce mutations into the Cdt1 D-box 1 (destruction box; see Figure 3A), oligonucleotide-directed mutagenesis (QuikChange Site-directed Mutagenesis Kit; Stratagene, La Jolla, CA) was performed with 5′-CACCCCCAGCCCCGCGGCGCCCGCAGCGCGCGCCCCGGCCTCC-3′ and a complementary oligonucleotide. The D-boxes 2 and 3 were mutated with 5′-CAGGCCAGGCCACCGGCCGCCAGGGCAGCCCCGGGCGTCGGTGGACGAGGTTTCC-3′ and a complementary oligonucleotide. Similarly, the T29A mutation was created with 5′-CTGGCCTGCCGCGCGCCCAGCCCCGCCAGG-3′ and a complementary oligonucleotide. Mutations in the conserved PIP-motif (PCNA-interaction protein motif) of Cdt1 were introduced with 5′- CGACCCATGGAGGCACGCCCGCGCAACCGACGCGTTCGCGCGCCGC-3′ and a complementary oligonucleotide. pEGFP-C1 was purchased from Clontech (Mountain View, CA). pCMV6-XL5-TOP2A expressing human topoisomerase IIα and pCMV6-XL4-SNF2H expressing human SNF2H were purchased from OriGene Technologies (Rockville, MD). GRWD1/WDR28 cDNA was synthesized according to the published sequences with codon optimization and confirmed by sequencing (GenScript, Scotch Plains, NJ). For convenience, it was tagged with hemagglutinin (HA) at its N-terminus, and recombination signals for the Gateway technology (Invitrogen, Carlsbad, CA) were also added to the either side. The cDNA was inserted into pGEX6P-2 for bacterial expression of GST-GRWD1 and into a retroviral vector pCLMSCVhyg (Tatsumi *et al.*, 2006) for expression in mammalian cells.

Transfection

Expression plasmids (total ~6 μg) were transiently transfected into 3×10^6 293T cells in 100-mm culture dishes with TransIT-293 reagents (Mirus, Madison, WI) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were lysed in 1× SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue) containing multiple protease inhibitors or extracted with appropriate buffers for immunoprecipitation. As appropriate, cells were supplemented with 40 μM MG132 (Calbiochem, La Jolla, CA) for 5 h before harvest.

Fluorescence-activated Cell Sorting

Cells were treated with a CycleTEST PLUS DNA Reagent Kit (Becton Dickinson, Franklin Lakes, NJ) for propidium iodide staining and then analyzed with a Becton Dickinson FACS (fluorescence-activated cell sorting) Calibur.

Establishment of HeLa, T98G, and 293T Cells Stably Expressing T7-tagged Cdt1

Cells were infected with recombinant retroviruses produced with retroviral vectors pCLMSCVhyg-T7-Cdt1 expressing wild-type or various mutants T7-Cdt1 (Sugimoto *et al.*, 2004; Tatsumi *et al.*, 2006) and then selected with hygromycin B. Detection of rereplication in HeLa cells was carried out ~4–6 d after infection. In other experiments, cells cultured for more than 2 wk after infection, in which rereplicated DNA had become virtually undetectable, were used.

Immunoprecipitation

For Figure 2B, 293T cells stably expressing T7-Cdt1 were lysed on ice in 1 ml of mCSK buffer containing 0.1% Triton X-100, 0.1 mM ATP, 1 mM DTT, and multiple protease inhibitors, and the soluble fractions were separated by centrifugation. Aliquots of the lysates were immunoprecipitated with anti-T7 antibody- or control IgG-fixed beads (Novagen, Madison, WI), and the beads were washed four times with 1 ml of the buffer. The immunoprecipitates were eluted with the elution buffer (100 mM glycine-HCl, pH 2.5) and subjected to immunoblotting.

For Figure 2, C–E, and Supplementary Figure S1A, 293T cells were transiently transfected with the indicated expression vectors and nuclear extracts were prepared with mCSK buffer containing 500 mM NaCl, 0.1% Triton X-100, 0.1 mM ATP, 1 mM DTT, and multiple protease inhibitors. Aliquots of the extracts were then immunoprecipitated with anti-Cdt1 antibody and protein G-Sepharose beads (Amersham).

For Figure 2F and Supplementary Figure S1B, transiently transfected 293T cells were lysed in NP-40 buffer (150 mM NaCl, 1% NP-40, 10 mM Tris-HCl, pH 7.4) containing multiple protease inhibitors. Aliquots of the lysates were immunoprecipitated with anti-HA or anti-Cdt1 antibodies.

For Figures 2G and 3C, 293T cells expressing T7-Cdt1 were first cross-linked with 1% formaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature. After washing with PBS, cells were lysed on ice in 250 μl RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid) containing multiple protease inhibitors and sonicated, and the soluble fraction was separated by centrifugation. Separate aliquots of the extracts were then immunoprecipitated with anti-Cdt1 antibody and protein G-Sepharose beads or with anti-T7 antibody beads. The beads were washed three times with 1 ml of NET gel buffer (150 mM NaCl, 50 mM Tris-HCl, pH7.4, 0.1% Triton X-100, 1 mM EDTA).

Small Interfering RNA Experiments

Small interfering RNA (siRNA) oligonucleotides (Dharmacon, Lafayette, CO) were synthesized with the following sequences (sense strand): Cdh1-1 (5′-UGAGAAGUCUCCCAGUCAGdTdT-3′), Cdh1–2 (5′-GAAGGGUCUGUUCACGUAUUCdTdT-3′), and GL2 (control; 5′-CGUACGCGGAAUACUUCGAdTdT-3′). For Figure 5A, HeLa cells (4×10^4/well in 12-well plates) were transfected with 180 pmol siRNA duplexes using Oligofectamine (Invitrogen) according to the manufacturer's instructions. For Figure 5B, HeLa cells (4×10^4/well in 12-well plates) were transfected with 12 pmol siRNA duplexes using HiPerFect transfection Reagent (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. For Figure 8B, T98G cells (4×10^4/well in 12-well plates) were transfected with 18 pmol siRNA duplexes using HiPerFect transfection Reagent.

In Vitro Ubiquitination Assay

APC/C^{Cdh1} was purified from HeLa cells synchronized in early G1 phase as follows. Cells were grown in the presence of 50 ng/ml nocodazole for 18 h, washed with PBS, grown in fresh medium without nocodazole for 4 h, lysed in 1 ml of hypotonic buffer (10 mM Tris-HCl, pH 7.5, 5 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM ATP, 1 mM DTT) containing multiple protease inhibitors, and homogenized by freeze-thawing and passage through a needle. The soluble fraction was separated by centrifugation, and aliquots were reacted with anti-Cdc27 antibody or control IgG for 5 h at 4°C. Immunoprecipitates were collected with protein G-Sepharose beads, washed twice with hypotonic buffer containing 0.1% Triton X-100, twice with hypotonic buffer containing 100 mM NaCl, and twice with hypotonic buffer. The beads were resuspended in 20 μl of ubiquitination buffer (25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) containing 2 μg recombinant Cdt1, 12 μg/ml E1 (BIOMOL, Plymouth Meeting, PA), 30 μg/ml E2 (UbcH10, Calbiochem), 0.8 mg/ml His-tagged ubiquitin (Sigma, St. Louis, MO), and 2 mM ATP. Recombinant Cdt1 was prepared from purified GST-Cdt1 (Sugimoto *et al.*, 2004) by digestion with PreScission Protease (Amersham). The reactions were incubated at 30°C for 180 min and subjected to SDS-PAGE followed by immunoblotting.

Immunostaining

Cells were briefly extracted with 1% Triton X-100 in PBS, fixed in 3.7% formaldehyde/10% methanol, and probed with anti-γ-tubulin mAb (GTU88, Sigma). The samples were then incubated with Alexa Fluor-594–conjugated goat anti-mouse IgG antibody (Molecular Probes, Eugene, OR), counterstained with DAPI, and analyzed with a Leica FW4000 microscope (Deerfield, IL).

Immunoblotting and Antibodies

Immunoblotting was performed as described previously (Sugimoto *et al.*, 2004), and antibody binding was visualized using the ECL system (Amersham). Chemiluminescent signals were captured by a cooled-CCD camera-directed detection system (LumiVision Imager, Aisin, Koshigaya, Japan) within the linear range, and the band intensities were quantified using the analyzing system. After subtracting the background signals, the obtained signals were further normalized to the signals of control proteins such as actin and topoisomerase I that do not oscillate during the cell cycle.

Preparation of polyclonal rabbit antibodies against human Cdt1, CDC6, MCM4, and MCM7 was described previously (Fujita *et al.*, 1998; Sugimoto *et al.*, 2004; Tatsumi *et al.*, 2006). Anti-human GRWD1 antibody was obtained by immunizing rabbits with a GST fusion protein containing full-length human GRWD1. Other antibodies were purchased from different companies: T7-tag (69522-3, Novagen), myc-tag (PL14, MBL, Nagoya, Japan), His-tag (70796-3, Novagen and 11-922-416-001, Roche, Indianapolis, IN), HA-tag (16B12, BabCO, Richmond, CA, and 3F10, Roche), geminin (SC-13015, Santa Cruz Biotechnology, Santa Cruz, CA), Skp2 (sc-7164, Santa Cruz), PCNA (PC10, DAKO, Carpinteria, CA), topoisomerase I (2012-2, TopoGEN, Columbus, OH), topoisomerase IIa (D081-3S, MBL), SNF2H (1B9/D12, Upstate Biotechnology, Lake Placid, NY), WSTF (2152, Cell Signaling, Beverly, MA), APC7 (sc-20987, Santa Cruz), APC8 (ab4172, Abcam, Cambridge, MA), Cdh1 (C7855, Sigma), Cdc20 (sc-13162, Santa Cruz), Cdc27 (C-7104, SIGMA), nucleophosmin (B0556, Sigma), cyclin A (6E6, Lab Vision, Fremont, CA), cyclin B (GNS-1, PharMingen, San Diego, CA), GFP(46-0092, Invitrogen), Chk2 (clone7, Cell Signaling), Thr68-phosphorylated Chk2 (number2661, Cell Signaling), ATM (2C1, Gene Tex, San Antonio, TX), Ser1981-phosphorylated ATM (200-301-400, Rockland Biosciences, Gilbertsville, PA), and E2F-1 (sc-251, Santa Cruz).

RESULTS AND DISCUSSION

Identification of Novel Cdt1-binding Proteins by a Combination of Cdt1 Affinity Chromatography and Mass Spectrometric Analysis

Cdt1-binding proteins enriched from HeLa cell nuclear extracts using a GST-Cdt1 column were eluted with buffer containing 0.5 M NaCl and analyzed on SDS-PAGE followed by CBB staining. Many specific proteins were detected, with some nonspecific protein bands, (Figure 1), concentrated, and subjected to preparative SDS-PAGE and LC/MS/MS analysis. The raw data were further processed to determine highly scored hits with protein sequence databases. We picked identified proteins with total probability scores from the Mascot search of more than 100. Among the proteins identified, we excluded membrane proteins, metabolic enzymes, cytoplasmic proteins, ribosomal proteins, splicing factors, heat shock proteins, and cytoskeletal proteins, which may have been nonspecifically retained in the Cdt1 column because of their abundance or low solubility. The remaining proteins are listed in Supplementary Table 1 in Supplementary Materials, designated as "primary data."

Among the proteins identified through our procedure, only MCM4 and MCM6 proteins were already known to bind to Cdt1 (Table 1; Yanagi *et al.*, 2002; Cook *et al.*, 2004). This was further confirmed by conventional GST-Cdt1 pull-down assay and immunoblotting (Figure 2A). It might appear anomalous that other known Cdt1-binding proteins such as geminin (Wohlschlegel *et al.*, 2000; Tada *et al.*, 2001) and Skp2 (Li *et al.*, 2003; Liu *et al.*, 2004; Sugimoto *et al.*, 2004) were not identified in our MS analysis. This might be explained by the fact that we used 0.5 M NaCl buffer to elute Cdt1-binding proteins in order to avoid GST-Cdt1 leaking out from the column and contaminating samples that were subjected to MS analysis. Under these experimental condi-

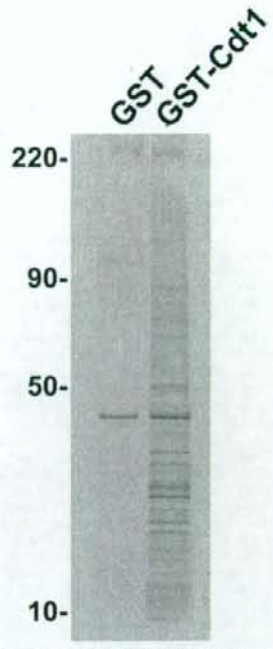

Figure 1. Resolution of Cdt1-binding proteins on CBB-stained SDS-PAGE. Nuclear extracts obtained from HeLa cells were loaded onto GST or GST-Cdt1 columns as described in *Materials and Methods*. The bound proteins were eluted and then separated in a 4–20% SDS-PAGE gel followed by CBB staining.

tions, it is conceivable that proteins binding to Cdt1 too tightly might not be eluted. Efficient enrichment of geminin, Skp2, and CDC6 (Cook *et al.*, 2004) was however observed when assessed by conventional GST-Cdt1 pulldown assay, in which bound proteins were eluted simultaneously with GST-Cdt1 by glutathione (Figure 2A).

On the basis of their potential involvement in cell cycle regulation, DNA replication, and Cdt1 function, we picked 12 potential novel Cdt1-binding proteins from the primary data for further analysis, as described below (Table 1). The other novel Cdt1-binding proteins identified by MS analysis, for example histones, are only listed in Supplementary Table 1. However, we could not exclude the possibility that some of them play crucial role(s) in Cdt1 functions or regulation.

Physical Interactions between the Newly Identified Proteins and Cdt1

PCNA is a well-known processivity factor for DNA polymerases involved in regulation machinery for many replication-associated processes (Tsurimoto, 1999). Conventional pulldown assay and immunoblotting showed that PCNA in fact binds to GST-Cdt1 with an efficacy comparable with those for known Cdt1-binding proteins such as MCM and geminin (Figure 2A; Nishitani *et al.*, 2006). PCNA binding to Cdt1 in vivo was demonstrated by immunoprecipitation assay (Figure 2B; Nishitani *et al.*, 2006). We and other groups have previously found that PCNA in fact mediates cullin4-DDB1 complex-directed ubiquitination and subsequent degradation of Cdt1 during S phase (Arias and Walter, 2006; Hu and Xiong, 2006; Nishitani *et al.*, 2006; Senga *et al.*, 2006).

Table 1. List of known and potential novel Cdt1 binding proteins from Supplementary Table 1

	Annotation	Gel slice number[a]
APC/C	APC8	20 (145), 21 (124)
	APC7	18 (115), 21 (132)
	APC5	24 (116)
Chromatin remodeling complex	SNF2H	30 (296, 128), 31 (260, 113)
	WSTF	31 (145)
PCNA	PCNA	11 (305), 12 (58)
DNA topoisomerase	Topoisomerase I	22 (407, 402, 270), 23 (731), 24 (233), 27 (148), 28 (48)
	Topoisomerase II α	32 (194), 33 (312), 34 (394), 35 (134)
Nuclear import	Importin α1	17 (203), 18 (186, 136)
	Importin β1	26 (274), 27 (91)
WD-repeat protein	GRWD1/WDR28	19 (105)
Nucleophosmin	Nucleophosmin	Many (for detail, see Supplementary Table 1)
MCM	MCM4	27 (142)
	MCM6	27 (59), 28 (257), 29 (139)

[a] Gel slice number, numbers of slices from the CBB-stained gels (see *Material and Methods*). Values in parentheses are the total probability scores from the Mascot search for identified proteins.

Topoisomerase I and topoisomerase IIα (Wang, 1996) were identified by MS analysis, and binding to GST-Cdt1 was confirmed by pulldown assay and immunoblotting. Although the binding efficacy appeared low compared with PCNA and APC subunits (Figure 2A), it was detected in multiple slices in the MS analysis (Table 1), suggesting that the absolute amounts of the bound proteins are high. We further confirmed these interactions by coimmunoprecipitation of topoisomerase I and IIα with Cdt1 (Figure 2, C and D). It has been suggested with the SV40 T antigen–directed replication system that topoisomerases may play a role not only in elongation but also initiation (Simmons *et al.*, 2004). It is also of interest that Tah11, a budding yeast Cdt1 homolog, genetically interacts with topoisomerase I (Devault *et al.*, 2002; Tanaka and Diffley, 2002). We therefore speculate possible involvement of topoisomerases in establishment of pre-RC, a possibility presently under investigation in our laboratory.

Imitation switch (ISWI)-type nucleosome remodeling factor SNF2H and WSTF (Williams syndrome transcription factor) were also identified by MS analysis and confirmed to bind to GST-Cdt1 by pulldown assay and immunoblotting, albeit at low efficacies (Figure 2A). In addition, SNF2H was coimmunoprecipitated with Cdt1 (Figure 2E and Supplementary Figure S1A). It has been suggested that the SNF2H-WSTF complex interacts with PCNA and thereby is targeted to replication foci to play a role in the maintenance of chromatin structure (Bozhenok *et al.*, 2002; Poot *et al.*, 2004). Therefore, recovery of the SNF2H-WSTF complex in our system might be due to the indirect binding to Cdt1 via PCNA. However, it also remains possible that SNF2H-based chromatin remodeling complexes could play a role in pre-RC formation and/or initiation step of DNA replication. For example, the CHRAC (chromatin accessibility complex), which contains SNF2H, allows binding of T-antigen and efficient initiation of replication in an in vitro replication system using SV40 DNA reconstituted into chromatin (Alexiadis *et al.*, 1998). It has been also reported that silencing of

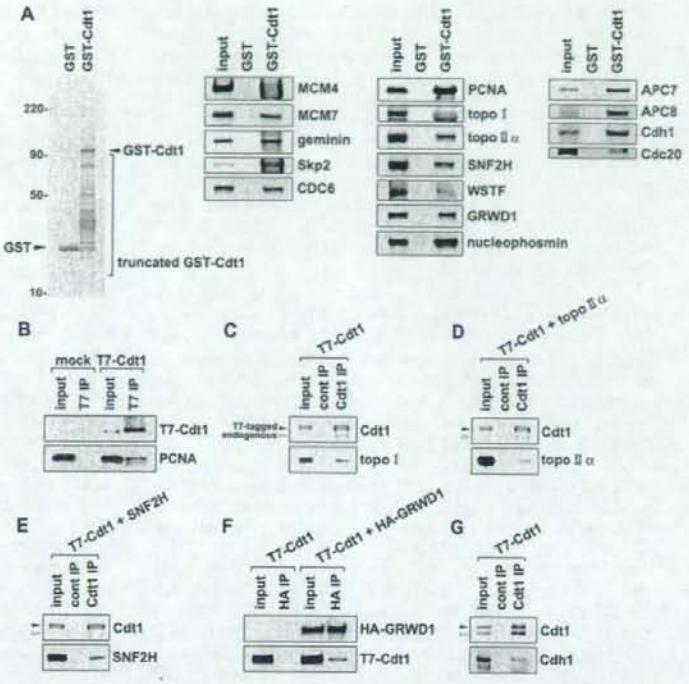

Figure 2. Confirmation of interactions between Cdt1 and proteins identified by the MS analysis. (A) GST-Cdt1 or GST were incubated with HeLa cell nuclear extracts, and bound proteins were analyzed by CBB staining (left panel) or immunoblotting with the indicated antibodies. Fifteen percent of the input was also loaded. (B) Cdt1-PCNA complexes were immunoprecipitated with anti-T7 antibody from 293T cells stably expressing T7-tagged Cdt1, and immunoprecipitates (IP) were subjected to immunoblotting with anti-T7 or anti-PCNA antibodies. Five percent of the input (for T7-Cdt1) or 0.5% of the input (for PCNA) were analyzed to show the precipitation efficiency. (C–E) 293T cells were transfected with the indicated expression vectors, and nuclear extracts were prepared. After immunoprecipitation with anti-Cdt1 antibody or control rabbit IgG, the precipitates were blotted with the indicated antibodies. Ten percent of the input was loaded. (F) 293T cells were transfected with the indicated expression vectors, and the soluble fractions were prepared. After immunoprecipitation with anti-HA antibody, the precipitates were blotted with anti-HA or anti-T7 antibodies. Five percent of the input was loaded. (G) The 293T cells stably expressing T7-tagged Cdt1 were first treated with formaldehyde and then solubilized with SDS and sonication. The lysates were immunoprecipitated with anti-Cdt1 antibodies or control IgG, and the immunoprecipitates (IP) were immunoblotted with anti-Cdt1 or anti-Cdh1 antibodies. Five percent of the input for Cdt1 or 0.5% of the input for Cdh1 were loaded.

SNF2H by siRNA impedes DNA replication in HeLa cells (Collins *et al.*, 2002). Recently, it was suggested that SNF2H is recruited to the Epstein-Barr virus origin of plasmid replication during G1 phase and that siRNA depletion of SNF2H reduces MCM3 loading at the origin (Zhou *et al.*, 2005). However, it remains unclear whether this is also the case for cellular replication origins and, if so, how SNF2H might be recruited. Further studies are required to address potential roles of Cdt1-SNF2H interactions in pre-RC formation.

GRWD1 (glutamate-rich WD-repeat protein 1), a human homolog of budding yeast RRB1, which is implicated in ribosome biogenesis and chromosome segregation (Iouk *et al.*, 2001; Schaper *et al.*, 2001; Killian *et al.*, 2004; Gratenstein *et al.*, 2005), was also identified by MS analysis. Interestingly, RRB1 is known to genetically interact with ORC6 and also physically with Yph1 (Killian *et al.*, 2004), a nucleolar protein required for ribosome biogenesis and involved in DNA replication probably through binding to ORC (Du and Stillman, 2002). Therefore, it is intriguing to speculate that GRWD1 might be involved in regulation of DNA replication initiation through binding to Cdt1 in mammalian cells. Both Cdt1 and GRWD1 proteins have a tendency to accumulate into nucleoli (Killian *et al.*, 2004; Nishitani *et al.*, 2001). As shown in Figure 2A, specific binging of GRWD1 to GST-Cdt1 could here be confirmed. Furthermore, in vivo interaction between GRWD1 and Cdt1 was confirmed by coimmunoprecipitation assay (Figure 2F and Supplementary Figure S1B). Very recently, GRWD1 was also identified as a candidate substrate-receptor of Cullin4-DDB1 ubiquitin ligase (Higa *et al.*, 2006). However, its biological significance remains to be elucidated.

Nucleophosmin/nucleoplasmin was identified by MS analysis, and binding to GST-Cdt1 was confirmed by pull-down assay and immunoblotting (Figure 2A). Its requirement for in vitro replication of *Xenopus* sperm nuclei in the egg extracts is of interest in this context (Gillespie and Blow, 2000).

Human Cdt1 protein has putative nuclear localization signals (Arentson *et al.*, 2002; Yanagi *et al.*, 2002), and it is indeed a nuclear protein. Therefore, it is conceivable that importinα1 and importinβ1 (Goldfarb *et al.*, 2004) bind to the nuclear localization signals, and they were indeed identified in our MS analysis.

APC/C^{Cdh1} Ubiquitin Ligase Physically Interacts with Cdt1

For this report, we further focused on APC/C as novel Cdt1-binding proteins and investigated its potential role in Cdt1 proteolytic regulation. APC/C is an ubiquitin ligase that regulates exit from mitosis by targeting several proteins to degradation through polyubiquitination (Vodermaier, 2004; Castro *et al.*, 2005; Pines, 2006). Important targets include cyclin A, cyclin B, geminin, and securin. In our initial MS analysis, APC5, 7, and 8 subunits were identified as Cdt1-binding proteins (Table 1). Binding of APC7 and APC 8 to Cdt1 was further confirmed by conventional GST-Cdt1 pulldown assay and immunoblotting (Figure 2A). APC/C is regulated by its association with one of two activator proteins, Cdc20 and Cdh1. APC/C^{Cdc20} is first activated at mitotic exit and APC/C^{Cdh1} is sequentially activated during G1 phase or when cells enter G0 phase (Vodermaier, 2004; Castro *et al.*, 2005; Pines, 2006; see also Figure 9). Therefore, we investigated whether these activator proteins were also

recoverable in Cdt1-bound fractions by GST-Cdt1 pulldown assay and immunoblotting. As shown in Figure 2A, Cdh1 was efficiently recovered in the Cdt1-bound fraction, and Cdc20 to a lesser extent, suggesting that APC/C^{Cdh1} rather than APC/C^{Cdc20} efficiently binds to Cdt1. Consistent with this conclusion, Cdh1 was coimmunoprecipitated with Cdt1 when cells were first cross-linked with formaldehyde and then subjected to immunoprecipitation (Figure 2G). So far, we could not observe their coimmunoprecipitation without formaldehyde cross-linking. Therefore, physical interaction between Cdt1 and Cdh1 may be relatively unstable. Nevertheless, as shown below, their interaction is specific and physiologically significant.

Identification of Destruction Boxes 1–3 in the Cdt1 N-Terminus Required for Recognition by APC/C^{Cdh1}

Substrates of APC/C are often characterized by the presence of a "destruction box" (D-box), which is a stretch of four amino acids (RxxL) first noted in cyclin B (Burton and Solomon, 2001; Vodermaier, 2004; Castro *et al.*, 2005; Pines, 2006). We have identified four RxxL motifs in human Cdt1 (amino acids 34–37, 67–70, 69–72, and 396–399; hereafter, termed D-boxes 1, 2, 3, and 4, respectively), but could not find any other known putative Cdh1 recognition motifs such as the KEN-box (Burton and Solomon, 2001; Pfleger and Kirschner, 2000), A-box (Littlepage and Ruderman, 2002), and O-box (Araki *et al.*, 2005). Two other known ubiquitin ligases that regulate proteolysis of Cdt1, SCF^{Skp2} and cullin4-DDB1, recognize the motifs in the Cdt1 N-terminus (Figure 3A; Fujita, 2006). We therefore reasoned that APC/C^{Cdh1} might also utilize D-boxes 1–3 in the Cdt1 N-terminus for recognition. Accordingly, we needed to prepare Cdt1 mutants in which the D-boxes 1, 2, and 3 in the N-terminus were individually or simultaneously mutated. A difficult problem in this regard is that the D-boxes 2 and 3 and the RXL cyclin-binding motif required for Skp2 binding overlap (Figure 3A). In principle, R67A L72A mutations might eliminate the function of the D-boxes 2 and 3 without affecting Cdt1 interaction with Skp2 because the Cy motif (R68 R69 L70) is conserved. However, we found the interaction with Skp2 (and also with cyclin A) to be severely impaired by the R67A L72A mutations (data not shown), and it appears impossible to abrogate D-boxes 2 and 3 without impairing the interaction with Skp2. In many experiments, we therefore used the Cdt1 Cy (R68A R69A L70A; Sugimoto *et al.*, 2004) as a mutant having disrupted D-boxes 2 and 3 (Figure 3A).

We investigated whether the mutations in D-boxes 1–3 indeed impair physical interaction of Cdt1 with APC/C^{Cdh1}. For this purpose, we first prepared GST-Cdt1 Cy and GST-Cdt1 Cy+D1m, in which the D-box 1 is mutated in addition to mutations in the D-boxes 2 and 3, as well as the wild type (Figure 3A), and performed GST pulldown assays. HeLa cell nuclear extracts were incubated with these GST-Cdt1 proteins and the bound proteins were analyzed. Binding of APC7, APC8, and Cdh1 to Cdt1 was partially impaired by the Cy mutation and severely impaired by the triple mutations (Figure 3B). As expected, PCNA binding to Cdt1, which is dependent on the N-terminal PIP-motif (Figure 3A), was not changed by the D-box mutations (Figure 3B). The data suggest that both the D-box 1 and D-boxes 2–3 are required for efficient APC/C^{Cdh1} binding.

We further investigated whether in vivo interactions between Cdh1 and Cdt1 detected by coimmunoprecipitation after formaldehyde cross-linking are dependent specifically on the D-boxes 1–3. 293T cells were transiently transfected with wild-type T7-Cdt1 or T7-Cdt1 Cy+D1m, cross-linked with formaldehyde, and then subjected to immunoprecipitation with anti-T7 antibody. While Cdh1 was coprecipitated with wild-type Cdt1, it was not with the Cdt1 Cy+D1m (Figure 3C). As expected, Skp2 coprecipitation was also lost with the mutations and geminin was coprecipitated, irre-

Figure 3. Interaction of Cdt1 with APC/C^{Cdh1} requires amino-terminal destruction boxes 1–3. (A) Top, schematic illustrations of the N-terminal amino acids of wild-type human Cdt1 and the mutants used in this study. Bottom, table summarizing the properties of the mutants. PIP-motif, PCNA-interaction protein motif (QxxI/L/VxxFF) required for PCNA binding and subsequent recognition by Cullin4-DDB1 ubiquitin ligase; Cy-motif, RXL-type cyclin-binding motif required for Cdt1 phosphorylation by cyclin A/Cdks and subsequent recognition by SCF^{Skp2} ubiquitin ligase; and T29, threonine 29 residue phosphorylated by cyclin A/Cdks. (B) Wild type and the indicated mutant GST-Cdt1 or GST were incubated with HeLa cell nuclear extracts, and bound proteins were analyzed by immunoblotting with the indicated antibodies. PCNA and geminin served as control proteins whose binding to Cdt1 is not affected by the mutations. Twenty five percent of the input was also loaded. (C) 293T cells were transfected with wild-type T7-Cdt1, T7-Cdt1 Cy+D1m, or control vector, cross-linked with formaldehyde, and lysed as described in Figure 2G. The lysates were immunoprecipitated with anti-T7 antibody and analyzed by immunoblotting with the indicated antibodies. Five percent of the input for Cdt1, geminin, and Skp2 or 0.5% of the input for Cdh1 and E2F-1 was analyzed to show the precipitation efficiency.

spective of the mutations (Figure 3C). In addition, we also confirmed that chromatin-associated proteins irrelevant to Cdt1 are not coprecipitated. As shown in Figure 3C, E2F-1, a transcriptional factor important for cell cycle progression, was never coprecipitated with T7-Cdt1 even after formaldehyde cross-linking. Taken together, these data demonstrate that APC/C^{Cdh1} binds to and recognizes Cdt1 via D-boxes 1–3.

In the following experiments, we have used Cdt1 Cy+D1m as a mutant Cdt1 with impaired APC/C^{Cdh1} binding and recognition. Because cyclin and Skp2 binding is inevitably impaired in Cdt1 Cy+D1m, we analyzed this mutant in comparison with Cdt1 Cy or Cdt1 T29A, in which Cdk phosphorylation and Skp2 binding are impaired (Sugimoto *et al.*, 2004; Takeda *et al.*, 2005), as well as the wild type. As a prerequisite for such experiments, it needed to be confirmed that the other known inhibitory machineries normally function in the Cdt1 Cy+D1m mutant. With geminin binding, both in pulldown and coimmunoprecipitation assays, it bound to Cdt1 Cy+D1m with comparable efficacy to the wild-type (Figure 3, B and C). For PCNA-dependent, cullin4-DDB1–mediated ubiquitination, we found that PCNA binds to Cdt1 Cy+D1m with efficacy comparable to that of the wild type in pulldown assays (Figure 3B). We further confirmed that the proteolytic pathway is functional in Cdt1 Cy+D1m by examining UV-induced Cdt1 degradation (Higa *et al.*, 2003; Hu *et al.*, 2004; Nishitani *et al.*, 2006; Senga *et al.*, 2006). As shown in Supplementary Figure S2A, the levels of Cdt1 Cy+D1m were decreased after UV irradiation to an extent comparable to the decrease in the wild-type Cdt1, although some residual protein was observed, probably because of the high basal level. As previously reported, a Cdt1 mutant having a disrupted PCNA-interaction protein motif (Cdt1 PIPm with Q3A V6A F9A mutations) proved resistant to UV-induced degradation (Supplementary Figure S2A).

Cdh1, but Not Cdc20, Overexpression Accelerates Cdt1 Destabilization

To examine effects of enforced expression of the activator proteins on Cdt1 stability, we transfected 293T cells with T7-Cdt1 expression vector, along with a construct expressing myc-Cdh1 or myc-Cdc20 (Figure 4A). Overexpression of Cdh1 resulted in a remarkable decrease in the steady-state levels of T7-Cdt1 proteins, whereas Cdc20 overexpression had only a limited effect, consistent with the fact that APC/C^{Cdh1} preferentially binds to Cdt1. Furthermore, addition of a proteasome inhibitor MG132 to the transfected cells significantly suppressed the Cdt1 reduction by Cdh1 (Figure 4A). Under our assay conditions, the steady-state levels of cotransfected GFP proteins were not affected by Cdh1 overexpression (Figure 4, B, C, and E). We also examined the effects of Cdh1 overexpression on endogenous Cdt1 levels. The steady-state levels of endogenous Cdt1 were decreased to an extent comparable to the decrease in cyclin A and cyclin B, whereas MCM7 protein levels were never affected (Figure 4B). These data suggest potential involvement of APC/C^{Cdh1} in proteolytic regulation of Cdt1.

Elimination of D-boxes 1–3 Stabilizes Cdt1 and Confers Partial Resistance to Cdh1 Overexpression

To address the potential roles of D-boxes 1–3 in APC/C^{Cdh1}-mediated Cdt1 regulation, we first created a construct expressing the Cdt1 N-terminal 2–101 amino acids tagged with 3HA (3HA-Cdt1[2-101]) and introduced mutations into the three D-boxes in the N-terminus (Figure 3A). Because the positions of D-boxes 2 and 3 overlap, we mutated them simultaneously. We transfected 293T cells with the 3HA-Cdt1[2-101] expression vectors, along with a construct expressing GFP for normalization of transfection efficiency. In these experiments, expression of both 3HA-Cdt1[2-101] and GFP was driven from a cytomegalovirus-derived promoter. Although mutations in D-box 1 alone did not affect the steady-state level of 3HA-Cdt1[2-101] protein and those in D-boxes 2 and 3 affected it only partially, significant increase was noted when all the three D-boxes were mutated (Figure 4C; lanes without Cdh1). As shown in Figure 4D, the half-lives of 3HA-Cdt1[2-101] with the mutations in all the D-boxes 1–3 were remarkably increased. 3HA-Cdt1[2-101] with the mutations in the D-boxes 2 and 3 was also stabilized but to a lesser extent. Because the mutations in the D-boxes 2 and 3 inevitably disrupt the Cy motif, simultaneous loss of SCF^{Skp2} binding undoubtedly could contribute to stabilization of the 3HA-Cdt1[2-101]. However, the fact that 3HA-Cdt1[2-101] was further stabilized with the mutations in all D-boxes 1–3 shows an importance for D-box 1. On the other hand, mutation limited to D-box 1 did not stabilize 3HA-Cdt1[2-101], suggesting that D-boxes 2 and 3 also function in proteolytic regulation of Cdt1 by APC/C^{Cdh1}.

Similar stabilization by mutations in D-boxes 1–3 was also observed in transient transfection assays with full-length Cdt1 (Figure 4, E and F). We then examined effects of enforced expression of Cdh1 on stability of the Cdt1 mutants. Unexpectedly, the steady-state levels of the full-length Cdt1 and 3HA-Cdt1[2-101] proteins with the triple mutations were still decreased upon coexpression of Cdh1, yet the remnant levels were highest (Figure 4, C and E). One possible reason why the triple D-box mutations confer only partial resistance on Cdt1 might be that aberrant overexpression of Cdh1 results in some chromosomal damage and this induces additional Cdt1 degradation. We found that phosphorylation of Chk2 kinase was induced upon Cdh1 overexpression in 293T cells (data not shown). This may be because Cdh1 overexpression can induce some rereplication (Supplementary Figure S3A), as reported previously (Sorensen *et al.*, 2000). However, addition of mutations in the PIP-motif could not confer further resistance on Cdt1 Cy+D1m (data not shown). It is now considered that the D-box sequence is more redundant than previously considered; namely, it is R/KxxL/I/M/V (Pines, 2006). Thus human Cdt1 potentially has eight additional D-boxes (amino acids 71–74, 189–192, 294–297, 311–314, 334–337, 368–371, 470–473, and 497–500). These D-boxes might operate with nonphysiological overexpression of Cdh1. Thus, the extent of the promotion of Cdt1 degradation by Cdh1 overexpression in 293T cells could be overestimated compared with physiological conditions. Nevertheless, the fact that elimination of D-boxes 1–3 stabilizes Cdt1 and confers partial resistance to Cdh1 overexpression also suggests the involvement of APC/C^{Cdh1} in proteolytic regulation of Cdt1.

Depletion of Cdh1 by siRNAs Stabilizes Mutant Cdt1 Proteins with Weakened Skp2 Binding

To further examine whether APC/C^{Cdh1} indeed participates in proteolytic regulation of Cdt1, we used siRNAs. Asynchronous HeLa cells were transfected with a siRNA targeting Cdh1 (termed Cdh1-1) or control siRNA, and harvested 48 h after transfection (Figure 5A). As expected, treatment with the siRNA Cdh1-1, but not control siRNA against luciferase, remarkably reduced its protein levels. Unexpectedly, we found that endogenous Cdt1 protein levels were rather decreased in Cdh1-silenced cells (Figure 5A). Cdt1 is targeted for degradation through polyubiquitination by SCF^{Skp2} (Li *et al.*, 2003; Liu *et al.*, 2004; Sugimoto *et al.*, 2004), and it was recently found that Skp2 is degraded via APC/

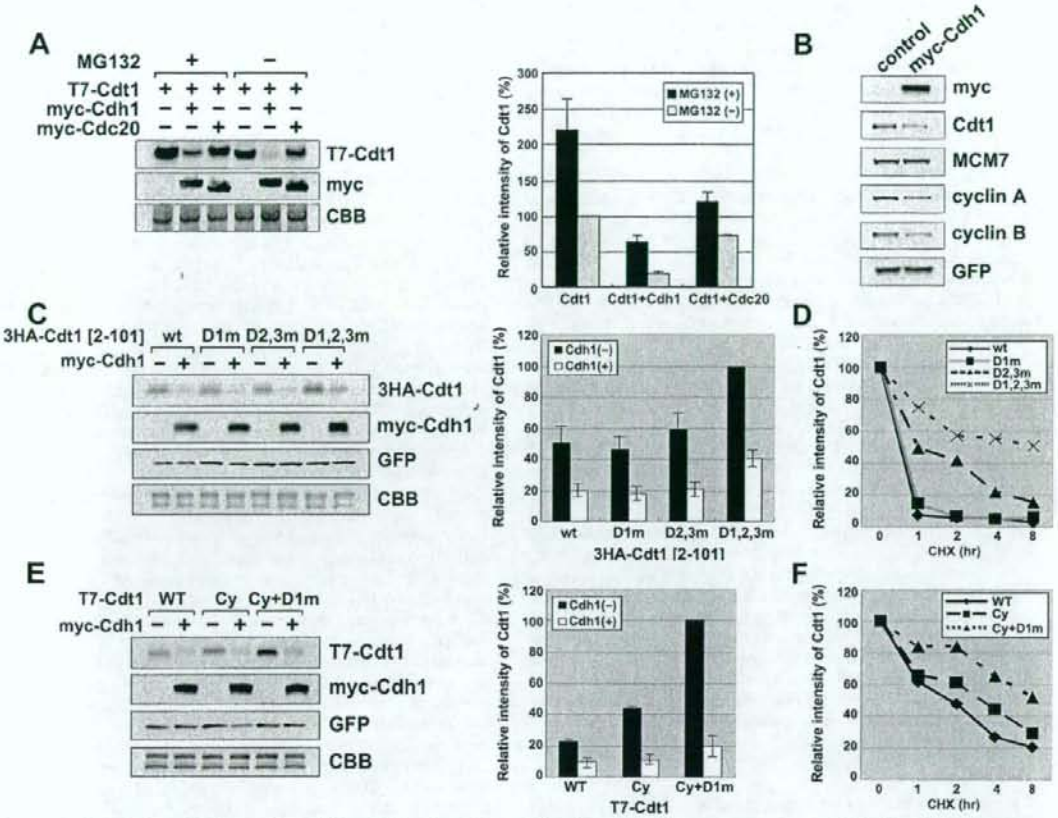

Figure 4. Elimination of D-boxes 1–3 stabilizes Cdt1 and confers partial resistance to Cdh1 overexpression in 293T cells. (A) Overexpression of Cdh1 promotes Cdt1 degradation in 293T cells. 293T cells were transfected with wild-type T7-Cdt1 expression vector (3 μg), along with a construct expressing myc-Cdh1 or myc-Cdc20 (3 μg), and whole cell lysates were prepared 48 h after transfection. When necessary, cells were supplemented with 40 μM MG132 for 5 h before harvest. Protein levels of T7-Cdt1, myc-Cdh1, and myc-Cdc20 were measured by immunoblotting with anti-T7 (left panel, top row) or anti-myc (middle row) antibodies. The membranes were also subjected to CBB staining to show equal loading (bottom row). The signal intensities of the bands were quantified, and the mean and SDs from two independent experiments are shown with Cdt1 alone without MG132 set at 100 (right panel). (B) Overexpression of Cdh1 promotes degradation of endogenous Cdt1. 293T cells were transfected with myc-Cdh1 (6 μg) and GFP (0.1 μg), and whole cell lysates were immunoblotted with the indicated antibodies. (C and D) Triple mutations in the D-boxes 1–3 increase the stability of 3HA-Cdt1[2-101] proteins. 293T cells were transfected with 3HA-Cdt1[2-101] expression vectors (3 μg), along with plasmids expressing myc-Cdh1 (3 μg) and GFP (0.1 μg). (C) Left panel, whole cell lysates were prepared 48 h after transfection. Protein levels of 3HA-Cdt1[2-101], myc-Cdh1, and GFP were measured by immunoblotting with anti-HA, anti-myc, or anti-GFP antibodies. The membranes were also subjected to CBB staining to confirm equal loading. The signal intensities of the bands were quantified, and the means and SDs from two independent experiments are shown with 3HA-Cdt1[2-101] D1,2,3m without Cdh1 set at 100 (right panel). (D) Cycloheximide (100 μg/ml) was added to the medium 48 h after transfection, and cells were sequentially harvested at the indicated time points for immunoblot analysis. (E and F) Triple mutations in the D-boxes 1–3 increase the stability of full-length Cdt1 proteins. Similar experiments to C and D were performed with full-length T7-Cdt1 expression vectors. For detection of T7-Cdt1, anti-T7 antibody was used.

C^{Cdh1}-mediated ubiquitination (Bashir *et al.*, 2004; Wei *et al.*, 2004). In fact, we found Skp2 protein levels to be up-regulated in Cdh1-silenced HeLa cells (Figure 5A). Therefore, the up-regulated Skp2 might have accelerated Cdt1 degradation even during the G1 phase when Cdk activities are low. Although Cdt1 ubiquitination by SCF^{Skp2} is enhanced when phosphorylated by Cdks, it is quite possible that unphosphorylated Cdt1 is also modified by SCF^{Skp2} with low efficiency. Indeed, ectopic Skp2 overexpression caused Cdt1 destabilization (Supplementary Figure S2B; Li *et al.*, 2003). On the basis of this hypothesis, we used a mutant Cdt1, Cdt1 Cy, whose sensitivity to Skp2-mediated destabilization is decreased (Figure 3A and Supplementary Figure S2B; Sugimoto *et al.*, 2004). We established HeLa cells stably expressing T7-Cdt1 Cy at levels comparable to the endogenous Cdt1 and silenced Cdh1 in these cells. As shown in Figure 5A, the amounts of T7-Cdt1 Cy protein were moderately but significantly increased upon Cdh1 silencing. As mentioned above, the Cy mutation also disrupted the two RxxL type D-boxes (Figure 3A). Thus, the extent of the increase in Cdt1 Cy upon

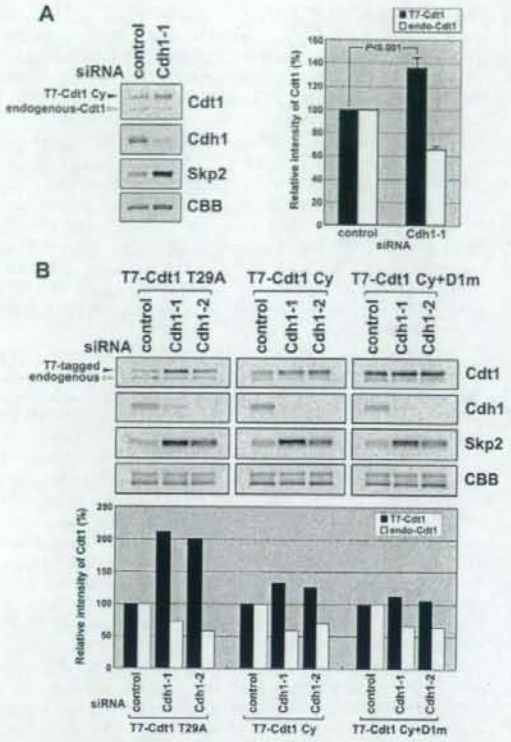

Figure 5. Silencing of Cdh1 stabilizes Cdt1 mutants with impaired binding to Skp2 in HeLa cells. (A) HeLa cells stably expressing the T7-Cdt1 Cy mutant were transfected with siRNA corresponding to a nonrelevant mRNA (control siRNA) or to a portion of Cdh1 mRNA (termed Cdh1-1). Forty-eight hours after transfection, whole cell lysates were subjected to immunoblotting (left). The signal intensities of the bands were quantified, and the mean and SDs from five independent experiments are shown with endogenous Cdt1 or T7-Cdt1 Cy in cells treated with control siRNA set at 100 (right panel). (B) HeLa cells stably expressing T7-Cdt1 T29A, Cy, or Cy+D1m were transfected with the control siRNA, siRNA Cdh1-1, and another siRNA against Cdh1 (termed Cdh1-2). Forty-eight hours after transfection, whole cell lysates were subjected to immunoblotting (top). The signal intensities of the bands were quantified and are shown with endogenous Cdt1 or each of the T7-Cdt1 mutants in cells treated with control siRNA set at 100 (bottom).

Cdh1 silencing could have been underestimated. We therefore performed a similar experiment with HeLa cells stably expressing another mutant T7-Cdt1 T29A, in which Skp2-mediated destabilization is impaired but D-boxes 1–3 are intact (Figure 3A and Supplementary Figure S2B; Takeda *et al.*, 2005). We also used a different siRNA against Cdh1 (termed Cdh1-2) in addition to the siRNA Cdh1-1. Cdh1 knockdown by either siRNA resulted in twofold increase in the amounts of T7-Cdt1 T29A protein (Figure 5B). Using flow cytometry, we investigated whether cell cycle profiles are influenced by Cdh1 silencing, but found no significant differences compared with the control (Supplementary Figure S3B), indicating that the observed up-regulation of the Cdt1 Cy and Cdt1 T29A upon Cdh1 silencing is not a result of changes in cell cycle distributions. To test more directly whether the increase of the Cdt1 mutants is mediated by APC/C^{Cdh1}, we repeated the experiments with HeLa cells stably expressing T7-Cdt1 Cy+D1m, in which the D-boxes 1–3 are disrupted in addition to Skp2 binding being impaired (Figure 3). The levels of T7-Cdt1 Cy+D1m protein were not changed upon Cdh1 silencing (Figure 5B), indicating that increase in T7-Cdt1 Cy and T29A protein levels by Cdh1 silencing is dependent on the D-boxes 1–3 recognized by APC/C^{Cdh1}. The steady-state level of each exogenous T7-Cdt1 mutant protein was different, with that of T7-Cdt1 Cy+D1m being highest among the mutants used. Because HeLa cells stably expressing various T7-Cdt1 mutants have been established by infection with the high-titer retroviruses, the steady-state level of the each protein may simply represent its stability. With regard to stable expression of exogenous Cdt1 in HeLa cells, including the point mentioned above, see the more detailed description and discussion below and Figure 7.

The above data indicate that inhibition of APC/C^{Cdh1} stabilizes mutant Cdt1 proteins with weakened Skp2 binding. Nevertheless, we wanted to know whether Cdh1 silencing can indeed increase endogenous Cdt1 protein levels. One possible way to address this is to test whether double silencing of Cdh1 and Skp2 causes Cdt1 stabilization. Unfortunately, we found that double silencing leads to obvious cell growth inhibition in HeLa and T98G cells (data not shown), and therefore it was difficult to assess the effects precisely. We also tried to determine the influence of Cdh1 silencing on Cdt1 in HeLa cells formally synchronized in G1 phase (Supplementary Figure S4). HeLa cells were first transfected with Cdh1 siRNAs and, 24 h after transfection, were further treated with nocodazole. Prometaphase-arrested cells were then collected, replated in fresh medium to progress through G1 phase, and harvested at the indicated time points. Already in the prometaphase-arrested cells, endogenous Cdt1 protein levels were decreased upon Cdh1 silencing. This was rather expected, because in these cells, Cdt1 is phosphorylated by Cdks, which is efficiently catalyzed by Skp2 up-regulated by Cdh1 silencing. Throughout the G1 phase examined, the Skp2 levels were increased up to 2–3-fold in Cdh1-silenced cells, and cyclin A levels were also increased (Supplementary Figure S4). Probably reflecting this, the Cdt1 levels were virtually unchanged upon Cdh1 silencing (Supplementary Figure S4). Thus, in cycling HeLa cells, we could not observe stabilization of endogenous Cdt1 protein by Cdh1 inhibition. However, we could show that destabilization of endogenous Cdt1 upon G0 entry is partially suppressed by Cdh1 silencing (see below).

APC/C^{Cdh1} Ubiquitinates Cdt1 In Vitro

We then tested whether APC/C^{Cdh1} can ubiquitinate Cdt1 in vitro. Recombinant Cdt1 was subjected to in vitro ubiquitination reaction using APC/C^{Cdh1} immunopurified from G1 phase HeLa cells with anti-Cdc27 antibody. A ladder of high-molecular-weight Cdt1 appeared when APC/C^{Cdh1} was added, but was hardly detectable with a control antibody (Figure 6A). The high-molecular-weight Cdt1 also reacted with antibody against 6xHis, tagged to ubiquitin for the assay (Figure 6A). In addition, the observed high-molecular-weight Cdt1 generated with APC/C^{Cdh1} was significantly reduced when D-boxes 1–3 were eliminated (Figure 6B). The data indicate that Cdt1 is specifically polyubiquitinated in vitro by APC/C^{Cdh1}.

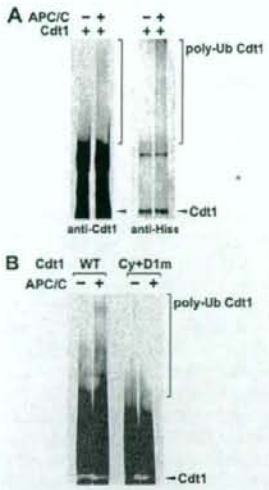

Figure 6. APC/C^{Cdh1}-dependent ubiquitination of Cdt1 in vitro. (A) APC/C^{Cdh1} complexes were purified from HeLa cells synchronized in G1 phase with anti-Cdc27 antibody. Recombinant Cdt1 proteins were incubated with E1, E2 (UbcH10), His-tagged ubiquitin, ATP, and the immunopurified APC/C or the control immunoprecipitate at 30°C for 180 min, separated by SDS-PAGE, and subjected to immunoblotting with anti-Cdt1 (left panel) or anti-His (right panel) antibodies. (B) The experiment was repeated using either the wild-type Cdt1 or the Cdt1 Cy+D1m mutant. The reacted samples were immunoblotted with anti-Cdt1 antibody.

Disruption of D-boxes 1–3 Stabilizes Cdt1 and Augments Its Function to Induce ReReplication and Chromosomal Damage

The line of evidence presented above led us to conclude that APC/C^{Cdh1} contributes to proteolytic regulation of Cdt1 during the cell cycle. APC/C^{Cdh1} plays a role in maintaining the G1 state in cycling cells. It is well established that overexpression of Cdt1 induces rereplication and/or chromosomal damage (Vaziri *et al.*, 2003; Tatsumi *et al.*, 2006). Therefore, appropriate levels of Cdt1 protein should be strictly maintained during the cell cycle. APC/C^{Cdh1}-mediated regulation of Cdt1 may have a role in balancing the levels. If proteolytic down-regulation of Cdt1 by APC/C^{Cdh1} is in fact biologically important, then its deregulation would lead to deleterious insults. We therefore examined the biological effects of Cdt1 Cy+D1m in different systems. In such experiments, we needed to compare its effects not only with the wild type but also with Cdt1 Cy or Cdt1 T29A, which induce stronger rereplication and chromosomal damage than the wild type (Takeda *et al.*, 2005; Tatsumi *et al.*, 2006), as discussed above.

First, we utilized transient Cdt1 overexpression-induced activation of the ATM/Chk2 DNA damage checkpoint pathway in 293T cells (Tatsumi *et al.*, 2006). As we reported previously, Cdt1 Cy induced stronger Chk2 phosphorylation than the wild type (Figure 7A). Notably, Cdt1 with mutations in the D-boxes 1–3 (Cdt1 Cy+D1m) was more stabilized and induced stronger checkpoint activation than Cdt1 Cy (Figure 7A).

We then investigated the effects of stable overexpression of Cdt1. HeLa cells were infected with the high-titer retroviruses and at 24 h after infection, cells were selected with hygromycin B. At 3 d after selection, when mock-infected cells were not viable, we found that cells with large nuclei (with a major axis larger than 26.6 μm, corresponding to the average + 2 SDs of cells infected with control virus without Cdt1) appeared with Cdt1 overexpression (Figure 7B). The frequency of such large-nucleus cells was ~20% with T7-Cdt1 T29A-infected cells and, notably, the percentage increased about twofold with T7-Cdt1 Cy+D1m (Figure 7B). We consider that such large nucleus may arise from rereplication and confirmed this by flow cytometry analyses. In agreement with the data of large-nucleus cells, cells with rereplicated DNA (the DNA content higher than 5N) were detected with T7-Cdt1 T29A and the percentage increased about twofold with T7-Cdt1 Cy+D1m (Figure 7C). There was no accumulation of 4N or 8N ploidy cells, which is indicative of mitotic failure.

To further confirm whether overexpression of T7-Cdt1 Cy+D1m induces rereplication within a single S phase or failed mitosis that can leads to increase in DNA content, we performed γ-tubulin immunostaining. If increase in DNA contents of T7-Cdt1 Cy+D1m-expressing cells results from mitotic failure, then these cells would be expected to contain multiple centrosomes detected by γ-tubulin staining. As shown in Figure 7D, most of the large-nucleus cells had one γ-tubulin spot, and the percentages of cells with 2 or >2 γ-tubulin spots was not increased remarkably, indicating that expression of T7-Cdt1 Cy+D1m can induce rereplication within a single S phase. Western blot analyses demonstrated that although the levels of the T7-Cdt1 T29A were higher than that of the wild type, the levels of T7-Cdt1 Cy+D1m were further increased (Figure 7E). Because HeLa cell populations stably expressing T7-Cdt1 proteins have been established with the high-titer retroviruses (at least 1×10^6 colony forming units per single infection experiment), differences in steady-state levels of proteins may simply represent stability rather than differences in transcription levels affected by the integration sites. In agreement with the strongest rereplication, T7-Cdt1 Cy+D1m induced the strongest checkpoint activation (Figure 7E). Thus, these data indicate that although expression of exogenous Cdt1 at levels comparable to those observed endogenously has only limited effect, disruption of D-boxes 1–3 leads to remarkable stabilization of Cdt1 and induction of strong rereplication and chromosomal damage under the same circumstances, well consistent with the findings of transient transfection assays.

Rereplicated cells gradually disappeared during continued culture and at 14 d after infection virtually no such cells were detectable (Supplementary Figure S5). This may be because cells with rereplicated DNA cannot grow and thus are diluted during the additional culture. In such remaining populations, we found no significant differences in the cell cycle profiles (Supplementary Figure S5) and cell growth rates (data not shown), yet T7-Cdt1 Cy+D1m was most stable (Figure 5B). The average levels of exogenous T7-Cdt1 proteins in remaining cell populations were found to be lower than in earlier populations containing rereplicated cells (compare Figure 7E with Figure 5B using the levels of endogenous Cdt1 as controls). Thus, rereplication may be induced in cells overexpressing T7-Cdt1 at high levels. It is also notable that only two- to threefold increase in Cdt1 levels compared with endogenous values evokes checkpoint activation (e.g., cells transfected with wild-type Cdt1 shown in Figure 7E), suggesting an importance for strict Cdt1 regulation.

Taken together, the above data indicate that proteolytic regulation of Cdt1 by APC/C^{Cdh1} through D-boxes 1–3

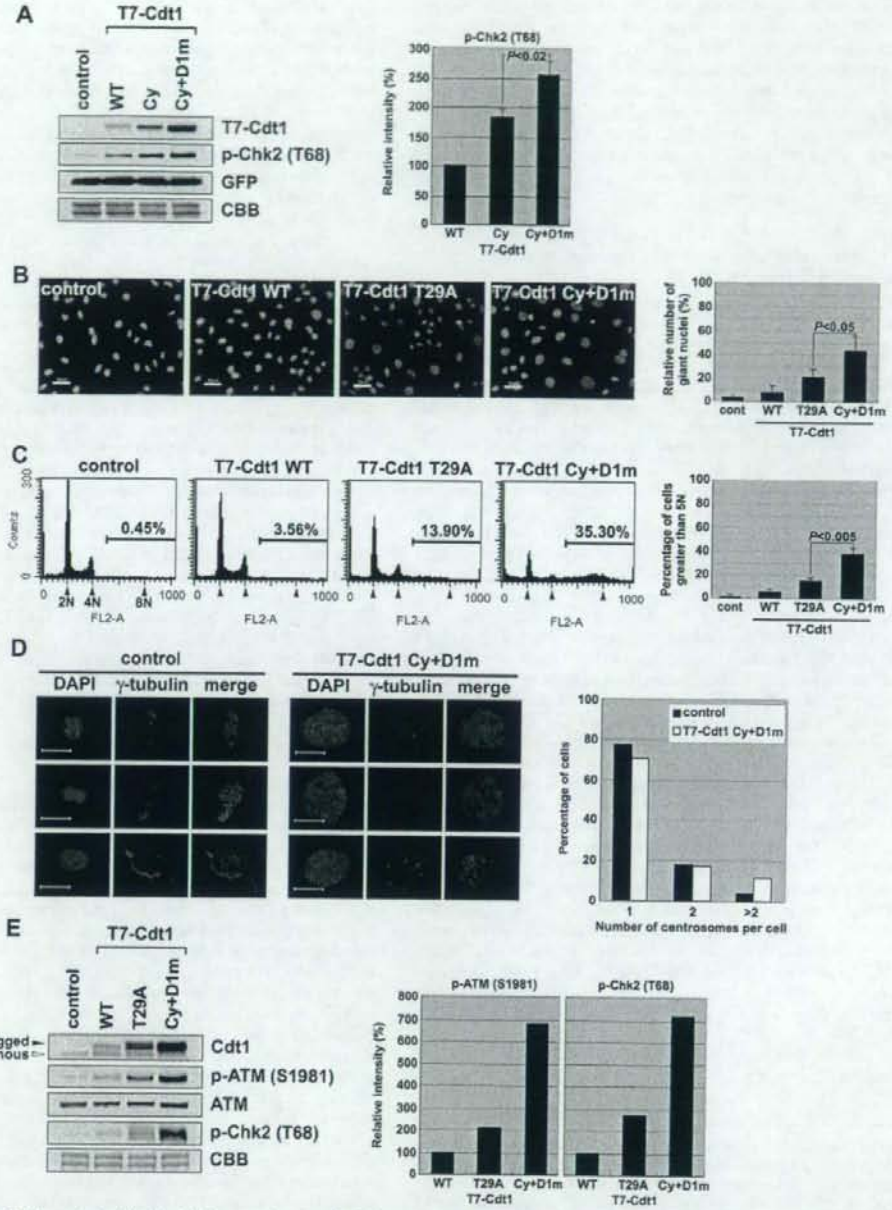

Figure 7. The Cdt1 mutant with the triple mutations in the D-boxes 1–3 induces the strongest rereplication, chromosomal damage, and checkpoint activation. (A) 293T cells were transiently transfected with T7-Cdt1 expression vectors (3 μg), along with a plasmid expressing GFP (0.1 μg) and an empty vector (3 μg) as a carrier. Whole cell lysates were prepared 48 h after transfection and immunoblotted with the indicated antibodies. The signal intensities of the bands were quantified, and the means and standard deviations from four independent experiments are shown with the Cdt1 wild type set at 100 (right panel). (B–E) HeLa cells were infected with the high-titer retroviruses expressing wild-type, T29A, or Cy+D1m T7-Cdt1 or control retroviruses and selected. (B) At 4 d after infection, cells were stained with DAPI to visualize nuclei. Scale bars, 50 μm. In the right panel, the percentage of cells with large nuclei in which the major axis of nuclei was larger than 26.6 μm, corresponding to the average + 2 SDs for that of cells infected with control viruses, is shown. Two hundred random nuclei were measured for each and the means and SDs from two independent experiments are shown. (C) At 4 d after infection, cells were collected and DNA content was analyzed by flow cytometry. The means and SDs of the percentage of rereplicated cells (the DNA content higher than

plays a crucial role in regulation of Cdt1 functions during the cell cycle. Although rereplication may occur in S, G2, and M phases, APC/C^{Cdh1} is not active during these periods. However, it is quite possible that if the levels of Cdt1 are kept high in G1 due to stabilization, then the level of remnant proteins after S phase degradation is also high. Indeed, even when HeLa cells stably expressing T7-Cdt1 were arrested in S phase by hydroxyurea treatment, the levels of T7-Cdt1 Cy+D1m were higher than in T29A mutant (Supplementary Figure S6).

Cdt1 Is Destabilized in G0 Depending on D-boxes 1–3 and Their Elimination Augments Its Function to Induce Chromosomal Damage in G0

APC/C^{Cdh1} also plays a role in inducing and maintaining quiescence (G0 phase). Cdt1 protein levels decrease as cells enter quiescence as well as CDC6, another target of APC/C^{Cdh1} (Petersen *et al.*, 2000; Xouri *et al.*, 2004; Mailand and Diffley, 2005; Tatsumi *et al.*, 2006). Importantly, inappropriate existence of Cdt1 in quiescent cells leads to chromosomal damage and activation of the ATM/Chk2 pathway (Tatsumi *et al.*, 2006). Therefore, it is conceivable that the APC/C^{Cdh1}-mediated destruction contributes to rapid clearance of Cdt1 when cells enter quiescence. To address this point, we used T98G cells that can be induced to enter quiescence (G0), where APC/C^{Cdh1} is active, by serum deprivation (Mailand and Diffley, 2005). T98G cells were infected with the high-titer retroviruses expressing T7-Cdt1 T29A or Cy+D1m and subjected to drug selection. During early passages, anomalous cells appeared in Cdt1-overexpressing cells, similar to the case with HeLa cells (data not shown). These cells might represent Cdt1-induced rereplication, although we did not examine this exactly. Such cells gradually disappeared during continued culture, like HeLa, and at 14 d after infection, virtually no such cells were detectable. In the remaining populations, we found no significant differences in the cell cycle profiles and cell growth rates (data not shown), yet T7-Cdt1 Cy+D1m induced stronger ATM/Chk2 phosphorylation than T29A in asynchronously growing cells (Figure 8A). Parental T98G cells and cells expressing T7-Cdt1 T29A or Cy+D1m were then serum deprived for 48 h. In all the cells tested, the levels of endogenous Cdt1 as well as cyclin A were similarly decreased as cells entered quiescence, as expected. The levels of T7-Cdt1 T29A were also decreased upon G0 entry although to a lesser extent compared with endogenous Cdt1. Importantly, the levels of T7-Cdt1 Cy+D1m were never decreased (Figure 8A), suggesting that Cdt1 decrease in G0 is at least partly due to protein destabilization dependent on the D-boxes 1–3 recognized by APC/C^{Cdh1}.

To gain further support for the role of APC/C^{Cdh1} in Cdt1 destabilization upon G0 entry, we examined whether the Cdt1 decrease is blocked by Cdh1 silencing. T98G cells expressing T7-Cdt1 T29A were first treated with Cdh1 siRNAs and then serum-deprived. In Cdh1-silenced cells, partial but significant stabilization of the endogenous Cdt1 was observed at 24 h after serum deprivation (Figure 8B). However, at 48 h, no significant difference was observed between control and Cdh1-silenced cells. Such apparently inefficient stabilization of endogenous Cdt1 upon Cdh1 knockdown may be due to counteracting Skp2 and cyclin A up-regulation (Figure 8B). As expected, we could observe more clear stabilization of T7-Cdt1 T29A in Cdh1-silenced and serum-deprived cells. Compared with cells treated with control siRNA, destabilization of the T7-Cdt1 T29A upon entry into quiescence was inhibited in cells treated with the Cdh1 siRNAs (Figure 8B). Together, these data provide further support for the role of APC/C^{Cdh1} in Cdt1 proteolysis upon G0 entry.

We then examined Cdt1-induced chromosomal damage in quiescent cells. Even in parental T98G cells, significant increase in ATM and Chk2 phosphorylation was observed upon entry into quiescence (Figure 8A). The reason is not clear at present. It is generally thought that entry into and exit from quiescence are accompanied by drastic changes in chromatin structures and this could be associated with the observed up-regulation of ATM/Chk2 phosphorylation. Consistent with previous reports (Takeda *et al.*, 2005; Tatsumi *et al.*, 2006), T7-Cdt1 T29A overexpression enhanced ATM/Chk2 phosphorylation upon G0 entry (Figure 8A). As expected from the high levels of the remnant protein, T7-Cdt1 Cy+D1m induced stronger ATM/Chk2 phosphorylation than the T29A mutant (Figure 8A). Together, these data indicate that proteolytic regulation of Cdt1 by APC/C^{Cdh1} via D-boxes 1–3 during exit from the cell cycle plays a crucial role in rapid clearance of Cdt1.

Biological Significance of Proteolytic Regulation of Cdt1 by APC/C^{Cdh1}

With the current paradigm, APC/C^{Cdh1} plays two major roles in regulating the cell cycle. One is inducing and maintaining quiescence. Our data indicate that Cdt1 destabilization by APC/C^{Cdh1} is required for rapid clearance of Cdt1 during exit from the cell cycle and that when this pathway is impaired, strong chromosomal damage and checkpoint activation are induced. Thus, the primary importance of APC/C^{Cdh1}-mediated regulation of Cdt1 may be in this context (see also Figure 9 showing a model for roles of APC/C^{Cdh1}-mediated Cdt1 proteolytic regulation), although molecular mechanisms by which deregulated Cdt1 damages chromatin in G0 are still unclear. APC/C^{Cdh1} also plays a role in maintaining the G1 state in cycling cells. Because Cdt1 levels do not remarkably oscillate from late mitosis through G1 phase (Supplementary Figure S4; Nishitani *et al.*, 2001), the efficacy of APC/C^{Cdh1}-mediated Cdt1 proteolysis may not be very robust. Indeed, we found that geminin is more efficiently catalyzed by APC/C^{Cdh1} than Cdt1 in our in vitro ubiquitination assay (data not shown). Nevertheless, this regulation may be also important for balancing the appropriate Cdt1 levels in G1 (Figure 9), because its impairment results in inappropriate Cdt1 accumulation, which in turn induces strong rereplication and chromosomal damage, as shown here.

APC/C^{Cdh1}-mediated proteolytic regulation appears to be a common feature of cell cycle regulation of replication initiation factors in metazoans, because human CDC6 and *Drosophila* ORC1 proteins are also under the control of this ligase (Petersen *et al.*, 2000; Araki *et al.*, 2005). Considering that CDC6 protein disappears more rapidly than Cdt1 dur-

Figure 7 (cont). 5N) from two independent experiments are shown (right panel). (D) Expression of T7-Cdt1 Cy+D1m induces rereplication without centrosome overduplication. At 4 d after infection, cells were stained with anti-γ-tubulin antibody and the number of γ-tubulin spots was counted at least in 200 cells. Cells infected with the control virus were analyzed randomly, and as to T7-Cdt1 Cy+D1m infection, cells larger than 26.6 μm were analyzed. The typical images of cells with 1 (top), 2 (middle), or >2 (bottom) centrosomes are shown in left panels. Scale bars, 20 μm. The percentages of cells with the indicated centrosome numbers are shown in right. (E) At 6 d after infection, whole cell lysates were immunoblotted with the indicated antibodies, and the signal intensities of the bands were quantified, here shown with the Cdt1 wild type set at 100 (right panel).

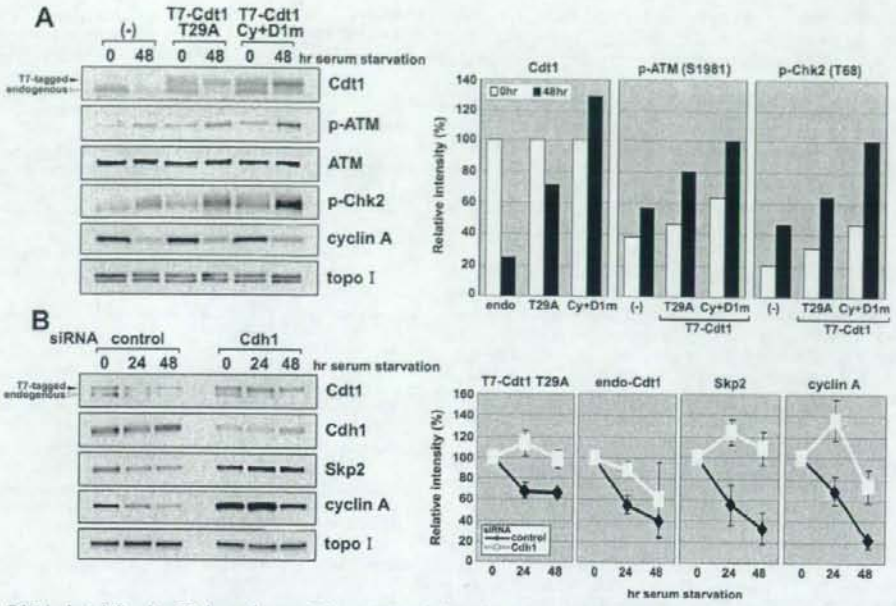

Figure 8. Cdt1 is destabilized in G0 dependent on D-boxes 1–3 and their elimination augments its function to induce chromosomal damage in G0. (A) T98G cells were infected with retroviruses expressing either T29A or Cy+D1m mutant T7-Cdt1. At 3 wk after infection, infected and parental cells were arrested in a quiescent state. Forty-eight hours after serum starvation, whole cell lysates were subjected to immunoblotting with the indicated antibodies (left). The signal intensities of the bands were quantified and shown with the values at 0 h set at 100 (for each of Cdt1 proteins) or with the maximum values set at 100 (for p-ATM and p-Chk2) (right). (B) T98G cells expressing T7-Cdt1 T29A were transfected with a mixture of siRNAs against Cdh1 (Cdh1-1 + Cdh1-2) and, 18 h after transfection, were deprived of serum for the indicated times. Whole cell lysates were then subjected to immunoblotting with the indicated antibodies (left). The signal intensities of the bands were quantified and shown with the values at 0 h set at 100 (right). The means and SDs from two independent experiments are shown.

ing G0 entry (Xouri *et al.*, 2004; Mailand and Diffley, 2005) and decreases remarkably in G1 phase (Petersen *et al.*, 2000), it may be a better substrate than Cdt1. However, the biological significance of APC/C^{Cdh1}-mediated CDC6 regulation remains to be clarified. By analogy with the Cdt1 case, this pathway might play a role in rapid clearance of CDC6 during G0 entry and disturbance might thus lead to deleterious insults. However, this has not been directly addressed.

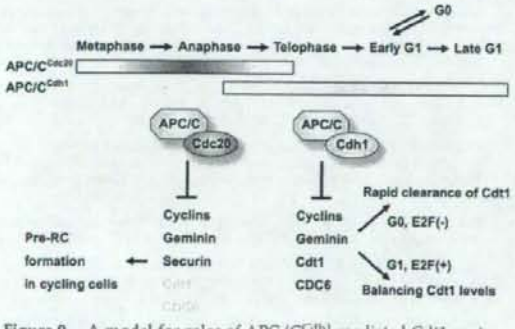

Figure 9. A model for roles of APC/C^{Cdh1}-mediated Cdt1 proteolytic regulation. For details, see the text.

In clear contrast to these initiation proteins that are regulated by APC/C^{Cdh1}, MCM proteins are relatively stable even after cells enter quiescence (Fujita *et al.*, 1996; Mailand and Diffley, 2005). In this context, it is noteworthy that expression of the ORC1, CDC6, Cdt1, and MCM genes is in all cases driven by the E2F transcription factor and, therefore, their transcription is rapidly shut down upon cell cycle exit (Fujita *et al.*, 1996; Xouri *et al.*, 2004; Fujita, 2006). Thus, differing from ORC1, CDC6, and Cdt1, MCM may not be subjected to specific proteolytic regulation during the cell cycle exit.

Previously, it was shown that an inhibitor of APC/C could interfere with Cdt1 degradation observed during exit from metaphase in a *Xenopus* egg extract system, and based on this finding, APC/C was suggested to be involved in Cdt1 proteolytic regulation (Li and Blow, 2005). Unfortunately, further studies to dissect the molecular mechanisms of APC/C-directed Cdt1 regulation were not performed at that time. In the *Xenopus* egg extract system, it is generally considered that APC/C^{Cdc20} but not APC/C^{Cdh1} is active during mitotic exit. In cycling mammalian cells, it is considered that pre-RCs are assembled around telophase, before which (i.e., from metaphase to anaphase) APC/C^{Cdc20} is active (Figure 9). This seems consistent with the fact that APC/C^{Cdc20} targets cyclins and geminin, critical inhibitors for pre-RC assembly, for proteolysis but does not efficiently function on CDC6 (Petersen *et al.*, 2000). Considering

that Cdc20 binds to Cdt1 only weakly and that Cdc20 overexpression has only a limited effect on Cdt1 stability, APC/C^{Cdc20} may not play a significant role in Cdt1 proteolysis, as is the case for CDC6.

In this report, we present data indicating that APC/C^{Cdh1} ubiquitin ligase controls the levels of Cdt1 via three destruction boxes in the Cdt1 N-terminus. Notably, elimination of these destruction boxes resulted in induction of strong rereplication and chromosomal damage. Similarly, the fact that Cy and T29A mutations, which impair Cdk phosphorylation and Skp2 binding, reinforce Cdt1 function demonstrates an importance of this pathway (Takeda *et al.*, 2005; Nishitani *et al.*, 2006; Tatsumi *et al.*, 2006; and this article). As to PCNA-dependent, cullin4-$DDB1^{Cdt2}$-mediated Cdt1 regulation in mammalian cells, although silencing of the components of this ligase induces rereplication (Jin *et al.*, 2006; Lovejoy *et al.*, 2006; Sansam *et al.*, 2006), mutations or deletion of PCNA-binding motif (PIP-motif) in Cdt1 rather reduce the ability to induce rereplication (Takeda *et al.*, 2005; Nishitani *et al.*, 2006). Thus, there are at least two possibilities (Fujita, 2006). One is that cullin4-$DDB1^{Cdt2}$ ligase may act on target proteins other than Cdt1. The other is that the Cdt1 N-terminal region including the PIP-motif could play some positive regulatory roles. These should be tested in future. In this regard, it should be also noted that in the *Xenopus* egg extract system, the mutant Cdt1 with impaired PCNA binding is stabilized and induces more rereplication than the wild type (Arias and Walter, 2006). In conclusion, in addition to SCF^{Skp2} and cullin4-based ubiquitin ligases, APC/C^{Cdh1} is a third ubiquitin ligase that plays a crucial role in proteolytic regulation of Cdt1 in mammalian cells.

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Functional analysis of Src homology 3-encoding exon (exon 2) of p130Cas in primary fibroblasts derived from exon 2-specific knockout mice

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p130Cas (Cas, Crk-associated substrate) is an adaptor molecule composed of a Src homology 3 (SH3) domain, a substrate domain (SD) and a Src binding domain (SBD). The SH3 domain of Cas associates with focal adhesion kinase (FAK), but its role in cellular function has not fully been understood. To address this issue, we established and analyzed primary fibroblasts derived from mice expressing a truncated Cas lacking exon 2, which encodes the SH3 domain (Cas Δexon 2). In comparison to wild-type cells, Cas exon $2^{\Delta/\Delta}$ cells showed reduced motility, which could be due to impaired tyrosine-phosphorylation of FAK and Cas, reduced FAK/Cas/Src/CrkII binding, and also impaired localization of Cas Δexon 2 to focal adhesions on fibronectin. In addition, to analyze downstream signaling pathways regulated by Cas exon 2, we performed microarray analyses. Interestingly, we found that a deficiency of Cas exon 2 up-regulated expression of CXC Chemokine Receptor-4 and CC Chemokine Receptor-5, which may be regulated by IκBα phosphorylation. These results indicate that the SH3-encoding exon of Cas participates in cell motility, tyrosine-phosphorylation of FAK and Cas, FAK/Cas/Src/CrkII complex formation, recruitment of Cas to focal adhesions and regulation of cell motility-associated gene expression in primary fibroblasts.

Introduction

Cas is composed of an N-terminal Src homology 3 (SH3) domain, a substrate domain (SD) that consists of a cluster of Tyr-Xaa-Xaa-Pro (YXXP) motifs (one YLVP, four YQXPs, nine YDXPs and one YAVP), a C-terminal Src binding domain (SBD) and other regions (Sakai *et al.* 1994). The SH3 domain binds to the proline-rich region of various signaling molecules, such as focal adhesion kinase (FAK) (Polte & Hanks 1995), PTP-1B (Liu *et al.* 1996), PTP-PEST (Garton *et al.* 1997), C3G (Kirsch *et al.* 1998) and CIZ (Nakamoto *et al.* 2000). The SD offers docking sites for the SH2 domain of several molecules including CrkII, Nck and an inositol 5′-phophatase, SHIP2 (SH2-containing inositol 5-phosphatase) in a tyrosine-phosphorylation-dependent manner (Mayer *et al.* 1995; Schlaepfer *et al.* 1997; Prasad *et al.* 2001). The SBD is rich in proline and serves as a binding site for the SH2 and SH3 domains of Src kinase (Nakamoto *et al.* 1996).

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Physiologically, Cas becomes tyrosine phosphorylated in response to various extracellular stimuli, such as integrin engagement (Nojima *et al.* 1995; Vuori & Ruoslahti 1995), which recruits Cas from cytoplasm to focal adhesions (Nakamoto *et al.* 1997). Tyrosine-phosphorylated Cas binds to CrkII, forming a Cas/CrkII complex (Vuori *et al.* 1996), which subsequently leads to the activation of the Rac–JNK pathway (Dolfi *et al.* 1998; Kiyokawa *et al.* 1998a). In addition, over-expression of Cas promotes cell motility, depending on its association with FAK and CrkII (Cary *et al.* 1998; Klemke *et al.* 1998).

To clarify biological roles of Cas, we generated Cas-deficient mice (Honda *et al.* 1998). Cas-deficient embryos died *in utero* at 12.5 dpc showing marked systemic congestion and growth retardation (Honda *et al.* 1998). Histologically, the heart was poorly developed and blood vessels were prominently dilated. Electron microscope analysis of the heart revealed disorganization of myofibrils and disruption of Z-disks (Honda *et al.* 1998). Cas-deficient fibroblasts showed impaired actin stress fiber formation, defects in cell migration, delayed cell spreading and resistance to Src-induced transformation (Honda *et al.* 1998, 1999). These results demonstrated that Cas is an actin-assembly molecule, which plays an essential role in embryonic development, cytoskeletal organization and Src-induced cellular transformation. Subsequently, to examine the role of each domain of Cas in these processes, we performed a compensation assay by expressing a series of Cas mutants in Cas-deficient fibroblasts (Huang *et al.* 2002). The results showed that motifs containing YDXP were indispensable for actin cytoskeleton organization and cell migration, suggesting that CrkII-mediated signaling regulates these biological processes (Huang *et al.* 2002). In contrast, C-terminal SBD was essential for cell migration, Src-induced transformation and membrane localization of Cas, but was dispensable for the organization of actin stress fibers (Huang *et al.* 2002). Although the above results provided insights in the roles of SD and SBD, the role of the SH3 domain of Cas, which has been shown to associate with various signaling molecules, remains unclear.

To address this issue, we generated mice deficient in Cas exon 2, which produce a truncated Cas protein lacking the SH3 domain. Heterozygous (Cas exon $2^{+/\Delta}$) mice, which were apparently normal, were intercrossed to produce homozygous (Cas exon $2^{\Delta/\Delta}$) mutants. Cas exon $2^{\Delta/\Delta}$ mice died *in utero* at 12.5–13.5 dpc and the detailed analysis of the embryonic lethality of the Cas exon $2^{\Delta/\Delta}$ mice is underway and will be published elsewhere. In this report, we established primary fibroblasts from Cas exon 2-deficient embryos and investigated the roles of Cas exon 2 in cellular functions.

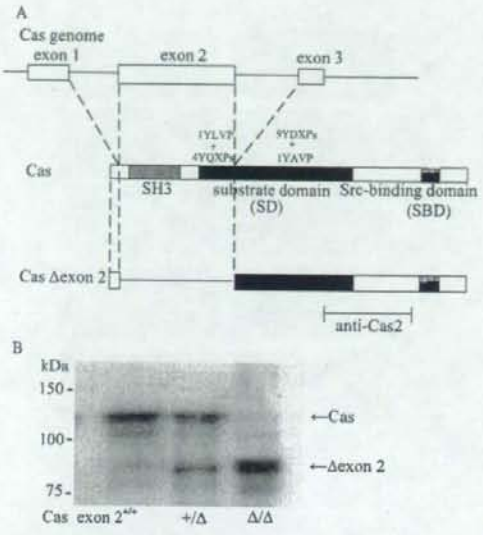

Figure 1 (A) Schematic illustration of Cas genome, Cas full-length product (Cas) and a truncated Cas protein lacking the exon 2-derived region (Cas Δexon 2). As compared to Cas, Cas Δexon 2 is deficient in the whole SH3 domain and one YLVP and four YQXP motifs. The position of the peptides for generating anti-Cas2 is also shown. (B) Western blot to detect Cas Δexon 2 protein. Thirty micrograms of cell lysates extracted from the wild-type (Cas exon $2^{+/+}$), heterozygous (Cas exon $2^{+/\Delta}$) and homozygous (Cas exon $2^{\Delta/\Delta}$) fibroblasts were separated by 7.5% SDS-PAGE, blotted to a nitrocellulose membrane and probed with 1:2000 diluted an anti-Cas antibody. Molecular weight markers are shown on the left.

Results

Cas exon $2^{\Delta/\Delta}$ cells are slower to initiate migration in the wound healing assay

To investigate functional defects caused by Cas exon 2-deficiency, we established primary fibroblasts from Cas exon 2-deficient (Cas exon $2^{\Delta/\Delta}$) embryos. Figure 1A shows the schematic diagram representing Cas Δexon 2. Cas exon 2 contains the entire SH3 domain and a part of the SD domain containing one YLVP and four YQXP motifs. It encodes 211 amino acids and the predicted molecular weight of Cas exon 2 is about 23 kDa. The expression of Cas Δexon 2 protein in Cas exon $2^{\Delta/\Delta}$ fibroblasts was detected almost as the expected size by Western blotting using an antibody against Cas, anti-Cas2 (Sakai *et al.* 1994) (Fig. 1B). Using the fibroblasts,

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