Chromatin Remodeler Sucrose Nonfermenting 2 Homolog (SNF2H) Is Recruited onto DNA Replication Origins through Interaction with Cdc10 Protein-dependent Transcript 1 (Cdt1) and Promotes Pre-replication Complex Formation

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THE CHROMATIN REMODELER SUCROSE NONFERMENTING 2 HOMOLOG (SNF2H) IS RECRUITED ONTO DNA REPLICATION ORIGINS THROUGH INTERACTION WITH CDC10-DEPENDENT TRANSCRIPT 1 (CDT1) AND PROMOTES THE PRE-REPLICATION COMPLEX FORMATION*

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Running title: SNF2H promotes pre-RC formation via Cdt1

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Background: Cdt1 is a DNA replication factor that loads MCM helicase onto chromatin. SNF2H is a chromatin remodeler.
Results: SNF2H is Cdt1-dependently recruited to replication origins and promotes MCM loading.
Conclusion: SNF2H may contribute to the maintenance of genome integrity through promotion of MCM loading.
Significance: Clarifying the roles of SNF2H in the process of MCM loading is crucial to understanding of DNA replication.

SUMMARY

From late mitosis to the G1 phase of the cell cycle, ORC, CDC6, and Cdt1 form the machinery necessary to load MCM2-7 complexes onto DNA. Here, we show that SNF2H, a member of the ATP-dependent chromatin remodeling complex, is recruited onto DNA replication origins in human cells in a Cdt1-dependent manner and positively regulates MCM loading. SNF2H was physically interacted with Cdt1. Chromatin immunoprecipitation assays indicated that SNF2H associates with replication origins specifically during the G1 phase. Binding of SNF2H at origins was decreased by Cdt1 silencing and, conversely, enhanced by Cdt1 overexpression. Furthermore, SNF2H silencing prevented MCM loading at origins and moderately inhibited S phase progression. Although neither SNF2H overexpression nor SNF2H silencing appeared to impact rereplication induced by Cdt1 overexpression, Cdt1-induced checkpoint activation was inhibited by SNF2H silencing. Collectively, these data suggest that SNF2H may promote MCM loading at DNA replication origins via interaction with Cdt1 in human cells. Because efficient loading of excess MCM complexes is thought to be required for cells to tolerate replication stress, Cdt1- and SNF2H-mediated promotion of MCM loading may be biologically relevant for the regulation of DNA replication.

In eukaryotes, DNA replication is strictly controlled so that the genome is replicated once per single cell cycle. From late mitosis to the G1 phases, the sequential assembly of multiple proteins, ORC1-6 (origin recognition complex 1-6), CDC6, Cdt1 (Cdc10-dependent transcript 1), and MCM2-7 (minichromosome maintenance 2-7),

results in the formation of a pre-replication complex (pre-RC) that is "licensed" for replication. In the latter period of the cell cycle, while MCM helicase is activated in a Cdk (cyclin-dependent kinase)-dependent manner, the activity of the MCM loaders is carefully regulated by multiple mechanisms so as to prohibit inappropriate reassembly of pre-RC and subsequent rereplication (1-3). Cdt1 strongly stimulates the licensing reaction in human cells (4, 5), and its activity is very tightly restricted by multiple mechanisms (3, 6-12). The overexpression of Cdt1, ORC1, or CDC6 alone induces no detectable re-replication. However, the overexpression of Cdt1+ORC1 or Cdt1+CDC6 yields a moderate level of rereplication, whereas joint overexpression of Cdt1+ORC1+CDC6 yields strong rereplication (5). In addition, the overexpression of Cdt1 induces ATM (ataxia telangiectasia mutated)/Chk2 checkpoint activation. This activation is apparently independent of rereplication induction, although its precise mechanism is unknown (13). Therefore, deregulation of Cdt1 is harmful to genome stability (13, 14). However, it is not fully understood how Cdt1 strongly stimulates the MCM loading reaction.

The initiation of DNA replication is strongly influenced by chromatin structure, as is expected for any molecular events involving DNA in eukaryotic cells. For example, histone acetylation is linked to the control of initiation of DNA replication. Early firing origins are typically localized in genomic regions that are transcribed and contain hyperacetylated chromatin, whereas late-firing origins lie in silenced heterochromatic domains (15-19). In addition, histone acetylation is involved in origin activation during early development in *Xenopus* (20) and at the chorion gene loci in *Drosophila* follicle cells (21, 22).

In pre-RC formation, the efficient loading of multiple MCM complexes is required for the toleration of replication stresses and activation of checkpoint pathways (23-25). In general, chromatin remodeling proteins, histone chaperones, and histone acetylation enzymes are thought to act synergistically to stimulate transcription on chromatin template (26). The situation may be the same for efficient MCM loading. In this regard, HBO1 (a MYST family histone acetyl transferase that binds to ORC), originally identified through its physical interactions with human ORC1 (27), was recently found to associate with replication origins through interaction with Cdt1 and to enhance licensing and DNA replication through its acetylation activity (28-30).

Chromatin remodeling complexes that utilize energy derived from ATP hydrolysis alter chromatin structure by disrupting and/or mobilizing nucleosomes. This large group of complexes can be subdivided into four subfamilies that include the SWI/SNF-type complex, the Imitation switch (ISWI)-type complex, INO80-type complex, and the CHD-type complex. Each complex contains a major catalytic component that possesses DNA-dependent ATPase activity, such as Brg1 (in the SWI/SNF-type complex) or SNF2H (in the ISWI-type complex) (31-35). The selection of catalytic ATPase subunits, combined with other complex components, defines the role of these complexes in various nuclear events including transcription, DNA replication and DNA repair.

The ISWI-type nucleosome remodeling factor SNF2H (sucrose nonfermenting 2 homolog) and WSTF (Williams syndrome transcription factor) were previously identified as novel human Cdt1-binding proteins (12). However, the biological significance of the interaction with Cdt1 remains unclear. SNF2H uses ATP hydrolysis to regulate chromatin structure and modulate nucleosome spacing (31-35). It is a constituent of several multiprotein remodeling complexes. These include WICH (WSTF/ISWI chromatin remodeling complex), ACF, CHRAC, RSF, and NoRC (31-35). The presence of these distinct complexes suggests that SNF2H performs multiple functions in chromatin regulation. Moreover, several previous reports implicate SNF2H as stimulating the initiation of DNA replication. For example, the CHRAC allows binding of T-antigen and efficient initiation in an *in vitro* replication system that employs SV40 DNA reconstituted into chromatin (36). Furthermore, SNF2H is apparently recruited to the Epstein-Barr virus (EBV) origin of plasmid replication (*OriP*) and that depletion of SNF2H with small interfering RNAs (siRNAs) reduces MCM3 loading at the *OriP* (37). However, it remains unclear whether this is also the case for

cellular replication origins and, if so, how SNF2H is recruited.

In this study, the hypothesis was explored that SNF2H proteins might play a role in the stimulation of MCM loading onto cellular replication origins and further that the Cdt1-SNF2H interaction is important in this context. Based on obtained data, we propose that SNF2H promotes MCM loading at cellular replication origins through interaction with Cdt1.

EXPERIMENTAL PROCEDURES

Cell culture and synchronization

HEK293T, T98G and HeLa cells were grown in Dulbecco's modified Eagle's medium with 8% fetal calf serum. For cell cycle synchronization, T98G cells were rendered quiescent by serum starvation for 48 hr and then released into the cell cycle by serum stimulation. Synchronization was verified by analysis of DNA contents with a flow cytometer.

Plasmids

Mammalian expression vectors, pCLMSCVhyg-T7-Cdt1, pcDNA3.1-3HA-Cdt1, and pcDNA3.1-zeo-Flag-ORC1 and bacterial expression vector pGEX-6P1-Cdt1 were described previously (5, 12). The pEGFP-C1 expression vector was purchased from Clontech (Mountain View, CA). pCMV-Flag-HBO1 was described previously (38). The pCMV6-XL4-SNF2H expression vector that expresses human SNF2H was purchased from OriGene Technologies (Rockville, MD). To construct the T7 promoter-driven SNF2H expression vector, pCMV-XL4-SNF2H was digested with Not I and the SNF2H fragment was subcloned into pCMV-XL5. The SNF2H protein was then synthesized by *in vitro* transcription-translation with rabbit reticulocyte lysate (T_NT T7 quick coupled transcription/translation system, Promega, Madison, WI) according to the manufacturer's instructions.

Immunoprecipitation

Nuclear extracts were prepared from HEK293T cells with modified CSK buffer containing 500mM NaCl, 0.1% Triton X-100, 0.1mM ATP, 1mM DTT, and multiple protease inhibitors as described previously (12, 39). Aliquots of the extracts were then immunoprecipitated using anti-Cdt1 antibody or anti-SNF2H antibody bound to protein G-Sepharose beads (Amersham Bioscience, Piscataway, NJ). The beads were washed four times with NET gel buffer (12) containing 200mM NaCl. The immunoprecipitates were eluted with 1 × SDS sample buffer (62.5mM Tris-HCl pH6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue) and subjected to immunoblotting.

GST-Cdt1 pull-down assay

For Fig. 1E, GST-Cdt1 (0.6μg) was mixed with the SNF2H produced by *in vitro* translation in 50μl of reaction mixture, diluted with binding buffer A (25mM Tris-HCl, pH7.4, 150mM NaCl, 0.01% Nonidet P-40 [NP-40], 1mM dithiothreitol [DTT], 0.5mM PMSF, 10% glycerol) containing 10mM glycerophosphate and 5mM NaF, and incubated at 4°C for 3hr. Then the GST-Cdt1 and associated SNF2H proteins were collected on glutathione beads and washed with binding buffer A. The bound proteins were eluted and analyzed by immunoblotting or Coomassie Brilliant Blue (CBB) staining.

For Fig. 1F, GST-Cdt1 was bound to glutathione beads, and the beads were mixed with diluted nuclear extracts prepared from HeLa cells. After washing three times with buffer A, the bound proteins were eluted and analyzed by immunoblotting.

Transfection

For Figs. 1B-1D, expression plasmids (total ~6μg) were transiently transfected into 3×10^6 HEK293T cells in 100-mm culture dishes using TransIT-293 reagents (Mirus, Madison, WI) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were harvested and subjected to immunoprecipitation analysis.

For Figs. 3D-3F, expression plasmids (total ~12μg) and 1.8μg of pMSCVpuro (Clontech) were transiently transfected into 6×10^6 HEK293T cells in 150-mm culture dishes using TransIT-293 reagents. Twenty-four hours after transfection, cells were selected with 2μg/ml puromycin for 2 days. Cells were then harvested and subjected to chromatin immunoprecipitation (ChIP) assay.

For Fig. 5, expression plasmids (total ~1.6μg) and siRNA (40pmol) were transiently transfected into 2×10^5 HEK293T cells in 12-well culture plates using Lipofectamine 2000 reagents (Invitrogen, Carlsbad, CA). Forty-eight hours after transfection, cells were lysed in 1 × SDS sample buffer containing multiple protease and phosphatase inhibitors and then subjected to immunoblotting.

Small interfering RNA (siRNA) experiments

HeLa and T98G cells were transfected with siRNA duplexes using the HiPerFect Transfection Reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. siRNA oligonucleotides were synthesized (IDT, Coralville, IA or Dharmacon, Lafayette, co) with the following sequences (sense strand): Cdt1 (5'-GCUGUUGUACUAUCAUGAGCCCUGGdAdC-3'); SNF2H (5'-GAGGAGGAUGAAGAGCUAUdTdT-3'); CDC6-1 (5'-AGACUAUAACUCUACAGAUUGUGdAdA-3'); CDC6-3 (5'-GGAGGACACUGGUUAAAGAAUUUdAdT-3'); ORC1 (5'-CUGCACUACCAAACCUAUAdTdT-3'); HBO1 (5'-GGGAUAAGCAGAUAGAAGAAAGGAT-3'); control DS scrambledNeg (5'-CUUCCUCUCUUUCUCUCCCUUGUdGdA-3'); and control GL2 (5'-CGUACGCGGAAUACUUCGAdTdT-3').

Fluorescence activated cell sorting (FACS)

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

Chromatin immunoprecipitation assay (ChIP)

ChIP assays were carried out essentially as described previously (40). Briefly, 6×10^6 cells were fixed with 1% formaldehyde and lysed in SDS lysis buffer (1% SDS, 10mM EDTA. 50mM Tris-HCl, pH 8.0). Chromatin was sonicated to yield an average fragment size of 0.5 kb. Each sample was incubated overnight at 4°C with 5 μg of the indicated antibodies or control IgGs and then collected using Dynabeads (Invitrogen). After formaldehyde reversal, phenol-chloroform extraction and ethanol precipitation, the DNA was dissolved in TE buffer.

Quantitative real-time PCR analysis

The SYBR Premix Ex Taq II (Takara, Kyoto, Japan) was used according to the manufacturer's instructions. All PCR reactions were carried out using an iCycler iQ Real-Time PCR Detection System (BIO-RAD, Berkeley, CA). The annealing temperature (*Ta*) was optimized for each primer (See Supplementary Table S1). For each reaction, the cycling parameters were set as follows: 1min at 95°C; 5 cycles of 95°C for 30 sec, *Ta* plus 4°C for 30 sec, and 72°C for 30 sec; 5 cycles of 95°C for 30 sec, *Ta* plus 2°C for 30 sec, and 72°C for 30 sec; and 50 cycles of 95°C for 30 sec, *Ta* for 30 sec, and 72°C for 30 sec.

Chromatin binding assay

The chromatin binding assay was performed as described previously (5, 39).

Immunoblotting and antibodies

Immunoblotting and quantification of the band signals were performed as described previously (12). Preparation of polyclonal rabbit antibodies against human Cdt1, ORC1, CDC6, MCM7, and MCM3 was described previously (10, 13, 41). Anti-human Brg1 antibody was obtained by immunizing rabbits with a peptide corresponding to the 50 amino acid C-terminal region of Brg1.

Other antibodies were purchased from various companies: green fluorescent protein (GFP) (46-0092, Invitrogen, Carlsbad, CA); Thr68-phosphorylated Chk2 (number 2661, Cell Signaling Technology, Danvers, MA); ATM (2C1, Gene Tex, San Antonio, TX); Ser1981-phosphorylated ATM (200-301-400, Rockland Biosciences, Gilbertsville, PA); SNF2H (1B9/D12, Upstate Biotechnology, Lake Placid, NY, or H-300, sc-13054, Santa Cruz Biotechnology, Santa Cruz, CA); WSTF (number 2152, Cell Signaling Technology); ACF1 (A301-319A, Bethyl Laboratories, Montgomery, TX); cyclin E (sc-247, Santa Cruz) Ser780-phosphorylated-Rb (555S, MBL); and HBO1 (sc-13284, Santa Cruz).

Statistical analysis

Statistical analysis was performed using the two-tailed Student's *t*-test to validate the data. *P*

values of <0.05 were considered statistically significant.

RESULTS

SNF2H interacts with Cdt1 in vivo and in vitro.

The chromatin modifying proteins, SNF2H and WSTF were previously identified as novel Cdt1 binding proteins by a combination of affinity chromatography and tandem mass spectrometry analysis (12). In addition, SNF2H and WSTF were shown to bind to GST-Cdt1 by pull-down assays with HeLa cell nuclear extracts, and ectopically expressed T7-Cdt1 was shown to co-immunoprecipitate endogenous SNF2H (12). Here, we designed to further confirm the association between endogenous SNF2H and Cdt1. As shown in Fig. 1A, SNF2H co-immunoprecipitated with Cdt1. Reciprocally, ectopically expressed T7-Cdt1 co-immunoprecipitated with SNF2H (Fig. 1B).

The WICH complex binds to PCNA through a PCNA-WSTF interaction (42). In addition, Cdt1 binds to PCNA (11). Therefore, the observed interaction between Cdt1 and SNF2H may be mediated by PCNA-WSTF binding. Indeed, WSTF was also detected in the T7-Cdt1 immunoprecipitates (Fig. 1C). However, WSTF was not enriched to the same extent as SNF2H (Figs. 1C & 1F). Moreover, the Cdt1 PIPm mutant, which is unable to interact with PCNA (11), also co-immunoprecipitated SNF2H (Fig. 1D). These results suggest that Cdt1 likely binds to SNF2H without mediation by other factors. To evaluate this hypothesis, purified GST-Cdt1 fusion proteins were incubated with SNF2H synthesized by *in vitro* transcription-translation with rabbit reticulocyte lysate. A direct interaction between the two proteins *in vitro* was demonstrated (Fig. 1E).

SNF2H is partnered with divergent proteins in various chromatin remodeling complexes, such as WSTF in the WICH complex (31-35). In addition to previous and current findings of WSTF co-purification with GST-Cdt1 (Ref. 12; Fig. 1F), this study showed that WSTF co-immunoprecipitated with T7-Cdt1 (Fig. 1C). The WSTF is also a subunit of the WINAC complex, a subclass of the Brg1-dependent chromatin remodeling complexes (43). Therefore the possibility that Cdt1 interacts with Brg1 was also investigated. As shown in Fig. 1F, Brg1 did not interact with GST-Cdt1. The ACF1 is another SNF2H partner, forming ACF or CHRAC complexes with SNF2H. However, ACF1 was not detected in either T7-Cdt1 immunoprecipitation or GST-Cdt1 pull-down assays (Figs. 1C & 1F). Finally, SNF2H and SNF2L are the two ISWI proteins found in human cells (44). However, considering that SNF2L protein is reportedly undetectable because of its low expression levels in HeLa cells (45), SNF2L binding to Cdt1 was not explored. Taken together, these data demonstrate that SNF2H specifically interacts with Cdt1 and that the WSTF-containing WICH complex may be the predominant SNF2H-related complex involved. However, the possibility cannot be ruled out that additional SNF2H-containing complexes may also interact with Cdt1.

SNF2H binds to replication origins in human cells

Cdt1 is a key component of the pre-RC that has been shown to be indispensable during the initiation steps of DNA replication. The interaction between Cdt1 and SNF2H observed in this work may thus indicate a functional link between chromatin remodeling and pre-RC formation. To directly assess this possibility, the presence of SNF2H at regions of known replication origins was first examined by the ChIP assay. Chromatin prepared from asynchronously growing cells was precipitated with the indicated antibodies, and the presence of the well-characterized *lamin B2* and *MCM4* origin sequences (46-50) was assessed by subsequent quantitative real-time PCR analysis using specific sets of primers (Fig. 2A). In two different human cell lines, HeLa and T98G, Cdt1-, MCM7-, or MCM3-specific antibodies efficiently immunoprecipitated DNA sequences that included origin regions of both loci (Figs. 2B & 2C). Although some enrichment for the regions distal to the origins was also observed, it was less efficient compared with the enrichment of the origin regions. In human cells, replication origins are not determined as strictly as replication origins in budding yeast (51). Thus, “origin regions” may in fact represent “preferential origins”. In other words, pre-RCs may be formed at the origin-distal regions with lower frequency than the origins. Overall, these data

are in good agreement with previous reports (49, 50, 52, 53).

On the other hand, the anti-SNF2H antibodies efficiently enriched both the origin and distal sequences of *lamin B2* and *MCM4* loci (Figs. 2B & 2C). The binding of SNF2H to the origin-distal regions may be due to its multifunctionality; that is, SNF2H binds to chromatin not only for the purpose of regulating replication but also to mediate transcription and other functions. Nevertheless, it seems clear that SNF2H does indeed bind to replication origins. Furthermore, SNF2H silencing with siRNAs decreased the origin enrichment in ChIP assay (See below, Fig. 4), confirming the validity of the assay. Therefore, the origin binding of SNF2H was the focus of further analysis.

SNF2H associates with replication origins specifically during the G1 phase

Chromatin association of licensing factors reaches its maximum in the G1 phase when DNA licensing is complete. Levels are reduced upon progression into the S phase or cell cycle exit. To monitor the cell cycle dependence of SNF2H localization to replication origins, ChIP assays were performed using T98G cells synchronized in G1/S phases and the data were compared with those from cells synchronized in the G0 or late S/G2/M phases (Fig. 2D). The specific enrichment of MCM7, MCM3, or Cdt1 at the *lamin B2* and *MCM4* origins was maximal in the G1/S phase (Fig. 2E), as reported previously (49, 50, 52, 53). Interestingly, SNF2H levels at replication origins were low in chromatin derived from cells synchronized in the G0 or late S/G2/M phase cells but were significantly elevated in chromatin derived from cells synchronized in the G1/S phase (Fig. 2E). The association of SNF2H at the origin-distal regions was not significantly altered during the cell cycle progression (Fig. 2E). The pattern of SNF2H binding was similar to that of licensing factors, a finding which is similar to previous results obtained with *OriP* of EBV (37). These results suggest that SNF2H is associated with cellular replication origins in the G1 phase in a cell cycle-dependent manner.

Origin binding of SNF2H is dependent on Cdt1

Given the potential role of a SNF2H-Cdt1 interaction in SNF2H origin binding, the dependence of SNF2H loading at the origins on Cdt1 was examined. Chromatin was prepared from HeLa cells treated with control siRNAs or siRNAs targeting Cdt1 and subjected to the ChIP assay. Down-regulation of Cdt1 with the siCdt1 was confirmed by immunoblotting (Fig. 3A). Cell cycle distribution pattern was not remarkably affected by Cdt1 silencing at this level (Fig. 3B). The specific enrichment of the origin sequences with the anti-Cdt1 antibody was also reduced by siRNA treatment (Fig. 3C and Supplementary Fig. S1), demonstrating the validity of the ChIP assay.

Cdt1 is essential for the loading of hexameric MCM 2-7 complexes onto chromatin (1-3). As expected, MCM7 binding at replication origins was decreased by Cdt1 silencing (Fig. 3C). Importantly, SNF2H binging at origins was also significantly decreased by Cdt1 silencing (Fig. 3C and Supplementary Fig. S1). The levels of Cdt1 and MCM7 at the origin-distal regions were also lowered by Cdt1 silencing (Fig. 3C). However, SNF2H binding at the origin-distal regions was not remarkably changed by Cdt1 silencing (Fig. 3C). Similar results were obtained with other siRNAs targeting Cdt1 (Data not shown).

The hypothesis that Cdt1 overexpression affects origin binding of SNF2H was also investigated. For this purpose, Cdt1 was overexpressed in HEK293T cells (Fig. 3D). Cell cycle distribution pattern was not remarkably affected by Cdt1 overexpression (Fig. 3E), as we reported previously (5). SNF2H binding at the MCM4 origin was enhanced upon Cdt1 overexpression (Fig. 3F). However, SNF2H binding at the distal region was not significantly affected (Fig. 3F).

Taken together, these results suggest that the origin binding of SNF2H is, at least partly, dependent on Cdt1. On the other hand, recruitment of SNF2H to the origin-distal regions may be mostly mediated by other factors.

Silencing of SNF2H suppresses MCM, but not Cdt1, loading at origins

The possibility that origin recruitment of SNF2H might affect MCM loading was next investigated. For this purpose, chromatin was prepared from HeLa cells treated with siRNA targeting SNF2H and then subjected to the ChIP assay with the indicated antibodies. The efficient depletion of

SNF2H was confirmed by both immunoblotting and ChIP assays (Figs. 4A & 4B). Remarkable change in cell cycle distribution pattern was not observed in SNF2H-silenced asynchronously growing HeLa cells (Fig. 4C). In line with the previous observation on the *OriP* of EBV (37), the binding of MCM7 and MCM3 binding to the cellular origins was suppressed by SNF2H silencing (Fig. 4B). On the other hand, Cdt1 binding at origins was not affected by SNF2H silencing. These results suggest that SNF2H association with cellular replication origins occurs after the loading of Cdt1 but prior to the loading of the MCM complex and that both Cdt1 and SNF2H are required for the efficient loading of MCM complexes.

The effect of SNF2H silencing on MCM loading was also investigated using a chromatin binding assay. Control and SNF2H-silenced T98G cells were synchronized in G0 by serum deprivation and then reentered into the cell cycle by serum stimulation. Cells were harvested sequentially at the indicated times and subjected to the chromatin loading assay to estimate the binding of pre-RC components to intact chromatin (Figs. 4D & 4E). In control siRNA-treated cells, Cdt1 as well as CDC6 were absent after serum starvation. Levels of both proteins increased approximately 10 hr after serum addition (Figs. 4D & 4E, whole cell extracts of siCont), coincident with the loading of MCM7 and MCM3 onto chromatin (Figs. 4D & 4E, chromatin/nuclear matrix-binding fraction). This result is in agreement with previous findings (12, 54).

Silencing of SNF2H modestly but significantly reduced the recruitment of MCM7 and MCM3 onto chromatin, whereas CDC6 and Cdt1 loading were not affected (Figs. 4D & 4E, chromatin/nuclear matrix-binding fraction of siSNF2H). Although SNF2H is also involved in transcription regulation (31-35), its silencing did not affect the expression levels of CDC6, Cdt1, MCM7, MCM3, or cyclin E proteins under these experimental conditions (Figs. 4D & 4E, whole cell extracts). In addition, Rb phosphorylation at Ser780 by cyclin D-Cdk4/Cdk6 was also investigated. Silencing of SNF2H had no notable effect on the kinetics of the Rb phosphorylation (Fig. 4D). These data give further support for the role of SNF2H in MCM loading.

In SNF2H-silenced cells, a slight delay of the S phase progression was observed (Fig. 4F), which could result from the partial reduction of MCM loading by SNF2H silencing. However, it is reported that cells with depleted MCM replicate at normal rates (25). SNF2H is also reportedly recruited onto replication foci via a physical interaction between WSTF and PCNA, where it stimulates replication fork progression (42, 55). In the absence of further experimental evidence, it is difficult to distinguish between these two possibilities.

SNF2H is involved in the activation of the ATM-Chk2 checkpoint pathway induced by Cdt1 overexpression.

Given that SNF2H promotes MCM loading via the interaction with Cdt1, manipulations in its level of expression could in turn influence Cdt1 overexpression-induced rereplication. As previously reported (5), Cdt1 overexpression alone did not induce detectable rereplication in HEK293T cells. However, simultaneous deregulation of Cdt1 plus ORC1 synergistically induced rereplication (Ref. 5; Supplementary Fig. S2). Under the same experimental conditions, co-overexpression of Cdt1 and SNF2H did not induce detectable rereplication (Supplementary Fig. S2). Although earlier work demonstrated that the co-overexpression of Cdt1 and HBO1 induced overt rereplication (29), no significant rereplication was observed with Cdt1 and HBO1 overexpression in this study (Supplementary Fig. S2).

Whether the silencing of SNF2H or HBO1 diminishes rereplication induced by the overexpression of the degradation-resistant Cdt1 mutant Cy+D1m (5, 12) was next examined. Cdt1 Cy+D1m-induced rereplication was not affected by the silencing of SNF2H or HBO1 (Supplementary Figs. S3A & S3B), but it was significantly reduced by ORC1 or CDC6 silencing (Supplementary Fig. S3C). These data suggest that SNF2H (and probably HBO1) may not be necessary for the minimal licensing reaction required for DNA replication. However, SNF2H may be essential for the maximal licensing reaction required for genome stability (discussed below).

We previously reported that the overexpression of Cdt1 induces ATM/Chk2

checkpoint activation (13), although the mechanism remains unclear. It has been proposed that ATM is activated not only by DNA strand breaks but also by inappropriate changes in chromatin structure (56). Therefore, when Cdt1 is overexpressed, the protein may recruit excess SNF2H. Excess SNF2H might in turn cause chromatin hyper-remodeling and subsequent ATM activation. To test this possibility, whether the overexpression of SNF2H activates ATM/Chk2 checkpoint was first examined. However, no significant checkpoint activation was induced by SNF2H overexpression (Supplementary Fig. S4). Also, co-expression of Cdt1 plus SNF2H did not enhance the Cdt1-induced checkpoint activation (Supplementary Fig. S4). This could be because endogenous SNF2H protein levels are high enough to support Cdt1. Therefore, whether the SNF2H silencing inhibits Cdt1-induced checkpoint activation was next investigated. HEK293T cells were transfected with T7-Cdt1 Cy+D1m expression vector along with control or SNF2H siRNAs, and cells were harvested and analyzed by immunoblotting (Fig. 5). When T7-Cdt1 Cy+D1m was overexpressed, ATM and Chk2 were phosphorylated. SNF2H silencing partially but significantly reduced Cdt1-induced ATM/Chk2 activation (Fig. 5). This result indicates the involvement of SNF2H in Cdt1 overexpression-induced ATM activation and an additional functional link between Cdt1 and SNF2H.

DISCUSSION

The licensing reaction for DNA replication occurs on chromatin. As such, diverse chromatin-modifying molecules may associate with pre-RC components to facilitate the reaction. Here, we demonstrate that Cdt1 physically interacts with the chromatin remodeling protein SNF2H (Fig. 1). SNF2H is recruited onto cellular replication origins in a cell cycle-dependent manner, with peak in the G1 phase (Fig. 2). The recruitment of SNF2H is impaired by Cdt1 silencing and stimulated by Cdt1 overexpression (Fig. 3). Furthermore, SNF2H is required for efficient MCM loading (Fig. 4). Thus, it is conceivable that SNF2H is recruited to DNA replication origins through interaction with Cdt1 and promotes MCM loading by regulating nucleosomal structure. This finding is consistent with previous reports that SNF2H is recruited onto *OriP* of EBV in late G1 and promotes MCM loading at *OriP* (37). In this case, however, the mechanism by which SNF2H is recruited onto *OriP* remains to be clarified.

On the other hand, SNF2H depletion by the siRNA had only a marginal effect on S phase progression in synchronized T98G cells, and neither its depletion nor its co-expression affected Cdt1 overexpression-induced rereplication. Thus, SNF2H may function to promote excess MCM loading via an interaction with Cdt1, but SNF2H may not be essential for the minimal loading of MCM helicase required for DNA replication. In accordance with this finding, SNF2H-WSTF complexes appear not to be required for DNA replication in *Xenopus* egg extracts (57). However, several previous studies demonstrate that excess MCM loading is crucial for maintaining the genome integrity. For example, although cells with depleted MCM replicate at normal rates, they are hypersensitive to replicative stress and defective in Rad17-dependent ATR-mediated checkpoint activation (23-25). Moreover, a mutation of MCM4 termed Chaos3 (chromosome aberrations occurring spontaneously 3) is a viable allele and causes adenocarcinoma (58-60). Similarly, although mice with reduced expression of MCM2 develop normally, their life span is greatly reduced because of lymphomas (61). In other studies, a reduction in MCM levels caused DNA damage with activation of ATR and ATM (62). These findings suggest that excess MCM loading is critical for the toleration of replication stress and activation of the checkpoint. Therefore, the SNF2H-mediated promotion of MCM loading reported herein may be biologically relevant for the regulation of DNA replication. On the other hand, SNF2H is a multifunctional protein involved in transcription (31-35), replication (36, 37, 42, 43, 55), and repair/recombination (63, 64). Therefore, the production of separation of function mutants of Cdt1 and/or SNF2H that specifically lose their interaction would be required for further analysis of the SNF2H-mediated promotion of MCM loading.

Several works indicate that HBO1, a histone H4-acetylase, is recruited on replication origins via interaction with Cdt1 and stimulates MCM loading by inducing H4 acetylation during

the G1 phase (28-30). In addition, the co-expression of HBO1 enhances Cdt1-induced rereplication (29). However, in our experimental system, co-expression of SNF2H or HBO1 did not enhance Cdt1-induced rereplication (Supplementary Fig. S1). The apparent difference in the impact of HBO1 overexpression on Cdt1-induced rereplication might be due to the difference in its expression level. In addition, silencing of SNF2H or HBO1 did not impact Cdt1-induced rereplication (Supplementary Fig. S2). Therefore, HBO1, like SNF2H, may be dispensable as far as the minimal MCM loading required for DNA replication is concerned but essential for excess MCM loading. Indeed, both DNA replication and cell cycle progression proceed normally in HBO1 knockout mice (65), and proliferation occurs in flies with reduced levels of HBO1 (66). Nevertheless, these results do not rule out the involvement of HBO1 in the promotion of MCM loading required for tolerance to replication stress.

It has been reported that SNF2H-WSTF complexes preferentially associate with acetylated histones in chromatin (67). Taking all of the data into consideration, one possible model is that 1) HBO1 is recruited onto replication origins through interaction with Cdt1 and acetylates histone H4, and then 2) SNF2H is recruited to the origin sites through interaction with both Cdt1 and acetylated H4, where it increases the accessibility or fluidity of chromatin. Finally, 3) MCM is efficiently recruited during the G1 phase. Further experimental analysis will be required to confirm this model.

Histone chaperones, in addition to histone acetylation and ATP-dependent chromatin remodeling, are thought to contribute to transcriptional control. In this regard, ubiquitylation of the histone chaperone FACT by Rtt101, the cullin subunit of E3 ubiquitin ligase, promotes MCM loading onto origins in *Saccharomyces cerevisiae* (68). On the other hand, FACT is reported to facilitate DNA replication elongation through interaction with MCM complexes in human cells (69). Furthermore, histone methylation is also involved in chromatin transactions. The histone methyltransferase PR-Set7/Set8 is the sole enzyme to catalyze monomethylation of histone H4 on lysine 20 (H4K20me1). Recently PR-Set7 was shown to be regulated by PCNA- and $CRL4^{Cdt2}$-dependent ubiquitylation and destruction during the S phase. This regulation of PR-Set7 was suggested to contribute to the disappearance of H4K20me1 at origins and the inhibition of licensing (70-74). However, it remains unclear how PR-Set7 is recruited to the replication origins and regulates the licensing reaction.

Although remodeling of chromatin structure is essential for several critical nuclear functions, aberrant activity in chromatin remodeling emerges as a contributor to checkpoint activation. Thus, as previously proposed, ATM is activated not only by DNA strand breaks, but also by inappropriate changes in the chromatin structure (56). In this study, Cdt1-induced ATM/Chk2 checkpoint activation (13) was shown to depend on SNF2H. Therefore, excess Cdt1 may recruit excess SNF2H and, cause alterations in chromatin architecture, thereby activating the ATM. This possibility is open to further investigation.

REFERENCES

1. Bell, S. P., and Dutta, A. (2002) *Annu. Rev. Biochem.* **71**, 333-374
2. Diffley, J. F. (2004) *Curr. Biol.* **14**, R778-86
3. Fujita, M. (2006) *Cell. Div.* **1**, 22
4. Vaziri, C., Saxena, S., Jeon, Y., Lee, C., Murata, K., Machida, Y., Wagle, N., Hwang, D. S., and Dutta, A. (2003) *Mol. Cell* **11**, 997-1008
5. Sugimoto, N., Yoshida, K., Tatsumi, Y., Yugawa, T., Narisawa-Saito, M., Waga, S., Kiyono, T., and Fujita, M. (2009) *J. Cell. Sci.* **122**, 1184-1191
6. McGarry, T. J., and Kirschner, M. W. (1998) *Cell* **93**, 1043-1053
7. Wohlschlegel, J. A., Dwyer, B. T., Dhar, S. K., Cvetic, C., Walter, J. C., and Dutta, A. (2000) *Science* **290**, 2309-2312
8. Tada, S., Li, A., Maiorano, D., Mechali, M., and Blow, J. J. (2001) *Nat. Cell Biol.* **3**, 107-113

9. Lee, C., Hong, B., Choi, J. M., Kim, Y., Watanabe, S., Ishimi, Y., Enomoto, T., Tada, S., Kim, Y., and Cho, Y. (2004) *Nature* **430**, 913-917
10. Sugimoto, N., Tatsumi, Y., Tsurumi, T., Matsukage, A., Kiyono, T., Nishitani, H., and Fujita, M. (2004) *J. Biol. Chem.* **279**, 19691-19697
11. Nishitani, H., Sugimoto, N., Roukos, V. *et al.* (2006) *EMBO J.* **25**, 1126-1136
12. Sugimoto, N., Kitabayashi, I., Osano, S., Tatsumi, Y., Yugawa, T., Narisawa-Saito, M., Matsukage, A., Kiyono, T., and Fujita, M. (2008) *Mol. Biol. Cell* **19**, 1007-1021
13. Tatsumi, Y., Sugimoto, N., Yugawa, T., Narisawa-Saito, M., Kiyono, T., and Fujita, M. (2006) *J. Cell. Sci.* **119**, 3128-3140
14. Arentson, E., Faloon, P., Seo, J., Moon, E., Studts, J. M., Fremont, D. H., and Choi, K. (2002) *Oncogene* **21**, 1150-1158
15. Kemp, M. G., Ghosh, M., Liu, G., and Leffak, M. (2005) *Nucleic Acids Res.* **33**, 325-336
16. Zhou, J., Chau, C., Deng, Z., Stedman, W., and Lieberman, P. M. (2005) *Cell. Cycle* **4**, 889-892
17. Karnani, N., Taylor, C., Malhotra, A., and Dutta, A. (2007) *Genome Res.* **17**, 865-876
18. Lucas, I., Palakodeti, A., Jiang, Y., Young, D. J., Jiang, N., Fernald, A. A., and Le Beau, M. M. (2007) *EMBO Rep.* **8**, 770-777
19. Goren, A., Tabib, A., Hecht, M., and Cedar, H. (2008) *Genes Dev.* **22**, 1319-1324
20. Danis, E., Brodolin, K., Menut, S., Maiorano, D., Girard-Reydet, C., and Mechali, M. (2004) *Nat. Cell Biol.* **6**, 721-730
21. Aggarwal, B. D., and Calvi, B. R. (2004) *Nature* **430**, 372-376
22. Hartl, T., Boswell, C., Orr-Weaver, T. L., and Bosco, G. (2007) *Chromosoma* **116**, 197-214
23. Tsao, C. C., Geisen, C., and Abraham, R. T. (2004) *EMBO J.* **23**, 4660-4669
24. Woodward, A. M., Gohler, T., Luciani, M. G., Oehlmann, M., Ge, X., Gartner, A., Jackson, D. A., and Blow, J. J. (2006) *J. Cell Biol.* **173**, 673-683
25. Ge, X. Q., Jackson, D. A., and Blow, J. J. (2007) *Genes Dev.* **21**, 3331-3341
26. Aalfs, J. D., and Kingston, R. E. (2000) *Trends Biochem. Sci.* **25**, 548-555
27. Iizuka, M., and Stillman, B. (1999) *J. Biol. Chem.* **274**, 23027-23034
28. Iizuka, M., Matsui, T., Takisawa, H., and Smith, M. M. (2006) *Mol. Cell. Biol.* **26**, 1098-1108
29. Miotto, B., and Struhl, K. (2008) *Genes Dev.* **22**, 2633-2638
30. Miotto, B., and Struhl, K. (2010) *Mol. Cell* **37**, 57-66
31. Varga-Weisz, P. (2001) *Oncogene* **20**, 3076-3085
32. Tsukiyama, T. (2002) *Nat. Rev. Mol. Cell Biol.* **3**, 422-429
33. Corona, D. F., and Tamkun, J. W. (2004) *Biochim. Biophys. Acta* **1677**, 113-119
34. Dirscherl, S. S., and Krebs, J. E. (2004) *Biochem. Cell Biol.* **82**, 482-489
35. Eberharter, A., and Becker, P. B. (2004) *J. Cell. Sci.* **117**, 3707-3711
36. Alexiadis, V., Varga-Weisz, P. D., Bonte, E., Becker, P. B., and Gruss, C. (1998) *EMBO J.* **17**, 3428-3438
37. Zhou, J., Chau, C. M., Deng, Z., Shiekhattar, R., Spindler, M. P., Schepers, A., and Lieberman, P. M. (2005) *EMBO J.* **24**, 1406-1417
38. Iizuka, M., Sarmento, O. F., Sekiya, T., Scrable, H., Allis, C. D., and Smith, M. M. (2008) *Mol. Cell. Biol.* **28**, 140-153
39. Fujita, M., Kiyono, T., Hayashi, Y., and Ishibashi, M. (1997) *J. Biol. Chem.* **272**, 10928-10935
40. Yugawa, T., Handa, K., Narisawa-Saito, M., Ohno, S., Fujita, M., and Kiyono, T. (2007) *Mol. Cell. Biol.* **27**, 3732-3742
41. Fujita, M., Yamada, C., Tsurumi, T., Hanaoka, F., Matsuzawa, K., and Inagaki, M. (1998) *J. Biol. Chem.* **273**, 17095-17101
42. Poot, R. A., Bozhenok, L., van den Berg, D. L., Steffensen, S., Ferreira, F., Grimaldi, M., Gilbert, N., Ferreira, J., and Varga-Weisz, P. D. (2004) *Nat. Cell Biol.* **6**, 1236-1244
43. Kitagawa, H., Fujiki, R., Yoshimura, K. *et al.* (2003) *Cell* **113**, 905-917
44. Lazzaro, M. A., and Picketts, D. J. (2001) *J. Neurochem.* **77**, 1145-1156
45. Bozhenok, L., Wade, P. A., and Varga-Weisz, P. (2002) *EMBO J.* **21**, 2231-2241

46. Giacca, M., Zentilin, L., Norio, P., Diviacco, S., Dimitrova, D., Contreas, G., Biamonti, G., Perini, G., Weighardt, F., and Riva, S. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 7119-7123
47. Abdurashidova, G., Deganuto, M., Klima, R., Riva, S., Biamonti, G., Giacca, M., and Falaschi, A. (2000) *Science* **287**, 2023-2026
48. Ladenburger, E. M., Keller, C., and Knippers, R. (2002) *Mol. Cell. Biol.* **22**, 1036-1048
49. Schaarschmidt, D., Ladenburger, E. M., Keller, C., and Knippers, R. (2002) *Nucleic Acids Res.* **30**, 4176-4185
50. Gerhardt, J., Jafar, S., Spindler, M. P., Ott, E., and Schepers, A. (2006) *Mol. Cell. Biol.* **26**, 7731-7746
51. Masai, H., Matsumoto, S., You, Z., Yoshizawa-Sugata, N., and Oda, M. (2010) *Annu. Rev. Biochem*. **79**, 89-130
52. Ghosh, M., Kemp, M., Liu, G., Ritzi, M., Schepers, A., and Leffak, M. (2006) *Mol. Cell. Biol.* **26**, 5270-5283
53. Rampakakis, E., and Zannis-Hadjopoulos, M. (2009) *Nucleic Acids Res.* **37**, 5714-5724
54. Mailand, N., and Diffley, J. F. (2005) *Cell* **122**, 915-926
55. Collins, N., Poot, R. A., Kukimoto, I., Garcia-Jimenez, C., Dellaire, G., and Varga-Weisz, P. D. (2002) *Nat. Genet.* **32**, 627-632
56. Bakkenist, C. J., and Kastan, M. B. (2003) *Nature* **421**, 499-506
57. MacCallum, D. E., Losada, A., Kobayashi, R., and Hirano, T. (2002) *Mol. Biol. Cell* **13**, 25-39
58. Shima, N., Alcaraz, A., Liachko, I., Buske, T. R., Andrews, C. A., Munroe, R. J., Hartford, S. A., Tye, B. K., and Schimenti, J. C. (2007) *Nat. Genet.* **39**, 93-98
59. Shima, N., Buske, T. R., and Schimenti, J. C. (2007) *Cell. Cycle* **6**, 1135-1140
60. Chuang, C. H., Wallace, M. D., Abratte, C., Southard, T., and Schimenti, J. C. (2010) *PLoS Genet.* **6**, e1001110
61. Pruitt, S. C., Bailey, K. J., and Freeland, A. (2007) *Stem Cells* **25**, 3121-3132
62. Orr, S. J., Gaymes, T., Ladon, D., Chronis, C., Czepulkowski, B., Wang, R., Mufti, G. J., Marcotte, E. M., and Thomas, N. S. (2010) *Oncogene* **29**, 3803-3814
63. Lan, L., Ui, A., Nakajima, S., Hatakeyama, K., Hoshi, M., Watanabe, R., Janicki, S. M., Ogiwara, H., Kohno, T., Kanno, S., and Yasui, A. (2010) *Mol. Cell* **40**, 976-987
64. Nakamura, K., Kato, A., Koboyashi, J., Yanagihara, H., Sakamoto, S., Oliveira, D. V.N.P., Shimada, M., Tauchi, H., Suzuki, H., Tashiro, S., Zou, L., and Komatsu, K. (2011) *Mol. Cell* **41**, 515-528
65. Kueh, A. J., Dixon, M. P., Voss, A. K., and Thomas, T. (2011) *Mol. Cell. Biol.* **31**, 845-860
66. Grienenberger, A., Miotto, B., Sagnier, T., Cavalli, G., Schramke, V., Geli, V., Mariol, M. C., Berenger, H., Graba, Y., and Pradel, J. (2002) *Curr. Biol.* **12**, 762-766
67. Hakimi, M. A., Bochar, D. A., Schmiesing, J. A., Dong, Y., Barak, O. G., Speicher, D. W., Yokomori, K., and Shiekhattar, R. (2002) *Nature* **418**, 994-998
68. Han, J., Li, Q., McCullough, L., Kettelkamp, C., Formosa, T., and Zhang, Z. (2010) *Genes Dev.* **24**, 1485-1490
69. Tan, B. C., Chien, C. T., Hirose, S., and Lee, S. C. (2006) *EMBO J.* **25**, 3975-3985
70. Abbas, T., Shibata, E., Park, J., Jha, S., Karnani, N., and Dutta, A. (2010) *Mol. Cell* **40**, 9-21
71. Centore, R. C., Havens, C. G., Manning, A. L., Li, J. M., Flynn, R. L., Tse, A., Jin, J., Dyson, N. J., Walter, J. C., and Zou, L. (2010) *Mol. Cell* **40**, 22-33
72. Lee, J., and Zhou, P. (2010) *Mol. Cell* **40**, 345-346
73. Oda, H., Hubner, M. R., Beck, D. B., Vermeulen, M., Hurwitz, J., Spector, D. L., and Reinberg, D. (2010) *Mol. Cell* **40**, 364-376
74. Tardat, M., Brustel, J., Kirsh, O., Lefevbre, C., Callanan, M., Sardet, C., and Julien, E. (2010) *Nat. Cell Biol.* **12**, 1086-1093

FOOTNOTES

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The abbreviations used are: Cdt1, Cdc10-dependent transcript 1; SNF2H, sucrose nonfermenting 2 homolog; pre-RC, pre-replication complex; ORC, origin recognition complex; Cdk, cyclin-dependent kinase; GST, glutathione S-transferase; DTT, dithiothreitol; MCM, minichromosome maintenance; WSTF, Williams syndrome transcription factor; CBB, Coomassie Brilliant Blue; ChIP, chromatin immunoprecipitation.

FIGURE LEGENDS

Figure 1. Cdt1 interacts with SNF2H. *A*. Nuclear extracts were prepared from HEK293T cells and immunoprecipitated with anti-Cdt1 antibody or control rabbit IgG. Immunoprecipitates were subjected to immunoblotting with the indicated antibodies. Three percent of the input was also loaded. *B*. HEK293T cells were transfected with T7-Cdt1 and SNF2H expression vectors and nuclear extracts were prepared. After immunoprecipitation with anti-SNF2H antibody, the precipitates were blotted with the indicated antibodies. One percent of the input for SNF2H or 0.3 percent of the input for Cdt1 was loaded. *C*. HEK293T cells were transfected with T7-Cdt1 and SNF2H expression vectors, and nuclear extracts were prepared. After immunoprecipitation with anti-Cdt1 antibody, the precipitates were immunoblotted with the indicated antibodies. One percent of the input was loaded. *D*. HEK293T 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 immunoblotted with the indicated antibodies. Three percent of the input was loaded. *E*. GST-Cdt1 or GST was incubated with SNF2H protein synthesized by *in vitro* translation-transcription with rabbit reticulocyte lysate. The GST-Cdt1 and associated SNF2H protein were then collected on glutathione beads and subjected to either immunoblotting with anti-SNF2H antibody (upper panel) or CBB staining (lower panel). Twenty percent of the input sample was analyzed. *F*. GST-Cdt1 or GST was incubated with HeLa nuclear extracts, and bound proteins were analyzed by immunoblotting with the indicated antibodies. Fifteen percent of the input was loaded.

Figure 2. SNF2H interacts with cellular replication origins during the G1 phase. The ChIP assay was performed as described in Experimental Procedures. Quantitative real-time PCR was performed with DNA templates extracted from chromatin precipitated with control IgGs or antibodies against SNF2H, MCM7, MCM3, or Cdt1. *A*. Schematic diagram of the human *MCM4* and *lamin B2* origin loci. Positions of primer pairs to detect the origins and distal sequences are shown above (black or gray box, respectively). Coordinates flanking the origin are given in bp. *B*. Results with asynchronously growing HeLa cells are shown as the percent of input DNA. The means and standard deviations are shown (n=8). *C*. Results with asynchronously growing T98G cells. The means and standard deviations are shown (n=6). **P*<0.05. *D & E*. T98G cells synchronized at G0 (serum starvation for 48 hr), G1/S (10 hr after serum addition), or late S/G2/M phases (24 hr in the presence of 50ng/ml nocodazole after serum addition) were examined by FACS analysis (D) or ChIP assay (E). For ChIP data, the means and standard deviations are shown (n=3).

Figure 3. SNF2H binding to origins is inhibited by Cdt1 silencing and enhanced by Cdt1 overexpression. *A-C*. HeLa cells transfected with control (DS scrambldNeg) or Cdt1 siRNAs for 48 hr were subjected to immunoblotting (A), FACS analysis (B), or ChIP assay (C). *D-F*. HEK293T cells transfected with the indicated expression vectors were analyzed by immunoblotting (D), FACS (E), or ChIP assay (F). CBB staining served as the loading control. For ChIP data, the means and standard deviations are shown (n=3). **P*<0.05.

Figure 4. Silencing of SNF2H suppresses MCM, but not Cdt1, loading at origins. *A & B*. HeLa cells transfected with control (GL2) or SNF2H siRNAs for 48 hr were subjected to immunoblotting (A),

ChIP assay (B), or FACS analysis (C). CBB staining served as the loading control. For ChIP data, the means and standard deviations are shown (n=3). *P<0.05. *D-F*. T98G cells were transfected with the siRNAs for 24hr, rendered quiescent by serum starvation for 48hr, and then stimulated to reenter the cell cycle by serum addition. Cells were harvested at the indicated times and subjected to the chromatin binding assay (D & E) or FACS analysis (F). *D*. Whole cell extracts and chromatin/matrix-binding fractions were immunoblotted with the indicated antibodies. *E*. The signal intensities of the pre-RC proteins were quantified, normalized to the signals of CBB staining, and shown with the maximum values set at 100 percent. The means and standard deviations are shown (n=2).

Figure 5. Cdt1 overexpression-induced ATM activation is decreased by SNF2H silencing. HEK293T cells were transfected with the T7-Cdt1 Cy+D1m expression vector and SNF2H siRNAs using Lipofectamine2000, along with a plasmid expressing GFP to allow monitoring of the transfection efficiency. GL2 siRNAs were used as the control. Whole cell lysates were prepared 48 hr after transfection and immunoblotted with the indicated antibodies. The signal intensity of each band was quantified, with the intensity of the band corresponding to cells transfected with T7-Cdt1 and treated with control siRNAs set at 100 percent. The means and standard deviations are shown (n=3).

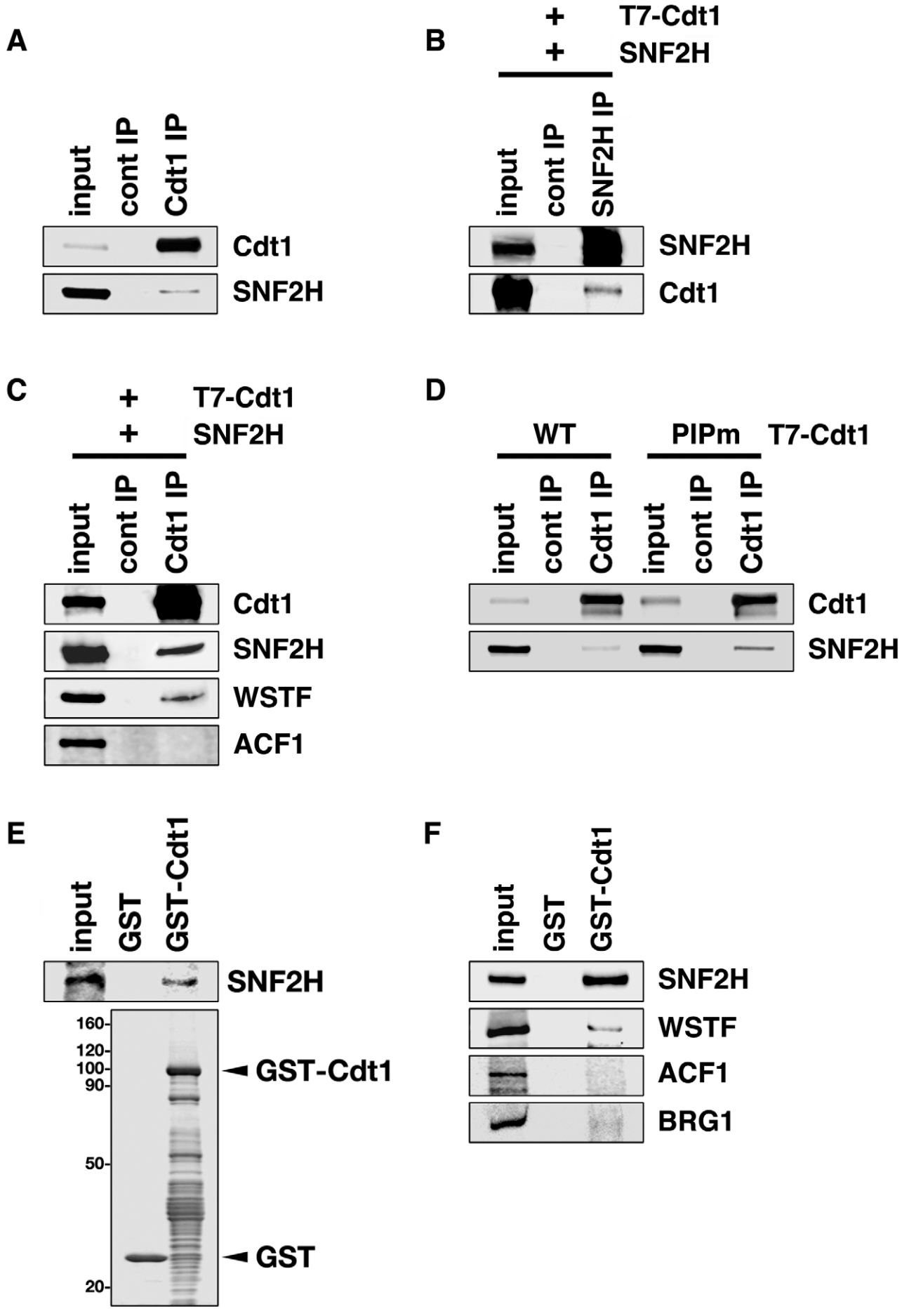


Figure 1. Sugimoto et al.

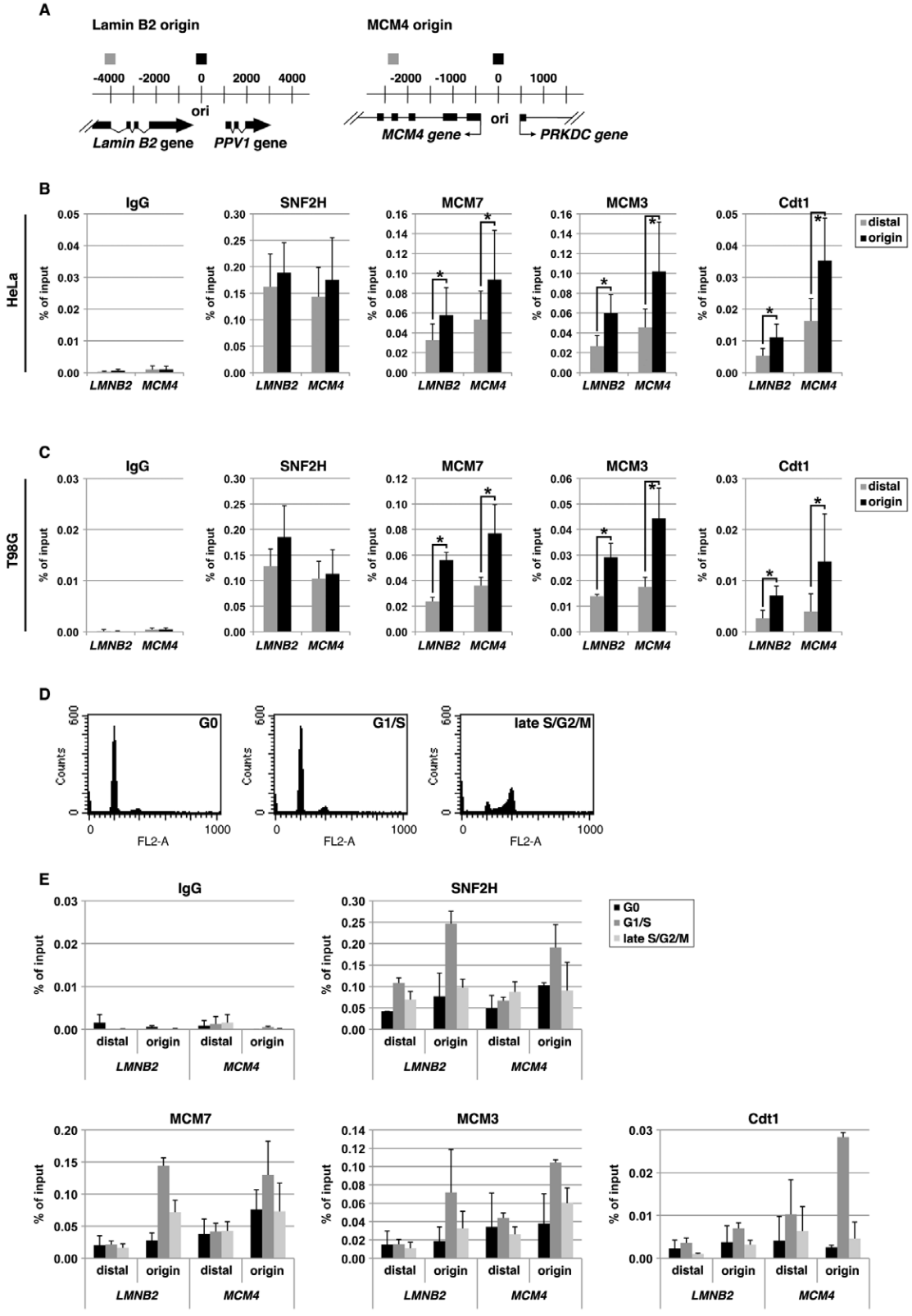
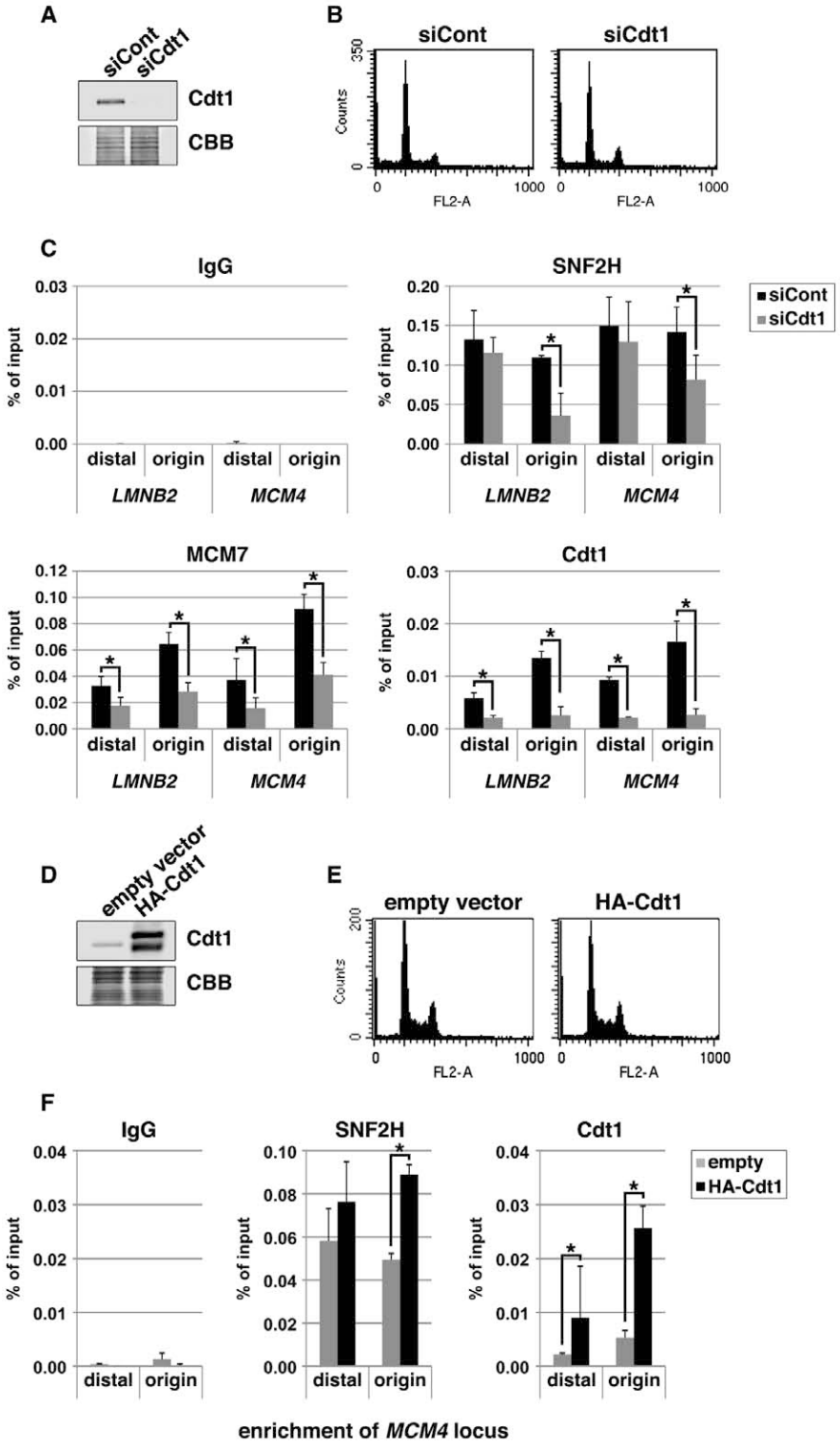


Figure 2. Sugimoto et al.



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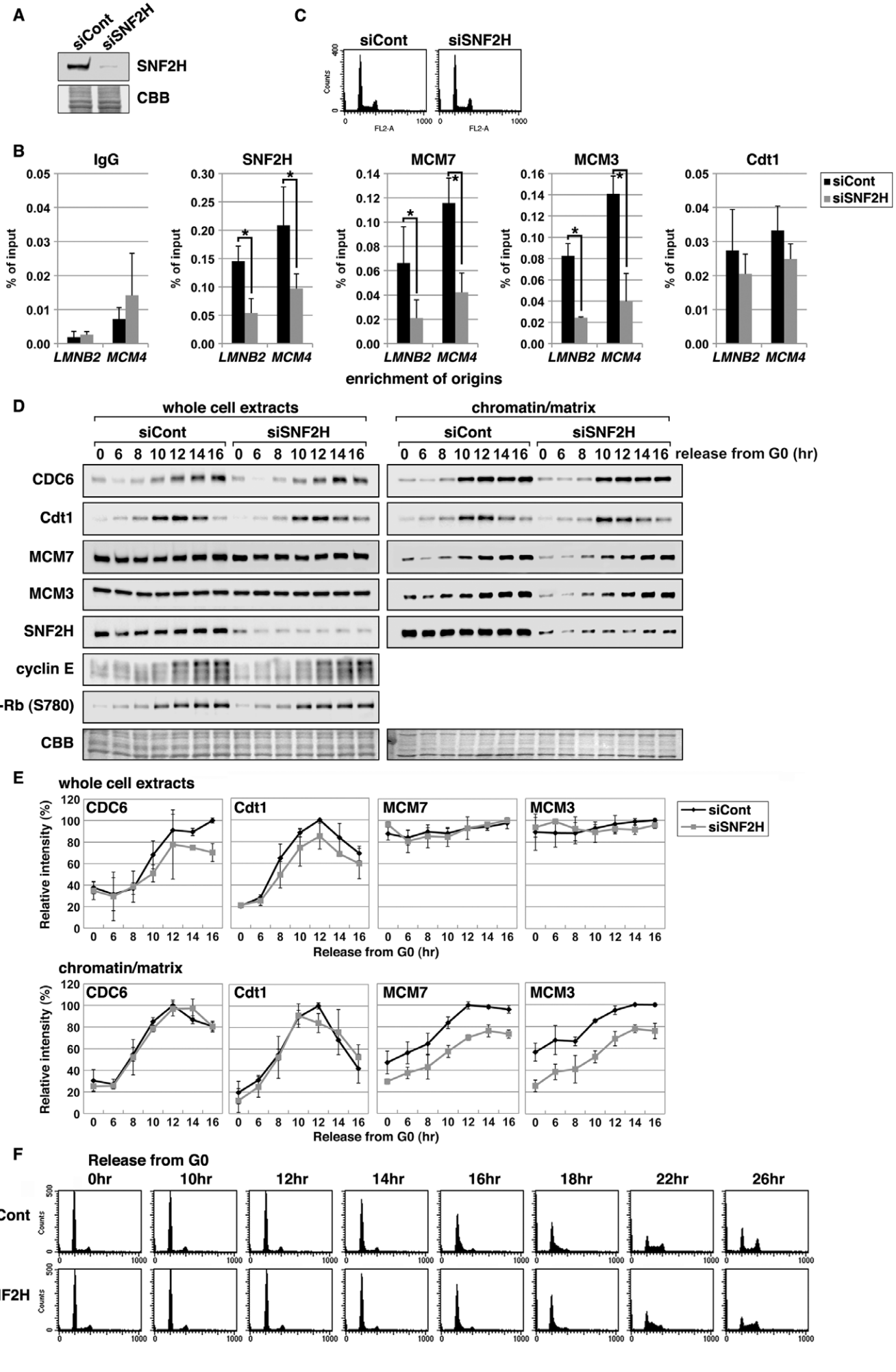

Figure 4. Sugimoto et al.

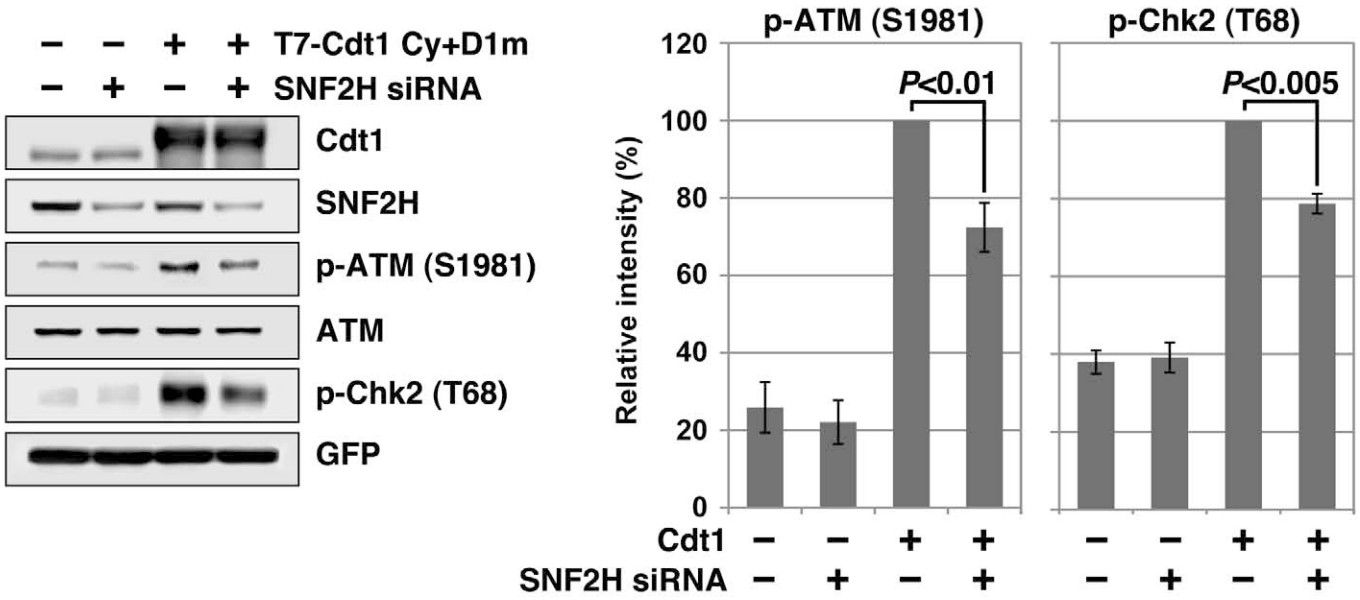

Figure 5. Sugimoto et al.