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Suppression of Cancer Cell Growth by Promoting Cyclin D1 Degradation

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Summary

The cyclin D1 proto-oncoprotein is a crucial regulator in cell cycle progression and aberrant overexpression of cyclin D1 is linked to tumorigenesis of many different cancer types. By screening ubiquitinated cyclin D1 as a substrate with a deubiquitinase library, we have identified USP2 as a specific deubiquitinase for cyclin D1. USP2 directly interacts with cyclin D1 and promotes its stabilization by antagonizing ubiquitin-dependent degradation. Conversely, USP2 knockdown destabilizes cyclin D1 and induces growth arrest in the human cancer lines where cell growth is dependent on cyclin D1 expression. Notably, cyclin D1 is not universally required for cell cycle progression. Inactivation of USP2 has either very mild effects on cell growth in normal human fibroblasts or no effect in the cancer cells that do not express cyclin D1. These findings suggest that targeting USP2 is an effective approach to induce growth suppression in the cancer cells addicted to cyclin D1 expression.

Keywords

cyclin D1; USP2; deubiquitination; cell cycle

INTRODUCTION

Cyclin D1, an important regulator of the G1 to S phase transition in normal cells, can also function as a proto-oncogene that is activated by chromosomal translocation in human B cell tumors or overexpressed in many types of human cancers, including lymphoid, breast, esophageal, lung, and bladder tumors (Diehl, 2002; Sherr, 1996; Landis et al., 2006; Lee and Sicinski, 2006; Li et al., 2006). Genetic lesions, such as chromosomal translocation or amplification of the cyclin D1 gene, can account for some, but not all, cases of tumor-specific cyclin D1 overexpression. However, there is growing evidence that inhibition of cyclin D1 turnover is responsible for the increased levels of cyclin D1 in many types of human cancers (Barbash and Diehl, 2008). In normal cells, cyclin D1 protein has a very short half-life (~20 min) due to rapid ubiquitin-dependent proteasome turnover. Recently, Diehl et al. showed that both the FBX4 F-box protein and its co-factor αB crystallin form a functional SCF complex that catalyzes cyclin D1 ubiquitination and that depletion of either

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component, as observed in some cancer cells, results in increased stability and accumulation of cyclin D1 (Lin et al., 2006). In addition, functional mutations of Fbx4 have been discovered in human cancer cell lines and tumor samples with high cyclin D1 levels (Barbash et al., 2008). An independent study identified FBXW8 as another F-box protein involved in cyclin D1 degradation, although potential alternations of FBXW8 in human cancers remain unclear (Okabe et al., 2006). Thus, understanding the regulation of cyclin D1 degradation should yield new insights into the mechanisms of tumor-associated cyclin D1 overexpression and reveal opportunities to target this pathway for therapeutic purposes.

Protein ubiquitination is a dynamic process balanced by removal of ubiquitin with deubiquitinating enzymes (Dubs) (Li et al., 2002; Ventuii and Wilkson, 2008; D'Andre and Pellman, 1998; Nijman et al., 2005a; Brooks and Gu, 2006). The approximately 90 potential Dub enzymes encoded by the human genome represent either cysteine- or metallo-proteases (Ventuii and Wilkson, 2008; Nijman et al., 2005a). These Dubs can be further divided into five subclasses based on the catalytic domain: ubiquitin-specific proteases (USP); ubiquitin C-terminal hydrolases (UC); Machado-Joseph disease proteases (MJ); ovarian tumor proteases (OT); and JAB1/MPN/Mov34 metallo-proteases (JA). A number of Dubs have been identified that specifically regulate the stability or function of various cellular factors, such as p53, Mdm2/Mdmx, c-Myc, TRAF2, BRCA2 and FANCD2 (Li et al., 2002; 2004; Cummins et al., 2004; Meulmeester et al., 2005; Popov et al., 2007; Brummekamp et al., 2003; Nijman et al., 2005b; Cohn et al., 2007; Schoenfeld et al., 2004; Hu et al., 2002; Wertz et al., 2004). Given the central role of ubiquitin-dependent turnover of cyclin D1 in normal cell cycle progression, it seems reasonable to propose that cyclin D1 function is also regulated by a specific Dub.

RESULTS

To identify Dubs for cyclin D1, we first generated a cDNA expression library encoding 76 distinct full-length human Dub proteins, accounting for 84% of predicted human Dubs (supplementary Figure S1A and S1B). The individual Dubs were then purified and used for *in vitro* screening assays on ubiquitinated cyclin D1. We chose to use monoubiquitinated, rather than polyubiquitinated, cyclic D1, as the substrate for Dub screening. This precaution was taken as many Dubs appear to depolymerize polyubiquitination chains *in vitro* with limited substrate specificity (data not shown). Therefore, to isolate monoubiquitinated-cyclin D1, we transfected human 293 cells with expression vectors encoding Flag-tagged cyclin D1 and HA-tagged ubiquitin. To enrich for monoubiquitinated proteins, cells were harvested in the absence of proteasome inhibitors and the cell extracts were subjected to two rounds of affinity purification. Thus, all Flag-tagged cyclin D1 polypeptides were captured on M2 (anti-Flag) agarose beads, and the eluate was then subjected to a second round of affinity purification using anti-HA agarose beads to enrich the ubiquitinated forms. As seen in Figure 1B, a prominent band corresponding to monoubiquitinated cyclin D1 migrated with a mobility approximately 10 kilodaltons larger than unmodified cyclin D1 (Figure 1A and 1B). The purified monoubiquitinated form (cyclin D1-Ub) was then used as a substrate for an *in vitro* deubiquitination reaction by incubation with each of the 76 purified Dubs (Figure S1C). Western blot analysis revealed a complete or partial removal of the ubiquitin moiety from cyclin D1 when it was incubated with 9 out of 76 Dubs (Figure 1C), suggesting that these candidates can deubiquitinating cyclin D1 directly *in vitro*. As a secondary assay, we then tested each of these candidates for cyclin D1 deubiquitination activity *in vivo*. Thus, 293 cells were transfected with expression vectors encoding Flag-tagged cyclin D1 and His-tagged ubiquitin, as well as each of the 9 candidate Dubs. As shown in Figure 2A, only one of these, namely USP2, was able to deubiquitinate polyubiquitinated cyclin D1 in human cells. These data identified USP2 as a potential deubiquitinase for cyclin D1.

We next investigated whether USP2 can directly interact with cyclin D1 upon co-expression of both proteins in 293 cells. As shown in Figure 2B, HA-cyclin D1 was clearly detected in immunoprecipitates of Flag-USP2 bound to M2 (α-Flag) beads (lane 3 vs. lane 4). Reciprocally, HA-USP2 was also readily precipitated with Flag-cyclin D1 on M2 beads (lane 7 vs. lane 8). We next examined the binding of endogenous cyclin D1 and USP2 in HCT116 cells. Cell extracts from HCT116 were immunoprecipitated with α-USP2 antibody or with control IgG. Indeed, Western blot analysis revealed that cyclin D1 was clearly detected in the immunoprecipitations obtained with the α-USP2 antiserum (lane 3, Fig. 2C) but not with the control antibody (lane 2). Conversely, endogenous USP2 was readily immunoprecipitated with cyclin D1 antibody (lane 5), but not with control IgG (lane 4). Finally, the interaction of USP2 and cyclin D1 was tested *in vitro* using GST pulldown assays. Thus, ^{35}S-labeled *in vitro*-translated USP2 bound immobilized GST-cyclin D1 but not to parental GST (Figure 2D) although similar amounts of the recombinant proteins were used (Figure S2). These data demonstrate a direct interaction between USP2 and cyclin D1.

As cyclin D1 ubiquitination has been implicated in proteasome-mediated cyclin D1 degradation, we examined whether USP2 affects cyclin D1 stability *in vivo*. Indeed, a dose-dependent increase in the steady-state levels of cyclin D1 was observed upon transfection of 293 cells with wildtype USP2, but not a catalytically inert form (USP2-CA) that harbors the C276A mutation (Figure 3A). In addition, expression of wildtype USP2, but not USP2-CA, dramatically reduced the cellular levels of polyubiquitinated cyclin D1 (Figure 3B). In accord with these observation, wildtype, but not mutant, USP2 significantly extended the half-life of cyclin D1 from 20 minutes to more than 90 minutes (Figure 3C). Taken together, these results demonstrate that USP2 can stabilize cyclin D1 *in vivo* by antagonizing the ubiquitin-mediated proteolysis.

We next investigated USP2 regulation of cyclin D1 under more physiological settings. We first examined the effect of USP2 knockdown on cyclin D1 stability in the HCT116 cells. Cells were transfected with either an USP2-specific (USP2#1) or a control (control) siRNA. As shown in Fig. 3D, the levels of USP2 polypeptides were severely reduced after three consecutive transfections (upper panels, lane 2 vs. lane 1) with USP2-siRNAi#1. Indeed, the steady-state levels of endogenous cyclin D1 were also drastically reduced. To avoid off-target effects, two additional siRNA oligos (#2 and #3) that target different regions of USP2 mRNA were used for knockdown of USP2 and again, reductions of cyclin D1 were also observed (lanes 3, 4, Fig.3D). Although USP2 knockdown did not change cyclin D1 mRNA levels (Figure 3E), the half-life of endogenous cyclin D1 was significantly decreased upon USP2 knockdown (Figure 3F and Figure S3); cell growth suppression was also observed (Fig. 3G).

Given the known requirement for cyclin D1 in normal G1/S phase progression, the growth repression of USP2 knockdown cells (Figure 3G) may reflect delayed cell cycle progression due to reduced cyclin D1 levels. To test this hypothesis, we first examined cell cycle distribution in cells treated with control versus USP2 siRNAs. The HCT116 cells were labeled with BrdU before harvest, and both BrdU staining (newly synthesized DNA) and PI staining (DNA content) were measured by FACS analysis. As seen in Figure 3H, USP2 knockdown significantly increased the proportion of cells in G1 phase (73% vs 60%) and decreased the proportion in S phase (26% vs 12%). To determine whether these changes in cell cycle distribution are due to an increase in the duration of G1, HCT116 cells treated with control siRNA or USP2 siRNA were blocked at prometaphase by nocodazole and released in medium containing BrdU at different time points. As shown in Figure 3I, USP2 knockdown reproducibly prolonged the G1 interval, leading to a significant delay in S phase entry after nocodazole release. These data demonstrate that inactivation of USP2

downregulates the levels of endogenous cyclin D1 and suppresses cell growth by delaying G1/S progression.

Recent studies indicate that high levels of cyclin D1 do not occur solely as a consequence of genetic lesions or cyclin D1 gene transcription, but that inhibition of cyclin D1 degradation is also critical for its accumulation in cancer cells (Barbash and Diehl, 2008). In accord with published results, we found that the levels of αB crystallin, which is a key cofactor for FBX4-mediated cyclin D1 degradation, are undetectable in several human cancer cell lines, including MCF-7 (breast adenocarcinomas) and PC3 (prostate cancer) (Figure 4A). The levels of USP2 are also relatively high in cell lines, such as MCF-7 and PC3 (Figure 4A), which display elevated cyclin D1 expression at the protein, but not the mRNA level. Again, knockdown of USP2 in MCF-7 and PC3 cells significantly reduced the steady state levels of cyclin D1 protein (Figures 4B, also in Figure S9) and also induced cell growth suppression (Figure 4C). As expected, the half-life of cyclin D1 protein in MCF-7 cells was longer than that of other cancer lines such as HCT116; however, USP2 knockdown drastically reduced the cyclin D1 half-life from 60 to 20 minutes (Figures 4D and Figure S4). As a control, the mRNA levels of cyclin D1 were not altered by USP2 knockdown (Figure 4E). These results suggest that USP2 control of cyclin D1 stability is effective in human cancer cells, regardless of the status of the E3 ligases that ubiquitinate cyclin D1. Our data also reveal that inactivation of USP2 can override defects in cyclin D1 ubiquitination, such as those resulting from αB crystallin depletion, and strongly induces cell growth suppression by promoting cyclin D1 degradation.

Notably, cyclin D1 is not universally required for cell cycle progression. Although *cyclin D1*$^{-/-}$ mice are about 50% smaller than their wild-type littermates, cyclin D1 is largely dispensable for development of many tissues and cyclin D1-null mouse embryo fibroblasts showed similar cell-cycle and growth characteristics under standard conditions (Fantl et al., 1995; Sicinski et al., 1995; Landis et al., 2006). In contrast to the results showed above, RNAi-mediated knockdown of endogenous USP2 had very mild effects on cell growth in normal human fibroblast cell lines IMR90 and NHF-1, although the levels of cyclin D1 were significantly reduced in these cells (Figure 4F and Figure S5A). Consistent with these results, RNAi-mediated knockdown of cyclin D1 in these cells also showed similar effects (Figure 4F). It was reported that cyclin D1 is not expressed in a number of cancer cell lines such as human osteosarcoma cell line SAOS-2 (Ewen et al., 1993). Although the levels of USP2 were largely reduced by USP2-RNAi, no obvious effect of cell growth was observed in SAOS-2 cells by knockdown of either USP2 or Cyclin D1 (Figure 4F and Figure S5B). Taken together, these data indicate that USP2 knockdown induces growth arrest only in the cells where cell growth is dependent on expression of cyclin D1.

Our results reveal that USP2 specifically modulates cyclin D1 function *in vivo* and demonstrate the importance of this regulation in human cancer cell growth. Nevertheless, to confirm that cyclin D1 is the major mediator/substrate for USP2 function, we examined (1) whether knockdown of endogenous cyclin D1 induces the same effects as that of USP2 knockdown and (2) whether the cellular effects induced by USP2 knockdown can be rescued by ectopic overexpression of cyclin D1. For this purpose, HCT116 cells were transfected with control siRNA, USP2 siRNA, and cyclin D1 siRNA. The cell cycle profile analysis revealed that both USP2 knockdown and cyclin D1 knockdown had very similar effects-- a significant increase in G1 population and a decrease in S population (Figures 5A, 5B). Similar results were also obtained with other cell types such as MCF-7 and PC3 (Figure S6). Strikingly, ectopic overexpression of cyclin D1 in USP2-knockdown cells effectively reverses the changes in cell cycle distribution caused by USP2 knockdown (Figure 5B). Moreover, previous studies indicate that USP2 may affect cell growth by modulating the levels of Mdm2, a critical regulator of p53, in certain cell types (Stevenson et al., 2007). As

such, USP2 knockdown could potentially reduce Mdm2 levels and thereby activate p53-mediated cell growth repression. Nevertheless, no obvious effects on the levels of either p53 or Mdm2 were observed by USP2 knockdown in these cells (Figures 5A, S7), suggesting that Mdm2 is not the major substrate of USP2 in these cells. By the same methods, no obvious reduction of the protein steady-state levels were detected for fatty acid synthase (FAS) in HCT 116 cells and MCF-7 cells upon USP2 knockdown (Figure S8), which was reported as a potential substrate for USP2 in prostate cells (Priolo et al., 2006; Graner et al., 2004). Thus, these data indicate that cyclin D1 is a major substrate of USP2 but USP2-mediated effects on Mdm2 and FAS are likely tissue specific.

To completely exclude the possibility that the cell growth repression induced by USP2 knockdown is p53-dependent, we performed the same experiments in isogenic p53 knockout cells (HCT116 p53-/-). While USP2 knockdown had no effect on Mdm2 levels in HCT116 p53-/- cells, it did reduce the steady-state levels and half-life of cyclin D1 (Figure 5C, 5D), delay cell cycle progression (Figure 5E), and suppress cell growth (Figure 5F). Moreover, the growth repression induced by USP2 knockdown in HCT116-p53-/- cells was again rescued by ectopic expression of cyclin D1 (Figures 5E and 5F). Taken together, these results demonstrate that USP2-mediated cell cycle control occurs primarily through its effect on cyclin D1, regardless of p53 status.

DISCUSSION

The emergence of deubiquitination enzymes (DUBs) has established that ubiquitination is a dynamic process in which cellular pathways driven by E3 ligases are commonly reversed or modulated by specific Dubs (Li et al., 2002; Ventuii and Wilkson, 2008; D'Andre and Pellman, 1998; Nijman et al., 2005a; Brooks and Gu, 2006: Cheon and Baek, 2006). Thus, certain Dubs are likely to play undefined but critical roles in tumorigenesis by specifically modulating the ubiquitination status of oncoproteins and tumor suppressors. In previous studies, the Dubs responsible for deubiquitination of specific substrates have been identified by screening Dub shRNA libraries (Brummelkamp et al., 2003; Nijman et al., 2005b). However, there are limitations to this method. First, since many Dubs are stable proteins with long *in vivo* half-lives, it is difficult to attain high efficiency knockdown evenly for each Dub protein during screening. Second, the phenotypes obtained from shRNA screening can arise from indirect effects of Dub depletion. To overcome these issues, we have established a complementary approach to screen for Dubs that deubiquitinate a defined substrate. Thus, the combination of these two approaches is likely to facilitate the identification of specific Dubs for many important cellular factors.

Overexpression of cyclin D1 is observed in various human cancers and the role of cyclin D1 in tumorigenesis is well established (Diehl, 2002; Sherr, 1996; Landis et al., 2006; Lee and Sicinski, 2006; Li et al., 2006). Despite of the redundant role of cyclin D1 in cell cycle progression in normal cells (Lee and Sicinski, 2006), some cancer cells are "addicted" to cyclin D1, that is, they require a greater amount of cyclin D1 to maintain their malignant phenotype than do most of normal cells or other cancer cells that never overexpressed cyclin D1; this phenomena is also called as "oncogene addiction" (Weinstein et al., 2002; Luo et al., 2009). Our study identifies USP2 as a critical regulator of cyclin D1 in vivo. Notably, inhibition of USP2 function induces growth suppression only in the cancer cells addicted to cyclin D1 expression whereas inactivation of USP2 has no major effect on cell growth of normal human fibroblasts, raising an interesting possibility that inhibition of USP2 may have differential effects on cancer cells vs. normal cells.

Thus, based on our study, inactivation of USP2 should have minimal effects on normal cells/tissues but induces dramatic growth inhibition on the cancer cells with cyclin D1

overexpression. We also observe a correlation between USP2 expression and high cyclin D1 protein levels in human tumor lines (Fig. 4C), but this result needs to be validated in larger panels of cancer cell lines and tumor samples. To this end, recent studies indicate that USP2 is overexpressed in certain types of prostate cancers (Priolo et al., 2006;Graner et al., 2004). Although ubiquitination events are thought to be promising targets for cancer therapy, identification of highly potent and specific inhibitors of ubiquitin ligases has not yet been successful owing to the complexity of the enzymatic cascade (e.g., E1, E2, and E3) involved in ubiquitin conjugation. However, since deubiquitination is a simpler process, much akin to proteolysis, Dubs such as USP2 may prove to be better targets to develop enzymatic inhibitors with therapeutic potential.

EXPERIMENTAL PROCEDURES

DUBs cloning and protein purification

The individual DUB cDNAs obtained from either Marathon-ready cDNA (Clontech) or commercially available cDNA clones (Open biosystem, RZPD) were amplified by PCR, and cloned into TOPO TA cloning vector (Invitrogen). Each DUB cDNA was then subcloned into Flag-HA (FH-) double-tagged expression vector, and its sequence was confirmed by DNA sequencing. The expression of each DUB plasmid was confirmed by Western Blot using α-HA antibody following transfection into 293 cells. To purify each DUB protein, 293 cells were transfected with each DUB plasmid, harvested 48 hours post transfection, and lysed in Flag lysis buffer (50 mM Tris-HCl, PH 7.9, 137 mM NaCl, 10 mM NaF, 1 mM EDTA, 1% Triton X-100, 0.2% Sarkosyl, 10% glycerol, and protease inhibitors). The cell extracts were subjected to immunoprecipitation using α-Flag M2 beads (Sigma), and proteins were eluted using Flag peptide (Sigma) in BC100 buffer (20 mM TRis-HCl, PH7.9, 100 mM NaCl, 0.2 mM EDTA, 20% glycerol, 0.2% Triton X-100). 1μl of each eluted protein was run on a 4-12% Tris-Glycine gradient gel, and detected by Western blot using α-HA antibody.

Cyclin D1 - Ub purification

To purify ubiquitinated cyclin D1 (Cyclin D1- Ub), 293 cells were transfected with Flag-cyclin D1 and HA-Ub. Forty-eight hours later, the cells were lysed with Flag lysis buffer. The cell extracts were subjected to immunoprecipitation using α-Flag M2 beads, followed by Flag peptide elution. The eluted Flag-Cyclin D1 was then subjected to immunoprecipitation using α-HA beads (Sigma), and Flag-cyclin D1-HA-Ub was eluted using HA peptide (Roche).

In vitro and *in vivo* deubiquitination assay for Cyclin D1-Ub

To perform the deubiquitination assay *in vitro*, the purified cyclin D1-Ub protein was incubated with each purified DUB protein in a deubiquitination buffer (50 mM Tris-HCl, PH 8.0, 50 mM NaCl, 1 mM EDTA, 10 mM DTT, 5% glycerol) for 2 hours at 37 °C. The reaction mixtures were then resolved on a 4-12% Tris-Glycine gradient gel (Invitrogen) for Western blot analysis using α-cyclin D1 antibody (Santa Cruz). To perform the deubiquitination assay *in vivo*, 293 cells were transfected with F-cyclin D1, His-HA-Ub, FH-DUB and GFP (an internal control for transfection efficiency). Forty-eight hours later, 10% of cells were lysed with Flag lysis buffer for input, and the rest were lysed with 6M Guanadine buffer and were subjected to Ni-NTA affinity purification. Proteins eluted by Imidazole were resolved on a 4-12% Tris-Glycine gradient gel for Western blot analysis using α-cyclin D1 antibody.

siRNA knockdown

Three different siRNA oligonucleotides purchased from QIAGEN were used to target human USP2 (#1: 5'-CAGATTGTGGTTACTGTTCTA; #2: 5'-CAGGAGAATGGCACACTTTCA; #3: 5'-CCGCGCTTTGTTGGCTATAAT). On-target plus smart pool siRNA for human cyclin D1 was purchased from DHARMACON. Cells were transfected with the indicated oligos according to the manufacturer's instructions. The transfection was repeated after 24 hours for a second round, and cells were harvested after another 48 hours.

Flow cytometry and cell cycle analysis

BrdU incorporation in DNA synthesizing cells were detected according to the manufacturer's protocol (BD Biosciences). In brief, HCT116 cells treated with the indicated siRNA oligos were incubated with 20 μM BrdU for 2 hours. Cells were then trypsinized and fixed in 70% ethanol. DNA was denatured in 2M HCl, followed by neutralization with 0.1 M $Na_2B_4O_7$ (PH=8.5). Incorporated BrdU was stained by immunofluorescence using FITC-conjugated anti-BrdU antibody. DNA was then stained with propidium iodide (PI) and analyzed by flow cytometry. Samples of 20,000 cells were analyzed for DNA histograms and cell cycle distributions using a FACSCalibur flowcytometer (Becton Dickinson), and the data were analyzed using CellQuest software (Becton Dickinson) according to the manufacturer's instruction.

Co-IP

293 cells transfected with Flag-USP2, Flag-USP2 and HA-cyclin D1, F-cyclin D1, or F-cyclin D1 and HA-USP2 were lysed with Flag lysis buffer. 10% of the cell extracts was kept for input, and the rest was subjected to immunoprecipitation using α-Flag M2 beads. Proteins eluted by Flag peptide were resolved on a 4-12% Tris-Glycine gradient gel for Western blot analysis using α-Flag and α-HA antibody. To detect the endogenous protein interaction between USP2 and cyclin D1, HCT116 cells were lysed in BC100 buffer. 10% of the cell extracts was kept for input, and the rest was incubated with α-USP2 (Abgent, C-term-L523), rabbit IgG (Santa Cruz Biotechnology), α-cyclin D1 (Cell signaling, #2926) or mouse IgG (Santa Cruz Biotechnology) antibody for 1 hour at 4°C. A/G PLUS-Agarose beads (Santa Cruz Biotechnology) were then added for overnight incubation at 4°C. After the beads were washed stringently, the bound proteins were eluted by boiling in SDS sample buffer, and detected by Western blot using α-USP2 and α-cyclin D1 antibodies.

GST pulldown

GST and GST-cyclin D1 were prepared by bacterial purification. ^{35}S-labeled USP2 was prepared by *in vitro* translation using the TNT reticulocyte lysate system (Promega). 1 μg GST or 2.5 μg GST-cyclin D1 was incubated with *in vitro* translated USP2 for an hour at 4°C in BC 100 buffer containing 1% bovine serum albumin. Glutathione-sepharose beads were then added, and the solution was incubated for another two hours at 4°C. After stringent washing, proteins were eluted by reduced glutathione, and resolved on a 9% SDS-PAGE gel for autoradiography.

Cell culture and antibodies

HCT116 cells were maintained in McCoy's 5a Medium supplemented with 10% fatal bovine serum (FBS). All other cells were maintained in DMEM supplemented with 10% FBS. Antibodies were obtained from the following suppliers: anti-cyclin D1 (Cell signaling, #2926), anti-USP2 (Abgent, #C-term-L523), anti-Flag (Sigma), anti-HA (Roche).

RT-PCR analysis

Total RNA was isolated from cells by using TRIzol Reagent (Invitrogen), and first-strand cDNA was synthesized from 1ug of total RNA by using the SuperScript First-Strand synthesis system (Invitrogen) with the oligo-dT primer. Prepared cDNA samples were amplified and analyzed by PCR. The following primers were used in the PCR reaction. Human cyclin D1 (forward): 5’ cctgtcctactaccgcctca; Human cyclin D1(reverse): 5’ tcctcctcttcctcctcctc; human USP2 (forward): 5’cttctgggacctctcactgc; Human USP2 (reverse): 5’ tctttgggaacctctggatg; Human GAPDH (forward): 5’ gaaggtgaaggtcggagt; Human GAPDH (reverse): 5’gaagatggtgatgggatttc; Human FBX4 (forward): 5’tcagaggaactttgcccaac; Human FBX4 (reverse): 5’ atcacactgtaccggcttcc; Human αB crystallin (forward): 5’ ttcttcggagagcacctgtt; Human αB crystallin (reverse): 5’ ttttccatgcacctcaatca.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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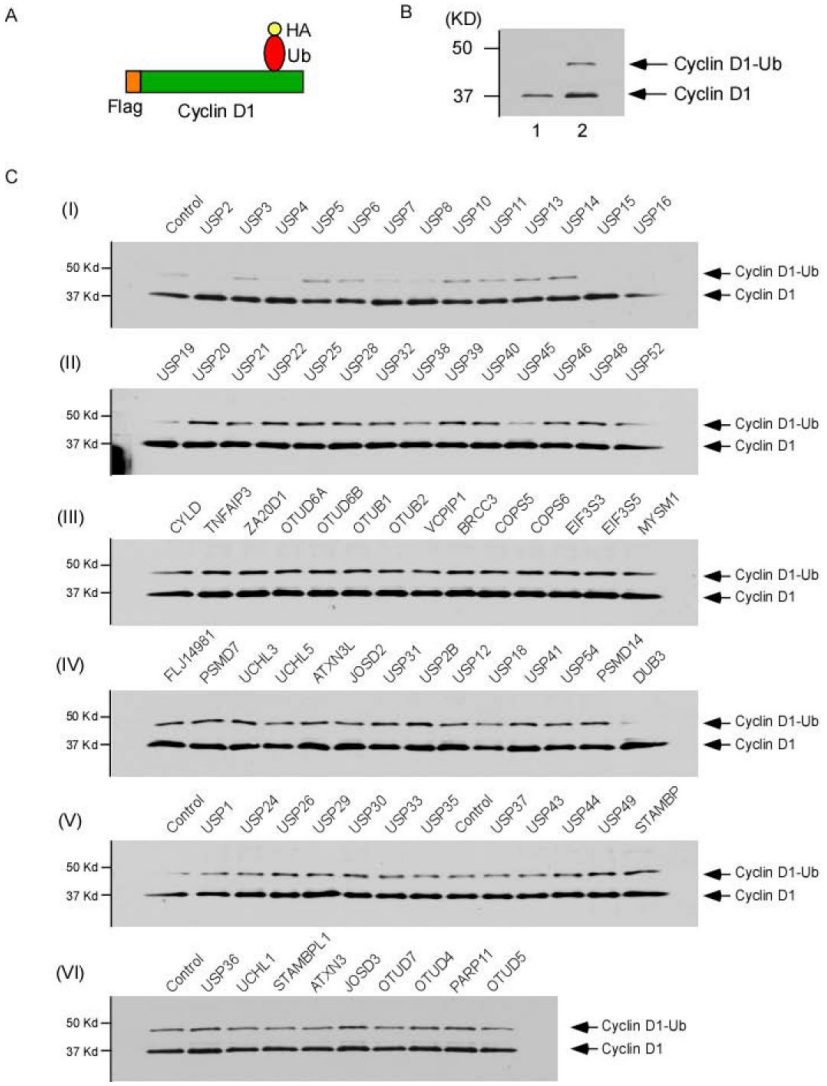

Figure 1. Screening of DUBs for cyclin D1-Ub by *in vitro* deubiquitination assay

(A) Schematic representation of Flag-cyclin D1 conjugated to one molecule of HA-ubiquitin (Ub), forming mono-ubiquitinated cyclin D1 (cyclin D1-Ub).

(B) Purification of mono-ubiquitinated cyclin D1 (cyclin D1-Ub). 293 cells were transfected with F-cyclin D1 and HA-Ub, and cell lysates were first subjected to α-Flag immunoaffinity purification, followed by α-HA immunoaffinity purification. Eluates after the first (lane 1) and second (lane 2) steps of purification were analyzed by Western blot using α-cyclin D1 antibody.

(C) *In vitro* deubiquitination assay for cyclin D1-Ub. Purified cyclin D1-Ub was incubated with each purified DUB as indicated, and the reaction mixture was subjected to Western blot analysis using α-cyclin D1 antibody to detect cyclin D1 (lower band) and cyclin D1-Ub (upper band).

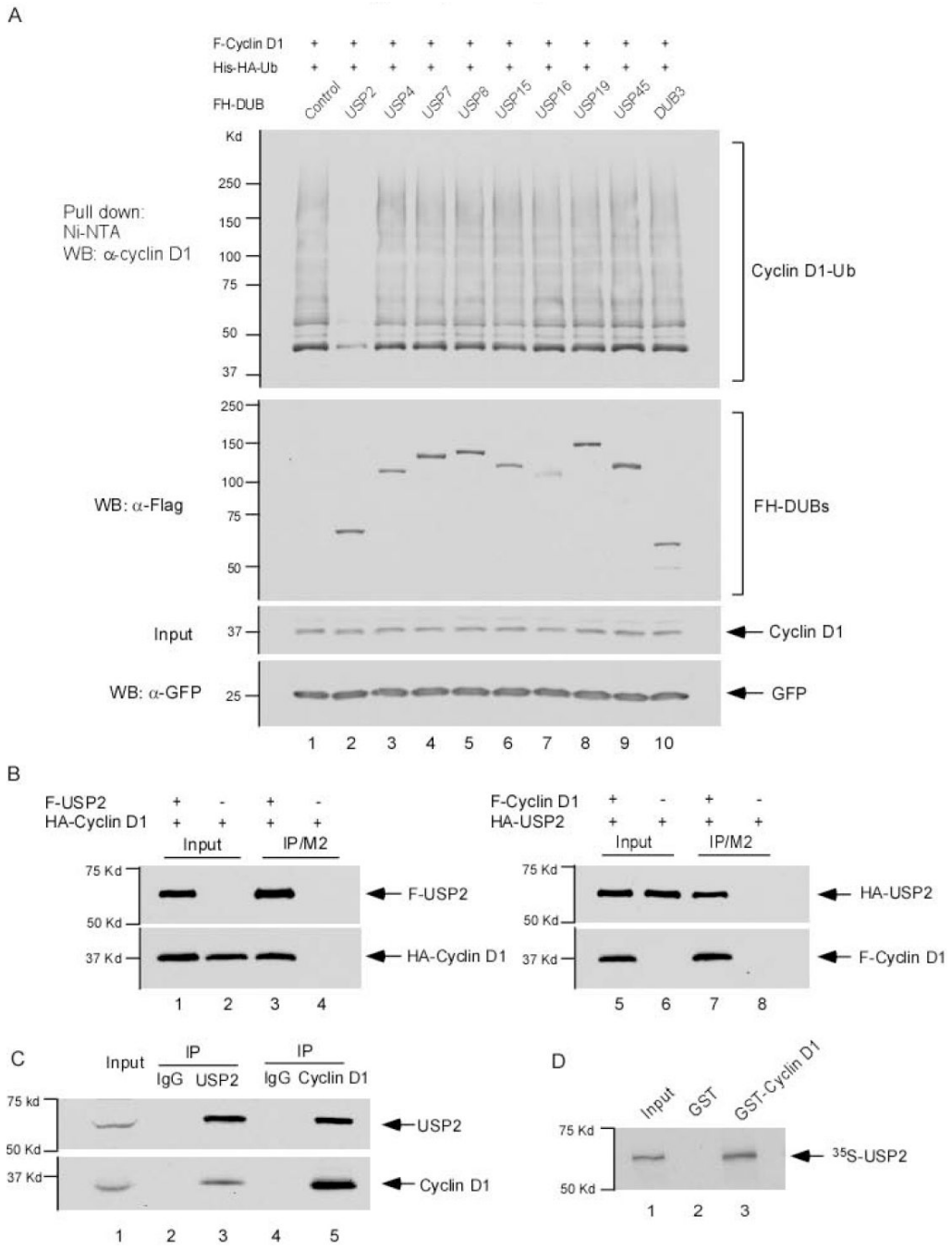

Figure 2. USP2 deubiquitinates cyclin D1 through their direct interaction

(A) *In vivo* cyclin D1 deubiquitination assay. 293 cells were transfected with cyclin D1, His-HA-Ub and DUBs as indicated, and Cyclin D1-Ub was detected by Western blot using α-cyclin D1 antibody following Ni-NTA pull down. For input controls, cells were treated with proteasome inhibitor before harvest, and cell lysates were subjected to Western blot analysis for FH-DUBs, cyclin D1 and GFP.

(B) Interaction of over-expressed cyclin D1 and USP2 in 293 cells. 293 cells were transfected with the indicated plasmids, and the cell lysates were subjected to immunoprecipitation using the M2 beads.

(C) Interaction of endogenous cyclin D1 and USP2 in HCT116 cells. Cell lysates were subjected to immunoprecipitation using rabbit IgG (lane 2), α-USP2 antibody (lane 3), mouse IgG (lane 4) or α-cyclin D1 (lane 5) antibody, followed by Western blot using α-USP2 and α-cyclin D1 antibody, respectively.
(D) Interaction of GST-Cyclin D1 and ^{35}S-USP2 *In vitro*. GST (lane 2) and GST-Cyclin D1 (lane3) were used in a GST pull down assay with *in vitro* translated ^{35}S-USP2.

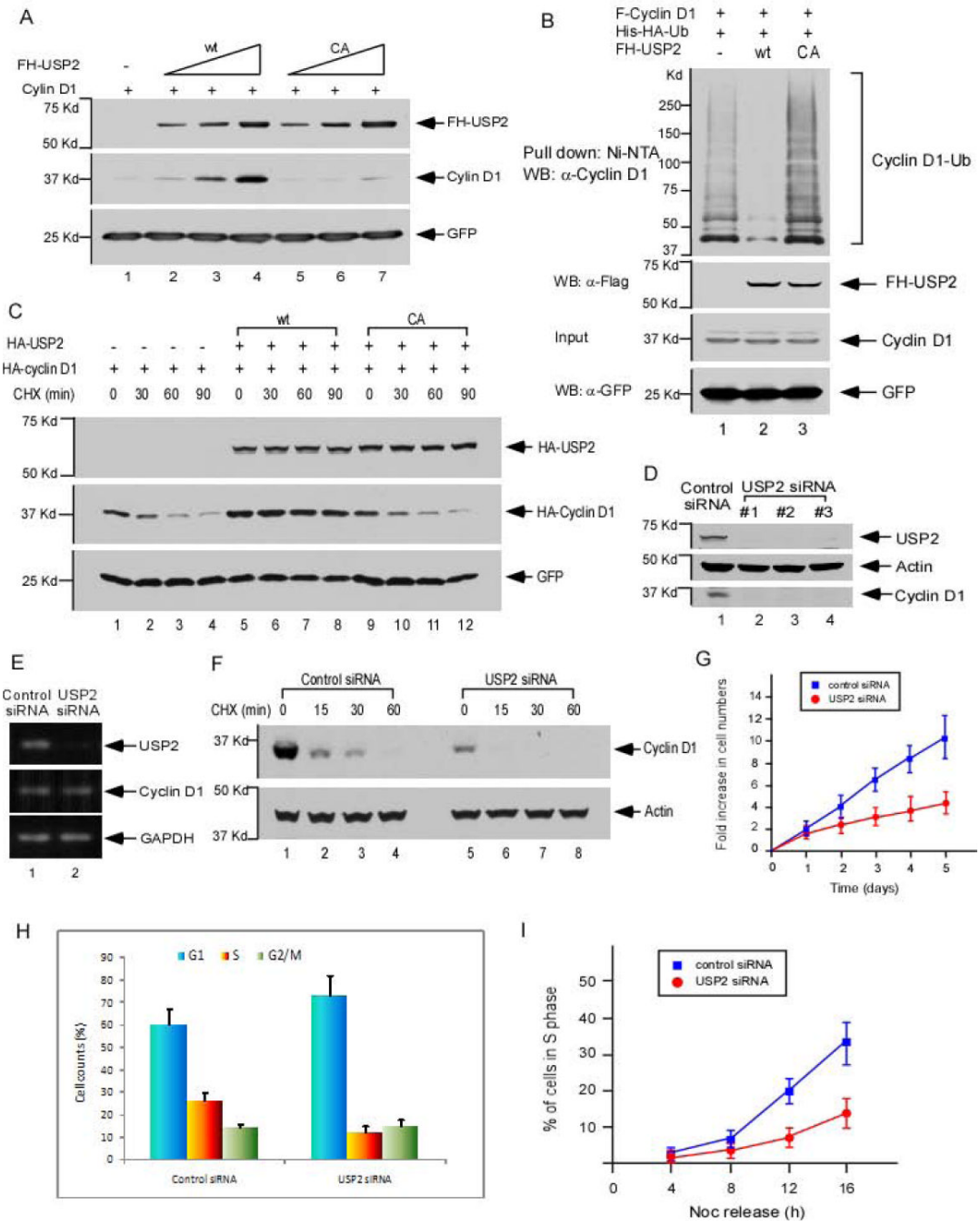

Figure 3. USP2 stabilizes cyclin D1 by preventing it from ubiquitination mediated degradation, thus accelerating the G1 to S phase progression

(A) Western blot analysis of cyclin D1 and USP2 in 293 cells transfected with the indicated plasmids.

(B) *In vivo* cyclin D1 deubiquitination assay by USP2 wt and CA mutant. 293 cells were transfected with the indicated plasmids, and Cyclin D1-Ub was detected by Western blot using α-cyclin D1 antibody following Ni-NTA affinity purification. For input controls, cells were treated with proteasome inhibitor before harvest, and cell lysates were subjected to Western blot analysis for FH-USP2, cyclin D1 and GFP.

(C) Western blot analysis of cyclin D1 and USP2 in 293 cells transfected with the indicated plasmids and treated with cycloheximide (CHX) for the indicated times.
(D) Knockdown of USP2 by different siRNA oligos. HCT116 cells were treated with control siRNA (lane 1) or USP2 siRNA (lanes 2, 3 and 4), and cell lysates were subjected to Western blot analysis for USP2, Cyclin D1 and Actin.
(E) RT-PCR analysis of USP2, Cyclin D1 and GAPDH in HCT116 cells treated with control siRNA or USP2 siRNA.
(F) Cyclin D1 half-life in HCT116 cells. Cells were incubated with cycloheximide for the indicated times following control siRNA or USP2 siRNA treatment.
(G) Growth curve of HCT116 cells after control siRNA or USP2 siRNA treatment. Error bars indicate standard deviations calculated from three independent experiments.
(H) Quantification of cell cycle distribution of HCT116 cells treated with control siRNA or USP2 siRNA by FACS analysis. Error bars indicate standard deviations calculated from three independent experiments.
(I) Kinetics of S phase entry following nocodazole treatment. HCT116 cells were treated with control siRNA or USP2 siRNA. 72 hours later, cells were incubated with nocodazole (Noc) for overnight and released into the normal growth medium containing BrdU for the indicated times. Error bars represent standard deviations calculated from three independent experiments.

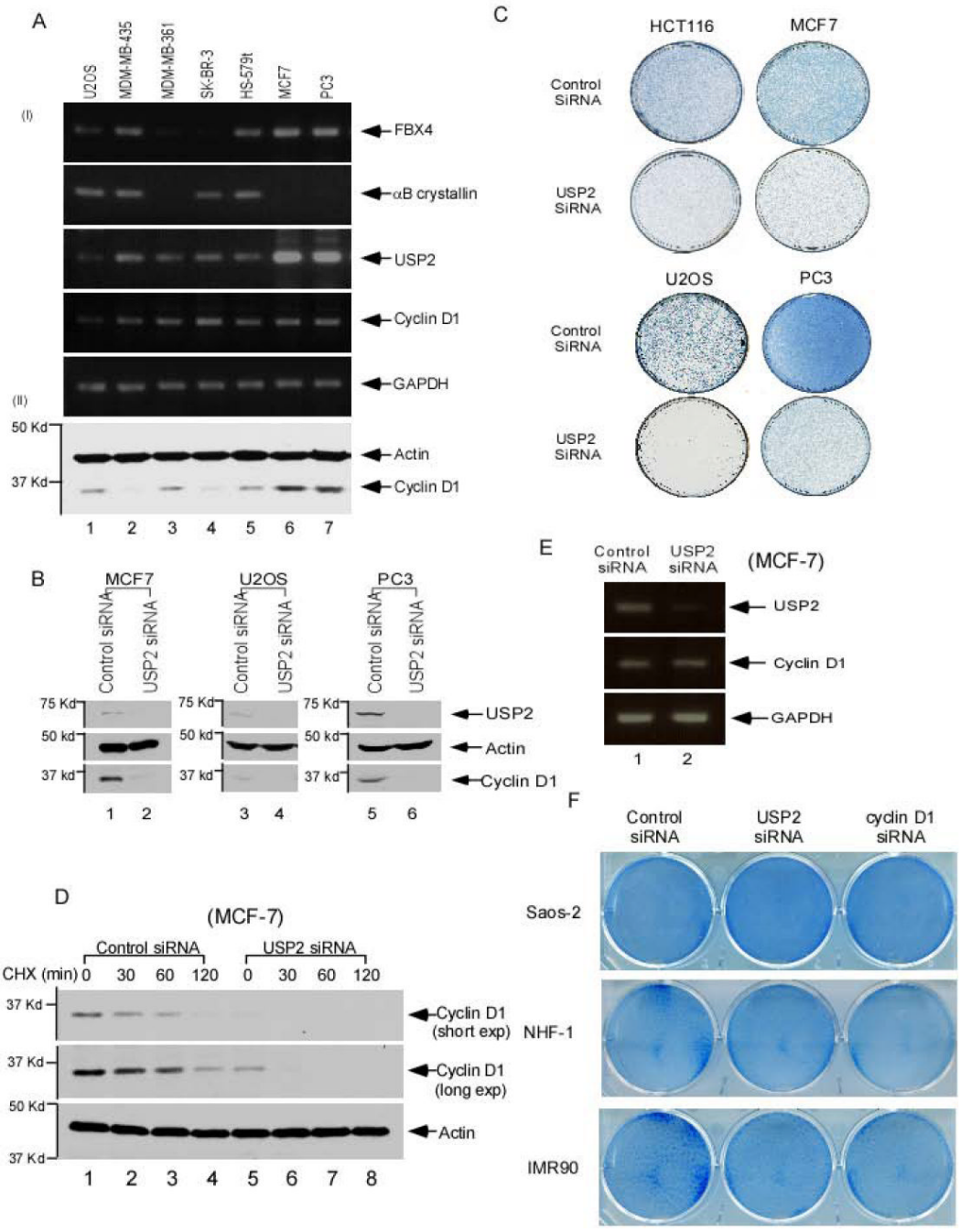

Figure 4. Knockdown of USP2 destabilizes cyclin D1 and suppresses cell growth in many human cancer cell lines where cell growth is dependent on expression of cyclin D1

(A) (I) RT-PCR analysis of FBX4, αB crystallin, USP2, Cyclin D1 and GAPDH in human cancer cell lines. (II) Western blot analysis of cyclin D1 and Actin in these cells.

(B)Western blot analysis of USP2, Cyclin D1and Actin in MCF7, U2OS and PC3 cells treated with control siRNA or USP2 siRNA.

(C) Cell proliferation assay of HCT116, MCF7, U2OS and PC3 cells treated with control siRNA or USP2 siRNA. The cells were stained with Methylene Blue four days after siRNA treatment.

(D) Western blot analysis of Cyclin D1 (short and long exposure time) and Actin in MCF7 cells incubated with cycloheximide for the indicated times following control siRNA or USP2 siRNA treatment.
(E) RT-PCR analysis of USP2, Cyclin D1 and GAPDH in MCF7 cells treated with control siRNA or USP2 siRNA.
(F) Cell proliferation assay of Saos-2, NHF-1, and IMR90 cells treated with control siRNA, USP2 siRNA or cyclin D1 siRNA. The cells were stained with Methylene Blue four days after siRNA treatment.

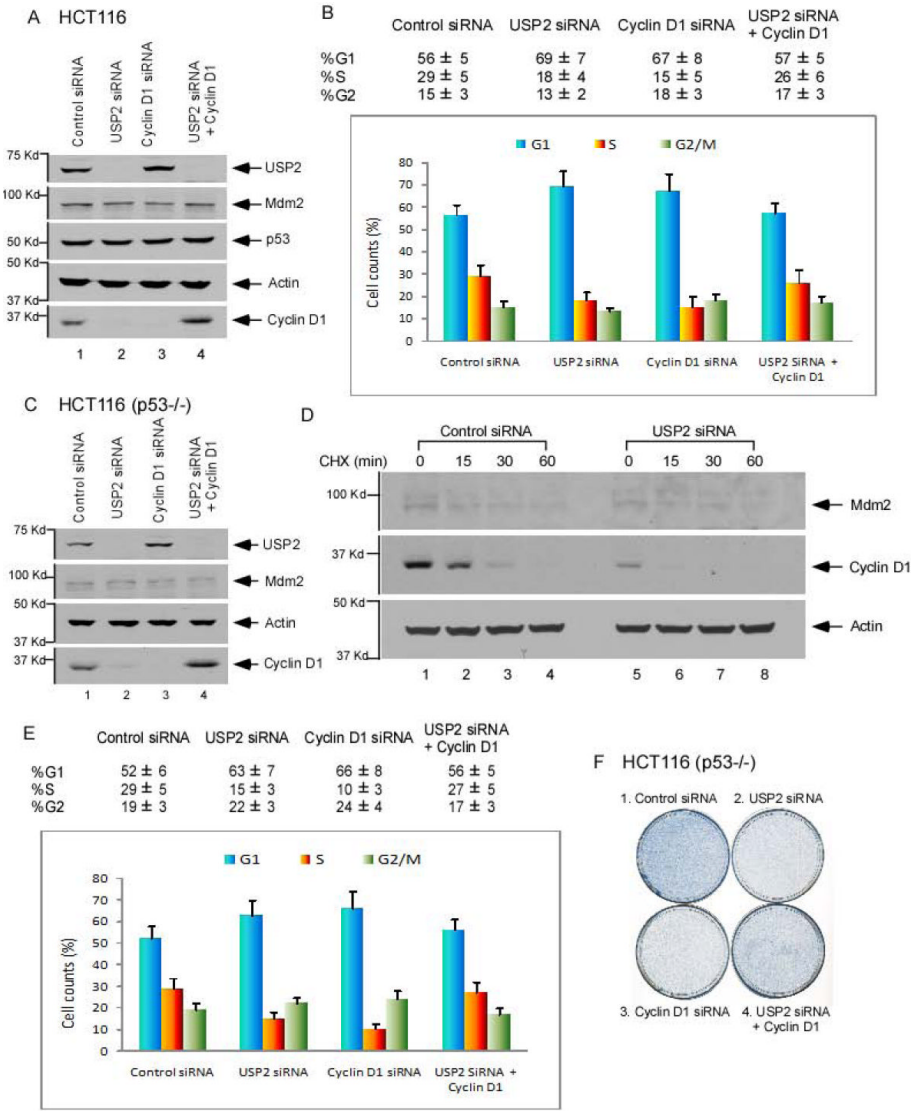

Figure 5. USP2 mediated cell cycle regulation is Cyclin D1 dependent, not p53 dependent

(A) Western blot analysis of USP2, mdm2, Cyclin D1, p53 and Actin in HCT116 cells treated with control siRNA, USP2 siRNA, cyclin D1 siRNA, or USP2 siRNA and cyclin D1.

(B) Quantification of cell cycle distribution of HCT116 cells treated with the indicated reagents by FACS analysis. Error bars indicate standard deviations calculated from three independent experiments.

(C) Western blot analysis of USP2, mdm2, Cyclin D1 and Actin in HCT116 p53-/- cells treated with control siRNA, USP2 siRNA, cyclin D1 siRNA, or USP2 siRNA and cyclin D1.

(D) Western blot analysis of mdm2, Cyclin D1 and Actin in HCT116 p53-/- cells incubated with cycloheximide for the indicated times following control siRNA or USP2 siRNA treatment.

(E) Quantification of cell cycle distribution of HCT116 p53-/- cells treated with the indicated reagents by FACS analysis. Error bars indicate standard deviations calculated from three independent experiments.

(F) Cell proliferation assay of the HCT116 p53-/- cells treated with the indicated reagents. The cells were stained with Methylene Blue four days after siRNA treatment.