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The Deubiquitinating Enzyme USP1 Regulates the Fanconi Anemia Pathway

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Summary

Protein ubiquitination and deubiquitination are dynamic processes implicated in the regulation of numerous cellular pathways. Monoubiquitination of the Fanconi anemia (FA) protein FANCD2 appears to be critical in the repair of DNA damage because many of the proteins that are mutated in FA are required for FANCD2 ubiquitination. By screening a gene family RNAi library, we identify the deubiquitinating enzyme USP1 as a novel component of the Fanconi anemia pathway. Inhibition of USP1 leads to hyperaccumulation of monoubiquitinated FANCD2. Furthermore, USP1 physically associates with FANCD2, and the proteins colocalize in chromatin after DNA damage. Finally, analysis of crosslinker-induced chromosomal aberrations in USP1 knockdown cells suggests a role in DNA repair. We propose that USP1 deubiquitinates FANCD2 when cells exit S phase or recommence cycling after a DNA damage insult and may play a critical role in the FA pathway by recycling FANCD2.

Introduction

Maintenance of genomic integrity is critical in the protection against malignant transformation. Genetic disorders that perturb the repair of DNA damage, induced by either exogenous agents or endogenous events, often lead to increased cancer susceptibility. One such disorder is Fanconi anemia (FA), a rare syndrome with predisposition to a variety of malignancies (D'Andrea, 2003). Genes mutated in FA have also been implicated in the carcinogenesis of sporadic tumors, underscoring the broad relevance of studying rare human genetic diseases (Taniguchi et al., 2003; Tischkowitz et al., 2003).

At the cellular level, FA is characterized by chromosomal instability and hypersensitivity to DNA-crosslinking agents, such as mitomycin C (MMC), cisplatin, diepoxybutane (DEB), and to a lesser extent, ionizing radiation. MMC and DEB hypersensitivity is a hallmark of FA and is used as a diagnostic test in the clinic (Auerbach et al., 1989).

The genetic basis for FA is diverse, and evidence exists for at least 11 complementation groups (Levitus et al., 2004). FA is clinically related to various other hereditary chromosomal instability syndromes, and recent work has shown that the protein products mutated in Bloom Syndrome, Nijmegen Breakage Syndrome (NBS), Ataxia Telangiectasia (ATM), and Seckel Syndrome (ATR) functionally intersect with the FA signaling pathway (Andreassen et al., 2004; Meetei et al., 2003b; Nakanishi et al., 2002; Taniguchi et al., 2002b). Furthermore, hypomorphic mutations in the *BRCA2* gene make up the Fanconi complementation group D1 (FANCD1) (Howlett et al., 2002).

Based on clinical, biochemical, and cellular phenotypes, the FA proteins appear to function in a common cellular signaling network. At least seven of these proteins, FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, and the ubiquitin E3 ligase FANCL form a nuclear multisubunit complex that is critical for the monoubiquitination of the FANCD2 protein (de Winter et al., 2000; Gordon and Buchwald, 2003; Meetei et al., 2003a; Meetei et al., 2004; Pace et al., 2002). Indeed, functional loss of any of these FA proteins abrogates S phase and DNA damage-induced FANCD2 ubiquitination.

Upon monoubiquitination on Lys561, FANCD2 relocalizes to nuclear DNA damage foci, where it binds to BRCA1 and the RAD51 recombinase and colocalizes with FANCD1/BRCA2 (Taniguchi et al., 2002a; Wang et al., 2004). It is thought that these nuclear foci mark the sites of DNA damage-induced double-strand breaks (DSB) in which DNA is repaired by means of homologous recombination. A role for FANCD2 and BRCA1 in homologous recombination is also suggested by their presence at sites of meiotic recombination in spermatogenesis (Garcia-Higuera et al., 2001). Although the monoubiquitination of FANCD2 appears to be a critical event in efficient DNA repair, the exact molecular function of FANCD2 is poorly understood.

As mentioned, FANCD2 is also monoubiquitinated during S phase, and this event is required for normal progression through this cell cycle phase. The monoubiquitinated form of FANCD2 (FANCD2-L) disappears when cells exit S phase and is transiently present in cells that have been exposed to DNA damage (Garcia-Higuera et al., 2001; Taniguchi et al., 2002a). Both forms of FANCD2 are stable and not subject to proteasomal degradation, indicating that the monoubiquitination does not serve to target FANCD2-L for degradation. Instead, it is more likely that a deubiquitinating enzyme (DUB) removes the ubiquitin moiety after DNA damage is repaired, and cells resume cycling. Like protein phosphorylation, ubiquitination is dynamic and reversible, involving numerous ubiquitin-conjugating enzymes and DUBs (Chung and Baek, 1999; D'Andrea and Pellman, 1998; Kim et al., 2003; Shackelford and Pagano, 2004; Wilkinson, 2000). Homology searches in human genome databases have yielded approximately 60 distinct genes

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coding for (putative) DUBs. Although DUBs have been functionally linked with various pathways and processes, surprisingly few mammalian DUB substrates have been identified (Brummelkamp et al., 2003; Cummings et al., 2004; Graner et al., 2004; Kovalenko et al., 2003; Li et al., 2002; Trompouki et al., 2003). To study the role of these enzymes in specific pathways, we have constructed a library of 220 independent vectors expressing short hairpin RNAs (shRNA) targeting 55 human DUBs. Using this library, we have previously identified the familial tumor suppressor CYLD as a negative regulator of TRAF2 poly-ubiquitination (Brummelkamp et al., 2002b; Brummelkamp et al., 2003). Here, we identify the deubiquitinating enzyme USP1 as a novel component of the FA pathway and propose that USP1 is the enzyme that deubiquitinates FANCD2.

Results

Identification of USP1 as a Regulator of FANCD2 Monoubiquitination

Previous experiments have indicated that during normal cell cycle progression, FANCD2 ubiquitination is dynamic (Taniguchi et al., 2002a). Therefore, we reasoned that inhibition of a DUB that cleaves the ubiquitin moiety from FANCD2 would lead to an overall increase of FANCD2-L (the monoubiquitinated isoform of FANCD2) in asynchronous cycling cells. To identify DUBs that have this effect on FANCD2 ubiquitination, we employed the DUB gene family RNA interference library, previously generated in our laboratory (Brummelkamp et al., 2003). The library currently consists of 55 pools of 4 independent shRNA-encoding plasmids targeting 55 DUBs for suppression by RNA interference (Figure 1A and see Supplemental Table S1 at http://www.molecule.org/cgi/content/full/17/3/331/DC1/). We electroporated each pool of DUB knockdown vectors separately in U2-OS cells and selected for shRNA expression. After 72 hr, we analyzed cell lysates by Western blot with an anti-FANCD2 antibody. As shown in Figure 1B, the pool targeting the ubiquitin-specific protease 1 (USP1, pool 47) significantly increased the FANCD2-L fraction (Fujiwara et al., 1998). The increase in FANCD2-L was comparable to the levels observed in MMC-treated cells (Figure 1B). Further validation showed that only the pool targeting USP1 reproducibly had this effect on FANCD2 (Figure 2A and data not shown).

Next, we tested the four independent USP1 shRNA vectors (A–D) present in the original pool for their ability to induce FANCD2-L accumulation (Figure 2A). Both MMC-treated and -untreated cells displayed enhanced FANCD2 monoubiquitination upon transfection of all four vectors (A–D). However, vectors A and C were more potent in inducing FANCD2-L than vectors B and D.

Retroviral delivery of an shRNA targeting USP1 by using the pRetroSuper vector (pRS) also enhanced FANCD2 monoubiquitination (Brummelkamp et al., 2002a). Compared to control cells, retrovirally transduced USP1 knockdown cells displayed enhanced FANCD2 ubiquitination when stimulated with MMC or left untreated (Figure 2B).

To verify that USP1 expression was indeed inhibited by the knockdown vectors, we cotransfected HEK293 cells with the four shRNA vectors and an expression vector containing a green fluorescent protein tagged version of USP1 (GFP-USP1). As expected, all four shRNA vectors efficiently suppressed GFP-USP1 expression (Figure 2C).

To study endogenous USP1 protein, a polyclonal antiserum directed against the N terminus of USP1 was generated (see Experimental Procedures) and tested on synthetic siRNA transfected or control HEK293 cells. Cells were also treated with the S phase inhibitor hydroxyurea (HU) to induce monoubiquitinated FANCD2. Two bands present in the control lanes were efficiently downregulated in lysates derived from the USP1 siRNA-transfected cells (Figure 2D, upper panel). As expected, the observed USP1 downregulation correlated with the upregulation of FANCD2-L (Figure 2D, lower panel). The predicted molecular weight of endogenous, full-length USP1 is 88 kDa, corresponding to the slower migrating USP1 species detected by Western blot and consistent with the size of ectopically expressed USP1 (see Figure 3D, lower panel). The faster migrating band is likely a proteolytic fragment of USP1. We conclude that in our experiments, both ectopically expressed and endogenous USP1 protein are efficiently inhibited by RNA interference.

Because FANCD2 monoubiquitination is activated in S phase, we investigated whether USP1 inhibition resulted in an altered cell cycle distribution or S phase delay. We retrovirally transduced U2-OS cells with a knockdown vector targeting USP1. After selection with puromycin, the cells were synchronized using a double-thymidine block. Cells were released and samples for FACS and protein analysis were taken at the indicated time points. Propidium-iodide (PI) staining of nuclei and subsequent FACS analysis indicated that cell cycle distribution and S phase progression of USP1 knockdown cells was unaffected, suggesting that USP1 inhibition does not activate a cell cycle checkpoint (Figure 2E). Furthermore, although FANCD2-L levels decreased significantly in the control cells about 4 hr after release, this decrease appeared to be strongly delayed in the USP1 knockdown cells (Figure 2F). USP1 inhibition did not induce p53 expression or γ-H2AX nuclear foci formation (data not shown), indicating that FANCD2-L upregulation was not an indirect effect of DNA damage.

Next we tested whether the effect of USP1 on FANCD2 was dependent on a functional FA core complex. We transfected a FANCA-deficient (FA-A) cell line or a FANCA-complemented derivative with a USP1 or control knockdown vector. Subsequently, HU-stimulated or -untreated cells were analyzed for FANCD2 ubiquitination (Figure 2G, upper panel). USP1 knockdown did not result in FANCD2-L accumulation in the FA-A cell line. However, in the complemented cell, FANCD2 ubiquitination was restored and enhanced in the USP1 knockdown cells, indicating that the ability of USP1 to affect FANCD2 monoubiquitination is dependent on a functional FA signaling pathway.

To investigate whether USP1 requires its protease activity to affect FANCD2 ubiquitination, we generated a catalytically inactive USP1 mutant in which the active site cysteine is replaced by a serine residue (GFP-USP1 C/S) (Papa and Hochstrasser, 1993). Overexpression of this mutant, similar to USP1 knockdown, led to the accu-

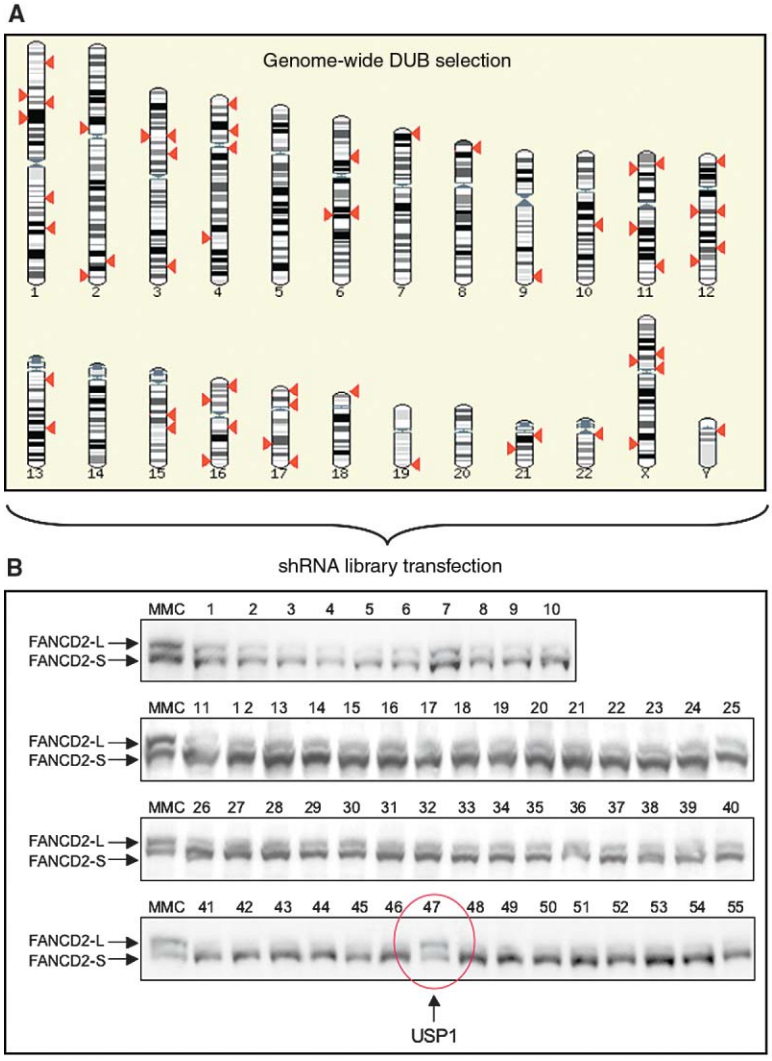

Figure 1. DUB Gene Family Screen

(A) Genome-wide chromosomal locations of selected DUBs (adapted from ENSEMBL).

(B) U2-OS cells were electroporated with the individual pools of the knockdown library and selected for shRNA expression with puromycin. 72 hr after transfection, whole-cell lysates were prepared and immunoblotted using a FANCD2 specific antibody. As a control for FANCD2 ubiquitination, cells were treated overnight with mitomycin C (50 ng/ml MMC). The unmodified and monoubiquitinated forms are indicated as FANCD2-S and FANCD2-L, respectively.

mulation of FANCD2-L (Figure 2H), likely by a dominant negative effect. Possibly due to efficient proteasomal degradation (see below), ectopic expression of wild-type USP1 had a minimal effect on monoubiquitinated FANCD2. We conclude that USP1 is a regulator of FANCD2 monoubiquitination and requires its deubiquitinating enzyme activity to exert this function.

USP1 Is a Cell Cycle-Regulated and Proteasomally Degraded Nuclear Protein

The USP1 protein sequence contains a putative nuclear localization signal (NLS). Analysis of GFP-USP1 localization by fluorescence microscopy confirmed that USP1 is a nuclear protein (Figure 3A).

Because FANCD2 monoubiquitination is cell cycle regulated, we investigated whether USP1 protein levels might also be regulated. HeLa cells were synchronized with a double thymidine block, and samples for protein and FACS analysis were taken at the indicated time points (Figure 3B). Whole-cell lysates were subsequently immunoblotted for FANCD2, USP1, cyclin A, and α-tubulin (loading control). USP1 protein levels were high in G1/S synchronized cells and remained high until FANCD2-L had disappeared in late S phase (8–10 hr after G1/S release, Figure 3B). We conclude that USP1 levels are cell cycle regulated and that USP1 protein is present when FANCD2-L is deubiquitinated.

USP1 data mining in the SOURCE microarray database indicated that USP1 mRNA is induced in S phase and coclusters in terms of its cell cycle regulation with other DNA repair proteins, suggesting a mechanism for the observed USP1 cell cycle regulation (Whitfield et al., 2002). The observation that USP1 is cell cycle regulated could be confirmed with an independent microarray dataset (Figure 3C). As indicated, USP1 mRNA levels followed a pattern similar to the known cell cycle-regulated genes Rad51 and PCNA.

Whereas USP1 mRNA levels decline when cells exit S phase, a marked decrease of USP1 protein levels is already noticeable in late S phase, just prior to cyclin A destruction (compare Figures 3B and 3C). This suggests that USP1 protein may be degraded by the ubiquitin-proteasome pathway. To explore this possibility, HEK293 cells were transfected with a HA-tagged murine USP1 (HA-mUsp1) expression construct and, 24 hr later, were synchronized with a double-thymidine block (Figure 3D).

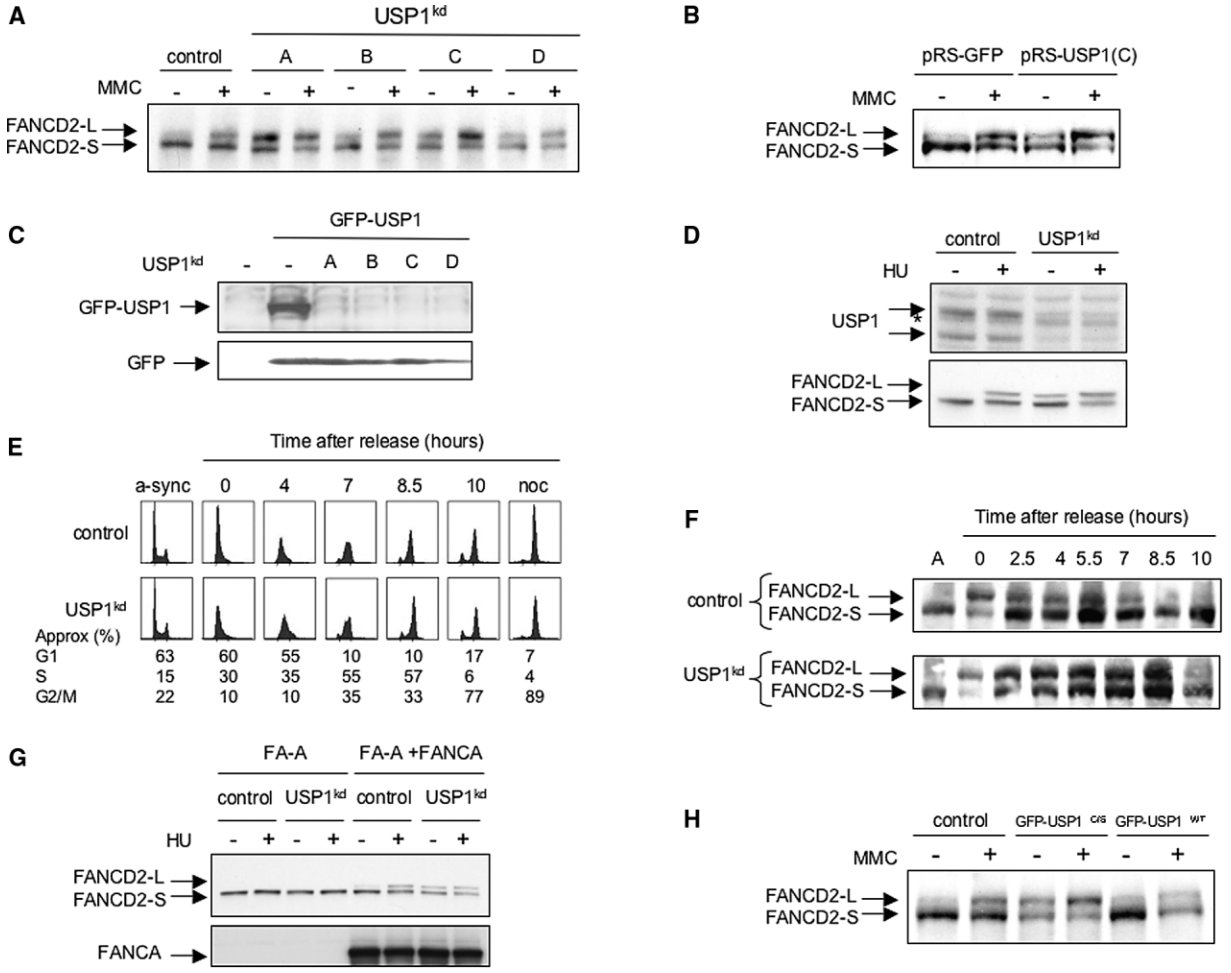

Figure 2. USP1 Is a Regulator of FANCD2 Monoubiquitination

(A) U2-OS cells were transfected with USP1 knockdown (USP1^{kd}) vectors as indicated (A–D), puromycin selected, and treated 72 hr later with MMC (50 ng/ml) overnight or left untreated. A pSUPER vector containing a hairpin targeting murine E2F3 served as a control. Whole cell lysates were analyzed by immunoblotting with a FANCD2 specific antibody.

(B) U2-OS cells expressing the ecotropic receptor were transduced with pRetroSuper-USP1 or pRetroSuper-GFP. After selection with puromycin, cells were stimulated with MMC (50 ng/ml) overnight or left untreated. Whole-cell lysates were analyzed by immunoblotting using a FANCD2-specific antibody.

(C) HEK293 cells were cotransfected with an expression plasmid containing GFP-USP1 and knockdown vectors as indicated (A–D). GFP served as a transfection control (lower panel). Whole-cell extracts were immunoblotted using a GFP antibody.

(D) HEK293 cells were transfected with a synthetic siRNA targeting USP1 or a control siRNA targeting LacZ. 72 hr later, cells were treated for 12 hr with hydroxy-urea (2 mM) or left untreated. Whole-cell lysates were prepared and analyzed with a FANCD2 specific antibody (lower panel) or USP1 polyclonal anti-serum (upper panel, the asterisk indicates a nonspecific background band).

(E) U2-OS cells expressing the ecotropic receptor were transduced with pRetroSuper-USP1 or pRetroSuper-GFP. After selection with puromycin, cells were synchronized and released, and PI-stained cells were analyzed by FACS. Indicated are the approximate average cell cycle phase distributions (G1, S, and G2/M) as percentage.

(F) As in (E), whole-cell lysates were immunoblotted with a FANCD2 antibody.

(G) A FANC-A-deficient patient cell line (GM6914) and a complemented derivative were transfected with a synthetic siRNA targeting USP1 or a control siRNA targeting LacZ. Cells were stimulated for 12 hr with hydroxyurea (2 mM HU) or left untreated. Whole-cell extracts were immunoblotted for FANCD2 (upper panel) or FANCA (lower panel).

(H) HEK293 cells were transfected with GFP-tagged wild-type USP1 (GFP-USP1^{WT}), a catalytically inactive USP1 mutant (GFP-USP1$^{C/S}$), or empty vector. Whole-cell lysates were prepared 72 hr after transfection and analyzed with a FANCD2 antibody.

After release, protein samples were taken at different time points and analyzed. Ectopically expressed murine Usp1 protein levels peaked between 3 and 9 hr after release, coinciding with FANCD2 deubiquitination. At 9–12 hr after release, mUsp1 levels decreased, similar to the results observed for endogenous USP1. This indicates that in addition to transcriptional control, USP1 protein levels may also be regulated posttranslationally. To investigate this further we transfected HEK293 cells with GFP-USP1 and treated the cells overnight with proteasome inhibitor. GFP-USP1 protein was stabilized upon incubation with CBZ-LLL (Figure 3E). Furthermore, immunoprecipitation of Flag-tagged-USP1 (Flag-USP1) from HA-ubiquitin-cotransfected cells indicated that USP1 is poly-ubiquitinated (Figure 3F). Taken together, these results indicate that USP1 levels are regulated in the cell cycle by transcriptional and posttranslational mechanisms.

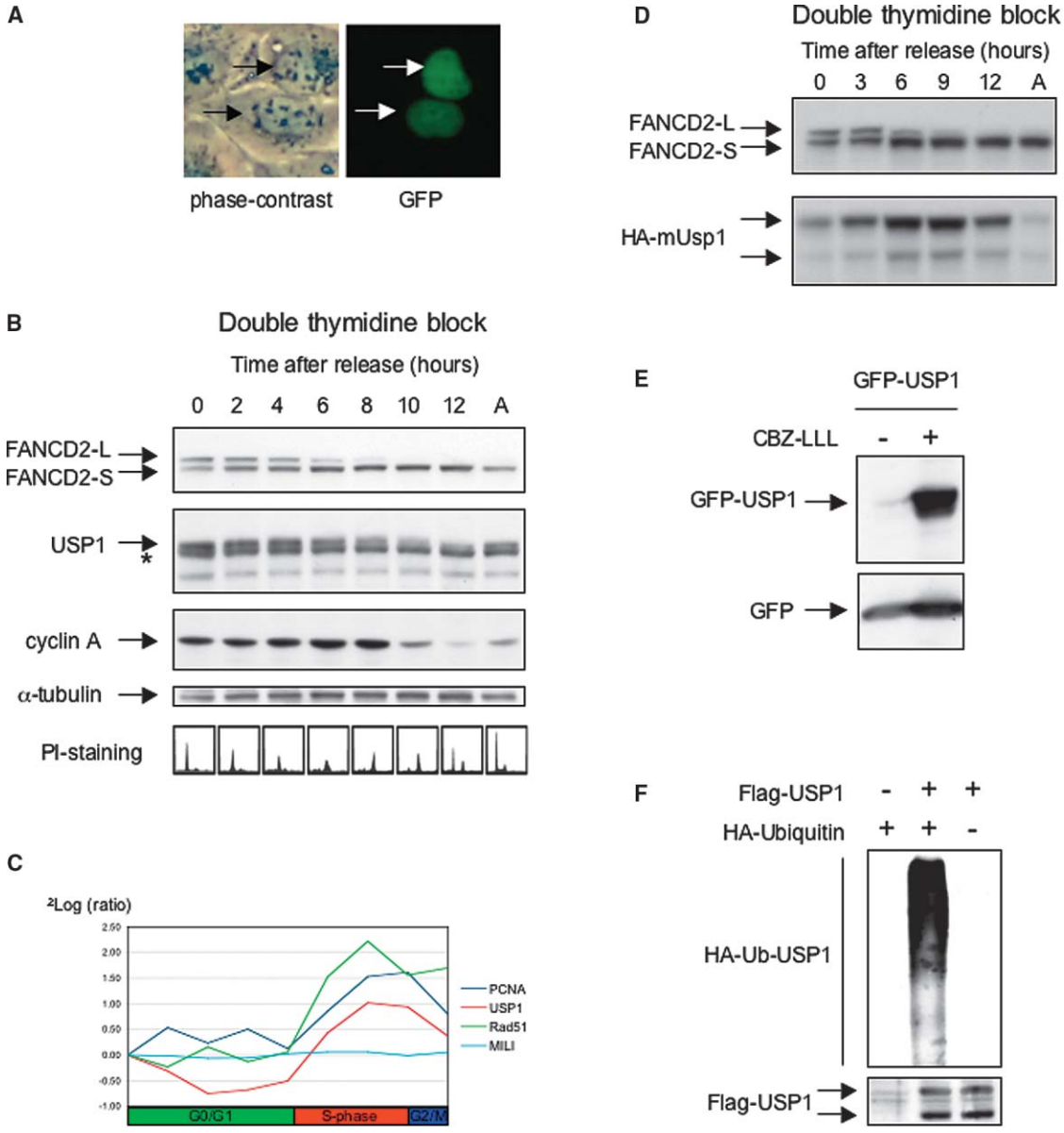

Figure 3. USP1 Is a Cell Cycle-Regulated Nuclear Protein

(A) U2-OS cells were transfected with GFP-tagged USP1, and 24 hr later, phase contrast and fluorescence (GFP) pictures were taken.

(B) HeLa cells were synchronized with a double-thymidine block and released. Samples were taken as indicated and analyzed by Western blot and FACS (PI staining, the asterisk indicates a nonspecific background band).

(C) Serum-starved Rat1 cells were stimulated by adding back 10% fetal calf serum. Samples for FACS and microarray analysis were taken at different time points. Indicated are the cell cycle phases and the ^{2}log relative mRNA levels of the cell cycle-regulated genes proliferating cell nuclear antigen (PCNA), Rad51, and USP1 and the cell cycle-unregulated MIWI-like gene MILI.

(D) HEK293 cells were transfected with HA-tagged murine Usp1 (HA-mUsp1) and synchronized by double-thymidine block. After release, cell extracts were prepared at the indicated time points and analyzed with a FANCD2 or HA antibody.

(E) HEK293 cells were transfected with GFP-USP1 and, 24 hr later, treated overnight with CBZ-LLL (10 uM). GFP served as transfection control. Whole-cell lysates were immunoblotted with a GFP antibody.

(F) HEK293 cells were transfected as indicated and treated overnight with CBZ-LLL (10 uM). Cells were lysed, and Flag-tagged USP1 was immunoprecipitated. Flag-USP1 present in the whole-cell lysates was detected with an anti-Flag antibody (lower panel). Ubiquitinated USP1 was visualized with an HA-specific antibody (upper panel).

USP1 Localizes in Chromatin and Associates with FANCD2

Monoubiquitination of FANCD2 is critical for its localization to chromatin after DNA damage (Taniguchi et al., 2002b; Wang et al., 2004). It is likely that this posttranslational modification on FANCD2 functions as a targeting signal, tethering it to nuclear DNA damage foci. Based on this model one would expect that the enzyme that deubiquitinates and thereby releases FANCD2 from DNA is either constitutively localized to chromatin or recruited to chromatin upon DNA damage. To study the subnuclear localization of USP1, cell fractionation

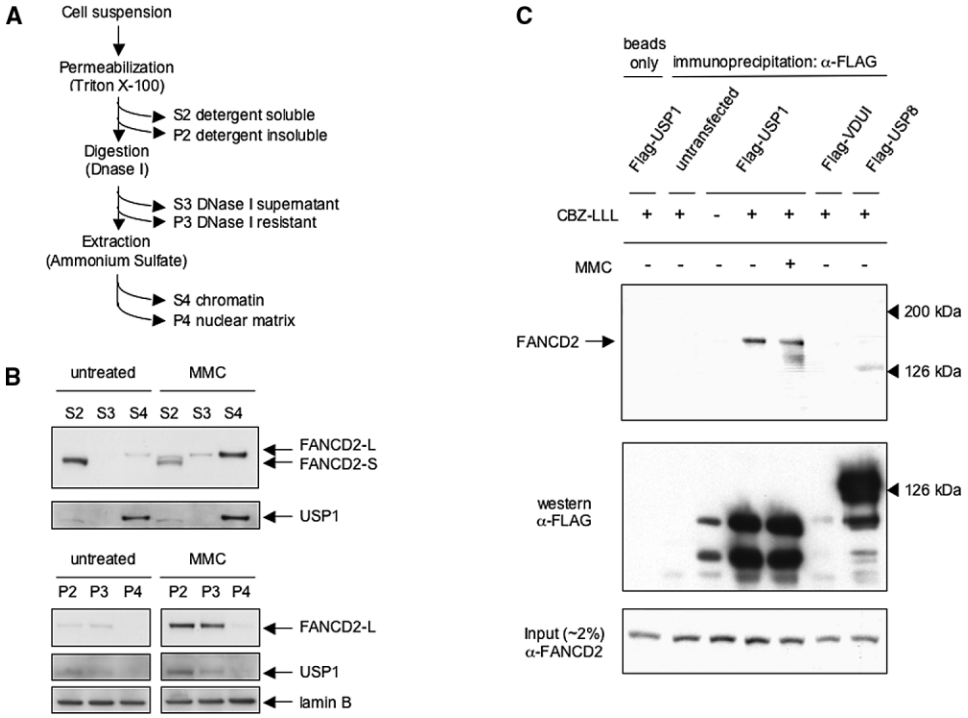

Figure 4. USP1 Localizes to Chromatin and Binds to FANCD2

(A) Schematic overview of nuclear fractionation of cells. Briefly, cytoplasm and nucleoplasm were extracted by permeabilization with detergent, the resulting nuclei were DNase I digested, and chromatin was extracted with ammonium sulfate.

(B) HeLa cells were treated overnight with MMC (160 ng/ml) or left untreated and subsequently fractionated as indicated. Supernatants (S) and pellets (P) were subjected to Western blot analysis with the indicated antibodies. Lamin B was used as a nuclear matrix (P4) loading control.

(C) HEK293 cells were transfected as indicated. 24 hr after transfection, the cells were left untreated, treated with CBZ-LLL (10 uM), or a combination of CBZ-LLL (10 uM) and MMC (50 ng/ml). The next day, cells were lysed in ELB, and Flag-tagged proteins were immunoprecipitated using a Flag antibody. Coimmunoprecipitated FANCD2 was visualized with a specific antibody (upper panel). The blot was subsequently reprobed with a Flag antibody to visualize the amount of immunoprecipitated Flag-tagged proteins (middle panel). Whole-cell lysates (input) were Western blotted for FANCD2 (lower panel).

experiments were performed (see schematic diagram, Figure 4A) (Wang et al., 2004). As expected, FANCD2-L was found primarily in chromatin fractions after MMC treatment (Figure 4B, compare S2 with S4 fraction). In agreement with a possible role for USP1 in the regulation of FANCD2 deubiquitination, endogenous USP1 protein was also found predominantly in the chromatin fraction in both MMC-treated and -untreated cells.

To investigate whether USP1 physically associates with FANCD2, we performed immunoprecipitation experiments. We transfected Flag-USP1 or control Flag-tagged DUBs (i.e., USP8 and VDUI) in HEK293 cells and immunoprecipitated these proteins with a Flag antibody. Because Flag-USP1 is being continuously degraded by the proteasome, the IP was done in the presence of a proteasome inhibitor. Under these conditions, endogenous FANCD2 was coimmunoprecipitated with USP1, suggesting that they interact in vivo (Figure 4C, upper panel). A FANCD2 interaction with the highly expressed Flag-USP8 or with the lower-expressed VDUI could not be detected, indicating that the observed USP1/FANCD2 interaction is specific (Figure 4C, middle and upper panels). Similar results were obtained with GFP-tagged USP1 and with a second, independent FANCD2 antibody (data not shown). Combination of proteasome inhibitor and MMC treatment did not significantly modulate the USP1/FANCD2 interaction. This suggests that DNA damage does not enhance or disrupt the interaction.

USP1 Inhibition Protects Cells from MMC-Induced Chromosomal Aberrations

Next, we asked whether USP1 inhibition, which increases the levels of FANCD2-L, has any functional consequences for DNA repair. To address this, we inhibited USP1 in HEK293 cells by RNAi, induced DNA damage by treating the cells with MMC (10 ng/ml), and analyzed metaphase spreads for chromosomal breakage. Under these conditions, many of the control siRNA (siLacZ)-treated cells displayed at least one chromosomal aberration (Table 1, column 4). Recent studies have suggested a critical upstream role for ATR in activating FANCD2 monoubiquitination. In agreement with this, the number of aberrations per cell and the number of cells with at least one triradial chromosome (a FA hallmark) was increased by siRNA-mediated inhibition of this DNA damage checkpoint kinase (Table 1, columns 3–5) (Andreassen et al., 2004). In contrast, inhibition of USP1 appeared to provide relative resistance against this type of DNA damage-induced aberrations in three independent experiments. Compared to control samples, the number of chromosomal aberrations per cell induced

Table 1. USP1 Inhibition Protects Cells from MMC-Induced Chromosomal Aberrations

Transfection	Treatment	Total Break Events per Cell	Cells with at Least One Triradial (n = 50)	Fraction of Cells with One Triradial (Control = 1)
siLacZ	−	0.09[a]	0	NA
siUSP1	−	0.16[a]	2	NA
siATR	−	0.21	0	NA
siLacZ	MMC	1.52[b] ± 0.47	19 ± 4	1
siUSP1	MMC	0.61[b] ± 0.28	10 ± 2	0.52 ± 0.02
siATR	MMC	3.54 ± 0.21	28 ± 11	1.42 ± 0.18

Table shows the total number of break events per cell and the number of cells with at least one triradial (displayed as an absolute number and as a normalized fraction where the value of the lacZ control is arbitrarily set as 1). Indicated are the mean number values from three independent experiments in which 50 metaphases were analyzed. Standard error values are shown. Cells were treated with 10 ng/ml MMC or left untreated (see Experimental Procedures).

[a] 2-sample chi-square test siUSP1 versus siLacZ $p > 0.3$.

[b] 2-sample chi-square test siUSP1 versus siLacZ $p < 0.0001$.

by MMC treatment was inhibited approximately by 50% when cells were treated with an siRNA targeting USP1 (Table 1, column 3). Also the number of cells displaying at least one triradial chromosome was lower when comparing USP1 knockdown cells to the control cells (Table 1, columns 4–5). This suggests that under these conditions, reduced levels of USP1 can protect cells from crosslinker-induced DNA damage.

Discussion

Using a gene family RNAi library, we have identified the deubiquitinating enzyme USP1 as a novel component of the Fanconi anemia pathway. We have found that USP1 inhibition increases the level of monoubiquitinated FANCD2 and protects cells against certain types of DNA damage. Furthermore, coimmunoprecipitation and cofractionation of endogenous USP1 and FANCD2 in chromatin after DNA damage suggest a direct role for USP1 in deubiquitination of FANCD2. However, we cannot exclude the possibility that USP1 has other substrates or that the observed effects are indirect. In addition, since DUBs have poor substrate specificity in vitro, it is difficult to obtain direct evidence for deubiquitination of FANCD2 by USP1 (Mason et al., 2004).

Inhibition of USP1 has allowed us to uncouple DNA damage or S phase arrest from the induction of FANCD2 monoubiquitination. Because we did not observe a significant defect in cell cycle progression of USP1 knockdown cells, FANCD2 monoubiquitination does not appear to be sufficient for activation of a cell cycle checkpoint.

Because USP1 levels are relatively constant throughout S phase, it is tempting to speculate on additional mechanisms regulating USP1 activity. USP1 deubiquitinating activity may be activated in late S phase by a yet-undefined posttranslational event(s). The predicted increase in USP1 activity in late S phase may account for (1) the rapid conversion of FANCD2-L to FANCD2-S and (2) the increased degradation of active USP1 by the ubiquitin-proteasome pathway.

Although we cannot exclude the presence of USP1 in FANCD2/BRCA DNA damage foci, we have not observed redistribution of USP1 upon treatment with DNA-crosslinking agents (data not shown). Possibly the dissolving of FANCD2-L foci by deubiquitination does not require massive redistribution of USP1. Furthermore, low levels or absence of USP1 in DNA damage foci during DNA repair processes would be in agreement with a role for USP1 in inhibition and recycling of FANCD2. The mechanisms regulating USP1 activity are a current focus of study.

The suggestion that USP1 knockdown has a protective effect against chromosomal aberrations induced by the DNA crosslinking agent MMC indicates that USP1 functions to inhibit or switch off FANCD2-L-mediated DNA repair. Furthermore, it may suggest that FANCD2-L availability—under these conditions—is a rate-limiting step in homologous recombination following high levels of DNA damage. However, in this context, it should be noted that the experimental conditions of MMC-breakage assays in general may not necessarily reflect a frequently occurring physiological situation. Therefore, from an evolutionary perspective, USP1 expression levels may not be optimal for dealing with this high level of DNA damage. Instead, USP1 levels may be more tuned to deal with low levels of DNA damage encountered during normal DNA replication. In addition, it should be noted that the constitutive presence of monoubiquitinated FANCD2 may lead to inappropriate DNA repair events not measured in our assays. For example, the inability to deubiquitinate FANCD2 may lead to homologous recombination in the absence of DNA damage, possibly leading to chromosomal instability. Thus, the inability to turn off or reset the FA pathway after the repair of specific DNA damage sites may have overall-deleterious effects on genome integrity or cause increased mutation frequency. Indeed, besides a defect in DSB repair by homologous recombination (HR) resulting in large deletions, FA cells display lower levels of mutational repair compared to normal cells (Papadopoulo et al., 1990; Sonoda et al., 2003). A functional link between error-prone translesion synthesis (TLS) and HR is also suggested by the observation that chicken DT40 cells deficient for Rev3, the TLS-associated DNA polymerase, display increased levels of sister-chromatid exchange and chromosomal breaks (Sonoda et al., 2003). Therefore, persistent FANCD2 monoubiquitination may lead to enhanced error-prone repair, perhaps through the heightened activity of translesion synthesis (TLS) polymerases (Niedzwiedz et al., 2004). Therefore, USP1 may also function to limit mutagenesis by its regulated

turn off of error-prone DNA repair processes. In conclusion, USP1 is a novel player in the DNA repair network by limiting FANCD2 activity and may play a critical role in the control of homologous recombination by the FA/BRCA pathway.

Experimental Procedures

Materials, Antibodies, and Plasmids

The generation of the DUB knockdown library has been described elsewhere (Brummelkamp et al., 2003). In short, four annealed sets of oligonucleotides encoding short hairpin transcripts corresponding to one DUB enzyme (see Table S1) were cloned individually into pSUPER (Brummelkamp et al., 2002b). The retroviral vector targeting USP1 (sequence C) was generated by ligating an EcoRI/XhoI-digested pSUPER fragment in pMSCV as described (Brummelkamp et al., 2002a). Human Flag- and GFP-tagged USP1 were generated by PCR amplification of Image clone 6473568 containing full-length USP1 and cloned into pEGFP (clontech) or a modified pcDNA3.1 plasmid containing a 5′ sequence coding for the Flag epitope (pVLAG). To generate human full-length. Flag-tagged VDUI, PCR-amplified Image clone 3874822 (a 3′-truncated fragment) was ligated in pVLAG together with a 3′ fragment acquired from a human cDNA library. Human Flag-tagged USP8 was cloned by ligating a BamHI-digested fragment in pVLAG. Mouse Usp1 was cloned by RT-PCR from NIH 3T3 cells and subsequently subcloned into an HA vector. The synthetic siRNA oligonucleotide sequence targeting human USP1 is 5′-TCGGCAATACTTGCTATCTTA-3′. Anti-FANCD2 (FI17), GFP (FL), HA-tag (Y-11), and lamin B (M-20) antibodies were acquired from Santa Cruz. Antibodies recognizing cyclin A (Ab-3) and α-tubulin were purchased from Oncogene research products and Calbiochem, respectively. Rabbit polyclonal Anti-USP1 antibody was generated by using an aminoterminal epitope corresponding to TDSQENEEKASEYRASEIC (Biosource). CBZ-LLL, mitomycin C, propidium iodide, thymidine, and hydroxyurea were purchased from Sigma.

Cell Cultures, G1/S Cell Synchronization, Transfections, and Chromatin Isolation

All cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. High-efficiency electroporation of U2-OS cells and FACS analysis was done as described (Agami and Bernards, 2000). Double-thymidine cell cycle block was done according to (Taniguchi et al., 2002a). SiRNA transfections were done using Lipofectamine 2000 (Invitrogen) (Andreassen et al., 2004). Chromatin fractions were isolated as described (Wang et al., 2004).

Immunoblotting and Immunoprecipitation

Western blots were performed with whole-cell extracts, separated on 7%–12% SDS-PAGE gels, Nupage 3%–12% Tris-Acetate, or 4%–12% Bis-Tris gradient gels (Invitrogen) and transferred to polyvinylidine difluoride membranes (Millipore). Western blots were probed with the indicated antibodies. For immunoprecipitation, HEK293 cells were transfected by calcium phosphate precipitation with the indicated plasmids; 48–72 hr after transfection, cells were lysed in ELB buffer (0.25 M NaCl, 0.1% NP-40, 50 mM HEPES [pH 7.3]) supplemented with "complete" protease inhibitors (Roche), and protein complexes were immunoprecipitated with 2 μg of the indicated antibody conjugated to protein G sepharose beads.

Chromosome Breakage Analysis

Chromosome breakage analysis was performed by the Cytogenetics Core Facility of the Dana-Farber Cancer Institute (Andreassen et al., 2004). For the analysis of HEK293 cells transfected with various siRNAs, cells were plated into T25 flasks 24 hr after transfection. Beginning the next day, cells were treated with 10 ng/ml MMC for 2 days. After treatment, cells were exposed to colcemid for 12 hr, swollen with 75 mM KCl, and fixed with 3:1 methanol/acetic acid. Slides were then stained with Wrights' stain, and a minimum of 50 metaphases were scored per treatment sample and averaged from three independent experiments using a blinded approach.

Microarray Analysis

For detailed information on the 15K cDNA microarrays used, visit http://microarrays.nki.nl. Rat 1A cells were serum starved in low-serum medium (DMEM, 0.01% FCS) and stimulated to reenter the cell cycle by adding back 10% serum. Time points were taken for RNA-extraction and FACS analysis. Total RNA was linear amplified by using the CMF-T7-RNA amplification method. Amplified antisense cRNAs were random prime-labeled with Superscript II reverse transcriptase (Invitrogen) to incorporate dUTP-Cy3 or dUTP-Cy5 (Amersham). The labeled nucleic acid molecules were dissolved in a hybridization mixture containing 5×SSC, 25% formamide, and 0.1% SDS, as well as blocking reagents (human cotDNA [Invitrogen], poly-dA [Pharmacia], and tRNA [Roche]) and hybridized to the array at 42°C for 18 hr. After subsequent washing and drying, the slides were scanned in the Agilent DNA microarray scanner (G2505B). Extended protocols can be found at http://microarrays.nki.nl/download/protocols.html. Two-dye swap pairs of hybridizations were performed as well as self-self experiments. The raw data were normalized and ^{2}log transformed using the Central Microarray Facility database software (http://cmfdb.nki.nl).

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