The Deubiquitinating Enzyme USP17 Is Highly Expressed in Tumor Biopsies, Is Cell Cycle Regulated, and Is Required for G1-S Progression

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Abstract

Ubiquitination is a reversible posttranslational modification that is essential for cell cycle control, and it is becoming increasingly clear that the removal of ubiquitin from proteins by deubiquitinating enzymes (DUB) is equally important. In this study, we have identified high levels of the DUB USP17 in several tumor-derived cell lines and primary lung, colon, esophagus, and cervix tumor biopsies. We also report that USP17 is tightly regulated during the cell cycle in all the cells examined, being abundantly evident in G1 and absent in S phase. Moreover, regulated USP17 expression was necessary for cell cycle progression because its depletion significantly impaired G1-S transition and blocked cell proliferation. Previously, we have shown that USP17 regulates the intracellular translocation and activation of the GTPase Ras by controlling Ras-converting enzyme 1 (RCE1) activation. RCE1 also regulates the processing of other proteins with a CAAX motif, including Rho family GTPases. We now show that USP17 depletion blocks Ras and RhoA localization and activation. Moreover, our results confirm that USP17-depleted cells have constitutively elevated levels of the cyclin-dependent kinase inhibitors p21<sup>cip1</sup> and p27<sup<kip1>, known downstream targets of Ras and RhoA signaling. These observations clearly show that USP17 is tightly regulated during cell division and that its expression is necessary to coordinate cell cycle progression, and thus, it may be considered a promising novel cancer therapeutic target.

Cancer Res; 70(8); 3329–39. ©2010 AACR.

Introduction

Ubiquitination is an important mechanism for regulating cell cycle progression. Numerous components of the cell cycle machinery, including various cyclins and cyclin-dependent kinase inhibitors (CDKI), are modified by ubiquitination (1). Ubiquitination is relatively well characterized and is mediated by either the E3 ligases SKP1-CUL-F-box complex or the anaphase-promoting complex/cyclosome at distinct stages of the cell cycle (2). Similar to other covalent modifications, such as phosphorylation or methylation, ubiquitination is a reversible posttranslational modification. Deubiquitinating enzymes (DUBs) catalyze the removal of ubiquitin by hydrolyzing the isopeptide bond linking ubiquitin to its substrate. Accumulating evidence suggests that DUBs also play an important role in the cell cycle as they help maintain the dynamic balance between ubiquitination and deubiquitination (3, 4).

Recently, several DUBs have been identified that regulate the cell cycle and control mitotic and spindle assembly checkpoints. For instance, anaphase initiation is facilitated by USP44, which regulates mitosis by deubiquitinating cdc20 (5). In addition, mitotic entry is facilitated by the gene responsible for cylindromatosis (CYLD), which modulates polo-like kinase 1 (Plk1) activity (6). Moreover, chromosomal alignment and segregation during mitosis is also regulated by the deubiquitination of survivin by USP9 (7).

DNA damage checkpoints are regulated by USP1 and USP7. USP1 catalyzes the deubiquitination of two important components of DNA damage repair pathways: FANCD2 and proliferating cell nuclear antigen (PCNA; refs. 8, 9). USP7 (HAUSP) deubiquitinates Mdm2 and Mdmx, which alters the stability and activity of p53 (10). DUBs can also modulate chromatin structure, thus altering gene transcription in a cell cycle–specific manner. Histone H2A is deubiquitinated by USP16 during mitosis and enables histone phosphorylation and chromosomal segregation (11). Additionally, USP3 catalyzes the removal of ubiquitin from histones in a cell cycle–dependent manner and is essential to promote S-phase entry (12).

The importance of DUBs for cell cycle control has been underscored by the strong association with tumorigenesis. Mutations in CYLD have been associated with cylindromatosis, Brooke-Spiegler syndrome, and familial trichoepithelioma,
all of which are predisposed to multiple skin tumors of the head and neck (13). Additionally, downregulation of USP7 has been linked with non–small cell lung carcinoma, and in conjunction with p53 status, USP7 was shown to be a prognostic marker for adenocarcinoma patients (14).

The DUB/USP17 family of cytokine-inducible DUBs was initially identified in murine hematopoietic cells. There are several murine family members (DUB-1, DUB-1A, DUB-2, and DUB-2A) and a human family member (DUB-3/USP17, subsequently referred to as USP17). USP17 family members are immediate-early genes, which are rapidly induced following cytokine stimulation (15–18). Several lines of evidence have implicated these enzymes in the regulation of cell growth and survival. Constitutive expression of murine DUB-1 has been shown to induce G1, cell cycle arrest, whereas DUB-2 expression can markedly inhibit apoptosis following cytokine withdrawal (15, 17, 19, 20). In addition, we have confirmed that constitutive overexpression of human USP17 blocks growth factor–dependent cell proliferation (20). More recently, we have also shown that USP17 regulates the activity of the GSTPase CAAX-box processing enzyme Ras-converting enzyme 1 (RCE1) to alter Ras processing and membrane localization, and confirmed that the ability of USP17 to block cell proliferation was RCE1 dependent (21).

In this study, we report high levels of USP17 expression in many primary tumor biopsies and tumor-derived cell lines. In addition, we find that USP17 expression is tightly regulated during the cell cycle and plays a fundamental role in mediating cell cycle progression. USP17 depletion markedly inhibits cell proliferation and prevents the passage of cells from G1 into S phase. We report that USP17 depletion blocks the translocation and proper activation of Ras and RhoA in G1, resulting in the accumulation of the CDKIs p21cip1 and p27kip1, which we suggest is the underlying cause of the G1 block. Our findings indicate that tight regulation of USP17 expression is essential for G1-S transition and that targeting USP17 could be a novel anticancer approach.

Materials and Methods

Immunohistochemistry. Immunohistochemical staining of tissue microarray (A301, Stretton Scientific, http://www.strettonscientific.co.uk) was done using 2 μg/mL of USP17 monoclonal antibody.

Cell culture, transfections, and synchronization. HeLa and MCF-7 cells were grown in 10% FCS, 1% penicillin/streptomycin, and 1% l-glutamine (DMEM) and maintained in a 37°C, 5% CO2 incubator. Cells were transfected with 3 μg DNA using FuGENE6 (Roche). The pSUPER-USP17shRNA constructs were a kind gift from Dr. Rene Medema (University Medical Centre, Utrecht, the Netherlands; shRNA#1, GCAGGAAAGATGCCCATGAA). The pRS-USP17shRNA (shRNA#2, GATGATTGTTGCTCTCGTGGCAAGACGCT) was from Origene Technologies. Cells were synchronized at G1-S by double thymidine block as described previously (22).

Propidium iodide staining and analysis by fluorescence-activated cell sorting. Cells were trypsinized, fixed in ethanol for 1 h at 4°C, stained with propidium iodide (PI) solution (10 μg/mL) with RNase A (250 μg/mL), incubated at 37°C for 30 min, and analyzed by FACSCanto II (BD Biosciences) and FlowJo.

Immunoblotting and immunoprecipitation. Cells were lysed in radioimmunoprecipitation assay buffer, and whole-cell lysates or immunoprecipitates were prepared as described previously (21). Antibodies were as follows: anti-USP17 (Fusion Antibodies); anti–pan-Ras, anti–extracellular signal-regulated kinase (ERK) 1/2, anti–phospho-ERK1/2, anti–mitogen-activated protein kinase (MAPK)/ERK kinase (MEK) 1/2, and anti–phospho-MEK1/2 (Cell Signaling); anti-RhoA, anti–cyclin D1, cyclin A, and cyclin B1 (Santa Cruz Biotechnology); anti-p21cip1 (Upstate Antibodies); and anti-p27kip1 (Calbiochem). Densitometry was carried out using ImageJ software (NIH).

Confocal microscopy. Cells were fixed with 10% trichloroacetic acid (TCA) as described previously (23). Ras and RhoA were stained according to antibody manufacturer’s instructions and detected with tetramethylrhodamine isothiocyanate (TRITC)–conjugated donkey anti-mouse (Jackson ImmunoResearch). Nuclei were counterstained with 300 μmol/L 4’,6-diamidino-2-phenylindole (DAPI; Molecular Probes). Slides were mounted using Vectashield (Vector Laboratories) and then viewed with LSM 510 META NLO confocal microscope (Zeiss).

GTPase activation assays. Ras and RhoA activation was determined by pull-downs using glutathione S-transferase (GST)–Raf–RBD and GST–Rhotekin, respectively, as described previously (21, 24).

RNA extraction and reverse transcription–PCR. RNA was extracted using STAT-60 according to the manufacturer’s instructions (Biogenes). Reverse transcription–PCR (RT-PCR) was performed on 1 μg of total RNA using OneStep RT-PCR kit (Qiagen) as described previously (25). The following primers were used: USP17, 5’-CAGTGATTCGTTGCGATGTGAGACCATGCTAC-3’ (forward) and 5’-AGTCATCGATCTGCGACACAAAGCATGCCCT-3’ (reverse); β-actin, 5’-GGACCTTCGAGCAAGAGATGG-3’ (forward) and 5’-AGCAGTGTTGGCCGTACAG-3’ (reverse).

Results

USP17 is highly expressed in tumor cells. Previous reports have linked USP17 with proliferation and survival, where constitutive USP17 overexpression in NIH3T3, mouse embryonic fibroblast, and bone marrow–derived pro–B-cell lines inhibited proliferation (20, 21). However, USP17 is also expressed at a high level in various hematopoietic tumor–derived cells (20). To further examine these apparently contradictory findings, USP17 expression was assessed in numerous cell lines derived from various solid tumors, including prostate (PC3 and DU145), breast (MDA-MB-231, MDA-MB-157, BT474, and T47D), and lung (A549, H1299, and H460; Fig. 1A and B; data not shown). USP17 mRNA and protein were clearly evident in all tumor-derived cell lines examined. The highest USP17 expression was observed in the PC3 prostate adenocarcinoma cell line, although high USP17 expression was also evident in the breast cancer cell lines.
derived from medullary (MDA-MB-231 and MDA-MB-157) and ductal (BT474 and T47D) carcinomas. To determine whether the expression of USP17 in cell lines was representative of USP17 levels in clinical samples, USP17 was also examined in primary tumor tissue. Consistent with our observations of the tumor-derived cell lines, examination of the intensity and distribution of USP17 revealed positive staining in colorectal adenocarcinoma and squamous cell carcinomas of the lung, cervix, and esophagus in comparison with corresponding normal tissue. The brown color represents USP17 protein expression and the blue (hematoxylin) counterstain shows nuclei. Magnifications, ×20 and ×40 (as specified).

**Figure 1.** USP17 is highly expressed in tumor cells. A, USP17 mRNA expression assessed by RT-PCR in various tumor-derived cell lines (PC3, DU145, MDA-MB-231, MDA-MB-157, BT474, and T47D). B, USP17 protein expression was determined by immunoprecipitation (IP) followed by immunoblotting (WB) for USP17. β-Actin was used as a loading control. C, Immunohistochemical staining of USP17 in colon adenocarcinoma and squamous cell carcinomas of the lung, cervix, and esophagus in comparison with corresponding normal tissue. The brown color represents USP17 protein expression and the blue (hematoxylin) counterstain shows nuclei. Magnifications, ×20 and ×40 (as specified).

these observations clearly confirmed that tumor cells expressed elevated levels of USP17, suggesting that it may play a role in tumor progression. Clearly, the initial observations suggested that USP17 was expressed in multiple tumors and was particularly evident in rapidly dividing tumor-derived cell lines. By contrast, however, it has previously been shown that constitutive overexpression of USP17 blocked proliferation (20). A possible explanation for these apparently contradictory observations could be that USP17 expression may be modulated during cell division in a similar manner to core components of the cell cycle machinery, including cyclins and CDKIs (26).

**USP17 expression is cell cycle regulated.** To examine USP17 expression during each cell cycle phase, HeLa cells were synchronized in G1 by double thymidine block and
the synchronous cell cycle progression was verified by PI staining (Fig. 2A, left). Following release from the double thymidine block, >90% of the cells had consistently entered S phase within 4 hours, successfully undergone mitosis by 10 hours, and completed the cell cycle within 12 hours. Consistent with the fluorescence-activated cell sorting (FACS) analysis, the elevated expression of cyclins E and A within 4 hours and increased cyclin B1 levels after 6 hours verified the synchronicity of cells in G1-S and G2-M, respectively (Fig. 2, right). USP17 expression was examined at each cell cycle

Figure 2. USP17 expression is cell cycle regulated. A, left, HeLa cells were synchronized in G1 by double thymidine block and cell cycle was assessed by PI staining and FACS analysis; right, cyclins E, A, and B1 were immunoblotted at indicated times following thymidine release. B, USP17 mRNA expression was assessed by RT-PCR (left) and USP17 protein was determined by immunoprecipitation followed by immunoblotting at indicated times following thymidine release (right). C, USP17 was stained using a TRITC-conjugated antibody (red staining and white arrows) and the nuclei were counterstained with DAPI (blue) at indicated times following thymidine release. D, MCF-7 cells were synchronized by double thymidine block and cell cycle was assessed by PI staining and FACS analysis (left) and USP17 mRNA expression was determined by RT-PCR (right) at indicated times following thymidine release.
phase and found to be exquisitely regulated (Fig. 2B). USP17 mRNA was not expressed in G1 (0 hour) but was abundant 2 hours after thymidine release as the cells entered the DNA synthesis phase. However, we consistently found negligible USP17 mRNA following 4-hour release from thymidine when >90% of the cells were in S phase (Fig. 2B). USP17 mRNA was also expressed on entry into G2-M and mitosis. Immunoprecipitation and Western blotting confirmed that USP17 protein levels reflected the tightly regulated expression of USP17 mRNA levels during cell cycle progression (Fig. 2B, right). This trend was also verified by confocal microscopy. Negligible levels of USP17 were consistently observed in G1 (Fig. 2C, left) and S phase (Fig. 2C, right), but USP17 was highly expressed during the G1-S transition (Fig. 2C, middle). To ensure that these findings were not cell type specific, MCF-7 cells were similarly examined (Fig. 2D, left). Consistent with our observation in HeLa cells, USP17 mRNA substantially increased within 2 hours of thymidine release and was undetectable by 4 hours when cells had entered S phase (Fig. 2D, right). Collectively, these findings clearly showed that USP17 expression was tightly regulated in a cell cycle–specific manner, and this may account for the elevated USP17 levels observed in rapidly dividing tumor cells (Fig. 1). Moreover, these findings indicated that cell cycle phase–specific expression of USP17 could underpin an important role in cell cycle control.

**USP17 silencing inhibits proliferation and impairs G1-S transition.** Cell cycle progression is driven by the oscillating activation of CDKs alongside precisely timed fluctuations in the synthesis and degradation of cyclins (26, 27). As USP17 displayed a similar cyclical expression pattern during cell division, we hypothesized that it also played a part in regulating cell cycle progression. To understand the role of USP17 during cell division and proliferation, we validated short hairpin RNA (shRNA) to silence endogenous USP17. Two independent USP17 shRNAs effectively depleted endogenous USP17 in a range of tumor-derived cell lines (Fig. 3A; data not shown). We next examined the effect of transient USP17 depletion on cell proliferation by MTT (Fig. 3B) and trypan blue exclusion assays (Fig. 3C). Silencing of USP17 had a
profound effect on cell growth in comparison with scrambled shRNA–transfected or mock-transfected cells. USP17 depletion blocked proliferation for up to 72 hours, after which proliferation gradually increased, probably due to transient transfection (Fig. 3B). Similar effects were determined with USP17 shRNA#2 (Supplementary Fig. S1A). The effect of USP17 depletion on cell growth was not due to increased cell death, as a sub-G0 peak was not detected in USP17 shRNA cells. In addition, we did not observe any profound S or G2-M cell cycle block in USP17-silenced cells. However, an increased number of USP17-depleted cells were consistently detected in G1 (62.6%), in comparison with scrambled shRNA cells (53.1%), which suggested that USP17 silencing may have caused cells to accumulate in G1 (Fig. 3D). These findings indicated that USP17 depletion had a significant cytopathic effect on tumor-derived cell lines, as USP17 shRNA markedly inhibited cell proliferation but had a negligible effect on cell death. In addition, these observations also indicated that USP17 had a role in cell cycle regulation, as USP17 silencing caused cells to accumulate in G1.

To further examine this effect, shRNA-transfected cells were synchronized in G1 and the effect on the cell cycle was monitored following thymidine release. We consistently found that USP17 silencing significantly impaired the transition from G1–S (Fig. 4A). Following release from the thymidine block, the vast majority (84%) of scrambled shRNA cells were synchronized in S within 4 hours. In contrast, only 50% of USP17-depleted cells had progressed into S and the remaining 47% had failed to exit G1 (Fig. 4A). The proportion of cells entering S phase were also examined by monitoring bromodeoxyuridine (BrdUrd) incorporation in synchronized cells following thymidine release, and the results of four independent experiments are summarized in Fig. 4B. Consistent with our previous observations (Fig. 2A), the vast majority (50%) of scrambled shRNA cells had incorporated BrdUrd following 4 hours of thymidine release, whereas USP17 depletion significantly delayed the entry into S phase with only 20% of the USP17-silenced cells incorporating BrdUrd over the same time period (Fig. 4B). Impaired G1–S-phase progression was also observed using USP17 shRNA#2 (Supplementary Fig. S1B). These observations clearly showed that USP17 silencing abrogated the passage of cells through G1 into S phase. Our previous observations confirmed negligible USP17 expression when cells exclusively resided in G1 (0 hour) or S (4 hours).
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however, USP17 was highly expressed during the G1-S transition (2 hours; Fig. 2C). Therefore, collectively, these results suggest that it is necessary to induce USP17 expression to enable cells to exit G1 and enter S phase.

**shRNA knockdown of USP17 impairs GTPase activation during the cell cycle.** We have previously documented that USP17 regulates the activity and intracellular localization of the GTPase Ras (21). Ras and Rho family GTPases play a major role in promoting G1-S progression through their modulation of cyclin and CDKI levels (28, 29). Because USP17 expression was also important for G1-S transition, we investigated whether USP17 silencing was causing G1 arrest by attenuating GTPase function. Ras and RhoA localization in scrambled or USP17 shRNA cells was examined by confocal microscopy in G1 (Fig. 5). Before thymidine release, Ras and RhoA to the plasma membrane was observed in scrambled shRNA cells 5 minutes following thymidine release (Fig. 5A and B, arrows; ref. 30). The distribution of GTPases at the plasma membrane dissipated after 15 minutes, and within 30 minutes of release, Ras and RhoA resided predominantly in the cytoplasm. A corresponding translation was not observed in the USP17-depleted cells; Ras and RhoA failed to relocalize to the plasma membrane following the thymidine release and were dispersed evenly throughout the cytoplasm. Membrane localization of GTPases is frequently used as a surrogate marker of activation; therefore, the lack of RhoA and Ras membrane association suggested attenuated GTPase activation in USP17-silenced cells. Ras and Rac activity in G1 were directly examined using GST-Rhotekin and GST-Raf pull-down assays, respectively (Fig. 5C and D). As expected, a significant induction of RhoA (Fig. 5C) and Rac (Fig. 5D) activation was observed in the scrambled shRNA cells immediately (5 minutes) following thymidine release. Consistent with the localization data, RhoA and Rac activation persisted for 15 minutes following thymidine release and diminished within 30 minutes. In comparison, USP17-silenenced cells were unable to regulate the activation of RhoA and Rac. Constitutive activation of RhoA and Rac was consistently observed in USP17-depleted cells, and yet, these GTPases failed to translocate to the plasma membrane (Fig. 5C and D). In agreement with our previous reports, these data show that USP17 is essential for coordinating the localization and activation of GTPases. Compellingly, these findings showed that USP17 silencing abrogates the rapid activation and plasma membrane localization of RhoA and Rac, following thymidine release, which are essential for driving G1-S progression and are likely to be the underlying cause of impaired cell cycle progression.

**Depletion of USP17 stabilizes the levels of the CDKIs p21cip1 and p27kip1.** It is well established that GTPase activation controls G1-S progression by regulating transcription, translation, and degradation of numerous proteins (28, 29). Our observations suggested that USP17 regulated GTPase localization and activation in a cell cycle–dependent manner (Fig. 5). Therefore, the effects of USP17 silencing were assessed by examining the effect on downstream effectors of GTPases in G1. Ras and RhoA activation stimulates the sustained ERK activation, which is necessary to drive cyclin D1 transcription in G1 (31, 32). We observed a corresponding activation of MEK and ERK in scrambled shRNA cells 1 hour following release from thymidine block, and this activation was sustained for 4 hours to drive entry into S. However, both MEK and ERK activation were markedly blunted in USP17-depleted cells (Fig. 6, top). This further supports our previous findings that USP17 depletion is associated with mislocalization and improper activation of GTPases, and that this in turn inhibits the downstream activation of MEK and ERK. The other major targets of GTPase signaling in G1 are the CDKIs p21cip1 and p27kip1, which are downregulated in response to GTPase activation (31, 33–35). CDKIs function as negative regulators of cell cycle progression by modulating the activities of cyclin-CDK complexes. Ras and RhoA stimulate the degradation of p27kip1 through the activation of cyclin E/CDK2, whereas Ras promotes the degradation of p27kip1 through the ERK/MAPK pathway (36, 37). The levels of p21cip1 are negatively affected by Ras and RhoA, which culminates in the repression of p21cip1 and increased protein turnover (31, 33, 35). These CDKIs have a potent inhibitory effect on the CDKs CDK2 and CDK4, which drive G1-S cell cycle progression. The levels of both p21cip1 and p27kip1 are downregulated in scrambled shRNA cells immediately following thymidine release to relieve the inhibition on the cyclin/CDK complexes and thus facilitate G1-S passage (Fig. 6). However, constitutively elevated levels of p21cip1 and p27kip1 are consistently observed in USP17-depleted cells (Fig. 6). It is widely accepted that elevated levels of p21cip1 and p27kip1 induce G1 arrest; therefore, it is highly likely that the impaired G1-S transition noted in USP17-depleted cells occurs as a result of derepression of p21cip1 and p27kip1 levels (31, 38). Collectively, these findings show that USP17 silencing inhibits signaling pathways that are integral to G1-S progression, resulting in constitutively high levels of p21cip1 and p27kip1 that block cell cycle progression.

**Discussion**

It is becoming increasingly apparent that cell cycle progression and proliferation are regulated by the dynamic balance between ubiquitination and deubiquitination (1, 2, 4). Much attention has been paid to ubiquitin-mediated cell cycle control and the role of DUBs (1, 4). In this study, we reveal that USP17 expression is tightly regulated during cell cycle progression and that temporal expression of this DUB is essential for cell division. We show that induction of USP17 expression in G1 is necessary for progression into S phase and that the G1 block observed in USP17-silenced cells occurs as a result of derepression of p21cip1 and p27kip1 levels (31, 38). Collectively, these findings show that USP17 silencing inhibits signaling pathways that are integral to G1-S progression, resulting in constitutively high levels of p21cip1 and p27kip1 that block cell cycle progression.
tumor-derived cell lines with the fact that constitutive over-expression blocks proliferation (20). Cell cycle progression is characterized by oscillating levels of numerous proteins involved in regulating cell cycle progression, such as cyclins, CDKIs, Plk1, securin, and Aurora A/B; as their levels are intimately regulated by ubiquitination, we wondered whether USP17 played a role in cell cycle control (2, 39–41). Consistent with this hypothesis, USP17 transcription and protein...
expression fluctuated significantly between each phase of the cell cycle, similarly to other cell cycle regulators. The tightly regulated expression of USP17 in a cell cycle–dependent manner is similar to other DUBs that have previously been implicated in cell cycle control. USP1 expression is induced in S phase and enables the deubiquitination of two important components of DNA damage repair pathways, FANCD2 and PCNA (8, 9), whereas DUBs with recognized roles in mitotic spindle assembly checkpoints, such as CYLD, USP9, and USP44, are also temporally expressed during cell division and levels are found to peak during mitosis (5–8).

Although the DUB/USP17 family has previously been implicated in proliferation, the mechanism underpinning USP17 control of cell cycle progression was unclear. Recent studies identified RCE1 as a substrate for USP17 and confirmed that RCE1 activity was regulated by deubiquitination, and more significantly, the ability of USP17 to block proliferation was completely dependent on RCE1 (21). In addition to Ras, RCE1 is also responsible for the posttranslational modification of numerous proteins that contain a CAAX motif, including the Rho family of GTPases. The role of Ras and Rho family GTPases in G1 progression has been comprehensively investigated and shown to modulate the levels of cyclins A, E, and D1 and the CDKIs p21^{cip1} and p27^{kip1} by distinct mechanisms (28, 29, 33, 34). Signals from Ras and RhoA interact to regulate the levels of p21^{kip1} and p27^{kip1} through the modulation of their transcription, translation, and degradation (34, 42). Moderate levels of Ras and Raf-MEK-ERK signaling increase transcription and stabilization of p21^{kip1} protein levels to promote cell cycle progression through its interaction with cyclin D1 (31, 35, 43). Cross talk between Ras and RhoA leads to downregulation of p21^{kip1} transcription, which is necessary for G1–S progression (24, 31). Ras and RhoA activation also promotes cell cycle progression by downregulating p27^{kip1} levels through enhanced degradation and decreased protein synthesis (34, 42).

Our findings suggest that the improper localization and activation of Ras and RhoA in the USP17 silenced cells could be responsible for the constitutively high levels of the CDKIs p21^{kip1} and p27^{kip1} observed.

The subcellular distribution of GTPases determines their interaction with corresponding guanine nucleotide exchange factors (GEF) and GTPase-activating proteins (GAP) and is critical in determining appropriate GTPase activation. Therefore, it was not surprising that improper GTPase activation was also observed in USP17-depleted cells, which had constitutively high levels of GTP-bound RhoA and Ras. Furthermore, recent advances in the field have identified pools of active Ras that signal from intracellular compartments, including the Golgi and endoplasmic reticulum (44). Indeed, it has been reported that the effectors downstream of ERK1/2 activation are dependent on the subcellular compartment

![Figure 6. Depletion of USP17 stabilizes the levels of the CDKIs p21^{kip1} and p27^{kip1}. HeLa cells transfected with scrambled or USP17 shRNA were synchronized by double thymidine block and lysates were collected at specified times following thymidine release. Cell lysates were immunoblotted with antibodies for phospho-ERK (pERK), ERK, phospho-MEK (pMEK), MEK, p21^{kip1}, and p27^{kip1}. Protein loaded was confirmed by immunoblotting for γ-tubulin. Right, densitometry of MEK and ERK activity and the relative levels of p21^{kip1} and p27^{kip1} standardized to γ-tubulin.](image-url)
from which the Ras signal originates (45). Therefore, it is plausible that constitutively active Ras is signaling from an intracellular compartment in USP17 depleted cells and driving an alternative signaling cascade that impairs cell cycle progression. In support of our hypothesis that improper localization and activation of GTPases causes USP17-depleted cells to accumulate in G1, it has recently been reported that constitutive activation of RhoA delays the entry of cells into S phase. This cell cycle defect was thought to have occurred as a result of suppressed ERK activation that was necessary to drive cyclin D1 expression (46). Consistent with these findings, we also observe attenuated ERK activation in association with constitutive GTPase activation in USP17-silenced cells. Therefore, we propose that USP17 must be required to facilitate proper localization and activation of GTPases in a cell cycle-dependent manner.

To date, overwhelming evidence has attributed the controlled timing of GTPase activation during the cell cycle to a range of complementary GEFs (such as Sos1/2, Dbl, Vav, and ECT2) and GAPs (such as p120 RasGAP and Cyk4). However, it has become increasingly apparent that GTPase processing enzymes are also implicitly involved in determining GTPase activation and consequent function. Indeed, enzymes involved in posttranslational modification of GTPases have been widely investigated as novel therapeutic targets to inhibit Ras and RhoA signaling and block tumor growth and progression. Farnesyltransferase (FT) and geranylgeranyltransferase I (GGT) are responsible for the prenylation of GTPases before CAAX-box cleavage by RCE1. Inhibitors of FT and GGT (FTI and GGTI) have been developed that block prenylation, and, therefore, GTPase activation. Attenuation of GTPase processing using these inhibitors causes cells to arrest in G1 (47, 48). In agreement with our observations in USP17-depleted cells, this was thought to occur as a result of improper GTPase activation that leads to elevated p21cip1 and p27kip1 levels (49, 50).

In summary, our findings clearly show that, like other cell cycle-regulated proteins, the expression of USP17 is tightly controlled during cell cycle progression. Additionally, we show that this rapid and transient expression of USP17 is necessary for successful cell cycle progression, as silencing of USP17 expression impairs G1-S transition and blocks proliferation. This would seem to occur through the deregulation of GTPase signaling and stabilization of CDKIs p21cip1 and p27kip1. These findings, coupled with the elevated levels of USP17 in primary tumor biopsies, suggest that this DUB may be an interesting target for cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Rene Medema for constructs and Drs. Shauna Hegarty, Suzanne McFarlane, and Perry Maxwell for their assistance.

Grant Support

Action Cancer and Biotechnology and Biological Sciences Research Council (BB/F013467).

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Received 11/11/2009; revised 01/28/2010; accepted 02/15/2010; published OnlineFirst 04/06/2010.

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Cancer Res 2010;70:3329-3339. Published OnlineFirst April 13, 2010.

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