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The ubiquitin-specific protease USP47 is a novel β-TRCP interactor regulating cell survival

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Abstract

Ubiquitin-specific proteases (USPs) are a subclass of cysteine proteases that catalyze the removal of ubiquitin (either monomeric or chains) from substrates, thus counteracting the activity of E3 ubiquitin ligases. Although the importance of USPs in a multitude of processes, from hereditary cancer to neurodegeneration, is well established, our knowledge on their mode of regulation, substrate specificity and biological function is quite limited. In this study we identify USP47 as a novel interactor of the E3 ubiquitin ligase, Skp1/Cul1/F-box protein β-transducin repeat-containing protein ($SCF^{β\text{-}Trcp}$). We found that both β-Trcp1 and β-Trcp2 bind specifically to USP47, and point mutations in the β-Trcp WD-repeat region completely abolished USP47 binding, indicating an E3-substrate-type interaction. However, unlike canonical β-Trcp substrates, USP47 protein levels were neither affected by silencing of β-Trcp nor modulated in a variety of processes, such as cell-cycle progression, DNA damage checkpoint responses or tumor necrosis factor (TNF) pathway activation. Notably, genetic or siRNA-mediated depletion of USP47 induced accumulation of Cdc25A, decreased cell survival and augmented the cytotoxic effects of anticancer drugs. In conclusion, we showed that USP47, a novel β-Trcp interactor, regulates cell growth and survival, potentially providing a novel target for anticancer therapies.

Keywords

ubiquitin; F-box proteins; β-Trcp; degradation

Introduction

The ubiquitin–proteasome system has a crucial role in regulating various processes, including cell growth, differentiation and apoptosis (Ciechanover and Schwartz, 1998; Melino, 2005; Bernassola *et al.*, 2008). Accordingly, aberrant protein ubiquitylation has been observed in numerous human tumors (Nakayama and Nakayama, 2006), and

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The authors declare no conflict of interest.

proteasome inhibitors are in use for the treatment of multiple myeloma (Orlowski and Kuhn, 2008).

Protein ubiquitylation is a multistep process requiring the sequential action of three enzymes: the ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin ligase (E3) enzyme (Varshavsky, 1997; Hershko and Ciechanover, 1998). The Skp1/Cul1/F-box protein (SCF) complexes, which are intimately involved in the regulation of cell growth and survival, are among the most important and best-understood RING finger-type E3s (Cardozo and Pagano, 2004; Nakayama and Nakayama, 2006; Frescas and Pagano, 2008). These multimeric complexes are composed of four subunits: S-phase kinase-associated protein 1 (Skp1), cullin 1 (Cul1), RING-box 1 (also known as regulator of cullins-1) and a F-box protein (Cardozo and Pagano, 2004). In humans, there are 69 different F-box proteins that provide specificity to the complex by recognizing different substrates, usually in a phosphorylation-dependent manner (Skowyra *et al.*, 1997; Jin *et al.*, 2004). β-Trcp is one of the best characterized mammalian F-box proteins. Mammals express two distinct β-Trcp paralogs (β-Trcp1/Fbxw1 and β-Trcp2/Fbw11; hereafter referred as β-Trcp), with indistinguishable biochemical properties (Kipreos and Pagano, 2000). β-Trcp consists of an N-terminal F-box domain, a C-terminal WD40-repeats domain and an α-helical domain linking the two (Suzuki *et al.*, 2000). The F-box domain is responsible for binding to Skp1, whereas the WD40- repeat domain mediates the interaction with the target proteins (Wu *et al.*, 2003). The DSGxxS destruction motif, or several related variants, is common in β-Trcp substrates and the two serine residues of this degron must be phosphorylated to allow recognition by β-Trcp (Fuchs *et al.*, 2004; Frescas and Pagano, 2008).

Work by many groups has shown the versatility of β-Trcp in regulating various cellular processes, including cell-cycle progression, DNA damage checkpoint response and apoptosis (Fuchs *et al.*, 2004; Frescas and Pagano, 2008). The β-Trcp-dependent control of the cell cycle is accomplished by targeting the degradation of proteins, such as Cdc25A, early mitotic inhibitor 1 (Emi1) and Wee1, that modulate cyclin-dependent kinase 1 activity in different cell-cycle phases (Guardavaccaro *et al.*, 2003; Jin *et al.*, 2003; Busino *et al.*, 2003; Watanabe *et al.*, 2004). Notably, β-Trcp has also emerged as an important player of the S and G2 DNA-damage response checkpoints. In response to genotoxic stresses, Cdc25A is rapidly degraded in a β-Trcp-dependent manner (Jin *et al.*, 2003; Busino *et al.*, 2003), keeping cdk1 activity low and instituting, and as a consequence, an efficient cell-cycle arrest. Interestingly, upon recovery from DNA damage, β-Trcp helps restore cdk1 activity by targeting Claspin and Wee1 for degradation (Watanabe *et al.*, 2004; Peschiaroli *et al.*, 2006).

Besides its role in cell-cycle regulation, β-Trcp also controls cell survival. Accordingly, inhibition by RNA interference or expression of a dominant-negative mutant of β-Trcp induces apoptosis in human malignant melanoma and breast cancer cells (Soldatenkov *et al.*, 1999; Tang *et al.*, 2005). The pro-apoptotic effect elicited by targeting β-Trcp activity is probably due to the accumulation of substrates that negatively regulate cell survival, such as IκBα (Yaron *et al.*, 1998), programmed cell death 4 (PDCD4) (Dorrello *et al.*, 2006), Cdc25A (Busino *et al.*, 2003) and BimEL (Dehan *et al.*, 2009).

The deubiquitylating enzymes are cysteine proteases that catalyze the removal or processing of ubiquitin, thus counteracting the activity of the E3 ubiquitin ligases (Nijman *et al.*, 2005). Based on their protease domains, these enzymes are subdivided into four classes, the largest of which is the ubiquitin-specific protease (USP) family (Nijman *et al.*, 2005). Despite the crucial role of some members of the USP family in a variety of biological processes, such as regulation of DNA damage checkpoint response (Zhang *et al.*, 2006), epigenetic regulation

(Moazed and Johnson, 1996) and protein stabilization (Li *et al.*, 2002), very little is known about the biological function of the majority of the USPs.

In this paper, we have identified the ubiquitin-specific protease USP47 as a novel interactor of β-Trcp. In contrast to other β-Trcp substrates, USP47 is a stable protein, whose protein levels are neither affected by silencing β-Trcp nor modulated in a variety of processes, such as cell-cycle progression, DNA damage or the inflammatory response. Notably, genetic or siRNA-mediated depletion of USP47 decreases cell survival and augments the anti-proliferative effect of anticancer drugs, providing a novel potential target for anticancer therapies.

Results

The ubiquitin-specific protease USP47 is a novel β-Trcp interactor

Using an immunoaffinity/enzymatic assay that enriches for ubiquitylated substrates followed by mass spectrometry analysis, we previously identified Claspin, PDCD4 and REST as new substrates of the $SCF^{\beta\text{-}TRCP}$ ubiquitin ligase (Dorrello *et al.*, 2006; Peschiaroli *et al.*, 2006; Guardavaccaro *et al.*, 2008). In the analysis of two independent purifications, we also recovered 37 unique peptides from USP47, a ubiquitin-specific protease whose biological function is unknown.

To confirm the specific binding between USP47 and β-Trcp, we screened 19 human F-box proteins. We found that the only F-box proteins who were able to co-immunoprecipitate endogenous USP47 were β-Trcp1 and its paralog β-Trcp2 (Figure 1a, lanes 2 and 10). In contrast, the other members of the FBXW family of F-box proteins, FBXW2, FBXW4, FBXW5, FBXW7α, FBXW8, FBXW9 and FBXW10, did not bind endogenous USP47. Similarly, various additional F-box proteins (FBXO4, FBXO6, FBXO7, FBXO9, FBXO1, FBXO17, FBXO19, FBXO21 and Skp2) and the substrate recognition subunits of the anaphase-promoting complex, cdh1 and Cdc20, did not co-immunoprecipitate USP47 either (Figure 1a and Supplementary Figure S1). These data showed that USP47 interacts specifically with β-Trcp.

The majority of β-Trcp substrates contain a canonical DSGxxS degron motif, in which the phosphorylation of the two serine residues is required to allow recognition by β-Trcp (Cardozo and Pagano, 2004). A canonical and conserved DSGxxS motif is present in the C-terminus of USP47 (encompassing amino acids 913–918 in humans, Figure 1b). To verify whether phosphorylation of the two serines in the USP47 degron motif is required for binding with β-Trcp, we generated a number of USP47 deletion mutants and point mutants in which Ser914 and/or Ser918 were mutated to alanine. Unexpectedly, the USP47 (S914/918A) mutant bound endogenous β-Trcp with the same efficiency as the wild-type protein (Figure 1b, lanes 2 and 3). Accordingly, a C-terminal deletion mutant, encompassing amino acids 623–1287 (CT), did not co-immunoprecipitate endogenous β-Trcp (Figure 1b, lanes 5 and 6), whereas an N-terminal deletion mutant, containing amino acids 1–623 of USP47 (NT), was able to co-immunoprecipitate endogenous β-Trcp (Figure 1b, lane 4). These results indicated that a domain in the N-terminus of USP47, and not the canonical DSGxxS, mediates binding to β-Trcp.

The WD40-repeat domain of β-Trcp is responsible for the interaction with the target proteins, and mutation of Arg434 in this domain completely abolished both the binding and the ubiquitylation of IκBα and β-catenin (Suzuki *et al.*, 2000; Wu *et al.*, 2003). To analyze whether the binding between β-Trcp and USP47 requires the WD40-repeat domain and an intact Arg434, we performed co-immunoprecipitation experiments using different β-Trcp mutants (all FLAG tagged). Although wild-type β-Trcp and an N-terminal deletion mutant

(ΔN) co-immunoprecipitated with endogenous USP47 (Figure 1c, lane 8), a C-terminal deletion mutant lacking the WD40-repeat domain (ΔW) did not (Figure 1c, lane 9). Importantly, mutation of Arginine 434 to alanine abrogated the binding of β-Trcp with both USP47 and β-catenin (Figure 1c, lane 10), indicating that the β-Trcp-USP47 association is comparable with other β-Trcp-substrate interactions.

β-Trcp activity does not control USP47 stability

To analyze whether the physical interaction between USP47 and β-Trcp resulted into a proteasome-dependent degradation of USP47, we first analyzed the effect of the proteasome inhibitor MG132 on USP47 protein levels. In contrast to p27 and c-Jun, USP47 protein levels were not affected by proteasome inhibition (Figure 2a). We also analyzed the half-life of endogenous USP47 and found that USP47 is a relatively high stable polypeptide, whose decay rate is not appreciable up to 8 h of protein synthesis blockade (Figure 2b). Subsequently, we used siRNAs to reduce the expression of β-Trcp in U-2OS cells, using a previously validated double-stranded RNA oligo that efficiently targets both β-Trcp1 and β-Trcp2 (Fong and Sun, 2002; Dorrello *et al.*, 2006; Peschiaroli *et al.*, 2006). Depletion of β-Trcp by siRNA did not induce stabilization of USP47 (Figure 2c). As positive controls, Cdc25A and β-catenin were efficiently stabilized in β-Trcp-depleted cells. Finally, attempts to ubiquitylate USP47 in the presence of purified $SCF^{\beta\text{-}Trcp}$, E1 and several E2s (UbcH5 and UbcH3) had negative results (data not shown).

Collectively, these findings indicated that endogenous USP47 degradation is a relatively slow process, and the activity of β-Trcp does not regulate USP47 protein levels *in vivo*.

USP47 expression is not regulated during cell cycle, DNA damage checkpoint response or tumor necrosis factor (TNF) treatment

The majority of β-Trcp substrates are degraded in response to specific stimuli, such as genotoxic stress, tumor necrosis factor-α (TNF-α) treatment or cell-cycle progression. To test whether USP47 protein levels might be regulated in response to these stimuli, we first conducted cell-cycle synchronization experiments in human cell lines to analyze the expression of USP47 during different phases of cell cycle. HeLa cells were arrested in prometaphase by nocodazole treatment, collected by mitotic shake-off and then allowed to progress through mitosis and into the next cell cycle. As shown in Figure 3a, USP47 protein levels did not change in cells exiting from mitosis, passing through G1 and entering S phase. As reported, Cdc25A and polo-like kinase 1 (Plk1) are efficiently downregulated in G1, whereas Claspin expression is induced when cells approach S phase (Peschiaroli *et al.*, 2006). We also analyzed USP47 expression during S and the G2/M transition by synchronizing HeLa cells at G1/S using a thymidine block before release into drug-free medium and progression toward mitosis. USP47 expression remained stable in S and G2/M phase, whereas Emi1, a β-Trcp substrate, was efficiently degraded in mitotic cells (Figure 3b). Furthermore, we found that not even mitogenic stimulation or growth factor withdrawal affected USP47 protein levels (Supplementary Figure S2). Collectively, these data showed that USP47, in contrast with established β-Trcp substrates, is not subjected to cell cycle-dependent degradation.

The DNA damage checkpoint response triggers the degradation of some β-Trcp substrates, such as Cdc25A (Busino *et al.*, 2003). To analyze the effect of the activation of the DNA damage checkpoint on USP47 protein levels, we treated U-2OS cells with hydroxyurea, doxorubicin or UV. As reported, Cdc25A is efficiently degraded in response to genotoxic stress (Busino *et al.*, 2003), whereas USP47 protein levels remained stable during the activation of the DNA damage checkpoint (Figure 3c).

Finally, we analyzed the expression of USP47 in HeLa cells treated with TNF-α, a stimulus triggering the β-Trcp-dependent degradation of IκBα (Yaron *et al.*, 1998). In contrast to IκBα, USP47 protein levels were not affected by TNF-α treatment (Figure 3d). All together, these results indicated that USP47 expression is not regulated under conditions that typically stimulate the β-Trcp-dependent degradation of target proteins.

Silencing of USP47 inhibits cell survival and sensitizes cells to chemotherapic agent-induced apoptosis

To analyze the biological function of USP47, we analyzed the effects of USP47 silencing in different cell lines. To this aim, we first tested two different siRNA oligos for their ability to downregulate USP47. Both oligos were able to decrease USP47 protein levels (Figure 4a, immunoblot panel). By FACS analysis, we found that depletion of USP47 significantly augments the apoptotic index, in concomitance with an increase in the percentage of cells in G2/M (Figure 4a).

To study the long-term effect of USP47 depletion on cell growth and survival, we generated cells stably expressing an shRNA directed against *USP47*. SAOS-2 and U-2OS cells were infected with lentiviruses encoding either a scrambled shRNA or a *USP47*-directed shRNA before plating at low density. Subsequently, cell numbers were quantified on different days. Silencing of *USP47* induced a cell growth defect that appeared on day 6 and was sustained through day 9 (Figure 4b), indicating that depletion of USP47 inhibits cell growth, likely by inducing apoptosis. The pro-apoptotic effect elicited by the silencing of *USP47* prompted us to analyze whether depletion of USP47 could increase cell death triggered by anticancer drugs in different tumor cell lines. To test this hypothesis, we performed siRNA-mediated silencing of *USP47* in both osteosarcoma (U- 2OS and SAOS-2) and breast cancer cell lines (T47D, BT-20 and MCF7). In all cell lines tested, the silencing of *USP47* markedly increased the cytotoxic effects elicited by chemotherapic agents (Figure 4c and Supplementary Figure S3). Apoptosis was confirmed at the biochemical level by the activation of caspase 3, caspase 7 and PARP cleavage in USP47-depleted cells compared with the control cells (Figure 4d).

To validate these results in a mouse model also, we tried to generate knockout mice for USP47, taking advantage of an embryonic stem cell line (RRJ301) carrying a ‘trap vector’ insertion in the mouse *USP47* gene. As previously described, the trap vector, encoding for a galactosidase-neomycin fusion protein (β-geo), is supposed to abolish the splicing events downstream of the insertion site (Stanford *et al.*, 2001). Using genomic PCR we found that the insertion site is located in the first intron of *USP47* gene at position 3715, interrupting the *USP47* coding sequence at position 39 (Figure 5a, upper panel). The mutant allele was transmitted through the germline to generate heterozygous mice which were intercrossed to recover homozygous mutant progeny. The genotype of embryos was determined by genomic PCR using a tri-primer method, as shown in Figure 5a, lower panel.

To determine whether the gene trap vector worked efficiently in splicing to upstream exons, quantitative real-time PCR analysis was performed on total RNA extracted from wild-type, heterozygous and homozygous embryos, using an oligo that covers the 5′ untranslated region as well as a primer that anneals to the position 1312 of the mouse USP47 cDNA. Wild-type transcripts were detected but only at a very low level, as shown in Figure 5b. We confirmed these data at protein levels also in mouse embryonic fibroblasts derived from the homozygous mice (see Figure 5c). Thus, as for other knockout mouse generated by the same strategy (Chen *et al.*, 2000; Lantinga-van Leeuwen *et al.*, 2004), it seems that the mice generated in this study carried an hypomorphic allele of the *USP47* gene. Mice homozygous for this mutant allele appeared viable, and homozygous males and females were fertile (data not shown).

To analyze the effects of this *USP47* hypomorphic allele on cell viability, we treated USP47$^{+/+}$, USP47$^{+/-}$ and USP47$^{-/-}$ mouse embryonic fibroblasts with ultraviolet radiation (30 J/m^2) and measured the apoptotic index using FACS analysis. As shown in Figure 5c, the genetic depletion of USP47 in mouse embryonic fibroblasts increased the sensitivity to the ultraviolet-induced cell death.

All together, these results indicated that USP47 depletion by either siRNA approach or hypomorphic mutation is effective in suppressing cell growth and survival alone or in combination with various chemotherapeutic agents.

Silencing of *USP47* increases Cdc25A protein levels

Silencing of β-Trcp induces apoptosis and augments the anti-proliferative effects of antitumor drugs in different cancer cell lines (Soldatenkov *et al.*, 1999; Tang *et al.*, 2005). The similarity of the phenotypes elicited by β-Trcp or USP47 depletion prompted us to analyze whether USP47 might control β-Trcp activity and, as a consequence, the protein levels of βTrcp substrates. To this aim, we transfected HeLa cells twice with siRNA targeting *USP47* and analyzed the protein levels of several βTrcp substrates by immunoblotting. We found that Cdc25A protein levels were markedly increased in cells transfected with either of the two oligos targeting *USP47*, whereas β-catenin, Emi1, Claspin, IκBα and BimEL were not significantly affected (Figures 6a and b and data not shown). Notably, Cdc25A upregulation was induced both in HeLa and U-2OS cells and correlated with the degree of USP47 depletion (Figure 6a and Supplementary Figure S4). Moreover, USP47 depletion induced upregulation of Cdc25A to similar levels as the silencing of βTrcp (Supplementary Figure S5). To analyze whether the Cdc25A upregulation was due to its protein stabilization, we analyzed the Cdc25A half-life in USP47-depleted cells. As shown in Figure 6c, silencing of USP47 did not affect Cdc25A stability. Moreover, we found that in response to hydroxyurea, Cdc25A was degraded in USP47-depleted cells to similar levels as in control cells (Figure 6d), implying a transcriptional regulation of Cdc25A induced by USP47 depletion. To verify this, we performed a quantitative real-time PCR and we found that USP47 but not β-Trcp depletion increases Cdc25A mRNA levels (Figure 6e).

Collectively, these results showed that USP47 potentially regulates Cdc25A expression at mRNA level, suggesting that USP47 does not regulate β-Trcp activity.

Discussion

In this study we identified the ubiquitin-specific protease USP47 as a novel interactor of the E3 ubiquitin ligase SCF$^{\beta\text{-Trcp}}$. A common event that mediates the interaction between β-Trcp and its substrates is the phosphorylation of the two serine residues in a DSGxxS motif (or variant) present in the majority of β-Trcp substrates. Although USP47 contains a canonical DSGxxS motif in the C-terminus, this sequence is dispensable for β-Trcp binding. Instead, we found that an N-terminal fragment of USP47 mediates binding to β-Trcp. This region does not contain any sequences resembling the DSGxxS motif, suggesting that other sequence determinants dictate binding to β-Trcp. A similar scenario has been described for heterogeneous nuclear ribonuclear protein- U (hnRNP-U), a nuclear phosphoprorein that binds β-Trcp in a specific and stoichiometric manner. A 198-amino-acid fragment of hnRNP-U, composed of long acidic and short glutamine-rich peptide segments, mediates hnRNP-U binding to β-Trcp (Davis *et al.*, 2002). Interestingly, the interaction between β-Trcp and hnRNP-U does not result in the ubiquitylation and subsequent proteasome-dependent degradation of hnRNP-U (Davis *et al.*, 2002). Our findings strongly indicate that USP47, similar to hnRNP-U, is not subjected to β-Trcp-dependent ubiquitylation and degradation. This statement is based on the following evidences: (i) USP47 is a stable protein, with a half-life of over 8 h; (ii) proteasome activity does not regulate USP47 protein

levels; (iii) silencing of β-Trcp does not induce stabilization of USP47; (iv) USP47 expression is constant during a variety of processes, such as cell-cycle progression, DNA damage checkpoint responses or TNF signaling; and (v) $SCF^{\beta\text{-}Trcp}$ is not able to mediate USP47 ubiquitylation *in vitro*. Collectively, these observations supported the idea that β-Trcp activity does not control USP47 protein degradation. However, we cannot rule out the possibility that β-Trcp might target USP47 for proteasome-dependent degradation in response to a stimulus that we did not analyze.

Currently, the substrate specificity and the biological function of USP47 remains unknown. We found that USP47 is involved in controlling cell growth and survival alone or in combination with various chemotherapeutic agents. Silencing of *USP47* decreases cell survival and augments the cytotoxic effects of antitumor drugs on a variety of tumor cells, including osteosarcoma and breast cancer cell lines. The pro-apoptotic effect elicited by USP47 depletion does not depend on p53 status, as both p53-positive (U-2OS) and p53-negative (SAOS-2) cell lines have a similar response to USP47 depletion.

Notably, we also confirmed the role of USP47 in controlling cell survival in mouse embryo fibroblasts derived from USP47 hypomorphic mutant mice. Although these mice appear normal and fertile, we cannot exclude the possibility that a complete loss of USP47 expression could determine mouse developmental defects due to the alteration of apoptotic cell death.

It has been previously shown that inhibition of β-Trcp induces apoptosis and augments the cytotoxic effects of anticancer drugs in various cancer cell lines (Soldatenkov *et al.*, 1999; Tang *et al.*, 2005). The specificity of the binding between USP47 and β-Trcp and the similarity of the phenotypes produced by targeting USP47 or β-Trcp suggested to us that USP47 could be a regulator of β-Trcp activity. However, our data do not support this hypothesis. In fact, in the absence of USP47, β-Trcp is still able to induce the efficient degradation of its substrates. In particular, we found that the steady-state levels of Claspin, β-catenin and Emi1 are unaffected by the silencing of USP47. Moreover, Claspin and Emi1, whose degradation in mitosis is mediated by β-Trcp, are efficiently degraded in prometaphase even in USP47- depleted cells (our unpublished results). In agreement with these data, we also found that IκBα degradation in response to TNF-α treatment is not altered in the absence of USP47. This finding also rules out the possibility that the suppression of the pro-survival signaling through NF-κB (due to the stabilization of the inhibitor IκBα) might be a mechanism underlying the high rate of apoptosis observed in USP47-depleted cells. In agreement with this result, we observed that silencing of *USP47* augments the apoptosis of MCF-7 breast cancer cells, which are known to be resistant to inhibition of NF-κB (Cai *et al.*, 1997).

Although the levels of the majority of the β-Trcp substrates are not affected by USP47 depletion, we did observe a marked increase in Cdc25A, both in HeLa and U-2OS cells, using two different siRNA oligos targeting *USP47*. Although we have not elucidated yet the precise mechanism through which USP47 depletion causes Cdc25A upregulation, our results suggest that USP47 could potentially regulate Cdc25A expression at a transcriptional level.

Different reports have established a pro-apoptotic effect for Cdc25A. In particular, it has been shown that Cdc25A overexpression triggers the DNA damage response by activating the ATR/Chk1 pathway due to the formation of double DNA breaks (Bartkova *et al.*, 2005). Moreover, chemical inhibition of Chk1 induces unscheduled DNA replication and DNA damage, probably due to Cdc25A stabilization (Syljuasen *et al.*, 2004). It is worth speculating that the Cdc25A upregulation observed in the absence of USP47 might induce unscheduled entry into mitosis in cells in which DNA damage has not been completely

repaired. Accordingly, we found that USP47 depletion significantly increases the percentage of cells in G2/M. We speculate that the regulation of Cdc25A by USP47 might explain the increased cytotoxic effects observed in USP47-depleted cells in response to different genotoxic agents. Therefore, our data are in agreement with the hypothesis that increasing, rather that inhibiting, Cdc25A might be useful as an adjuvant to radiotherapy or treatment with DNA-damaging drugs (Boutros *et al.*, 2007). In this context, the regulation of USP47 activity might be an additional tool to sensitize tumor cells to apoptosis triggered by chemotherapic drugs.

In conclusion, in this report, we identified the ubiquitin-specific protease USP47 as a novel β-Trcp interactor that regulates the anti-proliferative effects of chemotherapic agents in several tumor cell lines, providing a novel potential therapeutic target.

Materials and methods

Cell culture, synchronization and drug treatment

All cell lines used were maintained at 37 °C and 5% carbon dioxide in Dulbecco's modified Eagle's medium supplemented with 100 μg/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum. Transfections were performed using the calcium phosphate method for HEK293T. Where indicated, 10 μm MG132 was added for 10 h before harvesting the cells. Cell synchronization and half-lives experiments were performed as described (Peschiaroli *et al.*, 2006). Doxorubicin, cycloheximide and cisplatin were dissolved in dimethylsulphoxide and added to the cell medium in a final concentration of 1 μM, 80μg/ml and 10 μg/ml, respectively. Cells were UV irradiated (30 J/m^2) where indicated.

Generation and genotyping of *USP47* hypomorphic mutant mice

The BAYGenomics gene trap Embryonic stem cell line RRJ301, containing an insertion of the trap vector pGT0Lxf within an intron of the *USP47* gene, was purchased by the Mutant Mouse Regional Resource Center at University of California Davis. Founder chimeric males were generated and bred with C57Bl6/J females to generate heterozygous progeny. Genomic PCR was performed to genotype embryos and adults using a triple-primer set: 5′-CTTCACCTGTTCAAATCCTC CG-3′ (3015); 5′-GTTCCTTTCTGTTCATACCCG ATG-3′ (3921); and 5′-ATTCAGGCTGCGCAACTGTTGGG-3′ (vector). Quantitative real-time PCR analysis was performed using RNA extracted from the lungs of newborn mice using the RNAeasy Kit from Qiagen (Hilden, Germany). The following primers were used: 5′-TGTCGCTGCCGTTCTCTCCTGA GAC-3′ and 5′-CAACTGTAAGGTCAGCAGATAGGGG-3′ (USP47); 5′-TTATCGATGAGCGTGGTGGTTATGC-3′ and 5′-GCGCGTACATCGGGCAAATAATATC-3′ (β-Geo).

Plasmids

The N-terminal and C-terminal fragments of USP47 were amplified by reverse transcriptase–PCR using a cDNA generated from HEK293T cells. The reverse transcriptase–PCR products were sequentially inserted into pcDNA3.1- FLAG to obtain full-length USP47. USP47 mutants were generated using the QuickChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). All USP47 cDNAs were sequenced-confirmed. β-TrCP2 mutant constructs (ΔN, ΔW and R434A) were kindly provided by Dr Keiji Tanaka (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan).

Small interfering (si)RNA transfection and lentiviral infection

Cells were seeded at a density of 1.4×10^5 cells/well in a six-well plate and transfected with oligos twice (at 24 and 48 h after plating) using Oligofectamine (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. Two different 21-nucleotide RNA oligos were used, targeting the following USP47 cDNA sequences: 5′-CAGCAGC ATGA TGTACAAGAA-3′ (si 1) and 5′-CTGGATGGAGCACC AAA TAAA-3′ (si 2). The siRNA oligonucleotide sequences for β-Trcp or non-relevant gene (scramble) were previously described (Peschiaroli *et al.*, 2006). Cells were collected after 48 h, and lysates were subjected to immunoblotting. Lentiviral particles encoding for non-target shRNA or *USP47*-directed shRNA were purchased from Sigma (Taufkirchen, Germany) and used to infect U-2OS and SAOS-2 osteosarcoma cell lines at a multiplicity of infection of 5 for 12 h in a medium containing polybrene (8 μg/ml). After 48 h, cells were plated into puromycin-containing medium (1–2 μg/ml) for 3 days, and puromycin-resistant cells were seeded for colony formation.

Immunoblot analysis, immunoprecipitation and purification of β-Trcp2 interactors

Immunoblot analysis was performed using whole-cell extracts obtained by lysing cell pellets with Triton Buffer (50mM Tris-HCl pH 7.5, 250mM sodium chloride, 50mM sodium fluoride, 1mM EDTA 1 pH 8, 0.1% Triton), supplemented with protease and phosphatase inhibitors. Proteins were separated by SDS– polyacrylamide gel electrophoresis, transferred onto polyvinylidene fluoride membranes and blocked with phosphate-buffered saline and 0.1% Tween-20 containing 5% non-fat dry milk for 1 h at room temperature. The incubation with primary antibodies was performed for 2 h at room temperature, followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibody. Detection was performed with enhanced chemiluminescence Western Blot Reagent (PerkinElmer, Whaltam, MA, USA). Immunoprecipitation and purification of β-Trcp2 interactors were performed as previously described (Peschiaroli *et al.*, 2006).

Antibodies

Mouse monoclonal antibodies were from Zymed/Invitrogen (β-Trcp1, Cul1, Plk1, Emi1 and Skp1), Sigma (anti-FLAG and actin), Santa Cruz Biotechnology (Santa Cruz, CA, USA) (p21 and Cdc25A), Abnova (Taipei City, Taiwan) (USP47), BD Transduction Laboratories (San Jose, CA, USA) (c-Jun, p27 and β-catenin), BIOMOL (Farmingdale, NY, USA) (PARP) and Covance (Princeton, NJ, USA) (anti-HA). Rabbit polyclonal antibodies were from Zymed/Invitrogen (β-Trcp1), Sigma (anti-FLAG), Bethyl (Montgomery, TX, USA) (USP47) Upstate (Billerica, MA, USA) (phospho-Ser10 histone H3), Santa Cruz Biotechnology (IκBα), and Cell Signalling Technology (Beverly, MA, USA) (cleaved caspase 3, cleaved caspase 7). Mouse monoclonal antibody against Claspin was previously described (Peschiaroli *et al.*, 2006).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Bartkova J, Horejsi Z, Koed K, Kramer A, Tort F, Zieger K, et al. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature. 2005; 434:864–870. [PubMed: 15829956]

Bernassola F, Karin M, Ciechanover A, Melino G. The HECT family of E3 ubiquitin ligases: multiple players in cancer development. Cancer Cell. 2008; 14:10–21. [PubMed: 18598940]

Boutros R, Lobjois V, Ducommun B. CDC25 phosphatases in cancer cells: key players? Good targets? Nat Rev Cancer. 2007; 7:495–507. [PubMed: 17568790]

Busino L, Donzelli M, Chiesa M, Guardavaccaro D, Ganoth D, Dorrello NV, et al. Degradation of Cdc25A by beta-TrCP during S phase and in response to DNA damage. Nature. 2003; 426:87–91. [PubMed: 14603323]

Cai Z, Korner M, Tarantino N, Chouaib S. IkappaB alpha overexpression in human breast carcinoma MCF7 cells inhibits nuclear factor-kappaB activation but not tumor necrosis factor-alpha- induced apoptosis. J Biol Chem. 1997; 272:96–101. [PubMed: 8995233]

Cardozo T, Pagano M. The SCF ubiquitin ligase: insights into a molecular machine. Nat Rev Mol Cell Biol. 2004; 5:739–751. [PubMed: 15340381]

Chen H, Bagri A, Zupicich JA, Zou Y, Stoeckli E, Pleasure SJ, et al. Neuropilin-2 regulates the development of selective cranial and sensory nerves and hippocampal mossy fiber projections. Neuron. 2000; 25:43–56. [PubMed: 10707971]

Ciechanover A, Schwartz AL. The ubiquitin-proteasome pathway: the complexity and myriad functions of proteins death. Proc Natl Acad Sci USA. 1998; 95:2727–2730. [PubMed: 9501156]

Davis M, Hatzubai A, Andersen JS, Ben-Shushan E, Fisher GZ, Yaron A, et al. Pseudosubstrate regulation of the SCF(beta- TrCP) ubiquitin ligase by hnRNP-U. Genes Dev. 2002; 16:439–451. [PubMed: 11850407]

Dehan E, Bassermann F, Guardavaccaro D, Vasiliver-Shamis G, Cohen M, Lowes KN, et al. betaTrCP- and Rsk1/2-mediated degradation of BimEL inhibits apoptosis. Mol Cell. 2009; 33:109–116. [PubMed: 19150432]

Dorrello NV, Peschiaroli A, Guardavaccaro D, Colburn NH, Sherman NE, Pagano M. S6K1- and betaTRCP-mediated degradation of PDCD4 promotes protein translation and cell growth. Science. 2006; 314:467–471. [PubMed: 17053147]

Fong A, Sun SC. Genetic evidence for the essential role of beta-transducin repeat-containing protein in the inducible processing of NF-kappa B2/p100. J Biol Chem. 2002; 277:22111–22114. [PubMed: 11994270]

Frescas D, Pagano M. Deregulated proteolysis by the F-box proteins SKP2 and beta-TrCP: tipping the scales of cancer. Nat Rev Cancer. 2008; 8:438–449. [PubMed: 18500245]

Fuchs SY, Spiegelman VS, Kumar KG. The many faces of beta-TrCP E3 ubiquitin ligases: reflections in the magic mirror of cancer. Oncogene. 2004; 23:2028–2036. [PubMed: 15021890]

Guardavaccaro D, Frescas D, Dorrello NV, Peschiaroli A, Multani AS, Cardozo T, et al. Control of chromosome stability by the beta-TrCP-REST-Mad2 axis. Nature. 2008; 452:365–369. [PubMed: 18354482]

Guardavaccaro D, Kudo Y, Boulaire J, Barchi M, Busino L, Donzelli M, et al. Control of meiotic and mitotic progression by the F box protein beta-Trcp1 *in vivo*. Dev Cell. 2003; 4:799–812. [PubMed: 12791266]

Hershko A, Ciechanover A. The ubiquitin system. Annu Rev Biochem. 1998; 67:425–479. [PubMed: 9759494]

Jin J, Cardozo T, Lovering RC, Elledge SJ, Pagano M, Harper JW. Systematic analysis and nomenclature of mammalian F-box proteins. Genes Dev. 2004; 18:2573–2580. [PubMed: 15520277]

Jin J, Shirogane T, Xu L, Nalepa G, Qin J, Elledge SJ, et al. SCFbeta-TRCP links Chk1 signaling to degradation of the Cdc25A protein phosphatase. Genes Dev. 2003; 17:3062–3074. [PubMed: 14681206]

Kipreos ET, Pagano M. The F-box protein family. Genome Biol. 2000; 1:REVIEWS3002. [PubMed: 11178263]

Lantinga-van Leeuwen IS, Dauwerse JG, Baelde HJ, Leonhard WN, van de Wal A, Ward CJ, et al. Lowering of Pkd1 expression is sufficient to cause polycystic kidney disease. Hum Mol Genet. 2004; 13:3069–3077. [PubMed: 15496422]

Li M, Chen D, Shiloh A, Luo J, Nikolaev AY, Qin J, et al. Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. Nature. 2002; 416:648–653. [PubMed: 11923872]

Melino G. Discovery of the ubiquitin proteasome system and its involvement in apoptosis. Cell Death Differ. 2005; 12:1155–1157. [PubMed: 16094390]

Moazed D, Johnson D. A deubiquitinating enzyme interacts with SIR4 and regulates silencing in S. cerevisiae. Cell. 1996; 86:667–677. [PubMed: 8752220]

Nakayama KI, Nakayama K. Ubiquitin ligases: cell-cycle control and cancer. Nat Rev Cancer. 2006; 6:369–381. [PubMed: 16633365]

Nijman SM, Luna-Vargas MP, Velds A, Brummelkamp TR, Dirac AM, Sixma TK, et al. A genomic and functional inventory of deubiquitinating enzymes. Cell. 2005; 123:773–786. [PubMed: 16325574]

Orlowski RZ, Kuhn DJ. Proteasome inhibitors in cancer therapy: lessons from the first decade. Clin Cancer Res. 2008; 14:1649–1657. [PubMed: 18347166]

Peschiaroli A, Dorrello NV, Guardavaccaro D, Venere M, Halazonetis T, Sherman NE, et al. SCFbetaTrCP-mediated degradation of Claspin regulates recovery from the DNA replication checkpoint response. Mol Cell. 2006; 23:319–329. [PubMed: 16885022]

Skowyra D, Craig KL, Tyers M, Elledge SJ, Harper JW. F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. Cell. 1997; 91:209–219. [PubMed: 9346238]

Soldatenkov VA, Dritschilo A, Ronai Z, Fuchs SY. Inhibition of homologue of Slimb (HOS) function sensitizes human melanoma cells for apoptosis. Cancer Res. 1999; 59:5085–5088. [PubMed: 10537278]

Stanford WL, Cohn JB, Cordes SP. Gene-trap mutagenesis: past, present and beyond. Nat Rev Genet. 2001; 2:756–768. [PubMed: 11584292]

Suzuki H, Chiba T, Suzuki T, Fujita T, Ikenoue T, Omata M, et al. Homodimer of two F-box proteins betaTrCP1 or betaTrCP2 binds to IkappaBalpha for signal-dependent ubiquitination. J Biol Chem. 2000; 275:2877–2884. [PubMed: 10644755]

Syljuasen RG, Sorensen CS, Nylandsted J, Lukas C, Lukas J, Bartek J. Inhibition of Chk1 by CEP-3891 accelerates mitotic nuclear fragmentation in response to ionizing Radiation. Cancer Res. 2004; 64:9035–9040. [PubMed: 15604269]

Tang W, Li Y, Yu D, Thomas-Tikhonenko A, Spiegelman VS, Fuchs SY. Targeting beta-transducin repeat-containing protein E3 ubiquitin ligase augments the effects of antitumor drugs on breast cancer cells. Cancer Res. 2005; 65:1904–1908. [PubMed: 15753389]

Varshavsky A. The ubiquitin system. Trends Biochem Sci. 1997; 22:383–387. [PubMed: 9357313]

Watanabe N, Arai H, Nishihara Y, Taniguchi M, Watanabe N, Hunter T, et al. M-phase kinases induce phospho-dependent ubiquitination of somatic Wee1 by SCFbeta-TrCP. Proc Natl Acad Sci USA. 2004; 101:4419–4424. [PubMed: 15070733]

Wu G, Xu G, Schulman BA, Jeffrey PD, Harper JW, Pavletich NP. Structure of a beta-TrCP1-Skp1-beta-catenin complex: destruction motif binding and lysine specificity of the SCF(beta- TrCP1) ubiquitin ligase. Mol Cell. 2003; 11:1445–1456. [PubMed: 12820959]

Yaron A, Hatzubai A, Davis M, Lavon I, Amit S, Manning AM, et al. Identification of the receptor component of the IkappaBalpha- ubiquitin ligase. Nature. 1998; 396:590–594. [PubMed: 9859996]

Zhang D, Zaugg K, Mak TW, Elledge SJ. A role for the deubiquitinating enzyme USP28 in control of the DNA-damage response. Cell. 2006; 126:529–542. [PubMed: 16901786]

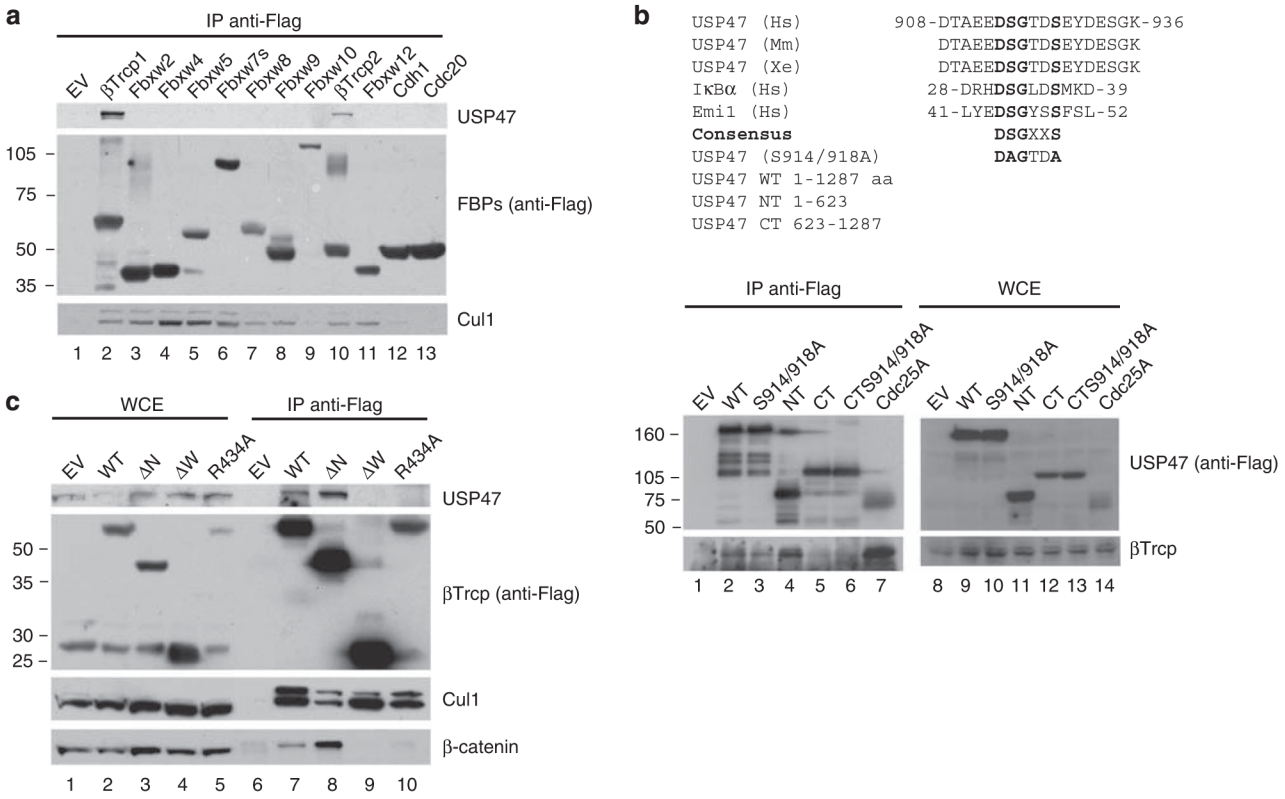

Figure 1.

USP47 is a novel β-Trcp interactor. (**a**) HEK293T cells were transfected with the indicated FLAG-tagged F-box protein constructs (FBPs). During the last 6 h before harvesting, cells were treated with the proteasome inhibitor MG132. Exogenous proteins were immunoprecipitated (IP) from cell extracts with anti-FLAG resin, and immunocomplexes were probed with antibodies to the indicated proteins. (EV, empty vector). (**b**) Upper panel: alignment of the amino acid regions corresponding to a potential β-Trcp binding motif in USP47 orthologs and DSGxxS motifs in previously reported β-Trcp substrates. Lower panel: HEK293T cells were transfected with an EV, FLAG-tagged wild-type USP47 (WT), FLAG-tagged USP47 (S914/918A), FLAG-tagged N-terminal deletion mutant (amino acids 1–623; NT), FLAG-tagged CT (S914/918A) mutant or a FLAG-tagged C-terminal deletion mutant (amino acids 623–1287; CT). Whole-cell extracts (WCE) were either subjected directly to immunoblotting to analyze the expression levels of wild-type and mutant USP47 proteins or to IP with an anti-FLAG resin followed by immunoblotting with antibodies to the indicated proteins. (**c**) HEK293T cells were transfected, as indicated, with an EV, FLAG-tagged wild-type β-Trcp (WT), FLAG-tagged N-terminal deletion β-Trcp mutant (amino acids 116–542; ΔN), Flag-tagged WD40 repeat deletion β-Trcp mutant (amino acids 1–259; ΔW) or FLAG-tagged β-Trcp (R434A) mutant. WCE were either subjected directly to immunoblotting to analyze the expression levels of wild-type and mutant β-Trcp proteins or to IP with an anti-FLAG resin followed by immunoblotting with antibodies to the indicated proteins.

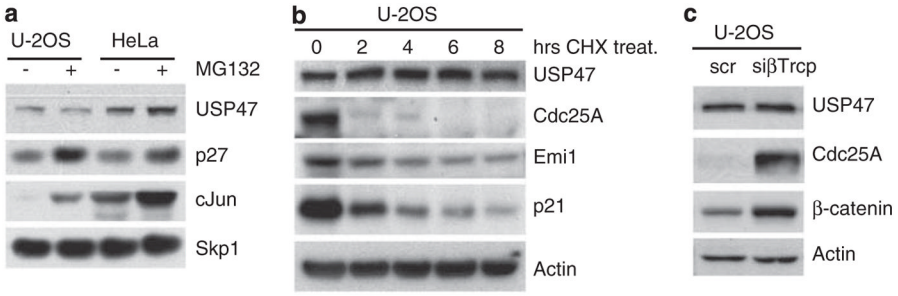

Figure 2.

USP47 protein levels are not regulated by β-Trcp. (**a**) U-2OS and HeLa cell lines were treated with 10 μM of proteasome inhibitor MG132 for 10 h. Cell extracts were subjected to immunoblotting with antibodies to the indicated proteins. (**b**) U-2OS cells were treated with cycloheximide (CHX, 80 ug/ml) for the indicated times. Cell lysates were collected and analyzed by western blot using antibodies to the indicated proteins. (**c**) U-2OS cells were transfected twice with small interfering RNAs (siRNAs) to a non-relevant mRNA (scr, scramble) or to both β-Trcp1 and β-Trcp2 mRNAs (siβTrcp). Protein extracts were probed with antibodies to the indicated proteins.

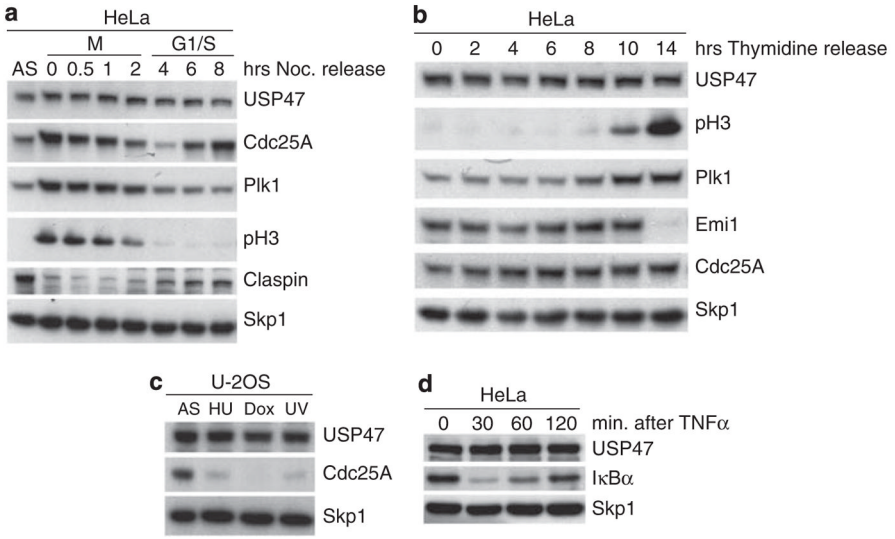

Figure 3.

USP47 protein levels are constant during the cell cycle, DNA damage checkpoint responses and tumor necrosis factor-α (TNF-α) treatment. (**a**) U-2OS cells were released from nocodazole-induced prometaphase arrest (indicated as time 0) and collected at the indicated times. Protein extracts were analyzed by immunoblotting with antibodies to the indicated proteins (AS, asynchronous cells). (**b**) U-2OS cells were synchronized at G1/S using thymidine for 24 h (indicated as time 0). Cells were subsequently washed and allowed to progress through the cell cycle for the indicated times in nocodazole-containing medium. Protein extracts were analyzed by immunoblotting with antibodies to the indicated proteins. (**c**) U-2OS cells were treated with 2mM hydroxiurea (HU) or 1 μM Doxorubicin (Dox) for 18 h. Alternatively, cells were ultraviolet (UV) irradiated (30 J/m^2). Protein extracts were analyzed by immunoblotting with antibodies to the indicated proteins. (**d**) HeLa cells were treated with TNF-α (10 ng/ml) for the indicated times, and protein extracts were subjected to immunoblotting using the indicated antibodies.

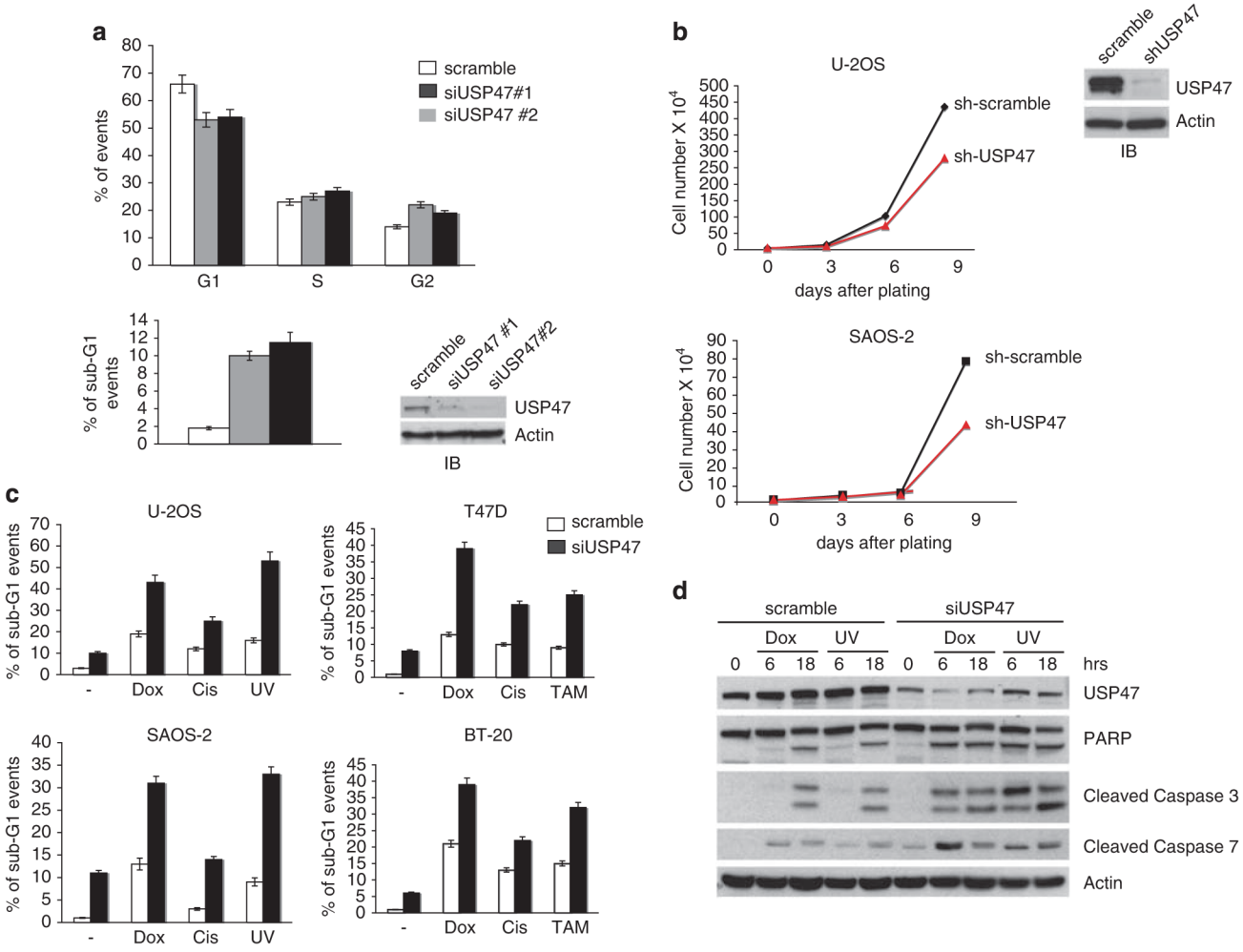

Figure 4.

Silencing of USP47 controls cell survival and augments the effects of antitumor drugs on several cancer cell lines. (**a**) U-2OS cells were transfected twice with two different small interfering RNA (siRNA) oligos targeting *USP47* mRNA (si 1 or si 2) or a non-relevant mRNA (scramble). The cell-cycle phase distribution and sub-G1 population were examined using fluorescence-activated cell sorting (FACS) analysis. USP47 protein levels were analyzed using immunoblot (IB). (**b**) U-2OS and SAOS-2 cells were infected with lentiviral particles encoding non-targeted shRNA or *USP47*-directed short hairpin RNA (shRNA). After puromycin selection, infected cells were seeded at low density, and cell numbers were quantified on different days. Concomitantly, cell extracts were subjected to immunoblotting using the indicated antibodies (IB). (**c**) U-2OS and SAOS-2, transfected with siRNA as in A, were left untreated or treated with doxorubicin (1 μM), cisplatin (10 μg/ml) or ultraviolet (UV) irradiation (30 J/m^2). Breast cancer cell lines (BT-20 and T47D) were transfected with siRNA as above and treated with doxorubicin (1 μM), cisplatin (10 μg/ml) and tamoxifen (2 μM). After 18 h, the percentage of sub-G1 cells was measured using FACS analysis. Representative results are shown (mean ± s.d. $n = 3$). (**d**) U-2OS cells were treated as in **c**, and cell lysates were collected and analyzed by western blotting using antibodies to the indicated proteins.

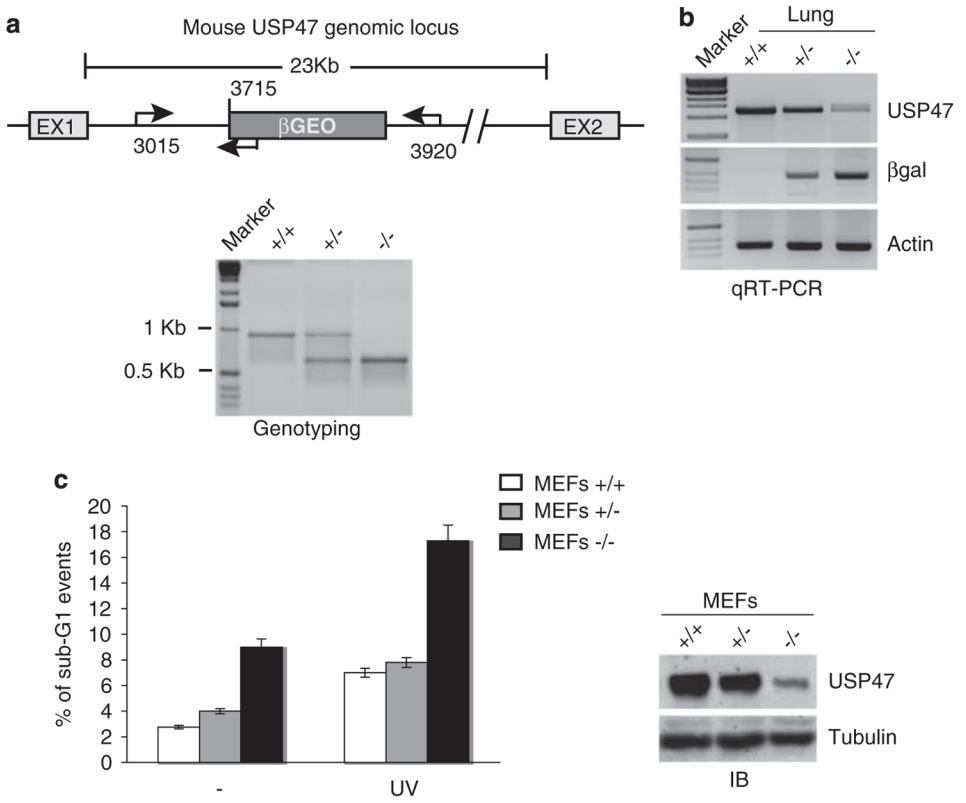

Figure 5.

Genetic depletion of USP47 increases DNA damage-induced apoptosis. (**a**) Upper panel: schematic representation of mouse USP47 genomic locus with the β-Geo insertion and the primers used for the embryos genotyping. Lower panel: genomic DNA was extracted from mice tails and PCR analysis was performed using a triple primer set to amplify products corresponding to the wild-type (905 bp) and mutant (700 bp) genes. (**b**) Quantitative real-time PCR (qRT-PCR) analysis was performed using RNA extracted from the lungs of newborn mice. See Materials and methods for the primers information. (**c**) Mouse embryonic fibroblasts (MEFs) generated from $USP47^{+/+}$, $USP47^{+/-}$ or $USP47^{-/-}$ mice were either left untreated or ultraviolet (UV) irradiated (30 J/m^2). After 18 h, the percentage of sub-G1 cells was measured using fluorescence-activated cell sorting (FACS) analysis. Representative results are shown (mean ± s.d. *n*=3). USP47 expression was analyzed using immunoblotting (IB).

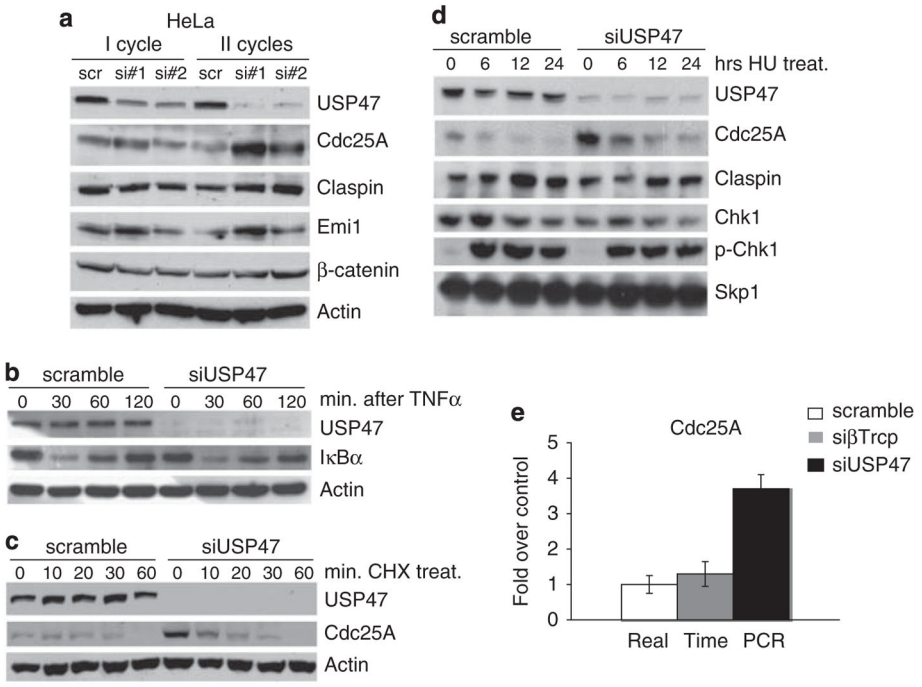

Figure 6.

Silencing of USP47 induces accumulation of cell division cycle 25 homolog A (Cdc25A). HeLa cells were transfected twice with small interfering RNA (siRNA oligos) targeting a non-relevant mRNA (scr) or *USP47* mRNA (si 1 or si 2). After 48 h of each siRNA oligo transfection, whole-cell extracts were subjected to immunoblotting with antibodies to the indicated proteins. (**b**) HeLa cells were transfected as in **a** and after 48 h cells were treated with tumor necrosis factor-α (TNF-α; 10 ng/ml) for the indicated times, and whole-cell lysates were analyzed using immunoblotting with antibodies to the indicated proteins. (**c**) U-2OS cells were transfected as in **b**, and then treated with cycloheximide (CHX, 80 ug/ml) for the indicated times. Cell lysates were collected and analyzed by western blot using antibodies to the indicated proteins. (**d**) U-2OS cells were transfected as in **b** and then treated with hydroxyurea (HU) for the indicated times. Cell lysates were collected and analyzed by western blot using antibodies to the indicated proteins. (**e**) HeLa cells were transfected as in **b** except that a siRNA oligo to β-Trcp was compared with an oligo targeting *USP47* mRNA. Total RNA was used for reverse transcription and quantitative real-time PCR was performed using specific primer for Cdc25A and β-Actin (for quantity normalization). Results are shown as mean ± s.d. from three independent experiments.