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p53 Stabilization can be Uncoupled from its Role in Transcriptional Activation by Loss of PTTG1/Securin

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HCT116 cells devoid of PTTG1/securin (*sec*$^{-/-}$ HCT116) show a stabilized yet transcriptionally latent form of p53 protein in the absence of DNA damage. Ser15, Ser20 phosphorylation and other post-transcriptional modifications of p53 resolved by 2D gel electrophoresis are comparable to that observed in *sec*$^{+/+}$ HCT116 cells. The difference in degradation was also shown to be independent of the ubiquitin system but reliant on calpains. However, the p53-mediated checkpoint response is active only after genotoxic stress in *sec*$^{-/-}$ HCT116 cells. These findings point to the calpain pathway as a key player to maintain steady state levels of p53 in resting cells without affecting its activity.

Key words: calpains, gene regulation, p53 tumour suppressor, protein stability, PTTG1/Securin.

Abbreviations: ATM, ataxia telangectasia mutated; ATR, ataxia telangectasia related; cdk, cyclin-dependent kinase; CHX, cyclohexymide; Dox, doxorubicin; LLnL, N-acetyl-L-Leucyl-L-leucyl-L-norleucinal; NP40, nonidet P40; PMSF, phenylmethylsulfonylfluoride.

The tumour suppressor p53 is a major player in the regulation of gene expression in conditions of stress in mammalian cells: under situations like hypoxia or DNA damage, p53 integrates intracellular signals to render either cell cycle arrest or apoptosis (*1*). Much of this function is brought about through up and down regulation of critical genes (*2*), although other functions for this protein have been described (*3*, *4*).

Under normal conditions, p53 is a latent cytosolic protein with relatively low affinity for its consensus sites in DNA. Also, it is a short-lived protein with a half-life of *ca* 30 min. In situations of stress, post-translational modifications increase its half-life to several hours (*5*, *6*). The study of the mechanisms leading to p53 stabilization is an active field of investigation. Stabilization is considered a prerequisite for p53 function and cancer cells often show alterations that affect p53 half-life, such as overexpression of the ubiquitin ligase HDM2 (*7*). Furthermore, the disruption of those mechanisms is the target for new promising anti-tumour drugs (*8*). The current line of thought is that p53 half-life is determined by the interplay of many pathways that converge on the HDM2-p53 regulatory loop: on the one hand the post-translational modification of p53 which alters its susceptibility for being ubiquitinated by HDM2, and on the other, the regulation of HDM2 itself (*9*). At any rate, it must be noted that other mechanisms such as intracellular localization or calpain proteolysis may be determinants of the fate of p53 (*10*, *11*).

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On the other hand, at the same time that they alter p53 stability, post-translational modifications activate p53 and translocates it rapidly to the nucleus, where it forms a tetrameric homocomplex that binds avidly to specific response elements (*12*, *13*). The study of these post-translational modifications of p53 have received much attention and, thus, the effects that phosphorylations at particular sites such as Ser15, or Ser33 have on gene expression are well known (*14*). The intracellular signaling pathways and proteins that converge in activation of p53 are still an open field. This is probably due to both the difficulty of the studies and the plethora of stresses to which p53 respond. Nevertheless, many of the mechanisms have been described. As an example, it is known that the DNA-PK and ATM pathways lead to phosphorylation on Ser15 and increases p53 transcriptional activity (*15*, *16*).

Our group is interested in the cellular functions of PTTG1/securin. This gene encodes for a 202 amino acid protein that was originally identified in rats as a proto-oncogene (*17*, *18*) and latter found to be a human securin. This protein is cell-cycle regulated with a peak at mitosis and down-regulated in response to serum starvation or cell confluence (*19*). On the other hand, securin levels are elevated in rapidly proliferating cells and many tumour types (*20*). Positive correlations between securin levels and proliferation *in vitro* (*19*) and metastasis by solid tumours (*21*) have been observed. Its best characterized cellular function is the regulation of sister-chromatid separation by separase protein, hence its name (*22*). On top of this, securin has been shown to be transcriptionally active (*18*) and to interact with Ku heterodimer of the Double Strand Break (DSB) repairing machinery (*23*). More significantly, it has been demonstrated that securin interacts and

modulates p53-transactivating functions *in vivo* and *in vitro* (*24*).

HCT116 is a near-diploid colorectal adenocarcinoma cell line (*25*). It has intact DNA damage and mitotic spindle checkpoints (*26*, *27*) and, mostly, an intact p53 response pathway, with the sole exception of being $p14^{ARF}$ deficient due to inactivation of its gene (*28*). Recently, both copies of the PTTG1/securin gene were deleted by homologous recombination in this background (*29*). Also, it has been shown to have a stable karyotype (*30*). Here we report that the knock-out of PTTG1/securin leads to a partial calpain-dependent stabilization of p53 and that this affects the speed of the response to a p53-dependent chemotherapeutic drug such as doxorubicin.

MATERIALS AND METHODS

Cell Lines, Reagents and Treatments—Wild-type HCT116 human colon carcinoma cells ($sec^{+/+}$ HCT116), which express normal securin, and a derivative in which both PTTG1/securin alleles have been deleted through homologous recombination ($sec^{-/-}$ HCT116) were kindly provided by Dr B. Vogelstein (Johns Hopkins University, Baltimore, MD) and have been described previously (*29*). HCT116 cells were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum and antibiotics.

Doxorubicin (Dox) and cycloheximide (CHX) were obtained from Sigma. For Dox treatment, subconfluent cells were treated with 0.2 μg/ml or 0.05 μg/ml in regular medium for 24 h, unless otherwise stated. CHX was added to the cells at a final concentration of 40 μg/ml and the cells were harvested at the indicated times.

Plasmids and Transfection—Introduction of a pCDNA3.1 carrying the intronless ORF of PTTG1/securin was carried out using JetPei (PolyPlus Transfections Inc.) at an N/P ratio of 5 and 3 μg as total amount of DNA, according to the manufacturer instructions. The actual amount of PTTG1/securin-plasmid (0.3 μg) was such as to minimize p53 stabilization due to overexpression stress. Interference of PTTG1/securin gene was done using duplex RNA oligonucleotides (100 nM) conforming to the sequence: GUCUGUAAAGACCAAGGG. Transfection of RNAi oligonucleotides was done using *Oligofectamine* (Invitrogen) according to manufacturer instructions.

Immunoblot Analysis—Cells were lysed and soluble proteins were harvested in NP40 buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol, 1% (v/v) NP40) containing a complete cocktail of protease inhibitors (Roche) and 1 mM PMSF except when extracts were destined to protease assays. Proteins were resolved on SDS-PAGE gels for detection of $p21^{WAF1/Cip1}$, securin, p53 and β-actin. Immunoblotting was performed using the following antibodies: rabbit polyclonal anti-human $p21^{WAF1/cip1}$ (C-19, Santa Cruz), rabbit polyclonal anti-human securin previously described (*18*), polyclonal rabbit anti-human pS-15 p53 (Cell Signaling Technologies), monoclonal mouse anti-human p53 antibodies (DO-1, Santa Cruz; DO-7, DAKO), mouse monoclonal anti-MDM2 (SMP14, Santa Cruz) and monoclonal mouse anti-human β-actin (AC-15, Sigma-Aldrich). Immunoblots were developed using a horseradish peroxidase-conjugated secondary antibody and chemiluminescence detection (ECL kit, Amersham Biosciences).

Indirect Immunofluorescence—Cells grown on cover slips for 24 h were fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 in PBS for 10 min. Cells were then blocked with 3% BSA in PBS for 1 h prior to incubate them with DO-1 antibody (1/500, 1 h.). After washing and incubation with fluorescence-labelled secondary antibody, cells were mounted and photographed under the microscope (40× magnification).

2-D Analysis—Protein samples (150 μg) were solubilized in 6 M Urea 4% CHAPS, 0.5% ampholytes (pH 3–10), 15 mM DTT and traces of bromophenol blue, loaded into immobilized isoelectrofocusing strips (BioRad) and focused on a Protean IEF-cell, according to manufacturer's instructions (BioRad). Proteins were then resolved on a 10% SDS-PAGE second dimension gel and transferred to nitrocellulose membrane for immunoblot analysis using DO-1 antibody to detect p53.

Northern Blot—Total RNA was isolated from subconfluent cultures (Trizol; Gibco BRL) according to manufacturer instructions. Ten micrograms of RNA loaded on 1.2% agarose–formaldehyde–formamide gels, electrophoresed, transferred to Hybond-N+ membranes (Amersham Biosciences) and probed according to standard procedures. p53 mRNA was detected using a radiolabelled fragment containing the entire coding sequence of human p53, also the human ribosomic 18S DNA was labelled with [^{32}P]dCTP and visualized by autoradiography.

Flow Cytometry—At indicated times, floating and adherent cells were processed for flow cytometry analysis on a Coulter Epics XL apparatus as described (*31*). Briefly, cells were fixed with 70% ethanol, treated with 200 μg/ml RNAse and stained with 20 μg/ml propidium iodide prior to analysis.

Other Methods—Protein contents were quantified by the method of Bradford (*32*) using bovine serum albumin as a standard. Western blots were quantified using a flat bed scanner and ScanAnalysis software (Biosoft).

RESULTS

p53 Status in Wild-type HCT116 and $sec^{-/-}$ HCT116 Cells—We have previously shown that securin binds to and modulates p53 transactivation both *in vivo* and *in vitro* (*24*). Since modifications in the securin-binding region in the *C*-terminal half of p53 often affects the stability of the protein, we investigated if the levels of p53 were altered in $sec^{-/-}$ HCT116 cells. Western blots showed that there was indeed an accumulation of p53 in $sec^{-/-}$ HCT116 cells grown under normal conditions (Fig. 1A). The level of p53 on these cells was *ca* 4-fold greater than that seen in HCT116 parental cells, as quantified by densitometry (Fig. 1B). A similar effect was also observed using a different antibody (Fig. 2C). Furthermore, re-introduction of PTTG1/securin into $sec^{-/-}$ HCT116 cells by transient expression brought p53 levels down to those observed in the parental cell line (Fig. 1C). To discard the possibility that altered levels of p53 protein is a consequence of clonal variation

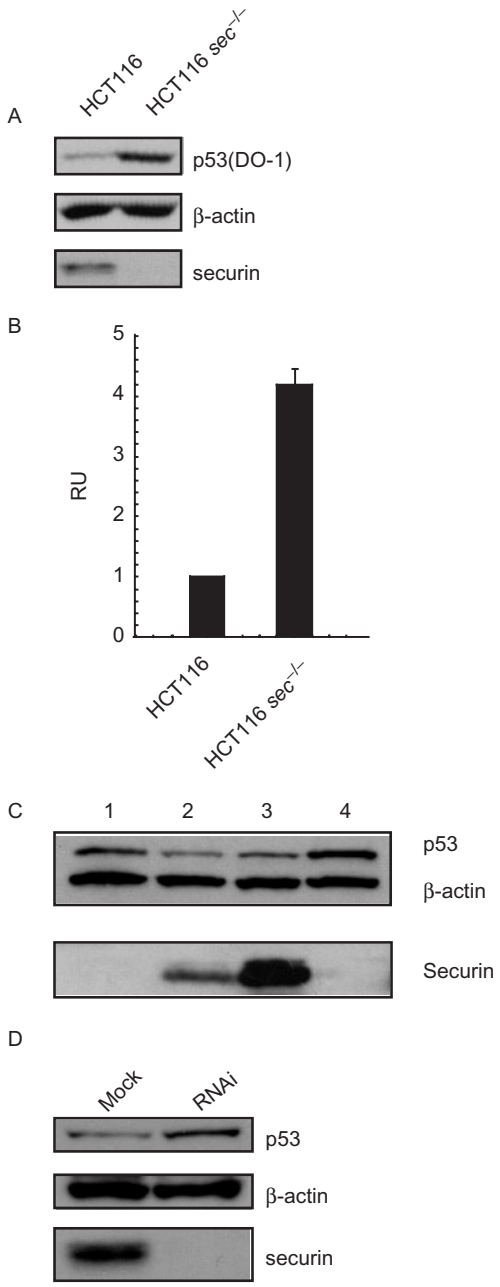

Fig. 1. **Levels of p53 in *sec*$^{+/+}$ HCT116 and *sec*$^{-/-}$ HCT116 cells.** (A) Western blots of total protein extracts from *sec*$^{+/+}$ HCT116 and *sec*$^{-/-}$ HCT116 cells grown under standard conditions. Equal amounts of protein were probed with antibodies against p53 (DO-1), β-actin and securin. (B) Quantitation of resting levels of p53 in *sec*$^{+/+}$ HCT116 and *sec*$^{-/-}$ HCT116 cells. Films from three independent western blots were scanned and intensity of bands quantified using ScanAnalysis program. Average levels found in *sec*$^{+/+}$ HCT116 cells were used as units. Bar corresponds to standard error of the mean. (C) Western blots of total protein extracts from *sec*$^{+/+}$ HCT116 and *sec*$^{-/-}$ HCT116 cells transfected with pCDNA3.1-hPTTG1, 1, pCDNA3.1 transfected *sec*$^{-/-}$ HCT116, 2, pCDNA3.1-hPTTG1 (0.3 μg) transfected *sec*$^{-/-}$ HCT116, 3, untransfected *sec*$^{+/+}$ HCT116, 4, untransfected *sec*$^{-/-}$ HCT116. (D) Levels of p53 in PTTG1/securin knock-down cells. Western blots of total protein extracts of cells transfected with RNAi oligonucleotides or mock treated.

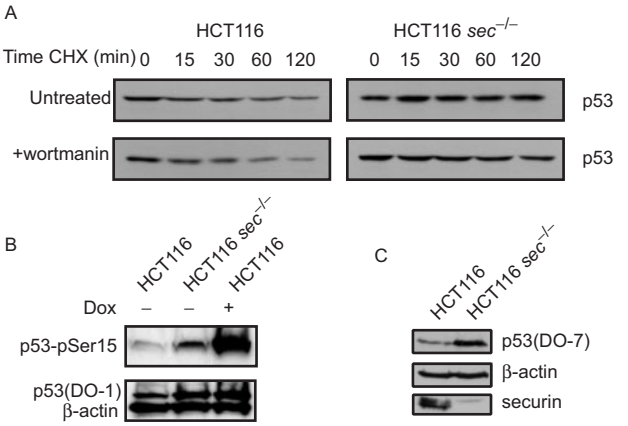

Fig. 2. **Activation state of p53 from *sec*$^{-/-}$ HCT116 cells.** (A) DNA-PK pathway is not involved in stabilization of p53. Cells grown under standard conditions were added wortmannin (5 μM) or DMSO (control) for 60 min prior to CHX treatment and samples taken at indicated times. Equal amounts of total protein extracts were probed with DO-1 antibody against p53. (B) Phosphorylation state at Ser15 on p53. Blots were probed with a polyclonal antibody specific for Ser15-phosphorylated p53 (p53-pS15) and anti-human β-actin antibodies. Extracts from *sec*$^{+/+}$ HCT116 cells treated with Dox were used as positive controls. (C) Phosphorylation state at Ser20 on p53. Total protein extracts were blotted after electrophoresis and probed with DO-7 antibodies against p53.

in the knock-out cells, securin was knocked down in HCT116 wild type cells using siRNAs. Silencing of *PTTG1/securin* gene resulted in no changes in the cell cycle profile of these cells under normal growth conditions, as observed for the *sec*$^{-/-}$ HCT116 knock-out cell line (see (*29*) and Fig. 3). Also a concomitant increase in p53 protein levels was observed (Fig. 1D), confirming a role of securin in p53 protein stability in resting cells.

To further characterize the PTTG1/securin knock-out-induced increase in p53, northern blots and CHX chase experiments were done. These experiments showed that the half-life of p53 in *sec*$^{-/-}$ HCT116 cells was affected (Fig. 4B), while the levels of mRNA coding for this protein remained unchanged in resting cells (Fig. 4A). In particular, p53 in HCT116 parental cells exhibited a measured half-life of *ca* 30 min, which was extended to nearly 2.5 h in *sec*$^{-/-}$ HCT116 cells (Fig. 4C). In addition, p53 protein localization was indistinguishable in both cell lines, indicating no mislocalization that could affect p53 functions (Fig. 5B).

Post-translational Modifications on p53—The observed increase in the p53 half-life in *sec*$^{-/-}$ HCT116 cells is reminiscent of its change during DNA damage response in wild-type cells. Therefore, we investigated if the extended half-life in the cell line under study was associated with features of activated p53. Securin has been shown to interact with Ku70/Ku80, the regulatory subunit of DNA-PK (*23*), and the latter is known to participate in the stabilization of p53 by phosphorylation of certain key residues (*33*). To check if the stability of p53 seen in our *sec*$^{-/-}$ cells was DNA-PK dependent, we did CHX chase experiments in the presence of wortmannin at concentrations known to inhibit

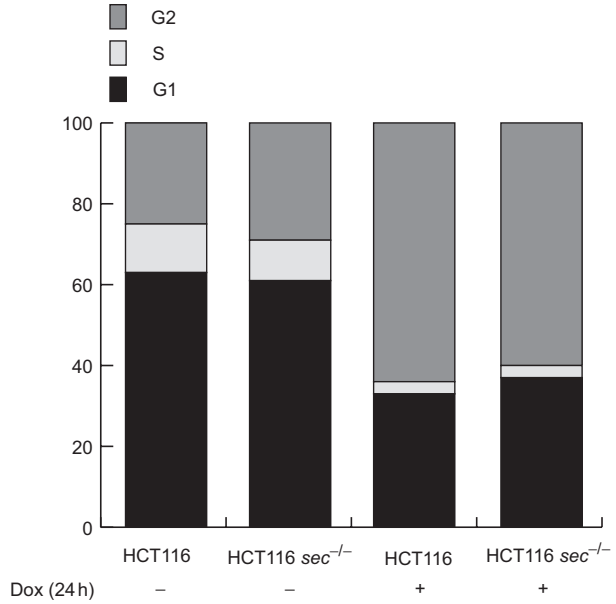

Fig. 3. **Cell cycle profiles.** Representative cell cycles before and after exposure to Dox (0.05 μg/ml) as obtained by flow cytometry. G1, S and G2 regions shown as a percentage of cells in each phase.

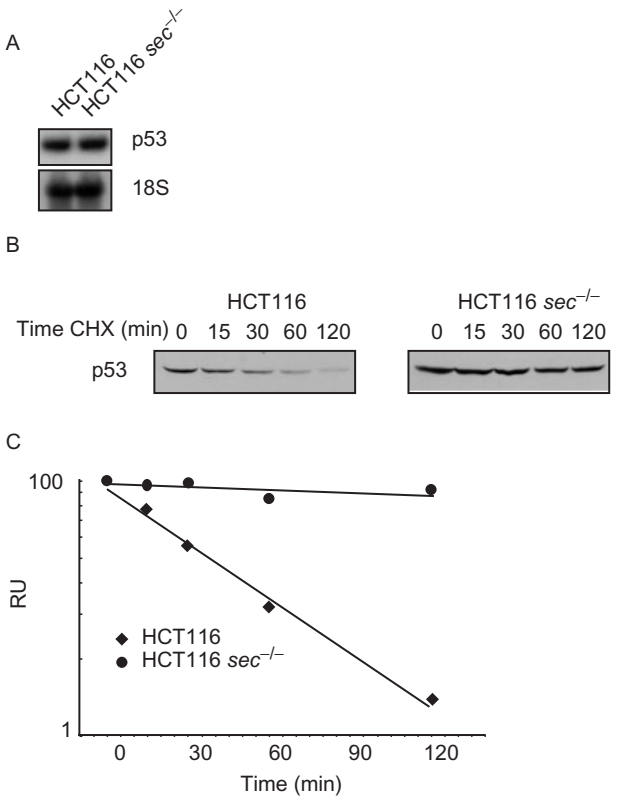

Fig. 4. **Increased half-life of p53 protein in *sec*$^{-/-}$ HCT116 cells.** (A) Northern blot of p53 gene expression. Equal amounts of total RNA from untreated cells were electrophoresed, transferred onto nylon membranes and hybridized to ^{32}P-labelled probes for p53 mRNA and 18S rRNA. (B) CHX chase. Cells grown under standard conditions were added CHX (40 μg/ml) and samples taken at indicated times, Dox treatment as indicated under experimental procedures. Equal amounts of total protein extracts were DO-1 antibody against p53. (C) Determination of half-life. Films from (B) were scanned and intensity of bands quantified with ScanAnalysis program. Data points were normalized using levels at the beginning of the experiment as 100 units.

DNA-PK activity (*34*). However, the addition of wortmannin had no effect on p53 stability in either PTTG1/securin knock-out or parental cells (Fig. 2A). To study whether the stabilization of p53 seen in *sec*$^{-/-}$ HCT116 cells was dependent on phosphorylation at Ser15, a known residue involved in p53 activation, western blots were done using a Ser15-phospho-specific antibody to check if the amount of total p53 in *sec*$^{-/-}$ HCT116 cells was related to the extent of phosphorylation on that residue. The levels were compared with wild-type HCT116 cells treated with Dox, a known elicitor of p53 stabilization through Ser15 phosphorylation. (Fig. 2B) In contrast to the marked increase in both total and Ser15-phosphorylated p53 in wild-type cells after DNA damage, untreated *sec*$^{-/-}$ HCT116 cells showed a high level of total p53 but no significant increase in the phosphorylated form, compared with untreated parental cells. A faint increase in the Ser15-phosphorylated form in *sec*$^{-/-}$ HCT116 cells can be explained by the fact that these cells have a 4-fold greater amount of p53. Indeed, when these same experiments were done using N-acetyl-L-Leucyl-L-leucyl-norleucinal (LLnL)-treated cells (a proteasome inhibitor which increases the levels of p53 in wild-type cells without affecting its phosphorylation state), no differences were observed (data not shown). Since DO-1 antibody is sensitive to phosphorylation on Ser20 (*35*), we cross-checked p53 levels using another antibody (DO-7) that recognized a non-phosphorylatable epitope (amino acids 37–45) to rule out an epitope masking effect. The results obtained using DO-7 were similar to those obtained with DO-1 (compare Fig. 1A with Fig. 2C).

To exclude the possibility of p53 being post-translationally modified at other residues in *sec*$^{-/-}$ HCT116, we did Western blots from 2D resolved gels. These experiments showed that p53 from *sec*$^{-/-}$ HCT116 cells is indistinguishable from that present in parental cells. Both cell lines exhibited three major forms differing in pI, in addition to others barely discernible (Fig. 5A). However, lack of securin did not promote the appearance of new dots corresponding to p53 modifications, and the only differences observed were in intensity, which can be ascribed to the presence of increased p53 levels in *sec*$^{-/-}$ HCT116 cells. Ubiquitination is a post-translational modification aimed mostly at proteolysis. Since the major pathway for p53 degradation is ubiquitin dependent, we then set out to find if the ubiquitination of p53 was affected in *sec*$^{-/-}$ HCT116 cells. DO-1 probed Western blots of untreated parental and *sec*$^{-/-}$ HCT116 cell extracts were overexposed to reveal the higher molecular weight ubiquitinated forms of this protein (*36*). There were no obvious differences between *sec*$^{-/-}$ HCT116 cells and its wild-type parental in terms of amount of high-molecular weight forms. Similarly, HDM2 levels were comparable in these cell lines.

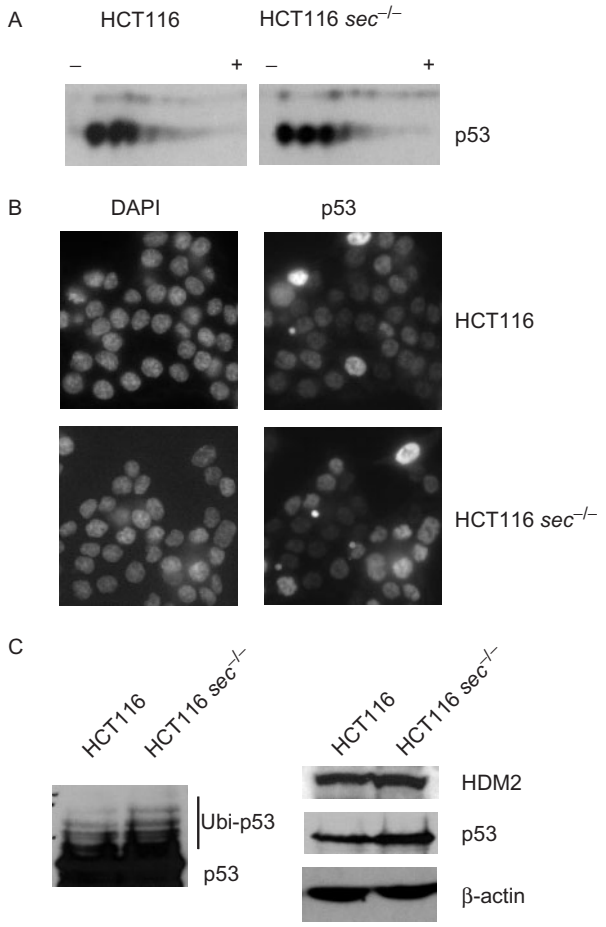

Fig. 5. **Post-translational modifications and localization of p53.** (A) Two-dimensional analysis of p53 protein from HCT116 and *sec*$^{-/-}$ HCT116 cells. Cell extracts were obtained from untreated cells and proteins separated as in experimental procedures. Blots were probed with DO-1 (p53). (B) Immunolocalization of p53. HCT116 (top panels) and *sec*$^{-/-}$ HCT116 cells (bottom panels) were immunodecorated with DO-1 to reveal p53 (left panels) and stained with DAPI to expose nuclei (right panels). Cells were micrographed at 40× magnification. (C) Western blot analysis of p53 ubiquitination using DO-1 and DO-7 antibodies. The right-hand side panel depicts a standard exposure. Left-hand side panel shows a longer exposure of the same extracts for visualization of ubiquitinated forms of p53 (*Ub-p53*).

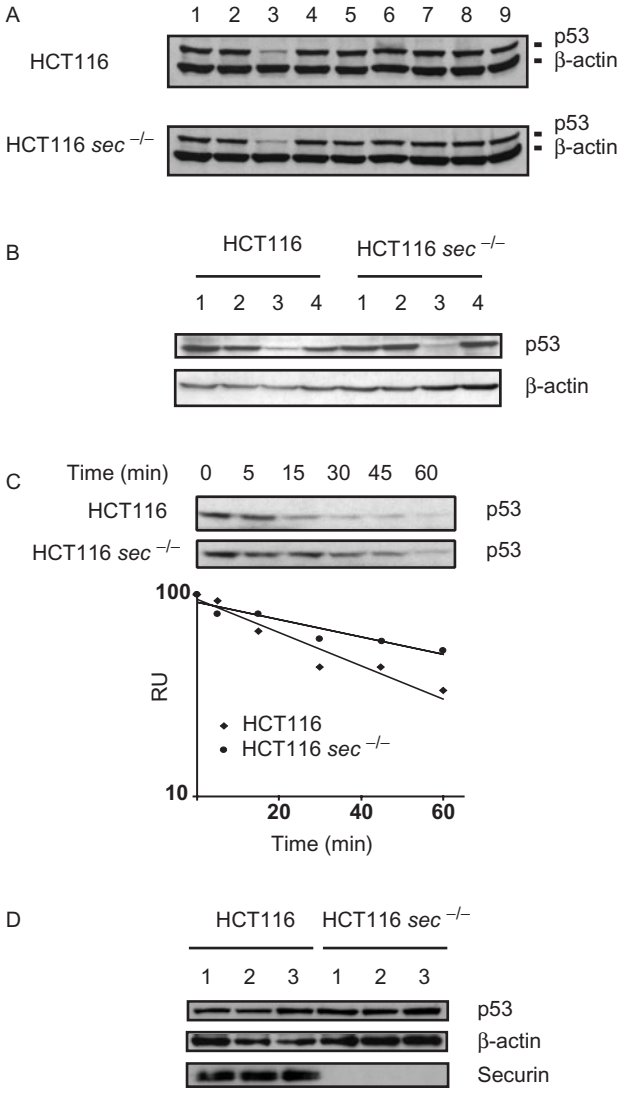

Fig. 6. **Analysis of degradation pathways on p53.** (A) Proteolytic activities capable of degrading p53. Cell extracts were incubated at 25°C for 1 h in the presence of: 1, no additions, prior to assay; 2, no additions, 1 h at 25°C; 3, $CaCl_2$ (1 mM); 4, EGTA (1 mM); 5, LLnL (0.1 mg/ml); 6, ATP·Mg (10 mM); 7, Pepstatin A (0.05 mg/ml); 8, PMSF (1 mM); 9, Leupeptin (0.05 mg/ml). Western blots were done using DO-1 (p53) and anti-β-actin antibodies. (B) Calcium-dependent proteolysis of p53. Cell extracts were incubated as in A in the presence of: 1, no additions; 2, EGTA (1 mM); 3, $CaCl_2$ (1 mM); 4, $CaCl_2$ (1 mM) and LLnL (0.1 mg/ml). (C) Time course of calcium-dependent p53 proteolysis. Cell extracts were incubated at 25°C for the indicated times in the presence of 1 mM $CaCl_2$. Top panel: Western blots probed with DO-1 (p53) and anti-β-actin antibodies. Bottom panel: densitometry of the Western blots above. (D) *In vivo* calpain inhibition. HCT116 and *sec*$^{-/-}$ HCT116 cells were treated with: 1, vehicle; 2, Calpain inhibitor XI (2.5 μM); 3, Calpastatin peptide (10 μM) for 24 h. Western blots were probed with DO-1 (p53), AC-15 (β-actin) and anti-securin antibodies.

These data ruled out ubiquitination-dependent proteasomal degradation as the major cause of increased levels of p53 in *sec*$^{-/-}$ HCT116 cells (Fig. 5C).

Proteolytic Degradation of p53—We probed other proteolytic pathways aiming to identify that responsible for the observed difference in p53 stability in *sec*$^{-/-}$ HCT116 cells. Whole cell extracts obtained in the absence of protease inhibitors were incubated in the presence of inhibitors or activators of selected proteolytic systems hypothesized to be involved in p53 regulation, including those mediated by calpain and the proteasome, the latter when independent of ubiquitin. However, degradation of p53 was only observed with addition of calcium, in concordance with published observations of calpain as a proteolytic system able to degrade p53. This effect was seen in both *sec*$^{-/-}$ and wild-type HCT116 cells (Fig. 6A). We further confirmed calpains as

the enzymes behind p53 proteolysis in these extracts by degradation assays in the presence of LLnL, a compound known to inhibit calpains (as well as the proteasome). In these experiments, LLnL successfully inhibited degradation of p53 in the presence of calcium in both *sec*$^{-/-}$ and wild-type cells, suggesting calpains as the responsible mechanism involved in stabilization of p53 in PTTG1/securin knock-out cells (Fig. 6B). Noteworthy, no proteasomal activity towards p53 was observed in these experiments (Fig. 6A, lanes 1, 2, 5 and 6). To ascertain if p53 is differentially sensitive towards calcium-dependent proteolysis in *sec*$^{-/-}$ HCT116 cells, we incubated extracts in the presence of 1 mM $CaCl_2$ and performed time-course experiments to assess the rate of p53 degradation. Thus, p53 was degraded *ca* 3-fold slower in extracts derived from *sec*$^{-/-}$ HCT116 cells compared with wild-type HCT116 cells (Fig. 6C). In order to confirm that calpains were indeed involved in the differential amounts observed in these cell lines, we treated cells for 24 h with two calpain peptide inhibitors: Calpain inhibitor XI (Z-L-Abu-CONH$(CH_2)_3$-morpholine) and Calpastatin peptide, a short (21-mer) peptide derived directly from the active domain of calpastatin, the natural *in vivo* inhibitor of calpains (Calbiochem). Calpastatin peptide was able to increase p53 levels in wild-type HCT116 up to those observed in untreated *sec*$^{-/-}$ HCT116 cells but produced no increases in this last cell line (Fig. 6D), demonstrating a direct role of calpains in the control of p53 protein in absence of securin in HCT116 cells. Calpain inhibitor XI had a less clear effect in these cell lines, probably due to its poor permeability across membranes ($IC_{50} = 41\,\mu M$ in platelets). We did not attempt to use a greater concentration of this last inhibitor due to the risk of inhibiting other proteases such as Cathepsin B (*37*).

We strived to know if there were any differences in overall calpain activity, but we saw no variation in m- or μ-calpain activity assayed as in (*38*) (data not shown). Alternatively, we attempted to find if there were differences in the conformation of p53 in *sec*$^{-/-}$ HCT116 cells that could make it more susceptible to calpains. We immunoprecipitated p53 using mutant conformation-recognizing antibody PAb240 but we observed no differences when using cell extracts from either *sec*$^{-/-}$ or wild-type HCT116 cell lines (data not shown), in agreement with (*39*).

p53-Dependent Induction of p21$^{WAF1/cip1}$ *in sec*$^{-/-}$ *HCT116*—Since p53 functions in the response to DNA damage as an inducible transcription factor, we examined the levels of $p21^{WAF1/cip1}$ in both *sec*$^{-/-}$ and parental HCT116 cells (Fig. 7A). High protein loads (100 μg) and overexposure of films were necessary in order to visualize this protein on Western blots of untreated cell extracts. Under these conditions, the relative amounts of this target of p53 were *ca* 2-fold greater in *sec*$^{-/-}$ HCT116 cells compared with its control, similar to that higher baseline level of p53 observed in this cell line. However, upon induction of DNA damage with Dox, *sec*$^{-/-}$ HCT116 cells showed and increased accumulation of p53 similar to the wild-type cell line, demonstrating the presence of a functional response (Fig. 7B), in concordance with our previous results (*24*), where the induction and p53

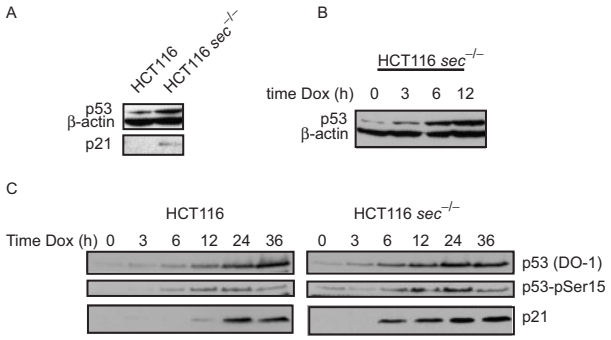

Fig. 7. **Activation of p53 in *sec*$^{-/-}$ HCT116 cells.** (A) Expression of $p21^{WAF1/cip1}$ under standard conditions. Western blots probed with DO-1 (p53), AC-15 (β-actin) and C-19 ($p21^{WAF1/cip1}$) antibodies. (B) Response of *sec*$^{-/-}$ HCT116 cells to DNA damage. Cells were treated with Dox and samples taken at indicated times thereafter. Western blots were probed with DO-1 (p53) and AC-15 antibodies (β-actin). (C) Time course induction of p53 response *sec*$^{+/+}$ HCT116 and *sec*$^{-/-}$ HCT116 cells were added to Dox (0.05 μg/ml) and samples taken at indicated times thereafter. Western blots were probed with DO-1 (p53), a polyclonal antibody specific for Ser15-phosphorylated p53 (p53-pS15) and C-19 ($p21^{WAF1/cip1}$) antibodies.

protein levels after 5-FU treatment are similar in both cell lines. To ascertain if there were differences in the way *sec*$^{-/-}$ HCT116 cells respond to genotoxic stress, these cells were treated with a sublethal dose of Dox and the levels of p53 and $p21^{WAF1/cip1}$ examined over time (Fig. 7C). Total p53 increased in both cell lines upon Dox treatment, albeit in *sec*$^{-/-}$ HCT116 cells this process was more conspicuous, due to an increased baseline p53 level. Functionality of the response was further validated by the finding that Ser15 phosphorylation of p53 in *sec*$^{-/-}$ HCT116 cells increased in accordance with accumulation of total p53. The induction of $p21^{WAF1/cip1}$ concurred with p53 activation in both cell lines but, interestingly showed an even earlier and stronger response in *sec*$^{-/-}$ HCT116 cells than in wild-type cells. This can be exemplified by comparing HCT116 cells at 12 h after Dox addition with *sec*$^{-/-}$ HCT116 cells at 6 h after treatment in Fig. 7C.

As $p21^{WAF1/cip1}$ is a major component of the G_1-checkpoint thorough its cdk regulating functions, we then studied the cell cycle profile of *sec*$^{-/-}$ HCT116 cells after DNA damage in comparison to the wild-type cell line. However, no major differences were observed 24 h after treatment (Fig. 3), showing no obvious checkpoint defects in these cells.

DISCUSSION

We have previously shown that securin interacts with p53 and is a negative regulator of its transcriptional activity (*24*). Here we describe for the first time that a consequence of the loss of PTTG1/securin is a change in half-life of the p53 protein. The functional activation of p53 is commonly linked to its stabilization within the cell. In *sec*$^{-/-}$ HCT116 cells, p53 shows an enhanced stabilization without a marked change in activity in normally cycling cells. The expected induction of G_1 cell cycle arrest that would be associated with elevated levels of p53 is not present in these cells (Fig. 3).

Post-translational modification of p53 affect its stabilization and hence its function as a transcriptional regulator. We demonstrate here that in *sec*$^{-/-}$ HCT116 cells, the stabilization of p53 was not the result of the modifications usually associated with activation of the DNA damage response mediated through kinases such as *ATM, ATR* or p38 (Fig. 2A and data not shown). In addition to these findings, no specific p53 post-transcriptional modifications that could explain the observed stabilization in untreated *sec*$^{-/-}$ HCT116 cells as opposed to the isogenic *sec*$^{+/+}$ HCT116 line were discernible on 2D Western blots (Fig. 5A) (*10*, *39*). The observed lack of differential post-transcriptional p53 modifications suggest a ubiquitin-independent degradation process such as that mediated by calpains (*10*, *39*). Indeed we demonstrate here that calpain activity on p53 degradation is affected by the presence of securin in resting cells. Calpains are a class of calcium-dependent cysteine proteases involved in p53 degradation (*10*) that recognize substrates on the basis of conformation rather than on sequence. Since securin binds p53 and affects its function, it is not unreasonable to think that this binding may also influence calpain degradation susceptibility. It springs to mind that securin binding may be masking the calpain recognition domain. However, this seems unlikely since calpain recognition site lies at the *N*-terminus of p53, and securin binding site is located at the *C*-terminus of the protein. Nevertheless, securin could still be impairing docking of calpain to p53 rather than the actual proteolytic process. Another possibility is that securin may promote a change in conformation in the p53 molecule. Conformational changes in p53 can be as subtle as not being discernible using the usual conformation-dependent antibodies (*10*, *39*). On the other hand, this kind of alterations do play a role in the regulation of a number of calpain-susceptible proteins such as the NMDA receptor (*40*) or brain spectrin (*41*). Further work would describe the fine mechanisms involved in this case.

In a parallel line of thought, our system here suggests that stabilization of p53 is not always accompanied by a decrease in its ubiquitination (Fig. 5C). In other words, this finding highlights the importance of calpains as a complementary pathway to the HMD2-p53 regulatory loop under normal growth conditions.

The p53 transcription factor is stabilized and activated in cells after genotoxic stress. It is often difficult to discern if a particular signalling pathway or post-transcriptional modification preferentially affects either stabilization of direct transcriptional activation or indeed both. A case in point is Ser15 phosphorylation. Modification of this residue was proposed to lead to stabilization and activation of the protein in a first instance (*42*) but later suggested to be involved in activation only (*43*). The present data adds weight to the latter hypothesis: in *sec*$^{-/-}$ HCT116 cells, phosphorylation at Ser15 is not required for stabilization, but emerges upon genotoxic insult known to activate p53. Concomitantly, expression of p21$^{WAF1/cip1}$, a typical p53 transcription target, is also observed after damage, indicating a functional checkpoint response (Fig. 7A and C, *sec*$^{-/-}$ HCT116 cells panels).

Regulation of p53 levels by securin and calpains is a subtle phenomenon (2- to 4-fold differences in protein levels). Other unknown mechanisms are undoubtedly at work. These mechanisms may alter the protein levels of p53 so as to mask at times the differences in p53 due to differences in securin. This may explain why we failed to see them in a previous report (*24*). However, under detailed experimental conditions, these differences are clearly revealed. Actually, differences in resting levels of p53 between wild-type and securin-null cells are patent in other researchers' work (*44–46*). Furthermore, similarly to the present study, differences in p53 speed of induction upon treatment with toxicological agents are reported in these same studies. These last observations strengthens the importance of securin down-regulation as a putatively important adjuvant strategy in p53-dependent chemotherapy.

Although the stabilization of p53 in *sec*$^{-/-}$ HCT116 cells results in some residual expression of p21$^{WAF1/cip1}$ in unstressed conditions, the levels of this last protein are almost undetectable as not to affect the cell cycle, in agreement with prior reports (*29*). In HCT116 cells, p21$^{WAF1/cip1}$ expression after dox treatment is dependent on the presence of p53 (*47*). Therefore, the difference in the total level of p21$^{WAF1/cip1}$ gene product upon dox treatment could be a direct consequence of an inhibition of p53 functions by securin, in accordance with our previous results (*24*); however, the observed difference in the speed of response can be attributed to differences in p53 resting levels.

The cell cycle arrest after DNA damage was quantitatively similar in both types of cells (Fig. 3). This, together with the ability of transactivating target genes, indicates that, on *sec*$^{-/-}$ HCT116 cells, the p53 response pathway is undamaged.

In summary, securin has an impact on p53 stability through modulation of its calpain susceptibility in a manner independent of post-translational modifications. This stabilization of p53 is uncoupled from its role as a transcription factor in the DNA damage response.

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