Published in final edited form as:
Cell Cycle. 2011 November 01; 10(21): 3719–3730. doi:10.4161/cc.10.21.17905.

CSA and CSB proteins interact with p53 and regulate its Mdm2-dependent ubiquitination

Paolo Latini[1], **Mattia Frontini**[2], **Manuela Caputo**[1], **Juraj Gregan**[3], **Lubos Cipak**[3,4], **Silvia Filippi**[1], **Vivek Kumar**[1], **Renier Vélez-Cruz**[5], **Miria Stefanini**[6], **Fabrizio Palitti**[1], and **Luca Proietti-De-Santis**[1,*]

[1]Unit of Molecular Genetics of Aging, DEB; University of Tuscia; Viterbo, Italy

[2]Clinical Sciences Centre; Medical Research Council Faculty of Medicine Imperial College London; Hammersmith Hospital Campus; London, United Kingdom

[3]Max F. Perutz Laboratories; University of Vienna; Vienna, Austria

[4]Laboratory of Cancer Genetics; Cancer Research Institute; Slovak Academy of Sciences; Bratislava, Slovak Republic

[5]Institut de Génétique et de Biologie Moléculaire et Cellulaire; CNRS/INSERM; Strasbourg, France

[6]Laboratorio Genetica Umana; Instituto Genetica Molecolare; Pavia, Italy

Abstract

Mutations in Cockayne syndrome (CS) A and B genes (*CSA* and *CSB*) result in a rare genetic disease that affects the development and homeostasis of a wide range of tissues and organs. We previously correlated the degenerative phenotype of patients to the enhanced apoptotic response, exhibited by CS cells, which is associated with the exceptional induction of p53 protein upon a variety of stress stimuli. Here we show that the elevated and persistent levels of p53 displayed by CS cells are due to the insufficient ubiquitination of p53. We further demonstrate that CSA and CSB proteins are part of a Cullin Ring Ubiquitin Ligase complex with p53 and Mdm2; this interaction greatly stimulates the ubiquitination of p53 in an Mdm2-dependent manner. Finally, we have found that p53 binds to the CSB promoter and transcriptionally controls the expression of *csb* gene allowing the establishment of a negative feedback loop that causes p53 to return at basal levels.

This study identifies CSA and CSB as the key elements of a regulatory mechanism that equilibrate beneficial and detrimental effects of p53 activity upon cellular stress. The deregulation of p53, in absence of either of the CS proteins, can potentially explain the early onset degeneration of tissues and organs observed in CS patients.

*Correspondence to: Luca Proietti-De-Santis; proietti@unitus.it.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Europe PMC Funders Author Manuscripts

Keywords

Cockayne syndrome; p53; Mdm2; ubiquitination; apoptosis; aging; cancer; DNA repair

Introduction

The tumor suppressor p53 is the main player of the cellular response organized to counteract the deleterious effects of many stress-inducing agents, including those damaging DNA.1 This response is aimed either to preserve genomic stability by pausing cell cycle and allowing DNA repair to occur or, alternatively, avoid malignant cell transformation by triggering the death of heavily damaged cells via apoptosis. Although p53 is very important to avoid cancer, its excessive accumulation can be highly detrimental because of the loss of non-renewable cells, which would lead progressively to tissue and organ degeneration.2 Therefore, cells need to fine-tune p53 levels in order to achieve a proper balance between the removal of highly damaged cells via apoptosis and the survival and proliferation of slightly damaged cells after proper repair. When this balance is lost two possibilities might arise: premature aging when p53 is over-activated3 or cancer proneness when p53 function is lost.4–6 The former has been recently proposed as one of the basis of the human Cockayne syndrome.7

Cockayne syndrome (CS) is an autosomal recessive disorder that affects the growth, development and maintenance of a wide range of tissues and organs. Among these, progressive neurological abnormalities including: demyelination, ataxia and cerebellar atrophy, are key features of this syndrome.8 Mutations in the *csa* and *csb* genes result in this syndrome.9,10 Both CSA and CSB proteins play a role in the transcription coupled repair pathway of nucleotide excision repair, which rapidly corrects certain transcription-blocking lesions located on the transcribed strand of active genes.11–13 However, it has become increasingly clear that some of the features exhibited by CS patients, including organ degeneration and growth failure, could hardly be attributed to the DNA repair deficiency,14,15 leading to the belief that some of the roles played by CS proteins are still to be unveiled. Indeed more recently, the CSB protein has been shown to interact and stimulate transcriptional protein complexes of all three classes of nuclear RNA polymerases, indicating a role for the CSB protein in transcription.16–20

Furthermore, we have recently shown that CS-B cells are unable to react to some stress stimuli, mainly because, the prosurvival transcriptional response is greatly overwhelmed by the pro-apoptotic transcriptional response, dictated by the vast accumulation of the p53 protein.21,22 As a consequence, the equilibrium between cell survival and apoptosis is tipped toward the latter in CS-B cells, that consequently manifest pronounced cell fragility when challenged with stress of broad nature, including UV irradiation, oxidative damage and hypoxia. This latter phenomenon might be at the basis of the failure to maintain tissues and organs and causative of the degenerative features observed in CS patients.

Here we show that the elevated and persistent levels of p53 pivoting the detrimental response in CS-A and CS-B cells are due to the lack of a proper ubiquitination of the p53 protein and are linked to the role played by CSA and CSB in this process. We show that CSA and CSB

Europe PMC Funders Author Manuscripts

proteins are in a complex with the p53 and Mdm2 proteins, and their presence stimulates the ubiquitination of p53, in vivo and in vitro, in an Mdm2-dependent manner. Finally, we show that CSB is under the transcriptional control of p53. We propose a model in which, under certain conditions the CS proteins (CSA and CSB) are responsible for the fine-tuning of the p53 response by its ubiquitination and subsequent degradation. This novel link between the CS proteins and p53 could help explain some of the elusive clinical and cellular phenotypes of Cockayne syndrome patients.

Results

CS cells display an increased stability of p53

In our previous analysis of the CS proteins' functions, it has become clear that cells derived from CS patients have increased basal levels of the p53 protein and this tips the cells response to several kinds of stresses toward the pro-apoptotic pathway.21,22 In this study we intend to investigate further into the relationship between the CS proteins and the elevated levels of p53 in CS cells.

A time-course analysis of p53 protein accumulation after UV irradiation revealed a clear difference when CS deficient primary fibroblasts were compared with WT primary fibroblasts (Fig. 1A and B). While in the two WT primary fibroblasts (C3PV and FB789) the accumulation of the p53 protein transiently peaks (7-fold induction relative to actin levels) between 3 and 6 h post UV irradiation and resumes basal levels within 12 h; in CS primary fibroblasts, belonging to CS-B (CS8PV, CS1PV and CS548PV) and CS-A (CS15PV) complementation groups, a dramatic and persistent increase of p53 protein levels (25–30-fold induction relative to actin levels, as quantified using ImageJ software) were observed. To compare the fold induction, among the different cells, the exposure times of the different protein gel blots were adjusted to show a comparable basal intensity. However, CS cells showed a higher basal p53 protein levels (Fig. 1C). Furthermore, treatments of cells with the protein synthesis inhibitor cycloheximide demonstrated that the half-life of the p53 protein was indeed extended in both CS-A and CS-B cells (CS15PV and CS8PV respectively), when compared with either WT cells (C3PV) or CS-B cells rescued with wt CSB overexpression (Fig. 1D).

CS cells are defective in p53 ubiquitination

In normal cells the amount of p53 protein is kept at low, barely detectable levels by continuous proteolytic degradation performed by the ubiquitin-proteasome pathway.23 We wondered whether the higher levels of p53 exhibited by CS cells were related to a defect on its ubiquitination. We therefore analyzed the ubiquitination pattern of p53 before and after UV irradiation. As shown in Figure 2A, in WT cells (C3PV) the increase in p53 protein levels (short exposition) was accompanied by an accumulation of ubiquitinated p53 (long exposition) which peaked at 6 h and then decreased, in agreement with the amount of p53 protein. In contrast, CS-B (CS8PV) cells showed a strong reduction in the ubiquitination pattern of p53 in both non-irradiated and irradiated cells. To ensure that the observed ladder was truly reflecting the ubiquitination status of p53 and not other post-translational modifications, we first immunoprecipitated p53 from WT cells and then we performed a

protein gel blot with anti-ubiquitin antibodies (Fig. 2A, left part). Alternatively, we overexpressed a Myc-tagged ubiquitin in WT cells and ubiquitinated p53 was detected by p53 IP followed by myc protein gel blotting (Fig. 2A, left part). As expected, non ubiquitinated p53 was not detected by myc antibody but only by p53 antibody (Fig. 2A, right part). In the latter p53 ubiquitinated forms are not visible because we exposed the film shortly to show the non-ubiquitinated p53 protein.

Extending the analysis to other cell lines we confirmed that CS cells, including CS-A cells, are unable to perform proper ubiquitination of the p53 protein (Fig. 2B). Additionally, transient transfection of a plasmid encoding wild-type CSB restored p53 protein ubiquitination in CS8PV cells (Fig. 2C). These analyses were performed without the use of any proteasome inhibitor. This indicates that the laddering visible in Figure 2A represents the multi-mono-ubiquitinated forms of p53. This form is the only one detectable without inhibiting the proteasome, since the proteasome immediately degrades the poly-ubiquitinated forms of p53.24

A different pattern of ubiquitination is obtained by inhibiting the proteasome with MG132 (Fig. 2D). In this case, a smear ranging from 100 to 200 kDa is observed in WT cells, which is representative of the poly-ubiquitinated forms of p53. Once again, CS cells showed a defect in the ubiquitination pathway that relates to both multi-mono- and poly-ubiquitination of p53.

As highlighted in Figure 2E, by using an antibody against ubiquitin we revealed that the problem with ubiquitination in CS cells is not widespread. Indeed WT and CS-B cells display a very similar pattern of overall protein ubiquitination, either before or after irradiation. Therefore the hitch in ubiquitination seems to be specific for p53 even if we cannot exclude that few other proteins, yet to be identified, may be affected as well.

The defect in p53 ubiquitination is not directly related to DNA repair. Primary fibroblasts belonging to a patient suffering from Xeroderma Pigmentosum complementation group A (XP-A), a disease caused by mutation in genes of the Nucleotide Excision Repair (NER) pathway that share some features with CS, does not display any defect in p53 ubiquitination although it does show a similar p53 accumulation (Fig. 2F). Other deficiencies, such as interference with the nuclear transport machinery or proteasome dysfunction might contribute to the p53 accumulation in XPA cells.25

p53 ubiquitination defect in CS is not related to a defective expression of Mdm2

It is well known that p53 is regulated by the E3 ubiquitin ligase Mdm2, a key mediator of p53 degradation whose levels are regulated through a p53-Mdm2 feedback loop.26–28 Previous work has suggested that the higher levels of p53 protein in CS cells are due to a dysregulation of the MDM2 gene.29 In order to explore this possibility we downregulated Mdm2 using siRNA. As seen in Figure 3A silencing of Mdm2 completely eliminated the ubiquitination pattern of p53 confirming irrefutably that Mdm2 is the main E3 ubiquitin ligase for p53. Additionally, as shown in Figure 3B and C, Mdm2 protein levels do increase in CS cells after UV treatment and basically, as evidenced in the graphic (Fig. 3C), its expression clearly mirrors the expression of p53 supporting the idea of a fully functional

feedback loop (even though the expression of Mdm2 is slightly delayed, when compared with WT cells). Also, protein gel blots showed the basal levels of Mdm2 evidenced a slight higher expression of Mdm2 in CS cells (Fig. 3D), in agreement to the higher basal expression of p53. We wondered whether a different post-translational modification that abolished the Mdm2-p53 interaction might occur in CS cells causing the inhibition of p53 ubiquitination. However, immunoprecipitation experiments showed that p53 co-immunoprecipitates efficiently with Mdm2 in both WT and CS-B cells, when using antibodies against p53 (Fig. 3E). This demonstrated that the interaction between p53 and Mdm2 is not perturbed in the context of the CS cells.

CSA and CSB proteins are in a complex with p53

Once verified that the Mdm2 p53 pathway/loop was functional, we decided to investigate the role of CSA and CSB in p53 ubiquitination by performing immunoprecipitation studies in order to establish whether these proteins were found in the same complex with p53 and Mdm2. In order to understand the composition of these putative complexes we designed reciprocal immunoprecipitations and we found that antibodies against p53 were able to co-immunoprecipitate CSA, CSB and as expected Mdm2 (Fig. 4A). Antibodies against CSA immunoprecipitated CSA together with p53 and CSB, but not Mdm2. Antibodies against CSB, immunoprecipitated CSB together with p53, Mdm2 and CSA and surprisingly antibodies against Mdm2 immunoprecipitated Mdm2 together with p53, CSB and also CSA. Therefore it appears that the mutual exclusivity of CSA and Mdm2, observed using anti CSA antibodies, is an artifact probably due to antigen accessibility. As a control we verified that an antibody for a TAP-tag is not able to precipitate any of our proteins. Altogether, these data suggest that p53, Mdm2, CSB and CSA exist as one or more complexes.

To confirm that these proteins are effectively part of the same complex, we expressed p53-flag protein and performed sequential immunoprecipitations. This experiment was performed in HeLa cells in order to achieve higher expression levels. First, we performed a Flag tag immunoprecipitations on Flag alone and Flag tagged WT p53 transfected HeLa cells; then we re-immunoprecipitated the eluted Flag immunocomplexes with either Mdm2 or CSB antibodies. As shown in Figure 4B, both Mdm2 and CSB immunocomplexes showed the presence of all 4 proteins and also other proteins such as DDB1 and Cul4, known to be part of CRL ubiquitin ligase complex. Interestingly, the stoichiometry between the proteins were different. In both immunocomplexes, we found a similar amount of both CSA and CSB proteins but a larger amount of p53 and Mdm2 in the Mdm2 immunoprecipitates thus suggesting that a large amount of p53 is interacting with Mdm2 but only a fraction of this complexes contains CSA and CSB proteins. At the moment we don’t know whether the association of p53-Mdm2 with CSs proteins is important and necessary only under some circumstance that need to be further exploited. However we cannot exclude the different stechiometry of immunoprecipitated might reflect a different kinetic of association with CSA and CSB proteins being engaded in this complex for a short time.

Previous works have illustrated an interaction between p53 and CSB,30 however, to our knowledge this is the first report demonstrating an interaction between CSA and p53. In order to establish whether CSA interacts directly with p53 or whether this interaction is

mediated by CSB, we immunoprecipitated p53 from different CS cell lines. As shown in Figure 5A, p53 co-immunoprecipitates with CSA and CSB (either the full length protein or the CSB-PGBD3 isoform, generated by an alternative splicing that join CSB exons 1 to 5 to the PiggyBac-like transposon PGBD3,31 in WT cells). Interestingly, CSA does not co-immunoprecipitate with p53 in CS8PV (CS-B) cells that do not express the full length CSB protein (but only the CSB-PGBD3 isoform) (Fig. 5B). Therefore, though CSB-PGBD3 is able to interact with p53, as it is found in the IP fraction, it is not able to interact with CSA, which was not detected in the IP. As a control, IP against p53 performed in CS15PV (CS-A) cells showed that CSB still interacts with p53 in the absence of CSA. Altogether these data demonstrate that CSA does not interact directly with p53, but their interaction is mediated by full length CSB or at least requires its presence.

The fact that the CSB-PGBD3 protein, whose expression is maintained in CS8PV (CS-B) cells, is still able to interact with p53 indicates that the N-terminal region of CSB is the one interacting with p53. In contrast the Carboxyl terminal region of CSB is fundamental for the CSA/CSB interaction as shown by the fact that CSB-PGBD3 does not co-immunoprecipitate with CSA in the p53 IP (Fig. 5C).

To confirm the essential role of CSB to bridge CSA and p53, we co-expressed p53 with CSA either in absence or in presence of CSB and then we immunoprecipitated p53 (using DO1 antibody that recognizes the p53 protein). An anti-flag protein gel blot demonstrated that CSA co-IP with p53 only when CSB was also expressed (Fig. 5D). UV irradiation does not seem to modify these interactions.

CSA and CSB proteins stimulates p53 ubiquitination in vitro

To determine whether CSB and CSA have intrinsic ubiquitin-protein ligase activity, we used an in vitro ubiquitination assay. Affinity purified CSA-flag and CSB-flag protein complexes were added alone or in combination to different amounts of Mdm2 E3 ligase to the reaction solutions containing E1, E2, p53 and ubiquitin recombinant proteins. As shown in Figure 6A, p53 ubiquitination pattern is directly proportional to Mdm2 concentration (lanes 1–3). Strikingly, the lowest of the p53 ubiquitination patterns obtained at the lowest concentration of Mdm2 (lane 3) is stimulated by adding either CSA or CSB immunopurified complexes (lanes 4 and 5, respectively). Addition of both CSA and CSB pushes the pattern of ubiquitination at the levels qualitatively similar to the ones seen using 50 times more Mdm2 (compare lanes 6 and 1). Remarkably, CS mediated stimulation depends on the presence of Mdm2. Indeed the pattern of ubiquitination significantly decreases in the absence of Mdm2 even if the first two bands are still clearly visible (compare lanes 7 and 8). As negative control, Figure 6B shows that withdrawal of either E1 or E2 (lanes 9 and 10, respectively) abolishes p53 ubiquitination. Also, absence of p53 from the reaction mix (lane 11) eliminates any ladder confirming that the ubiquitinated protein is indeed p53. Finally, comparison of ubiquitination reactions performed with normal or methylated ubiquitin, the latter being unable to generate polyubiquitin chains, highlighted that the laddering represents genuine multi-monoubiquitination. Figure 6C shows that CSA and CSB proteins are truly responsible of the formation of the first two bands of ubiquitination (lanes 8 and 14) since CSA and CSB knock down abolished the activity of the cognate IP (lane 15).

Also when we overexpressed CSA and CSB proteins in C3PV (WT) cells that were then irradiated and recovered 6 h post-UV, we observed a significant increase in the ubiquitination and degradation of p53 (Fig. 6D lane 17). Interestingly, knockdown of Mdm2 in CSA/CSB overexpressing cells (lane 18) reduces p53 ubiquitination and results in higher levels of p53 indicating that Mdm2 expression is fundamental for p53 ubiquitination even if higher levels of CS proteins are expressed.

Altogether, these data demonstrate that the CSA/CSB proteins clearly stimulate the ubiquitination of p53, which, however, relies on Mdm2.

CSB protein expression is induced by p53

Several proteins implicated in the degradation of p53 have been shown to be part of a negative feedback loop mechanism in which p53 is the transcriptional activator of a factor implicated in its negative regulation.32–35 To determine whether CSB expression is under the control of p53 we transfected HeLa cells with increasing amounts of a vector expressing p53-flag protein and we analyzed the endogenous CSB expression by protein gel blot. Figure 7A clearly shows that CSB protein was induced by the increased expression of p53 in the absence of DNA damage. Next, by using software for the analysis of DNA sequence elements of transcription factors (GENOMATIX, Germany), we identified a peculiar putative p53-responsive element in the promoter region of CSB gene, stretching around 3 Kb (-3,139 bp) upstream of the transcription initiation site (Fig. 7B). To confirm that this was a bona fide p53 binding site we performed chromatin immunoprecipitation (ChIP). As shown in Figure 7C, the occupancy of p53, increases significantly and accordingly to the increased expression of p53-flag proteins, at the region sited 3 Kb upstream of the transcription start (set 2) but not at another region sited 7 Kb upstream of the transcription start (set 1). ChIP experiments also showed a significant increase in RNA polymerase II occupancy on the CSB promoter (Fig. 7D) and increased acetylation of H4 (Fig. 7E), all hallmarks of transcriptional activation.

In order to prove that such regulation also occurs under a physiological stress, which is known to be fatal in CS cells, we performed chromatin immunoprecipitations in normal fibroblasts (C3PV), after UV irradiation; and to validate the exclusive role of p53 in such regulation we compared the transcriptional activation of CSB gene in p53 proficient and p53 knock-down fibroblasts (by siRNA). Figure 7F shows the induction of p53 in primary fibroblasts after UV irradiation which does not occur in the p53 knock-down cells. Consistent with our hypothesis, we were able to detect an enrichment of p53 protein (Fig. 7G) at the previously identified p53's binding site in the promoter of CSB gene. Accordingly, we observed a p53-dependent accumulation of CSB both at mRNA (Fig. 7H) and at protein (Fig. 7I) levels in response to UV irradiation.

Discussion

We have recently shown that CS-B cells are unable to react to some stress stimuli, probably because pro-survival transcriptional pathways are overcome by the pro-death transcriptional response, the latter dictated by an abnormally high accumulation of the p53 protein.21,22 In this study we confirmed the massive induction of p53 in CS-B cells and we extended the

investigation to CS-A cells. Indeed, both CS complementation groups showed a higher and prolonged p53 response when compared with WT cells. This abnormal p53 response occurred not only after DNA damage, but also when CS cells were stressed with other agents, such as chelators (DFO and CoCl2) and inhibitors of RNA synthesis (α-amanitin), both known to induce p53 as well (data not shown). Furthermore, we linked the higher and prolonged accumulation of p53 to a defect on its ubiquitination, the crucial step required to drive p53 protein to degradation via the proteasome pathway. CS cells were unable to perform either multi-mono- or poly-ubiquitination as shown by the ubiquitination analysis performed with the inhibitors of the proteasome pathway.

Though other E3 ligases have been associated in recent years to the ubiquitination of p53, Mdm2 remains the main E3 ligase responsible for its ubiquitination. Accordingly, our experiments showed that siRNA knockdown of Mdm2 abolished p53 ubiquitination. However, in contrast to what was previously hypothesized, Mdm2 expression was normal in CS cells. Furthermore, we demonstrated that binding of Mdm2 to p53 is preserved in CS cells, thus excluding any potential problem in post-translational modifications on either protein that could eventually disturb p53-Mdm2 interaction within the context of these cells. Therefore, though Mdm2 is fundamental for the ubiquitination and degradation of p53, our work shows that, under certain circumstances, this ubiquitination process seems to depend on other proteins as well. This is the case for CSA and CSB proteins, since when these proteins are mutated the ubiquitination of p53 does not occur properly, leading to a massive accumulation of the p53 protein due to a faulty degradation.

What is the role/s of these proteins in the ubiquitination process? Ubiquitination assays showed that CSA/CSB proteins stimulated the E3 ligase activity of Mdm2. Indeed, the addition of the CSA/CSB proteins, either in vivo or in vitro, increased the efficiency of Mdm2 dependent ubiquitination of p53. Mdm2 is a ring protein able to interact with both the E2 and the ubiquitination substrate and thus directly catalyze the ubiquitination of p53. It is possible that, under specific circumstances, Mdm2 requires to be part of a multi-subunit Cullin-RING ubiquitin ligases (CRLs) complex, containing a Cullin, an adaptor and an F-box protein. We hypothesize that this complex is more efficient in transferring ubiquitin onto p53 either in general or onto specific lysine residues. The assembly of Mdm2 in a CRL complex has been described recently in reference 36. It is possible that the F-box protein CSA, known to be part of a CUL4/DDB1 complex,37 might be part of a CRL complex, also containing the ring protein Mdm2. In agreement with this model, we have found that CSA and CSB form a complex that contains p53, Mdm2 and also DDB1 and Cul4 (Fig. 4B).

What is the role played by CSB? CSB has been previously described as the substrate of a CUL4/DDB1 complex.37 However, in the present study we demonstrated that CSB is playing an active role in p53 ubiquitination, and its absence dramatically decreases the ubiquitination of p53 after UV irradiation in cells. Furthermore, we have shown that the CSA-p53 interaction depends on the presence of full-length CSB. Therefore, CSB may serve a structural role and stabilize the interaction between the F-box protein CSA and p53. Alternatively, but not mutually exclusive, CSB may have a function in the stabilization of the E3 ubiquitin ligase complex. Indeed, due to the rapid turnover of some of its subunits, CRL-type complexes are highly unstable.38 This phenomenon seems to have evolved to guarantee

a fine modulation of their activity. Intriguingly, using tandem affinity purification combined with mass spectrometry, we found that CSB interacted with both CAND1 and COP9 signalosome (Sup. Mat.), known to maintain the stability of CRL based complexes39 and UPS7 (HAUSP), which has been shown to deubiquitinate and stabilize specific factors, including Mdm2.40 Noteworthy, CSB also interacts with DET1, which has been shown to form a complex with DDB1 and COP9 signalosome in Arabidopsis (both also identified the present study as bona fide interactors of CSB) enhancing E2 activity.41 This led us to speculate that CSB may enhance the activity of ubiquitin conjugating enzymes in agreement with our in vitro assay data. Accordingly, very recently, an ubiquitin-binding domain has been discovered in CSB protein.42

Finally, we demonstrated that the p53 protein binds to the CSB promoter and transcriptionally controls CSB expression. Other proteins involved in the degradation of p53 have been demonstrated to be under the transcriptional control of p53 itself. This allows the establishment of a negative feedback loop whereby p53 returns to basal levels. Though p53 does not control the CSA gene, we highlighted that the functionality of the latter depends on CSB, which therefore is the main regulator loop. Importantly, CSA and CSB are also the key components of the TCR, the DNA repair pathway evolved to guarantee the gene expression after DNA damage. The apoptotic cell death response triggered upon stress in the absence of one of these proteins led us to envision an evolutionary selection mechanism by which a defect in DNA repair of the transcribed strand leads to the upregulation of p53 response in order to maintain the genomic stability. Recently, it has been described how p53 nuclear accumulation and apoptosis following a transcription blockage due to a DNA lesion might be part of a transcription stress response (TSR), which plays an important role in safeguarding the genome from DNA damage and suppressing tumorigenesis.43 By removing the blocking lesions and restoring mRNA synthesis, TCR effectively removes the trigger that activates TSR. Our findings suggest a redundant mechanism, either alternative or synergistic to the one of the transcription blockage that elicits p53 activity. In this case, the absence or transient unavailability of CSA and CSB proteins (due to them being engaged in DNA repair), key components of TCR would turn off p53 ubiquitination, thus triggering the activation of the TSR response in a way independent of the transcription blockage. This mechanism would appear particularly important in case of lesions like those induced by oxidative damage, that despite being highly mutagenic, do not substantially block elongation by RNA polymerase II.44 Therefore, CSA and CSB proteins by modulating the activity and the amount of p53, do help the “system” find a reasonable equilibrium between the beneficial and the detrimental effects of p53 activity.

Our work links the absence of CS proteins (CSA and CSB) to the over activation of p53, even in the absence of DNA damage. This deregulation of p53 could potentially explain some of the segmental progeria and degenerative clinical symptoms displayed by CS patients.

Materials and Methods

Cell lines

Primary human fibroblasts were grown in Minimal essential medium (MEM) containing 15% Foetal Calf Serum (FCS) and antibiotics. CS1AN cells were grown in DMEM/F10 (1:1) medium containing 10% FBS and antibiotics. HeLa cells were grown in DMEM medium containing 10% serum and antibiotics. UV irradiation treatments (10 J/m^2) were done with UV-C (254 nm) lamp. Proteasome inhibitor experiments were conducted with 25 μM MG132 (Sigma).

Antibodies

The monoclonal antibodies against actin (C2), Mdm2 (SMP14), p53 (DO-1) and polyclonal antibodies against CSB (H300), DDB1 (H300), ubiquitin (FL76) were purchased from Santa Cruz. The monoclonal antibody against the Flag epitope (M2) was purchased from Sigma Aldrich. The monoclonal antibody against Cul4 (2699) was purchased from Cell Signaling. The monoclonal antibody against CSA (96780) was purchased from Abcam.

Protein gel blot analysis

Cells were lysed for 10 min on ice in RIPA buffer. The cell lysates were centrifuged at 13,000 rpm for 5 min and the supernatant containing the proteins was recovered. Protein concentration was determined by Bradford protein assay kit (BioRad). Fifty micrograms of proteins were separated on polyacrylamide gradient gel (4–20%) electrophoresis and blotted onto PVDF membrane (Amersham) following standard protocols. The membrane was incubated with TBST (20 mM Tris-HCl, pH 7.4, 137 mM NaCl; 0.2% Tween 20) buffer containing 5% NFDM for 60 min at RT and subsequently incubated with primary antibodies and HRP conjugated secondary antibody (Vector). The signal was detected using the enhanced chemiluminescence method (ECL) following the manufacturer's instructions (Amersham).

RNA interference

A pool of four RNA oligonucleotides (Dharmacon) forming a 19 base-duplex, specifically designed to target Mdm2 mRNA was transfected in C3PV cells at the concentration of 50 nM. A pool of RNA oligonucleotides, without any target mRNA, was used as control. RNA transfection were performed by using Lipofectamine 2000 reagent (InVitrogen).

Construction of tagged vector, transfection and selection

CSA-Flag, CSB-Flag and p53-Flag tagging vectors were obtained by cloning the respective ORF into the p3xFLAG-CMV-10 mammalian expression vector (Sigma-Aldrich). Transient transfection was performed using Lipofectamine 2000 reagent (InVitrogen) according to the manufacturers instructions.

The C-terminal TAP tagging (TAP-CSB) vector was obtained by inserting the ORF of CSB into a pBABE based mammalian expression vector where the C-TAP domain (protein A + TEV site + calmodulin binding domain) had been previously inserted. Stable transfection selection was performed with puromycin (0.3 μg/ml).

Preparation of cellular extract

The cells were scraped from plates into ice-cold PBS and pelleted by centrifugation at 2,000 x g for 10 min at 4°C. After removal of excess PBS, the cell pellet (30 ml) was resuspended in 60 ml of ice-cold IPP150 lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol, 0.1% NP-40, complete protease inhibitors, 1 mM PMSF). The cells were homogenized with 40 strokes in a Dounce homogenizer with a tight-fitting pestle and incubated on ice for 5 min. Insoluble material was removed by centrifugation at 16,000x g for 20 min at 4°C.

Chromatin immunoprecipitation

Cells were cross-linked with a 1% formaldehyde solution for 10 min at RT. Cross-linking was stopped by addition of glycine to 125 mM final concentration. Samples were sonicated to generate DNA fragments below 500 bp. For immunoprecipitations, 1 mg of protein extract was precleared for 2 h with 50 ml of a 50% slurry of 50:50 protein A/G-sepharose before addition of the indicated antibodies. Then, 2 mg of p53 antibody was added to the reactions and incubated over night at 4°C in the presence of 50 ml of protein A/G beads. After serial washings, the immunocomplexes were eluted twice for 10 min at 65°C and cross-linking was reversed by adjusting to 200 mM NaCl and incubating 5 h at 65°C. Further proteinase-K digestion was performed for 2 h at 42°C. DNA was purified by using Qiagen columns (QIAquick PCR purification Kit). Immunoprecipitated DNA was quantified by real-time quantitative PCR. Primers sequences are available upon request.

Ubiquitination in vitro assay

Reactions were performed in a 50 µl mixture containing 1x Ubiquitin Conjugation reaction buffer (B70), 1x Energy Regeneration Solution (B-10), 1 mM ubiquitin (U-100H), 400 nM His-p53 (SP450), different amount of His-Mdm2 (E3-200) as indicated in the figure legends, 50 nM Ubiquitin Activating Enzyme (UBE1-E305), 250 nM UbcH5c (E2-627) purchased from BostonBiochem (USA) and where indicated immunoprecipitated CSA- and/or CSB-complexes. CSA-Flag and CSB-Flag containing complexes were immunoprecipitated by using anti-Flag affinity gel (Sigma-Aldrich) and eluted with 3xFlag peptides.

After 2 h of incubation at 37°C, reaction mixtures were separated by SDS-PAGE and analyzed by immunoblotting using p53 antibody.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Girish Rajendraprasad for critical reading of the manuscript. This study has been supported by funds from “Associazione Italiana per la Ricerca sul Cancro” (AIRC) and MISE. L.P.D.S. is a Brain Gain Italian program (Rientro dei Cervelli-MIUR) recipient and has been also supported by the European Molecular Biology Organization (EMBO-Short-term Fellowship). J.G. was supported by Austrian Science Fund grants (P18955, P20444, F3403) and L.C. was supported by the (European Community’s) Seventh Framework Programme (FP7/2007–2013) under grant agreement number PERG07-GA-2010-268167. We thank Dr. Ammerer and his team for the mass-spectrometry analysis.

References

1. Llanos S, Serrano M. Depletion of ribosomal protein L37 occurs in response to DNA damage and activates p53 through the L11/MDM2 pathway. Cell Cycle. 2010; 9:4005–12. DOI: 10.4161/cc.9.19.13299 [PubMed: 20935493]
2. Feng Z, Hu W, Rajagopal G, Levine AJ. The tumor suppressor p53: cancer and aging. Cell Cycle. 2008; 7:842–7. DOI: 10.4161/cc.7.7.5657 [PubMed: 18414039]
3. Tyner SD, Venkatachalam S, Choi J, Jones S, Ghebranious N, Igelmann H, et al. p53 mutant mice that display early ageing-associated phenotypes. Nature. 2002; 415:45–53. DOI: 10.1038/415045a [PubMed: 11780111]
4. Brown JG, Familiari U, Papotti M, Rosai J. Thymic basaloid carcinoma: a clinicopathologic study of 12 cases, with a general discussion of basaloid carcinoma and its relationship with adenoid cystic carcinoma. Am J Surg Pathol. 2009; 33:1113–24. DOI: 10.1097/PAS.0b013e3181a2443b [PubMed: 19461509]
5. Band M, Ashur-Fabian O, Avivi A. The expression of p53-target genes in the hypoxia-tolerant subterranean mole-rat is hypoxia-dependent and similar to expression patterns in solid tumors. Cell Cycle. 2010; 9:3347–52. DOI: 10.4161/cc.9.16.12712 [PubMed: 20703075]
6. Inuzuka H, Fukushima H, Shaik S, Wei W. Novel insights into the molecular mechanisms governing Mdm2 ubiquitination and destruction. Oncotarget. 2010; 1:685–90. [PubMed: 21317463]
7. Frontini M, Proietti-De-Santis L. Cockayne syndrome B protein (CSB): linking p53, HIF-1 and p300 to robustness, lifespan, cancer and cell fate decisions. Cell Cycle. 2009; 8:693–6. DOI: 10.4161/cc.8.5.7754 [PubMed: 19221478]
8. Weidenheim KM, Dickson DW, Rapin I. Neuropathology of Cockayne syndrome: Evidence for impaired development, premature aging and neurodegeneration. Mech Ageing Dev. 2009; 130:619–36. DOI: 10.1016/j.mad.2009.07.006 [PubMed: 19647012]
9. Troelstra C, van Gool A, de Wit J, Vermeulen W, Bootsma D, Hoeijmakers JH. ERCC6, a member of a subfamily of putative helicases, is involved in Cockayne's syndrome and preferential repair of active genes. Cell. 1992; 71:939–53. DOI: 10.1016/0092-8674(92)90390-X [PubMed: 1339317]
10. Henning KA, Li L, Iyer N, McDaniel LD, Reagan MS, Legerski R, et al. The Cockayne syndrome group A gene encodes a WD repeat protein that interacts with CSB protein and a subunit of RNA polymerase II TFIIH. Cell. 1995; 82:555–64. DOI: 10.1016/0092-8674(95)90028-4 [PubMed: 7664335]
11. Licht CL, Stevnsner T, Bohr VA. Cockayne syndrome group B cellular and biochemical functions. Am J Hum Genet. 2003; 73:1217–39. DOI: 10.1086/380399 [PubMed: 14639525]
12. Lainé JP, Egly JM. When transcription and repair meet: a complex system. Trends Genet. 2006; 22:430–6. DOI: 10.1016/j.tig.2006.06.006 [PubMed: 16797777]
13. Fousteri M, Vermeulen W, van Zeeland AA, Mullenders LH. Cockayne syndrome A and B proteins differentially regulate recruitment of chromatin remodeling and repair factors to stalled RNA polymerase II in vivo. Mol Cell. 2006; 23:471–82. DOI: 10.1016/j.molcel.2006.06.029 [PubMed: 16916636]
14. Cleaver JE, Hefner E, Laposa RR, Karentz D, Marti T. Cockayne syndrome exhibits dysregulation of p21 and other gene products that may be independent of transcription-coupled repair. Neuroscience. 2007; 145:1300–8. DOI: 10.1016/j.neuroscience.2006.08.074 [PubMed: 17055654]
15. Brooks PJ, Cheng TF, Cooper L. Do all of the neurologic diseases in patients with DNA repair gene mutations result from the accumulation of DNA damage? DNA Repair (Amst). 2008; 7:834–48. DOI: 10.1016/j.dnarep.2008.01.017 [PubMed: 18339586]
16. Selby CP, Sancar A. Cockayne syndrome group B protein enhances elongation by RNA polymerase II. Proc Natl Acad Sci USA. 1997; 94:11205–9. DOI: 10.1073/pnas.94.21.11205 [PubMed: 9326587]
17. Tantin D, Kansal A, Carey M. Recruitment of the putative transcription-repair coupling factor CSB/ERCC6 to RNA polymerase II elongation complexes. Mol Cell Biol. 1997; 17:6803–14. [PubMed: 9372911]

18. van Gool AJ, van der Horst GT, Citterio E, Hoeijmakers JH. Cockayne syndrome: defective repair of transcription? EMBO J. 1997; 16:4155–62. DOI: 10.1093/emboj/16.14.4155 [PubMed: 9250659]
19. Bradsher J, Auriol J, Proietti de Santis L, Iben S, Vonesch JL, Grummt I, et al. CSB is a component of RNA pol I transcription. Mol Cell. 2002; 10:819–29. DOI: 10.1016/S1097-2765(02)00678-0 [PubMed: 12419226]
20. Yuan X, Feng W, Imhof A, Grummt I, Zhou Y. Activation of RNA polymerase I transcription by cockayne syndrome group B protein and histone methyltransferase G9a. Mol Cell. 2007; 27:585–95. DOI: 10.1016/j.molcel.2007.06.021 [PubMed: 17707230]
21. Proietti-De-Santis L, Drane P, Egly JM. Cockayne syndrome B protein regulates the transcriptional program after UV irradiation. EMBO J. 2006; 25:1915–23. DOI: 10.1038/sj.emboj.7601071 [PubMed: 16601682]
22. Filippi S, Latini P, Frontini M, Palitti F, Egly JM, Proietti-De-Santis L. CSB protein is (a direct target of HIF-1 and) a critical mediator of the hypoxic response. EMBO J. 2008; 27:2545–56. DOI: 10.1038/emboj.2008.180 [PubMed: 18784753]
23. Maki CG, Huibregtse JM, Howley PM. In vivo ubiquitination and proteasome-mediated degradation of p53(1). Cancer Res. 1996; 56:2649–54. [PubMed: 8653711]
24. Li M, Brooks CL, Wu-Baer F, Chen D, Baer R, Gu W. Mono- versus polyubiquitination: differential control of p53 fate by Mdm2. Science. 2003; 302:1972–5. DOI: 10.1126/science.1091362 [PubMed: 14671306]
25. Ljungman M, O'Hagan HM, Paulsen MT. Induction of ser15 and lys382 modifications of p53 by blockage of transcription elongation. Oncogene. 2001; 20:5964–71. DOI: 10.1038/sj.onc.1204734 [PubMed: 11593403]
26. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. Nature. 2000; 408:307–10. DOI: 10.1038/35042675 [PubMed: 11099028]
27. Zdzalik M, Pustelny K, Kedracka-Krok S, Huben K, Pecak A, Wladyka B, et al. Interaction of regulators Mdm2 and Mdmx with transcription factors p53, p63 and p73. Cell Cycle. 2010; 9:4584–91. DOI: 10.4161/cc.9.22.13871 [PubMed: 21088494]
28. Miliani de Marval PL, Zhang Y. The RP-Mdm2-p53 pathway and tumorigenesis. Oncotarget. 2011; 2:234–8. [PubMed: 21406728]
29. Conforti G, Nardo T, D'Incalci M, Stefanini M. Proneness to UV-induced apoptosis in human fibroblasts defective in transcription coupled repair is associated with the lack of Mdm2 transactivation. Oncogene. 2000; 19:2714–20. DOI: 10.1038/sj.onc.1203583 [PubMed: 10851071]
30. Wang XW, Yeh H, Schaeffer L, Roy R, Moncollin V, Egly JM, et al. p53 modulation of TFIIH-associated nucleotide excision repair activity. Nat Genet. 1995; 10:188–95. DOI: 10.1038/ng0695-188 [PubMed: 7663514]
31. Newman JC, Bailey AD, Fan HY, Pavelitz T, Weiner AM. An abundant evolutionarily conserved CSB-PiggyBac fusion protein expressed in Cockayne syndrome. PLoS Genet. 2008; 4:1000031.doi: 10.1371/journal.pgen.1000031
32. Wu X, Bayle JH, Olson D, Levine AJ. The p53-mdm-2 autoregulatory feedback loop. Genes Dev. 1993; 7:1126–32. DOI: 10.1101/gad.7.7a.1126 [PubMed: 8319905]
33. Leng RP, Lin Y, Ma W, Wu H, Lemmers B, Chung S, et al. Pirh2, a p53-induced ubiquitin-protein ligase, promotes p53 degradation. Cell. 2003; 112:779–91. DOI: 10.1016/S0092-8674(03)00193-4 [PubMed: 12654245]
34. Dornan D, Wertz I, Shimizu H, Arnott D, Frantz GD, Dowd P, et al. The ubiquitin ligase COP1 is a critical negative regulator of p53. Nature. 2004; 429:86–92. DOI: 10.1038/nature02514 [PubMed: 15103385]
35. Fuchs SY, Adler V, Buschmann T, Yin Z, Wu X, Jones SN, et al. JNK targets p53 ubiquitination and degradation in nonstressed cells. Genes Dev. 1998; 12:2658–63. DOI: 10.1101/gad.12.17.2658 [PubMed: 9732264]
36. Banks D, Wu M, Higa LA, Gavrilova N, Quan J, Ye T, et al. L2DTL/CDT2 and PCNA interact with p53 and regulate p53 polyubiquitination and protein stability through MDM2 and CUL4A/DDB1 complexes. Cell Cycle. 2006; 5:1719–29. DOI: 10.4161/cc.5.15.3150 [PubMed: 16861890]

37. Groisman R, Kuraoka I, Chevallier O, Gaye N, Magnaldo T, Tanaka K, et al. CSA-dependent degradation of CSB by the ubiquitin-proteasome pathway establishes a link between complementation factors of the Cockayne syndrome. Genes Dev. 2006; 20:1429–34. DOI: 10.1101/gad.378206 [PubMed: 16751180]

38. Wee S, Geyer RK, Toda T, Wolf DA. CSN facilitates Cullin-RING ubiquitin ligase function by counteracting autocatalytic adapter instability. Nat Cell Biol. 2005; 7:387–91. DOI: 10.1038/ncb1241 [PubMed: 15793566]

39. Wu JT, Chan YR, Chien CT. Protection of cullin-RING E3 ligases by CSN-UBP12. Trends Cell Biol. 2006; 16:362–9. DOI: 10.1016/j.tcb.2006.05.001 [PubMed: 16762551]

40. Li M, Brooks CL, Kon N, Gu W. A dynamic role of HAUSP in the p53-Mdm2 pathway. Mol Cell. 2004; 13:879–86. DOI: 10.1016/S1097-2765(04)00157-1 [PubMed: 15053880]

41. Yanagawa Y, Sullivan JA, Komatsu S, Gusmaroli G, Suzuki G, Yin J, et al. Arabidopsis COP10 forms a complex with DDB1 and DET1 in vivo and enhances the activity of ubiquitin conjugating enzymes. Genes Dev. 2004; 18:2172–81. DOI: 10.1101/gad.1229504 [PubMed: 15342494]

42. Anindya R, Mari PO, Kristensen U, Kool H, Giglia-Mari G, Mullenders LH, et al. A ubiquitin-binding domain in Cockayne syndrome B required for transcription-coupled nucleotide excision repair. Mol Cell. 2010; 38:637–48. DOI: 10.1016/j.molcel.2010.04.017 [PubMed: 20541997]

43. Ljungman M. The transcription stress response. Cell Cycle. 2007; 6:2252–7. DOI: 10.4161/cc.6.18.4751 [PubMed: 17700065]

44. Charlet-Berguerand N, Feuerhahn S, Kong SE, Ziserman H, Conaway JW, Conaway R, et al. RNA polymerase II bypass of oxidative DNA damage is regulated by transcription elongation factors. EMBO J. 2006; 25:5481–91. DOI: 10.1038/sj.emboj.7601403 [PubMed: 17110932]

Europe PMC Funders Author Manuscripts

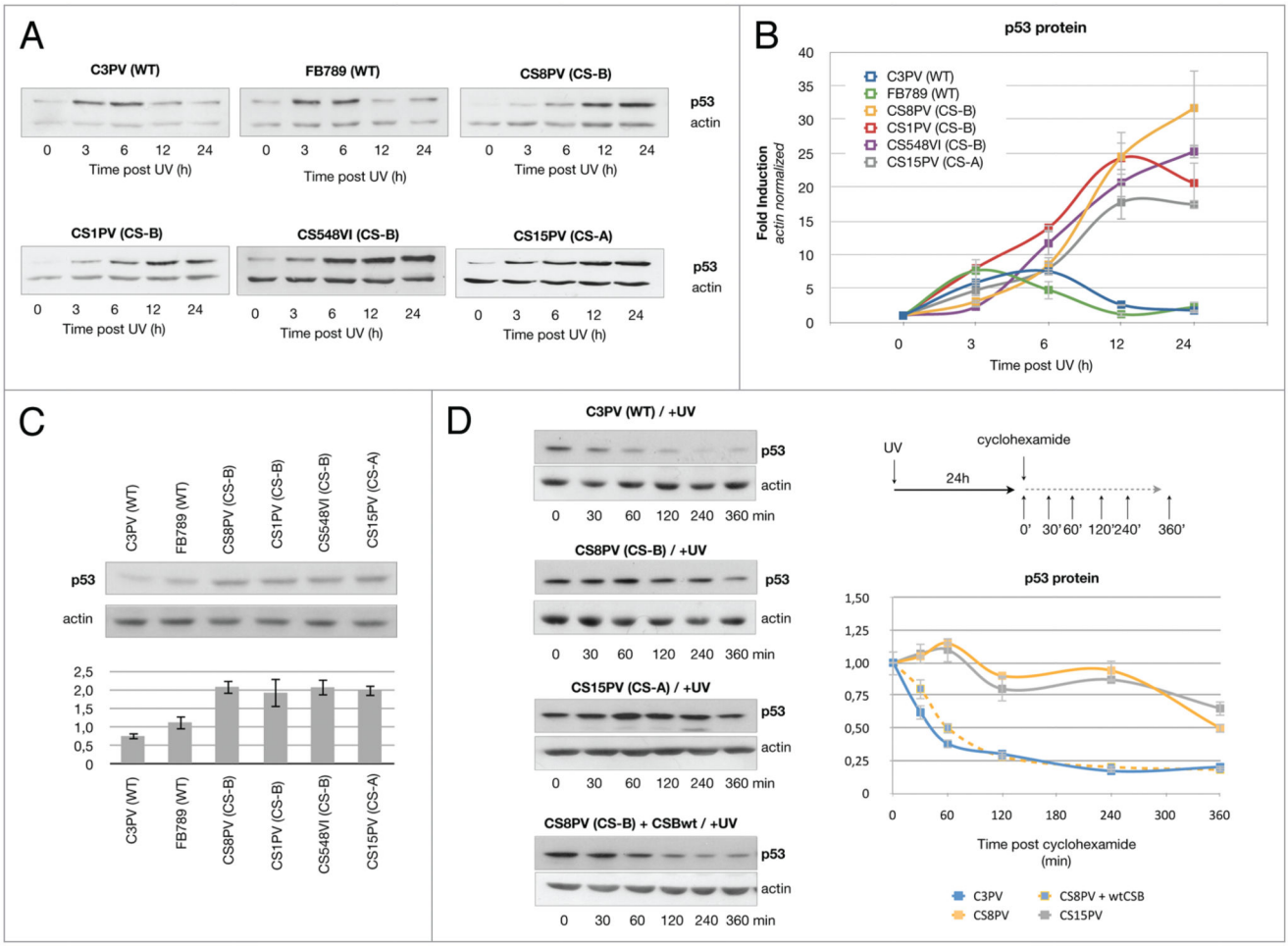

Figure 1.

(A) Protein gel blot analysis of p53 and β-actin protein amount from whole cellular extracts of WT (C3PV and FB789), CS-B (CS8PV, CS1PV and CS548VI) and CS-A (CS15PV) cells upon UV irradiation (10 J/m^2). Protein extracts were collected at the indicated times. Exposure times were adjusted to show similar basal levels of p53. (B) Graph represents quantification of p53 protein amounts (mean ± SD of three independent experiments) after UV irradiation (10 J/m^2) using ImageJ software (NIH). Data have been normalized by β-actin. (C) Protein gel blot analysis of basal p53 and β-actin protein amount from whole extracts of WT (C3PV and FB789), CS-B (CS8PV, CS1PV and CS548VI) and CS-A (CS15PV). Bottom part showed the relative quantification obtained by normalizing p53 by β-actin protein amount. (D) Protein gel blot analysis of p53 and β-actin protein amount from whole cellular extracts of cycloheximide treated WT (C3PV), CS-B (CS8PV), CS-A (CS15PV) and CS-B cells rescued with wtCSB overxpression, upon UV irradiation (10 J/m^2). As illustrated in the figure cells were UV-irradiated, then 24 h later treated with cyclohexamide (10 mg/ml) and harvested at 30, 60, 120, 240 and 360 min. Graph illustrated p53 levels in cyclohexamide treated cells vs. time.

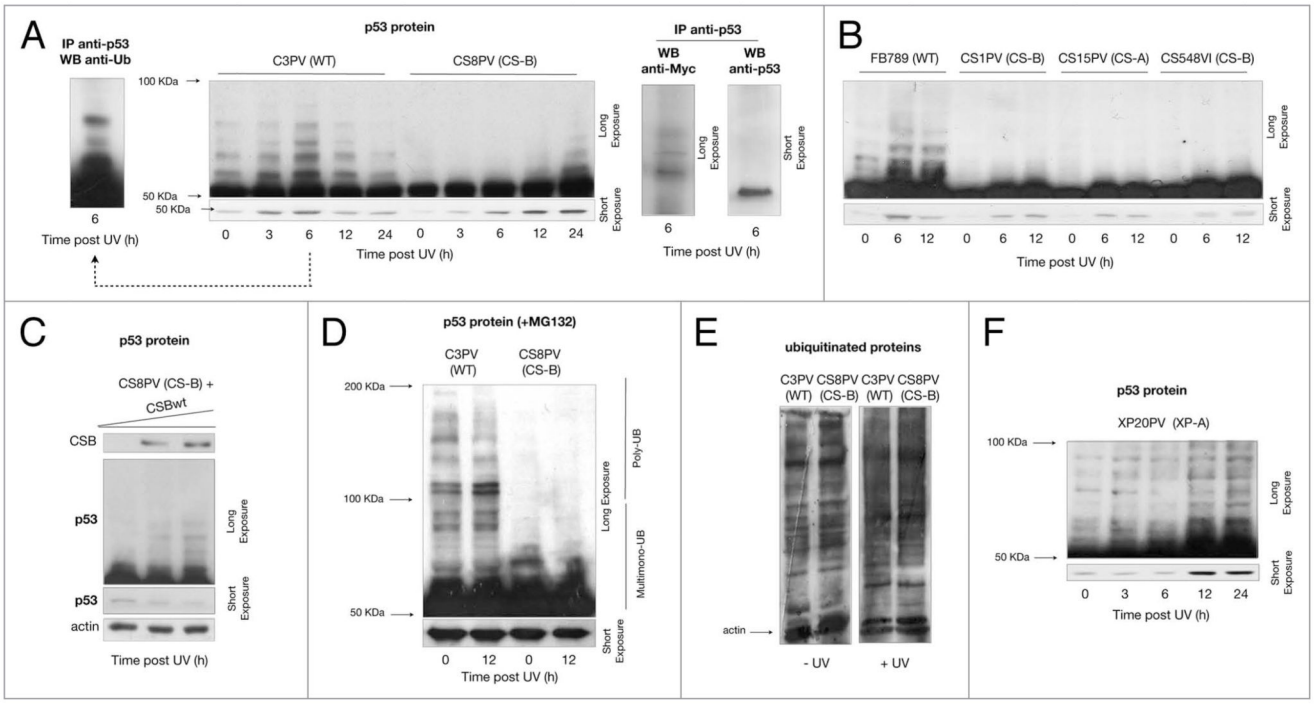

Figure 2.

(A and B) Protein gel blot analysis of p53 ubiquitination (long exposure) and p53 protein amount (short exposure) upon UV irradiation (10 J/m^2). Protein extracts were collected at the indicated times. Long and short exposure indicate the length of time to which the film was exposed during the chemiluminescent detection. The left part of (A) shows protein gel blot analysis, using an antibody against ubiquitin, of p53-immunoprecipitates of C3PV cells 6 h after irradiation. (A′) shows protein gel blotting analysis using antibodies against either myc-tag or p53, of p53-immunoprecipitates of ubiquitin-myc overexpressing C3PV cells 6 h after irradiation. In the right part of (A') the secondary antibody recognizes only the light chain of the antibody. (C) Protein gel blot analysis of p53 ubiquitination in CS-B (CS8PV) cells after UV irradiation (10 J/m^2) of protein extracts, collected at the time indicated. Cells were transfected with increasing amount of CSB-expression vector (0, 1 and 2 μg) and three days of puromycin selection was performed to enrich for CSB expressing cells before the UV irradiation. (D) Protein gel blot analysis of p53 ubiquitination (long exposure) and p53 amount (short exposure) in either unirradiated and UV-irradiatated (10 J/m^2) cells that were preventively exposed to the proteasome inhibitor MG132 (50 μM) 3 h before the irradiation. (E) Protein gel blot analysis of ubiquitinated proteins from whole cellular extracts of both unirradiated and UV-irradiated WT and CS-B cells using an antibody against ubiquitin. (F) Protein gel blot analysis of p53 ubiquitination (long exposure) and p53 amount (short exposure) after UV irradiation (10 J/m^2) in DNA repair deficient cells belonging to Xeroderma pigmentosum complementation group A (XP-A).

Europe PMC Funders Author Manuscripts

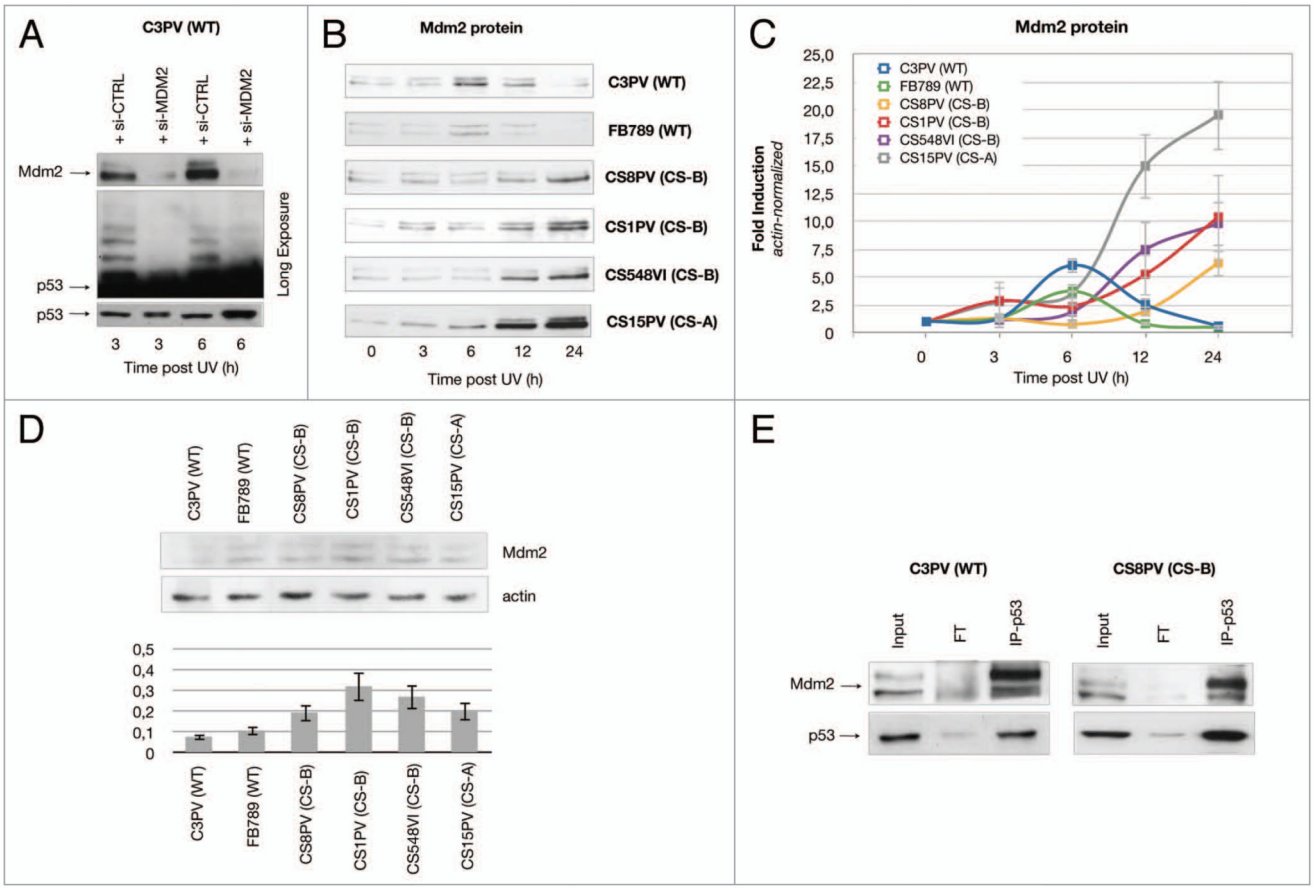

Figure 3.

(A) Protein gel blot analysis of p53 ubiquitination upon UV irradiation (10 J/m^2), in WT cells transfected with either control siRNA oligonucleotides (si-CTRL) or targeting Mdm2 mRNA (si-Mdm2). Both p53 and Mdm2 protein amount are also showed. Protein extracts were collected at the indicated times. The experiments were performed 72 h after siRNA transfection. (B) Protein gel blot analysis of Mdm2 protein amount from whole cellular extracts of WT (C3PV and FB789), CS-B (CS8PV, CS1PV and CS548VI) and CS-A (CS15PV) cells upon UV irradiation (10 J/m^2). Protein extracts were collected at the indicated times. (C) Graph represents quantification of Mdm2 protein amounts (mean ± SD of three independent experiments) after UV irradiation (10 J/m^2). (D) Protein gel blot analysis of basal Mdm2 and β-actin protein amount from whole extracts of WT (C3PV and FB789), CS-B (CS8PV, CS1PV and CS548VI) and CS-A (CS15PV). Bottom part showed the relative quantification obtained by normalizing p53 by β-actin protein amount in three different experiments. (E) Whole cell extracts from unirradiated WT (C3PV) and CS-B (CS8PV) cells were immunoprecipitated (IP) using antibodies against p53. The input, the flow through (FT) and the IP fractions were analyzed by protein gel blotting using antibodies that recognize the proteins indicated on the left.

Europe PMC Funders Author Manuscripts

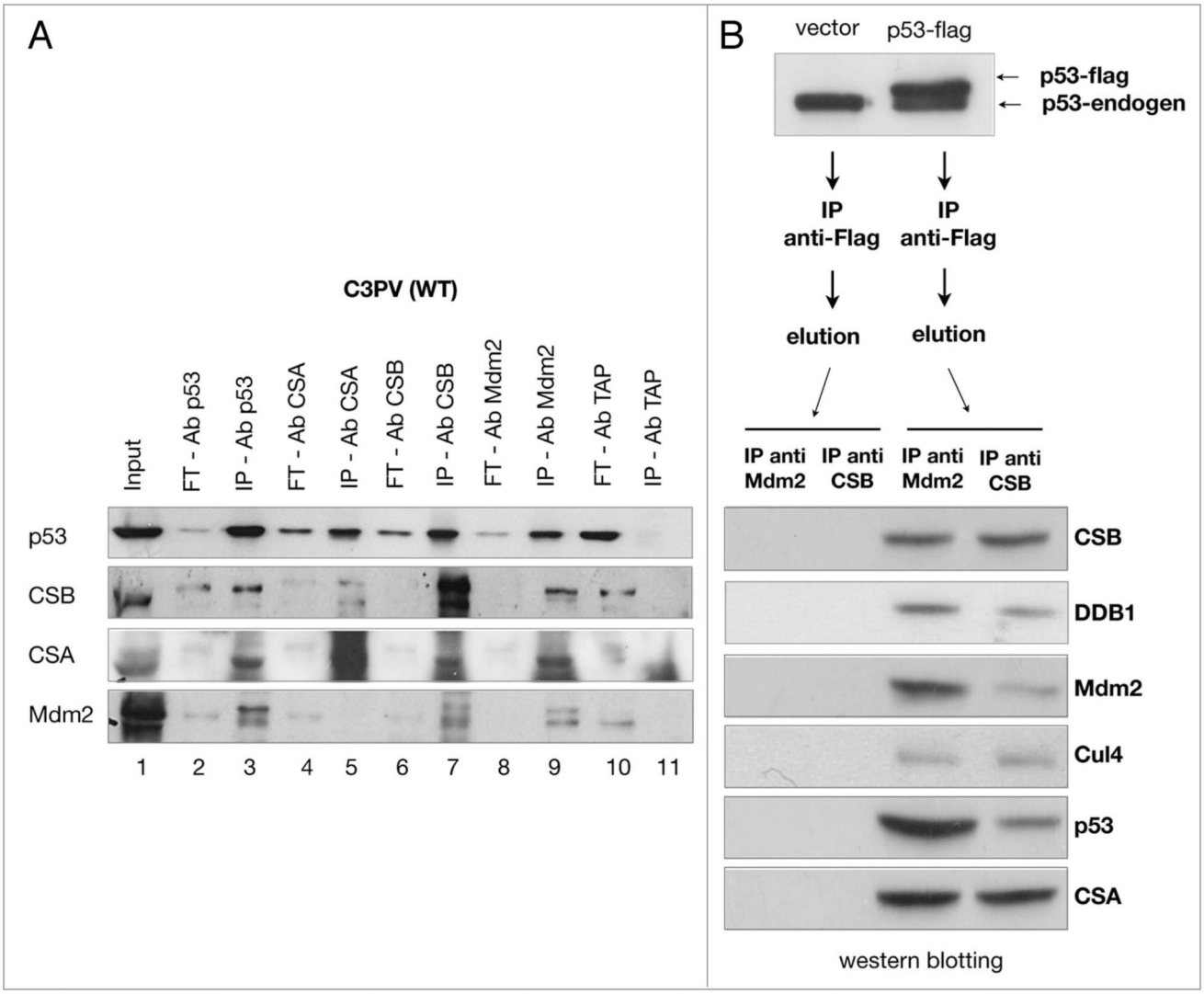

Figure 4.

(A) Whole cell extracts from unirradiated WT (C3PV) cells were immunoprecipitated (IP) using antibodies against p53, CSA, CSB, Mdm2 and TAP proteins. The IP and the flow through (FT) fractions were analyzed by protein gel blotting using antibodies that recognize the left indicated proteins. (B) Immunopurification of p53 complexes was performed using a double-immunoprecipitation strategy by transfecting Hela cells with plasmids expressing either Flag peptide or Flag-tagged p53 protein. The amount of the exogeneous (p53-flag) and endogenous (p53 endogen) p53 proteins was analyzed by protein gel blotting before immunoprecipitation (upper part). The Flag protein containing complexes were immunoprecipitated by using an antibody against the Flag epitope and the eluted complexes were re-immunoprecipited using either Mdm2 or CSB antibodies. The lower part shows the protein composition of p53/Mdm2 and p53/CSB sequential double-purifications.

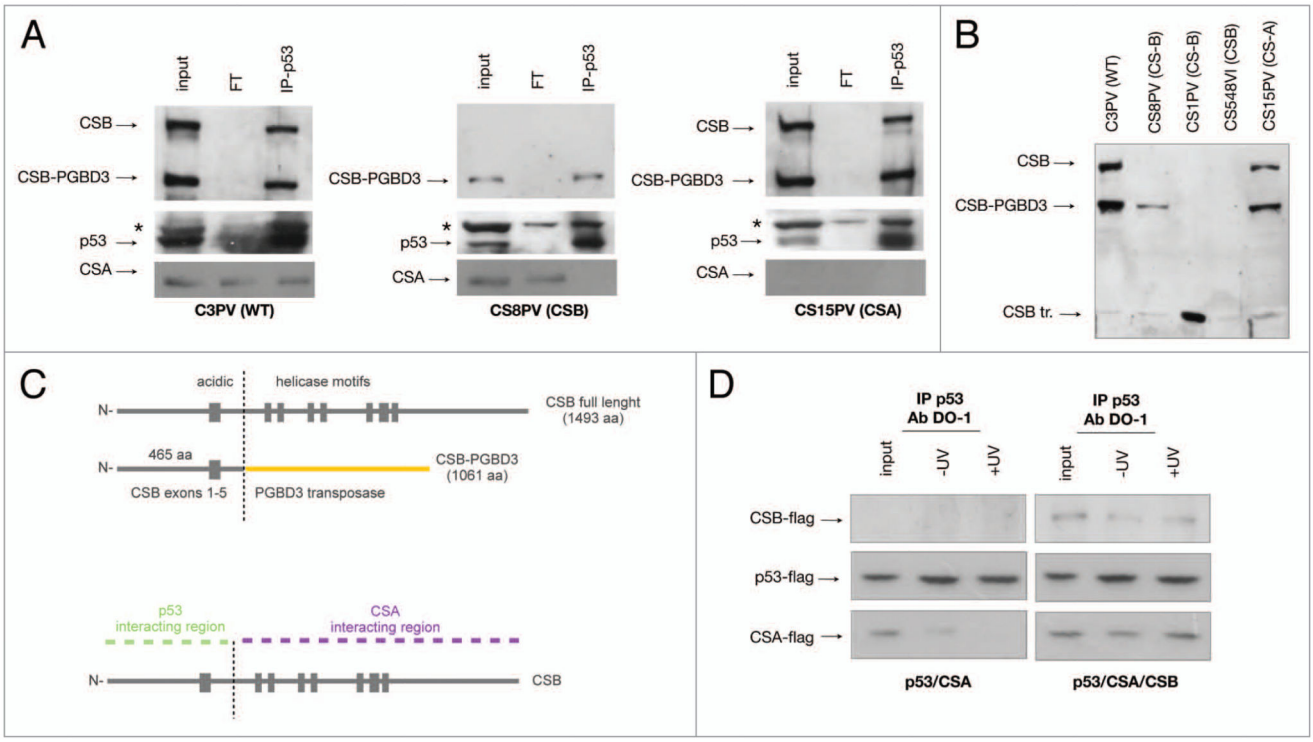

Figure 5.

(A) Whole cell extracts from WT (C3PV), CS-B (CS8PV) and CS-A (CS15PV) cells were immunoprecipitated using p53 antibody. Immunoprecipitated (IP) and flowthrough (FT) fractions have been analyzed by protein gel blot with antibodies against CSB, p53 and CSA proteins. *p53 isoform (B) protein gel blot analysis of CSB protein from whole cell extracts of WT (C3PV), CSB (CS8PV, CS1PV and CS548PV) and CSA (CS15PV) cells. (C) Diagram showing the two CSB isoforms expressed in WT cells: the full length CSB protein (1,493 aa) and the CSB-PGBD3 fusion protein (1,061 aa) that includes the 465 N-terminal residues of CSB and the entire PGBD3 transposase. CS1PV (CS-B) cells due to a homozygous stop codon express a truncated proteins of 452 aa (see also lane 3 of part C). CS8PV cells (CS-B) due to different mutations sited downstream of the 465 N-terminal residues of CSB maintain the expression of the CSB-PGBD3 fusion proteins but do not show any other truncated proteins. On the bottom, the putative interacting regions of CSB with p53 and CSA as proposed from the data showed in (A). (D) Whole cell extracts of HeLa cells co-expressing flagged CSA-CSB- and p53-proteins were immunoprecipitated using an antibody against p53 (DO-1) and analyzed by protein gel blotting using an antibody against Flag. Where indicated the samples have been irradiated with UV (10 J/m^2) and lysed 6 h later.

Europe PMC Funders Author Manuscripts

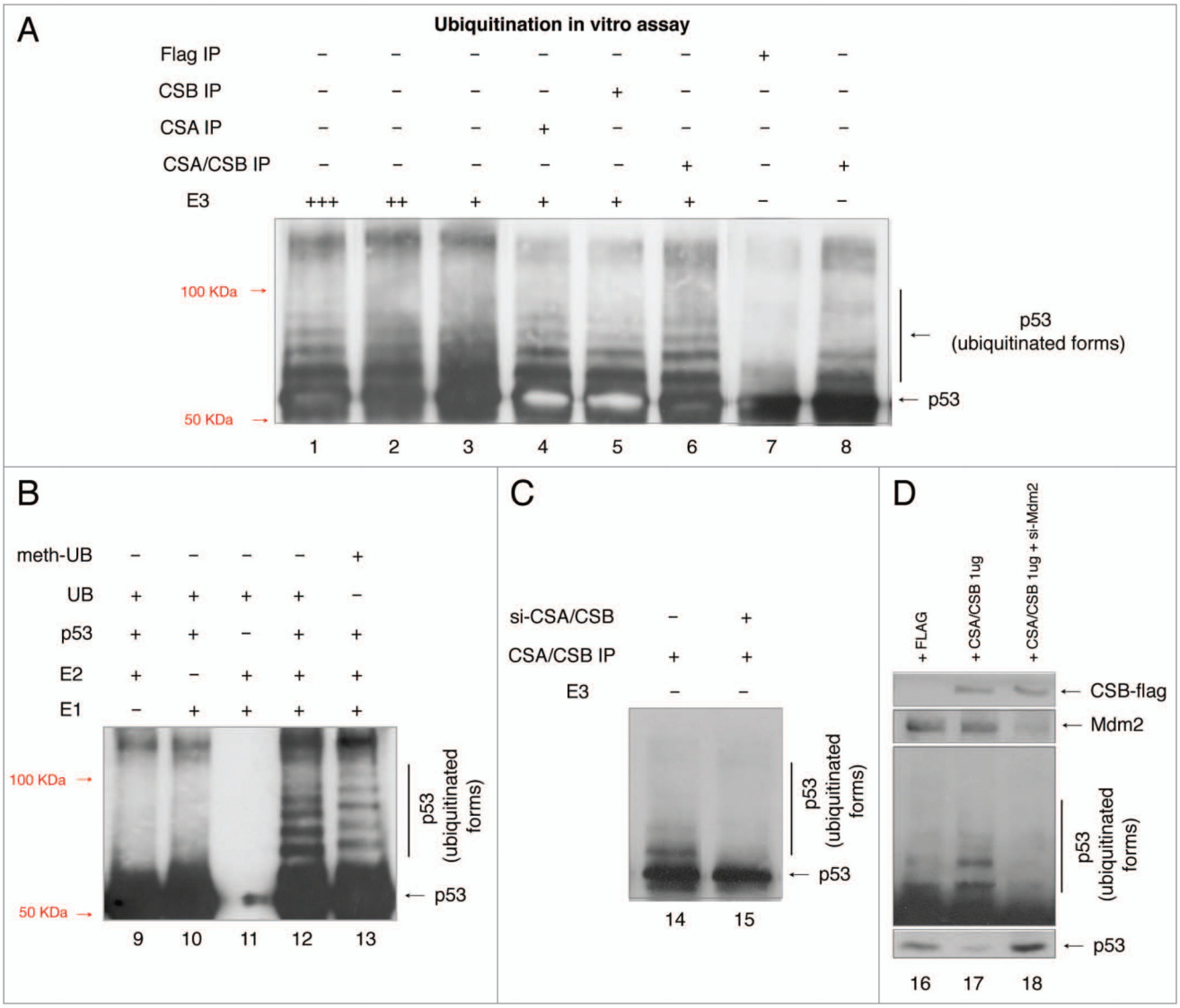

Figure 6.

(A–C) In vitro ubiquitination reactions were performed in the presence of recombinant E1, E2 and Ub proteins and where indicated, recombinant Mdm2 (E3) protein and/or CSA and CSB immunocomplexes, obtained as indicated in Material and Methods. Reaction were performed for 2 h at 37°C and resolved by SDS-PAGE followed by immunoblotting with anti-p53 antibody. +, ++ and +++ indicate 10 nM, 100 nM and 500 nM Mdm2, respectively. Mass-spectrometry analysis confirmed the presence of p53, mdm2, CSA and CSB proteins in the sample analyzed in the lane 6 (data not shown). In (C) lane 15 in vitro reaction was performed as indicated in lane 6 with the exception that the cells used to immunopurify CSA or CSB complexes had been silenced for CSA and CSB for 48 h before performing immunoprecipitation. (D) Protein gel blot analysis of p53 ubiquitination upon UV irradiation (10 J/m^2), in WT cells transfected with either control Flag, or CSA-Flag and CSB-Flag expression vectors. Where indicated cells were also transfected with siRNA

Europe PMC Funders Author Manuscripts

oligonucleotides targeting Mdm2 mRNA (si-Mdm2). p53 protein amount is also showed after short exposure of the film. Protein extracts were collected at the indicated times. The experiments were performed 48 h after siRNA transfection.

Europe PMC Funders Author Manuscripts

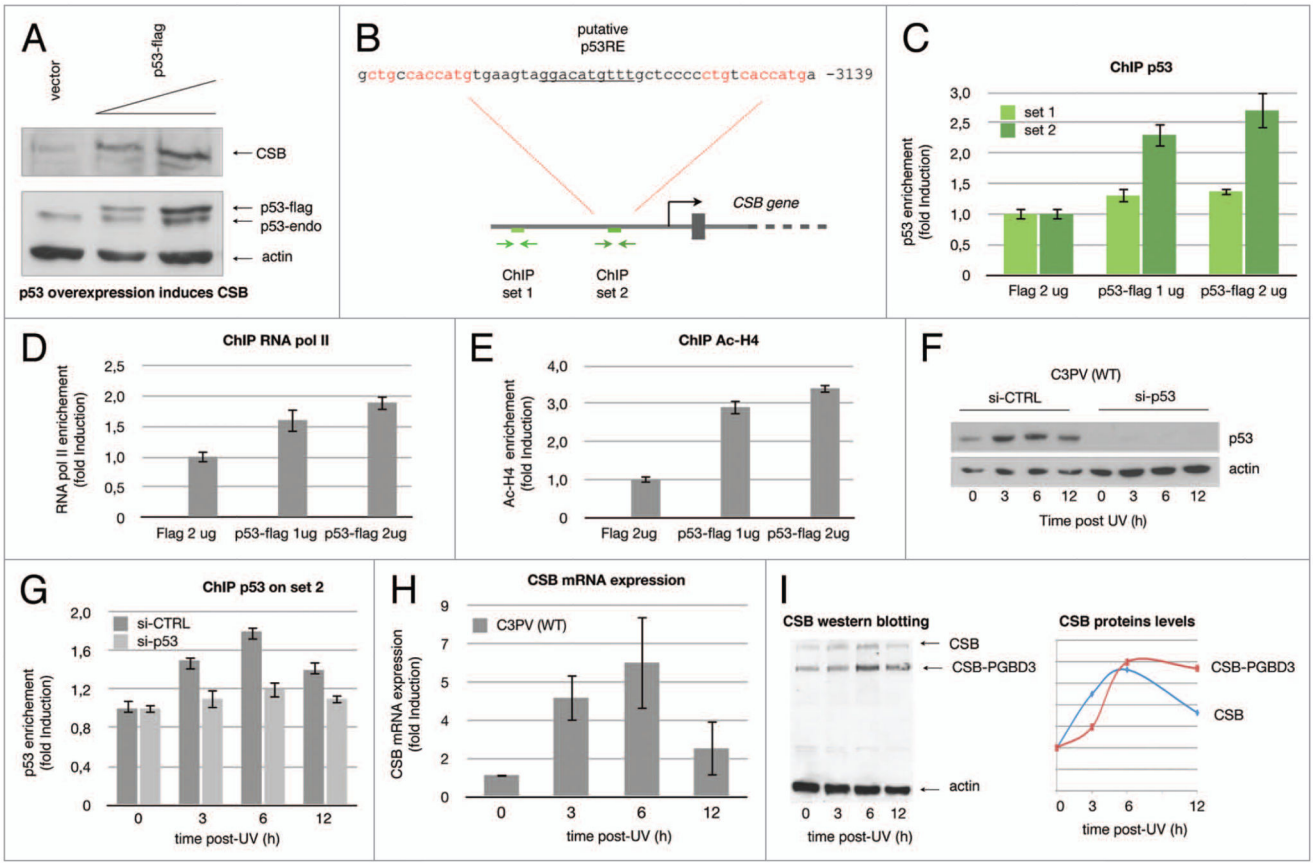

Figure 7.

(A) Protein gel blot from whole cellular extracts of HeLa cells transfected with increasing concentrations of p53-flag expression vector (0, 1 and 2 μg). The enrichment of p53 proteins on the in silico predicted response element region (B) was analyzed by chromatin immunoprecipitation. Soluble chromatin was prepared from HeLa cells transfected with increasing concentrations of p53 expression vector, and subjected to ChIP assay using antibodies against p53 (C), RNA pol II (D) and Acetylated histone H4 (E). Real-time PCR using specific primers was performed to test the relative enrichment for: the responsive region (primers set2) of the gene compared with either the non transfected sample or the upstream region (primers set1) in (C) and the promoter region sited immediately upstream of the transcription initiation site (D and E). (F) Protein gel blot analysis of p53 and β-actin protein amount from whole cellular extracts of WT (C3PV) cells transfected with either control RNA oligonucleotides (si-CTRL) or targeting p53 mRNA (si-p53) cells upon UV irradiation (10 J/m^2). Protein extracts were collected at the indicated times after UV irradiation (10 J/m^2). Soluble chromatin was prepared from WT (C3PV) cells transfected with either control RNA oligonucleotides (si-CTRL) or targeting p53 mRNA (si-p53), and subjected to ChIP assay using antibodies against p53 (G). CSB mRNA expression (H) and CSB protein expression (I) in C3PV cells after UV irradiation.

Europe PMC Funders Author Manuscripts