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Bimodal regulation of p21^{waf1} protein as function of DNA damage levels

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Abbreviations: BLM, bleomycin; DDR, DNA damage response; DSBs, double strand breaks; FCS, fetal calf serum; IF, immunofluorescence.

Human p21^{Waf1} protein is well known for being transcriptionally induced by p53 and activating the cell cycle checkpoint arrest in response to DNA breaks. Here we report that p21^{Waf1} protein undergoes a bimodal regulation, being upregulated in response to low doses of DNA damage but rapidly and transiently degraded in response to high doses of DNA lesions. Responsible for this degradation is the checkpoint kinase Chk1, which phosphorylates p21^{Waf1} on T145 and S146 residues and induces its proteasome-dependent proteolysis. The initial p21^{Waf1} degradation is then counteracted by the ATM-Chk2 pathway, which promotes the p53-dependent accumulation of p21^{Waf1} at any dose of damage. We also found that p21^{Waf1} ablation favors the activation of an apoptotic program to eliminate otherwise irreparable cells. These findings support a model in which in human cells a balance between ATM-Chk2-p53 and the ATR-Chk1 pathways modulates p21^{Waf1} protein levels in relation to cytostatic and cytotoxic doses of DNA damage.

Introduction

The DNA damage response (DDR) is a sensitive and complex network of pathways protecting the integrity of the genome.[1] The apical kinases of the DDR cascade are ATM and Chk2 for the response to DSBs, and ATR and Chk1 for other lesions like pyrimidine dimers or replication fork arrest, while one of the main common effectors is the transcription factor p53.[1] DDR-dependent post-translational modifications enable p53 to escape degradation and direct this protein on specific targets.[2]

A key transcription target of p53 is the p21^{Waf1} gene, whose encoded protein was initially described as a negative regulator of CDKs, with a role in G1 and G2 arrest maintenance after DNA damage.[3,4] In addition, p21^{Waf1} has been found to repress apoptosis,[5] an observation well correlating with p21^{Waf1} accumulation detectable in several tumors.[6] Furthermore, p21^{Waf1} was described as an important factor in DNA damage-induced senescence[7] and, more recently, as a regulator of DSBs repair.[8]

It is generally assumed that, in response to DNA damage, p53 induces the transcription and accumulation of the p21^{Waf1} protein, thus promoting cell cycle arrest and, eventually, apoptosis repression. However, p21^{Waf1} protein poorly accumulates in S phase cells also in presence of DSBs, as a consequence of proteasome-dependent p21 degradation.[9] Yet, conditions like replication forks progression impairment or exposure to high UV doses cause a reduction, rather than an increase, in the steady state levels of p21^{Waf1}. Particularly, p21^{Waf1} is reduced in human cells after DNA replication arrest, in an ATR- and Chk1-dependent manner and through the repression of p21^{Waf1} mRNA elongation.[10] Furthermore, in response to UV irradiation, the decrease of p21^{Waf1} protein level occurs through a proteasome-dependent pathway. However, contrasting data have been reported about the correlation with the UV dose,[11,12] the role of ubiquitin[12,13] and p21^{Waf1} translocation to cytoplasm.[13,14] Other DNA damaging agents are supposed to reduce p21^{Waf1} levels, but a precise correlation with the presence of DSBs is still lacking, since ionizing radiation was described not to affect[11] or reduce p21^{Waf1} in a dose-dependent manner.[14,15] The biological consequence of a p21^{Waf1} decrease remains to be defined.

Here we show that p21^{Waf1} transiently decreases at any cell cycle phase above a certain level of DNA damage. The degree and duration of this decrease is dose-dependent and cell type-specific, although more evident and irreversible in the absence of a functional p53. p21^{Waf1} downregulation in response to DBSs is due to protein degradation and specifically it is mediated by the 26S proteasome. This degradation is not linked to the ATM-Chk2 signaling, that actually counteracts this pathway. Instead, p21^{Waf1} is targeted for degradation by Chk1, which phosphorylates p21^{Waf1} on residues known to be involved in its stability. The outcome of this transient reduction of basal p21^{Waf1} seems linked to p21^{Waf1} anti-apoptotic activity.

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Results

Since $p21^{Waf1}$ levels strongly decrease in UV-irradiated cells, while contrasting results have been reported for treatments with DSB-inducing agents, we analyzed the $p21^{Waf1}$ protein in U2OS cells treated with different doses of bleomycin (BLM), a chemotherapeutic drug that primarily generates DSBs. We found that the basal levels of $p21^{Waf1}$, while steadily increase by 3 hrs onwards after treatment with 6 μM BLM, transiently decrease by 50% and 75% after 3 and 6 hrs of treatment with 120 μM BLM and re-accumulate at 24 hrs (**Fig. 1A** top). To quantify the cytotoxic effect of the treatments, we determined by FACS analysis the percentage of subdiploid apoptotic cells at 24 hrs and cell viability by trypan blue assay at 48 hrs. Whereas treatment with 6 μM BLM had a minor effect on cell survival (**Fig. S1A** left and middle), exposure to 120 μM BLM resulted in 14% of cell death at 24 hrs and about 50% of cell viability at 48 hrs (**Fig. S1A** left and middle). Both conditions activated the G2/M checkpoint, as evaluated by FACS analysis (data not shown). Long-term survival and reversibility of cell-cycle arrest were tested by colony-formation assay (**Fig. S1A** right) and it was found that cells pre-treated for 3 hrs with 6 μM BLM were able to form colonies when cultured in the absence of drug (>80% compared to the untreated cells, **Fig. S1A** right), whereas cells pre-treated with 120 μM BLM only yielded <10% colonies, **Fig. S1A** right). These results suggest a cytostatic effect by 6 μM and cytotoxic effect by 120 μM BLM exposure.

The transient decrease of $p21^{Waf1}$ was also seen in response to other DSB-inducing agents like neocarzinostatin (40 nM), H_2O_2 (800 μM) and IR (50 Gy) (**Fig. S1B**). An equitoxic dose of etoposide (10 μM, **Fig. S1C**), a topoisomerase II inhibitor yielding limited amounts of DSBs as secondary lesions,[16] caused instead $p21^{Waf1}$ accumulation already at 3 hrs (**Fig. 1A** bottom and **Fig. S1B**). All these treatments invariably enhanced p53 accumulation (**Fig. 1A**; **Fig. S1B**) and, except for etoposide, increased the γ-H2AX signal linked to DSBs production (**Fig. S1D**). Furthermore, 120 μM BLM elicited the accumulation of the p53 transcriptional target Mdm2, confirming full p53 activation under such experimental conditions (**Fig. S1E**). Immunofluorescence (IF) analysis showed decreased $p21^{Waf1}$ protein after BLM treatment in most of the cell population (**Fig. 1B**) and, given the absence of significant changes in cell cycle distribution at 6 hrs of treatment with each BLM doses or etoposide (**Fig. S1F**), we can exclude that $p21^{Waf1}$ reduction could be linked to cell cycle phase redistribution. To further confirm that $p21^{Waf1}$ decrease is independent of cell cycle phase, cells replicating DNA were marked by EdU incorporation for 3 hrs in presence or absence of 120 μM BLM. Immunostaining analyses showed that a fraction of EdU positive cells are negative for $p21^{Waf1}$ also before drug addition, as expected since this protein is at very low level during S-phase.[9] However, a significant decrease of $p21^{Waf1}$ protein levels is detectable in both EdU-positive and -negative cells in presence of BLM (**Fig. 1C**), supporting the observation that this event is not limited to S-phase cells. The independence of $p21^{Waf1}$ decrease from cell cycle phase was further confirmed by cyclin B/$p21^{Waf1}$ co-immunostaining (**Fig. S1G**), since cyclin B is a positive marker of S-G2 cells.

The genotoxic dose-dependent $p21^{Waf1}$ decrease was detectable also in a normal lymphoblastoid cell line (LCL) as well as in several cancer cell lines carrying wild type p53 (HCT116, SH-SY5Y) and mutant p53 (T47D, Hela and Saos-2) (**Fig. 2A**). No $p21^{Waf1}$ decrease was detectable in BJ-hTERT fibroblasts and IGROV-1 cancer cells treated for 6 hrs with up to 360 μM BLM (**Fig. 2A**; **Fig. S2A**), and actually, in the case of BJ-hTERT, IR and H_2O_2 strongly induced $p21^{Waf1}$ protein accumulation (**Fig. S2B**). Notably, in HCT116 cells, loss of p53 resulted in a lower basal expression of $p21^{Waf1}$, strongly reduced after BLM treatment and that did not recover at 24 hrs (**Fig. 2B**). A similar behavior was observed in a time course analyses of the p53 *null* Saos-2 cells (**Fig. S2A**), confirming that p53 is important for $p21^{Waf1}$ recovery at later time points after BLM exposure.

Stalled forks can induce a Chk1-dependent reduction of $p21^{Waf1}$ mRNA owing to repression of transcription elongation.[10] We thus quantified $p21^{Waf1}$ mRNA by RT-PCR and observed an increased signal in response to all dose of BLM tested, and after 10 μM etoposide treatment (**Fig. 3A**), excluding that $p21^{Waf1}$ protein downregulation could be due to decreased transcription. To determine the involvement of protein degradation or impaired translation in these events, cells were pre-incubated with the 26S proteasome inhibitor MG132 or the translation elongation inhibitor cycloheximide (CHX) prior to treatment with 120 μM BLM. As expected, CHX treatment reduced $p21^{Waf1}$ protein in untreated cells, but an additional decrease was detectable in presence of BLM. On the contrary, MG132 increased $p21^{Waf1}$ accumulation in untreated cells but prevented its decrease after BLM treatment (**Fig. 3B**). To confirm that $p21^{Waf1}$ decrease was due to protein degradation, CHX was added to U2OS cells to block the *de novo* protein synthesis and the remaining $p21^{Waf1}$ levels were monitored at different time points in presence or absence of 120 μM BLM. The half life of $p21^{Waf1}$ was reduced to <30 min in presence of 120 μM BLM (**Fig. 3C** and **S3**). It has been suggested that $p21^{Waf1}$ degradation correlates with its nuclear/cytoplasmic localization,[13] but in BLM-treated cells the nuclear localization of $p21^{Waf1}$ was not affected by pre-incubation with MG132 (**Fig. 3D**).

The main regulator of the response to DSBs is the ATM-Chk2 pathway, backed-up by the ATR-Chk1 pathway.[17,18] To verify the involvement of these kinases in $p21^{Waf1}$ expression, we pre-treated U2OS cells with KU55933 and VRX0466617, the chemical inhibitors respectively of ATM[19] and Chk2.[20] Both compounds enhanced $p21^{Waf1}$ degradation (**Fig. 4A**), indicating that the ATM-Chk2 pathway promotes $p21^{Waf1}$ accumulation at any dose of BLM. Similar results were obtained after Chk2 silencing (data not shown). These observations suggest that the ATM-Chk2-p53 pathway is active in presence of severe DNA damage and partially counteracts $p21^{Waf1}$ decrease, perhaps by modulating $p21^{Waf1}$ transcription. To confirm this possibility and according to the previously reported attenuation of the ATM-Chk2 pathway in U2OS cells,[21] we observed that Chk2 overexpression significantly counteracts $p21^{Waf1}$ reduction after severe DNA damage (**Fig. 4B**). This finding is consistent with previous observations implicating the ATM-Chk2-p53 pathway in $p21^{Waf1}$ accumulation after DSBs induction.[22]

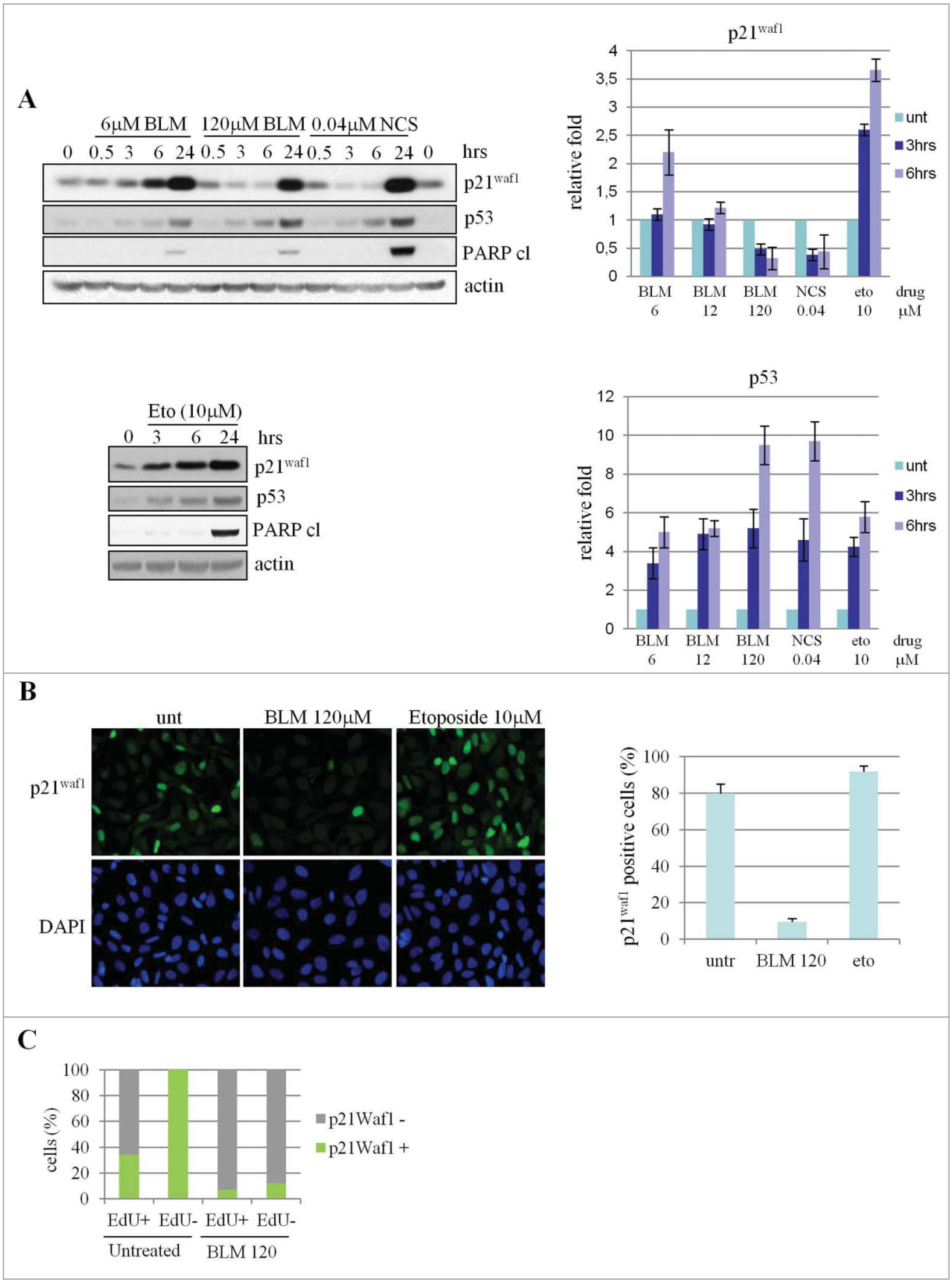

Figure 1. For figure legend, see page 2904.

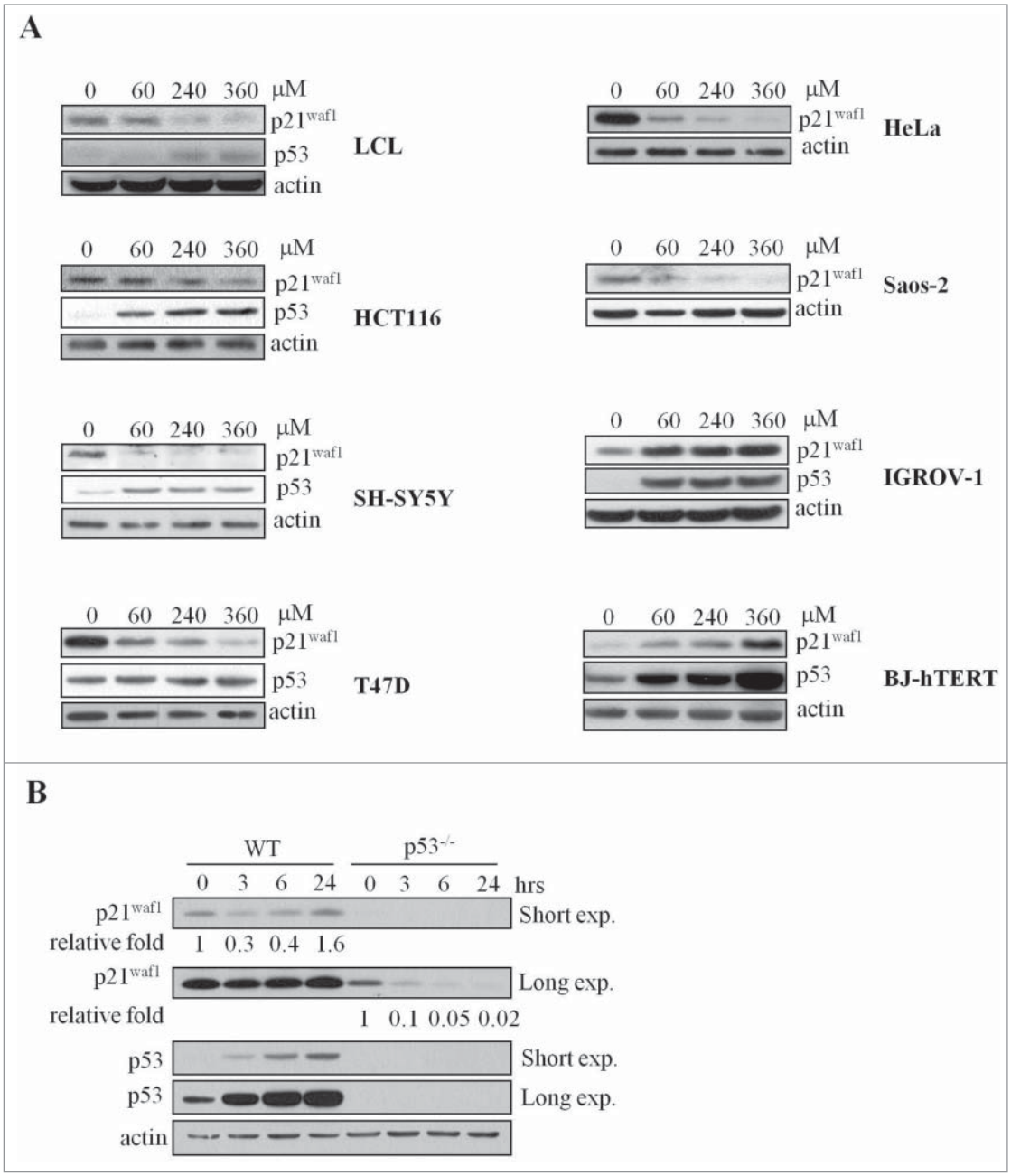

Figure 2. p21^{Waf1} regulation in normal and cancer cell lines. (**A**) Human normal lymphoblastoid cells (LCL), normal fibroblast h-TERT immortalized (BJ-hTERT), ovarian carcinoma (IGROV-1), colon carcinoma (HCT116), neuroblastoma (SH-SY5Y), breast cancer (T47D), cervix adenocarcinoma (HeLa) and osteosarcoma (Saos-2) cell lines were tested by immunoblot for p21^{Waf1} and p53 before and after 3 hrs of treatment with increasing concentrations of BLM. HeLa and Saos-2 are negative for p53, T47D cells have a stabilized, but not inducible form of p53. (**B**) p53 wt or p53 KO HCT116 cells were analyzed by protein gel blot for p21^{Waf1} and p53 before and after treatment for up to 24 hrs with 240 μM BLM. Relative quantification of band intensities, obtained by densitometric analyses, normalized to actin for loading, is shown.

Figure 1 (See previous page). p21^{Waf1} downregulation in U2OS cells following treatment with DNA damaging agents. (**A**) Western blot analysis of p21^{Waf1}, p53 and cleaved PARP in U2OS cells exposed to increasing doses of bleomycin (BLM), neocarzinostatin (NCS) and etoposide (Eto). The histograms for p21^{Waf1} and p53 proteins were obtained from densitometric analysis of the bands, normalized against actin, from 3 independent western blot experiments. (**B**) p21^{Waf1} protein detected by immunofluorescence in cells exposed for 3 hrs to genotoxic agents. The intensity of single cell signal was assessed by ImageJ software analysis on microscope images. Cells with p21^{Waf1} signal intensity lower than 10% of the average signal detectable in the positive control (undamaged cells), were considered as p21^{Waf1} negative. (**C**) EdU and p21^{Waf1} double staining. To mark DNA replication, EdU was added to cell cultures 15 minutes before BLM treatment. p21^{Waf1} protein levels were evaluated as described in (**B**), in untreated and treated cells (120 μM BLM, 3 hrs) and in EdU-positive (cells that transit in S-phase during the time of the experiment) and negative populations.

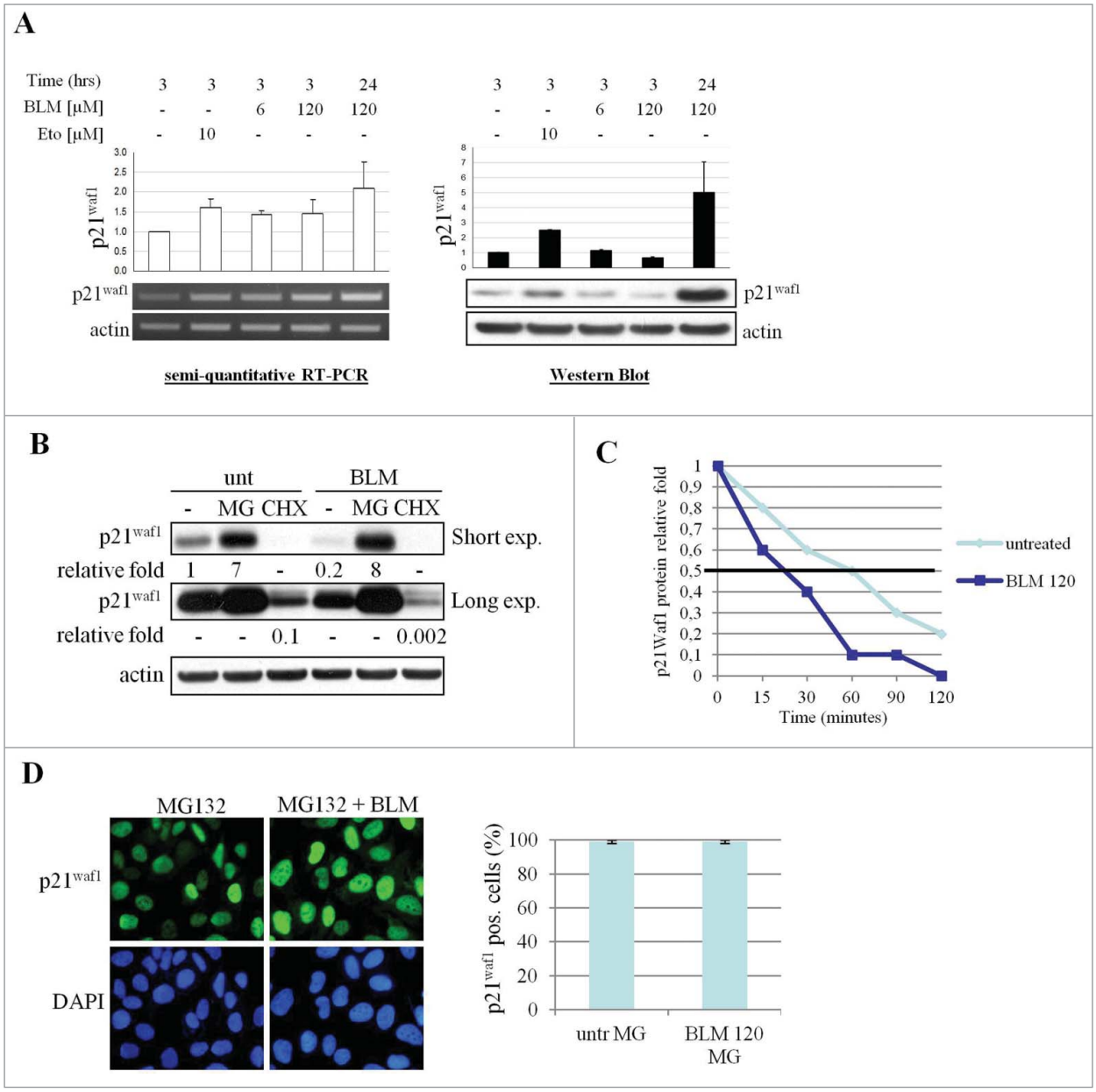

Figure 3. p21^{Waf1} downregulation is due to protein degradation. (**A**) p21^{Waf1} transcript and protein levels following BLM or Eto treatments were analyzed on the same U2OS samples by semi-quantitative RT-PCR and western blot. Relative quantification of band intensities was carried out considering the untreated sample as 1. The experiment shown is representative of 3 independent experiments. (**B**) U2OS were pre-treated for 30 min with 10 μM MG132 or 10 μg/ml cycloheximide (CHX) before addition of 120 μM BLM for 3 hrs. Total lysates were analyzed by protein gel blotting for p21^{Waf1}. Relative quantification of band intensities, obtained by densitometric analyses normalized to actin loading is shown. (**C**) p21^{Waf1} protein half life was assessed in presence of CHX alone or CHX and 120 μM BLM. The data plotted in the graph were obtained by western blot and densitometric analysis of the bands. Untreated samples were considered as 1. (**D**) Immunofluorescence analysis of p21^{Waf1} protein localization in U2OS cells pretreated for 30 min with 10 μM MG132 and treated for 3 hrs with 120 μM BLM. Nuclei were visualized by DAPI staining. Data in the graph were obtained analyzing the microscope images as in **Fig. 1B**.

Notably, the Chk1 inhibitors UCN-01 and PF-477736[23] prevented p21^{Waf1} degradation in cells treated with 120 μM BLM (**Figs. 4C and D**) and neither of these inhibitors alone had any significant effect on cell cycle phase distribution, p53 and p21^{Waf1} accumulation and γ-H2AXγ phosphorylation up to 6 hrs after treatment (**Figs. S4A** and **B** and data not shown). However, markers of DDR were detectable at 24 hrs, probably as a consequence of DNA replication stress,[24] but no increase in DNA damage, evaluated by γ-H2AXγ staining was evident for up to 6 hrs of treatment with PF-477736 and BLM combination (**Fig. S4C**). Furthermore, no changes in p21^{Waf1} mRNA were induced by Chk1 inhibitors alone or in combination with BLM (**Fig. 4E**), excluding a significant activity of Chk1 on p21^{Waf1} mRNA synthesis, stability and translation, under these experimental conditions, and also excluding the contribution of p53 transcriptional activity. Additionally, Chk1 silencing prevented

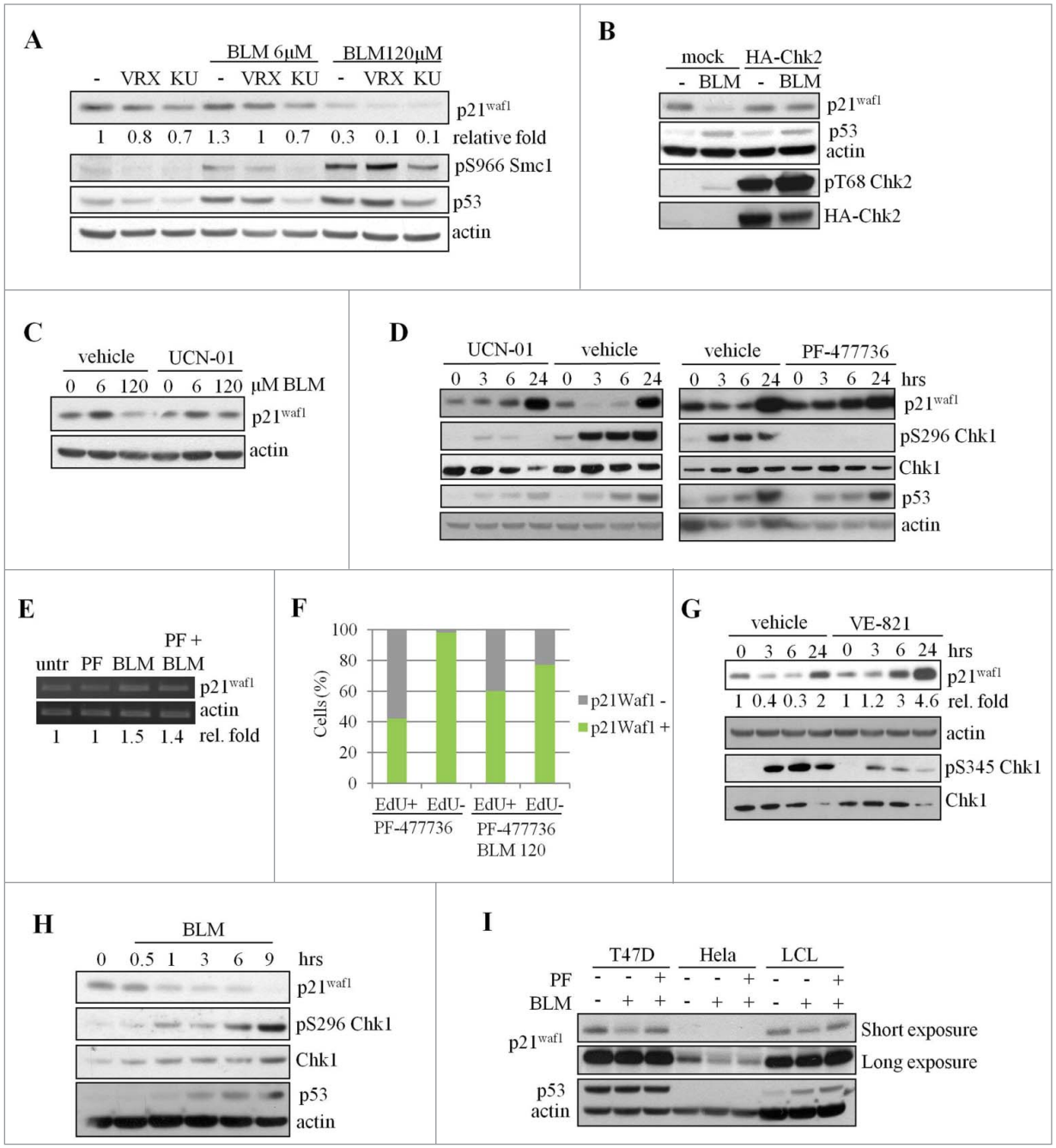

Figure 4. Chk1-dependent degradation of p21^{Waf1} by bleomycin. (**A**) U2OS cells were pre-treated for 1 hr with inhibitors of ATM (KU55933, KU, 10mM) or Chk2 (VRX0466617, VRX, 10 μM), prior to treatment with BLM for 3 hrs. Cell lysates were analyzed by protein gel blot. pS966-Smc1 was used as a reporter of the ATM/ATR kinase activity. Relative quantification of band intensities, obtained by densitometric analyses normalized to actin loading sample, is shown. The samples with no inhibitors and no BLM were considered as 1. (**B**) U2OS were transfected with either mock or HA-Chk2 vector and 48 hrs later treated or not with 120 μM BLM for 3 hrs. (**C**) U2OS cells were pre-treated for 1 hr with 300 nM of the Chk1 inhibitor UCN-01, then treated for 3 hrs with BLM and analyzed by western blot. (**D**) U2OS cells were pre-treated with UCN-01 (300 nM, 1 hr) or PF-477736 (400 nM, 1 hr) and then exposed to 120 μM BLM for the indicated times. Total Chk1 and the pS296 fraction, p21^{Waf1}, p53 and actin were evaluated by protein gel blotting. (**E**) p21^{Waf1} mRNA levels in presence of 120 μM BLM and PF-477736 (PF). Relative quantification of band intensities, obtained by densitometric analyses normalized to actin mRNA is shown. (**F**) Edu and p21^{Waf1} double staining in presence of PF-477736. p21^{Waf1} positivity was evaluated in EdU-positive and negative cells as described in **Fig. 1B**. PF-477736 was added 1 hr before BLM treatment, EdU 15 min before BLM. The image is representative of 3 independent experiments. (**G**) The ATR inhibitor VE-821 (1 μM) was added 1 hr before 120 μM BLM. Total lysates were obtained at the indicated time point after BLM addition. Total Chk1 and the pS345 fraction, p21^{Waf1} and actin were evaluated by western blotting. Relative quantification of band intensities, obtained by densitometric analyses normalized to actin loading is shown. Untreated samples were considered as 1. (**H**) Time course analysis of p21^{Waf1} degradation and Chk1 autophosphorylation in U2OS cells exposed to 120 μM BLM. (I) Pre-treatment with PF-477736 (PF, 400 nM, 1 hr) of T47D, HeLA and LCL cells partially or completely represses p21^{Waf1} downregulation 3 hrs following 120 μM BLM.

$p21^{Waf1}$ degradation by BLM (**Fig. S4D**), even though markedly increased the basal levels of p53 and $p21^{Waf1}$ (**Fig. S4D**), possibly as a consequence of cell cycle perturbation[24] or DNA damage occurrence, as also detectable 24 hrs after Chk1 inhibition (**Fig. S4A**). Moreover, the inhibitory effect of UCN-01 on $p21^{Waf1}$ degradation was detectable at all stages of the cell cycle, as shown by EdU (**Fig. 4F**, compare with **Fig. 1C**) and cyclin B (**Fig. S4E**) stainings. Since Chk1 activation in response to UV or fork stalling occurs through ATR phosphorylation, we tested the role of this kinase in $p21^{Waf1}$ degradation. Before the addition of BLM we treated U2OS cells with VE-821, a specific inhibitor of ATR activity[25] and we evaluated $p21^{Waf1}$ protein levels. As a reporter of ATR inhibition we evaluated, with a phosphospecific antibody, Chk1 phosphorylation on S345, a known ATR target site.[26] A robust but not complete reduction of S345 phosphorylation was detectable in VE-821 treated cells, suggesting a residual ATR activity or, alternatively, that this event and therefore Chk1 activation, is partially independent by ATR in presence of 120 μM BLM. However, a reduction of $p21^{Waf1}$ degradation was detectable in VE-821 treated cells both 3 and 6 hrs after BLM addition (**Fig. 4G**).

The role of Chk1 on $p21^{Waf1}$ degradation was further investigated by measuring its autophosphorylation on S296 (a marker of Chk1 activity). We observed a time-dependent Chk1-pS296 increase already at 1 hr of treatment with 120 μM BLM, which inversely correlated with $p21^{Waf1}$ degradation (**Fig. 4H**). Similar data were obtained by IF analysis of Chk1-pS345 (**Fig. S4F**), demonstrating the presence of Chk1 activity throughout the cell cycle after treatment with 120 μM BLM, since more than 90% of the cells was positive for pS345. Accordingly, 24 hrs after BLM treatment, when $p21^{Waf1}$ reaccumulates (**Fig. 1A**), Chk1 is dephosphorylated and partially degraded (**Fig. S4G**). Furthermore, while p53 accumulates similarly at 3hrs of treatment with 6 μM and 120 μM BLM, Chk1 phosphorylation strongly increases at the 120 μM dose (**Fig. S4H**). Altogether, these observations support a correlation between Chk1 activation and $p21^{Waf1}$ degradation after BLM exposure. However, since the Chk1 inhibitor PF-477736 prevented $p21^{Waf1}$ degradation more effectively in T47D and LCL than in HeLa (**Fig. 4I**) or HCT116 $p53^{-/-}$ cells (**Fig. S4I**), it is possible that alternative pathways might contribute to $p21^{Waf1}$ degradation in some cell lines.

We additionally investigated the role of REGγ, which regulates $p21^{Waf1}$ half life in unstressed cells,[27] and of caspases, known to cleave $p21^{Waf1}$ in response to genotoxic agents.[28] For this, we analyzed cells that were silenced for REGγ or pre-treated with the pan-caspase inhibitor z-VAD-fmk[29] and found that REGγ depletion increases the basal level of $p21^{Waf1}$, but nevertheless BLM induced $p21^{Waf1}$ degradation in siREGγ as in siCtrl cells (**Fig. S5A**). Similarly, z-VAD-fmk had no effect on $p21^{Waf1}$ protein levels in our experimental conditions (**Fig. S5B**). Moreover, since GSK3β is involved in $p21^{Waf1}$ degradation after UV damage,[12] we inhibited this kinase with LiCl,[30] but no effect on $p21^{Waf1}$ protein decrease after BLM was observed (**Fig. S5C**). Altogether, our data suggest that high doses of DSBs elicit a specific Chk1-dependent degradation of $p21^{Waf1}$.

Chk1 kinase might promote $p21^{Waf1}$ degradation by direct phosphorylation. Chk1 (and Chk2) preferentially phosphorylates proteins carrying an RXXS/T motif[31], which in $p21^{Waf1}$ is present on T97, T145, S146. We thus performed *in vitro* kinase assays and found that Chk1, but not Chk2, was able to phosphorylate recombinant GST-$p21^{Waf1}$ (**Fig. 5A**). Moreover, using specific antibodies against the phosphorylated RXXS/T consensus motif or against phospho-S146, we were able to demonstrate that Chk1 *in vitro* phosphorylates these residues (**Fig. 5B**), but unfortunately, these antibodies did not work on immunoprecipitated $p21^{Waf1}$, also in presence of MG132 (data not shown). However, T145, S146, were of interest because of their involvement in $p21^{Waf1}$ protein stability,[32,33] and therefore Ala substitutions were introduced in these residues to test their effect in $p21^{Waf1}$ degradation. While the exogenous wild type $p21^{Waf1}$ protein was reduced after BLM and Chk1 inhibitor repressed this decrease (**Fig. 5C**), the $p21^{Waf1\text{-}T145A/S146A}$ double mutant was only slightly degraded and unaffected by the Chk1 inhibitor (**Fig. 5C**). These data strongly suggest that the phosphorylation of $p21^{Waf1}$ on T145, S146 by Chk1 promotes its degradation.

To investigate the biological impact of $p21^{Waf1}$ protein degradation, we initially evaluated the effects on the $p21^{Waf1}$-regulated G1/S and G2/M checkpoints in the LCL cells. Enumerating EdU-labeled S-phase cells 3 and 6 hrs after genotoxic treatments, we observed G1/S checkpoint activation, independent of the used doses, inducing (360 μM BLM or 50 Gy IR) or not (30 μM BLM or 5 Gy IR) $p21^{Waf1}$ degradation (**Fig. 6A**). Therefore $p21^{Waf1}$ degradation does not impair the G1/S checkpoint, at high dose of damage. The G2/M checkpoint was analyzed in cells pre-incubated with nocodazole (used as a mitotic trap to evaluate the G2/M transition rate avoiding M phase exit) and exposed to BLM or etoposide for 3 and 6 hrs. Mitotic cells were then enumerated and whereas control untreated cells showed a time-dependent increase in mitotic figures (**Fig. 6B**), cells treated with genotoxic agents showed less mitosis irrespective of dose and type of damage (**Fig. 6B**), indicative of a G2 arrest. Since G1 and G2 checkpoints were fully active at all doses of BLM, inducing or not $p21^{Waf1}$ degradation, it is clear that the decrease of $p21^{Waf1}$ is not involved in DNA damage checkpoints regulation.

Since $p21^{Waf1}$ exerts an anti-apoptotic effect against genotoxic agents, we analyzed this phenomenon in response to BLM. As determined by cytofluorimetric analyses, knockdown of $p21^{Waf1}$ increased of about threefold the amount of sub-diploid cells at 6 μM BLM doses, but less at 120 μM BLM (**Fig. 6C**). Similar observations on the anti-apoptotic activity of $p21^{Waf1}$ were obtained with a biochemical approach testing PARP cleavage (**Fig. 6D**). However, in sip21^{Waf1} cells treated with 6 μM BLM, apoptosis does not reach the level obtained with 120 μM in both assays, thus demonstrating that besides $p21^{Waf1}$ downregulation other pro-apoptotic signals are necessary to conduct cells to suicide. Moreover, cells expressing $p21^{Waf1\ T145A/S146A}$, which is not degraded after damage, showed reduced apoptosis, as evaluated by cytofluorimetric analysis and PARP protein cleavage, compared to cells expressing wild type $p21^{Waf1}$(**Figs. 6E and F**).

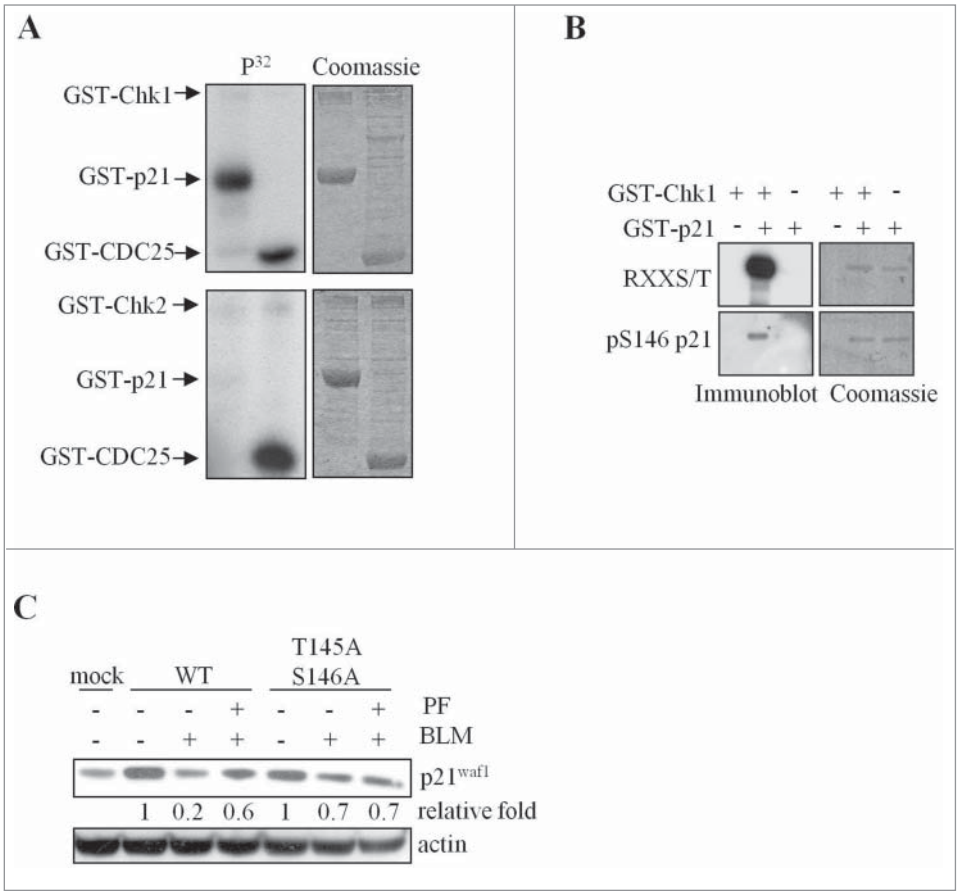

Figure 5. *Chk1 phosphorylates in vitro p21^{Waf1} at sites involved in p21^{Waf1} degradation.* (**A**) *In vitro* kinase assays performed with recombinant GST-Chk1 (upper) or GST-Chk2 (lower) kinases and GST-p21^{Waf1} or GST-Cdc25C fragment as substrates. Low amounts of kinases were added to increase the specificity of the assay. Left panel: autoradiography of incorporated ^{32}P; right panel: Coomassie staining. (**B**) The non radioactive *in vitro* kinase assay containing GST-Chk1 and GST-p21^{Waf1} proteins was immunoblotted with an anti-RXXpS/T specific antibody or an anti-pS146- p21^{Waf1} antibody. Normalization was done by Coomassie staining. (**C**) U2OS cells were transfected with wt or T145A/S146A p21^{Waf1} double mutant. Tests were conducted 72 hrs after transfection, when the expression levels of the exogenous proteins were reduced to less than fold2- of the endogenous p21^{Waf1} and to avoid possible effects of the overexpression on cell cycle progression. Cells were pre-treated or not with 400 nM PF-477736 for 1 hr and then exposed to 120 μM BLM for 3 hrs. Whole cell extracts were analyzed by protein gel blot. The densitometric analyses of the experiment, normalized to actin and to mock value, are shown.

Data about apoptosis were not influenced by cell cycle perturbations since p21^{Waf1} silencing does not significantly alter the cell cycle distribution of the cellular population (**Fig. S6A**). Furthermore, also cells expressing low levels of the wild type or mutant form of p21^{Waf1} show comparable cell cycle profiles (**Fig. S6B**). These results suggest that the early downregulation of p21^{Waf1} protein following severe DNA damage might facilitate apoptosis.

Discussion

The notions that the human ATR-Chk1 pathway is solely involved in the response to stalled replication forks or UV-induced DNA damage and that p21^{Waf1} is up-regulated after damage have been recently re-evaluated.[11,15,17,18]

Here, we demonstrate that p21^{Waf1} is transiently degraded in response to high yields of DNA DSBs induced by BLM. BLM is an anticancer drug used at high doses (10-27 mg/m^2, peak in the blood = 10 μg/ml; $t_{1/2}$ = 21 hrs) in combination with other chemotherapeutic drugs or radiotherapy, or at 0.25-1 mg/cm^2 in local electrochemotherapy for skin metastases of different tumor types. It is important to note that the highest dose of BLM used in this study induces in U2OS less than 50% of cell death at 48 hrs, with about 5-10% survival in long-term colony assays.

Transient degradation of p21^{Waf1} is cell line-specific and more pronounced in cells with reduced activation of the ATM-Chk2 pathway, and somewhat dependent on the type of DNA lesion since this phenomenon is not observed in response to the topoisomerase 2 inhibitor etoposide, which strongly activates DDR and apoptosis. We found that p21^{Waf1} downregulation is not restricted to S phase, as previously demonstrated after HU treatment[10] or as suggested by PCNA-dependent degradation upon UV irradiation.[34] Indeed, a relation with cell cycle is unlikely since p21^{Waf1} downregulation occurs in the whole population, in cells with an active (LCL) or impaired G1 checkpoint (i.e. U2OS), and only in response to a severe damage, although the transition through S phase in the latter is similar at high or low doses of BLM early after damage (data not shown).

Furthermore, we found that in U2OS p21^{Waf1} mRNA induction after DNA damage is detectable at all doses of BLM tested and after etoposide, excluding mRNA synthesis or stability events in p21^{Waf1} downregulation. Therefore, we can rule out the possibility that specific post-translational modifications on p53 could repress p21^{Waf1} transcription. Regulation of p21^{Waf1} at the translational level was also described, but we found that in the presence of a translational repressor p21^{Waf1} decrease was still detectable. Indeed, we demonstrated that the expression of p21^{Waf1} is regulated after BLM treatment by 26S proteasome-mediated degradation and not by transcription or translation, but with a mechanism different from that described for UV treatment[35] as it does not involve significant translocation of p21^{Waf1} to the cytoplasm.

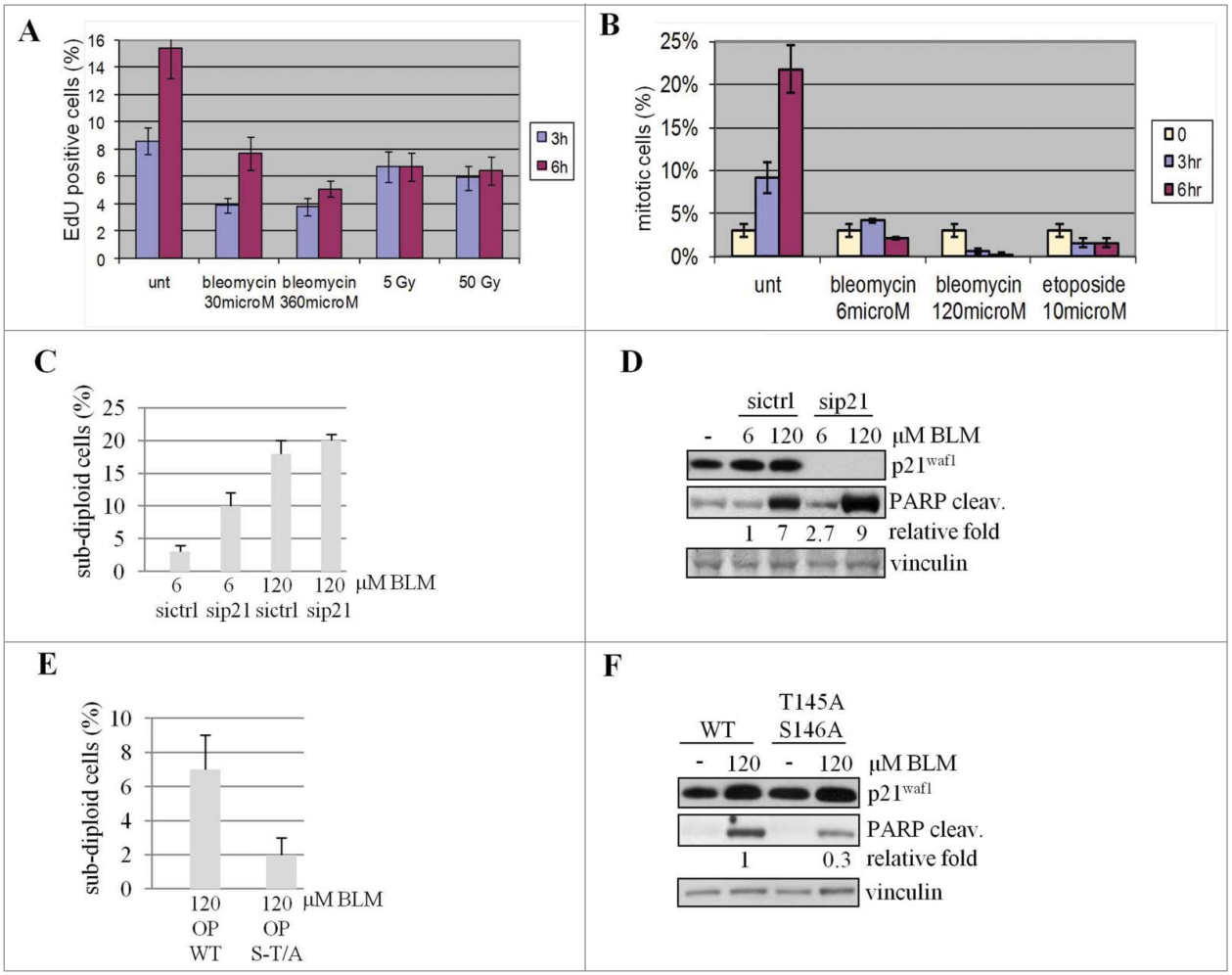

Figure 6. *p21^{Waf1} degradation impacts on apoptosis.* (**A**) To assess G1 to S transition, LCL replicating cells were labeled with EdU and evaluated before and 3 and 6 hrs after the indicated DNA damaging treatments. The fraction of EdU positive cells before drugs addition or IR exposure was subtracted from each time point. (**B**) The G2 to M transition was assessed in U2OS cells pre-treated with 100 ng/ml nocodazole before exposure to genotoxic agents to trap cells in M phase to correctly enumerate cells entering in M phase avoiding mitosis exit. (**C**) U2OS cells were transfected with siRNA to knock down p21^{Waf1} (sip21) or a control sequence (sictrl). 48 hrs after transfection, cells were treated for 24 hrs with the genotoxic agents, stained with propidium iodide and the subdiploid apoptotic fraction quantitated by DNA flow cytofluorimetry. (**D**) The same cells were also lysed and tested for PARP cleavage by immunoblotting. The densitometric analysis of the cleaved form of PARP is shown after normalization on the 6 µM BLM sample. (**E**) U2OS cells transiently transfected with wild type (OP WT) or mutated (OP S-T/A) forms of p21^{Waf1}, were exposed (72 hrs after transfection to reduce the amount of exogenous p21^{Waf1} protein and limit the impact of overexpression on cell cycle progression) for 24 hrs to BLM. The subdiploid apoptotic fraction quantified by DNA flow cytofluorimetry was evaluated as above. (**F**) The same cells were also lysed and tested for PARP cleavage by immunoblotting. The densitometric analyses of the cleaved form of PARP, normalized with vinculin and relative to the wt sample, are shown.

Since the main regulators of the DDR are the ATM-Chk2 and ATR-Chk1 pathways, we determined their impact on BLM-induced p21^{Waf1} degradation. We found that inhibition of ATM or Chk2 does not prevent, but rather enhance, p21^{Waf1} degradation, in accordance with previous findings demonstrating that the ATM-Chk2 pathway promotes p53-dependent transcription of p21^{Waf1} upon DSBs.[22]

Unexpectedly, selective inhibition of Chk1 kinase prevented p21^{Waf1} degradation in several human cell lines, with a more evident effect in cells with active p53. However, in cells with inactive p53 and low p21^{Waf1} protein levels (e.g. HeLa or HCT116 p53$^{-/-}$), Chk1 inhibition cannot completely rescue p21^{Waf1} degradation. Two factors could explain these findings: i) other pathways could contribute to p21^{Waf1} downregulation; ii) impaired p53 transcriptional activity cannot promote p21^{Waf1} accumulation. Notably ATR inhibition was also able to significantly reduce p21^{Waf1} degradation, suggesting that Chk1 activation, in presence of a severe amount of DSBs, is mainly imputable to ATR.

It was previously demonstrated that Chk1 activation is restricted to S and G2 cells upon IR.[36] Furthermore, previous studies indicate that in S phase cells, experiencing DSBs or stalled forks, p21^{Waf1} protein levels are low but stable and that, in Chk1$^{-/-}$ cells, p21^{Waf1} activates a strong S phase entry block.[37]

Differently, our data demonstrate that in presence of a severe damage, specifically DSBs, Chk1 is activated at all cell cycle phases and directly targets, by phosphorylation, $p21^{Waf1}$ for degradation. We also found that Chk1 phosphorylates $p21^{Waf1}$ *in vitro*, possibly on the T145 and S146 consensus sites, and that a $p21^{Waf1\text{-}T145A\text{-}S146A}$ double mutant was more stable than the ectopically expressed wt counterpart in cells treated with high doses of BLM. Notably, these sites have been previously implicated in $p21^{Waf1}$ protein turnover,[32,33] and furthermore, in murine cells treated with a mitogenic factor, Chk1 phosphorylates $p21^{Waf1}$on T140 and S141 (homologues of human T145 and S146), leading to its degradation.[38] Since these Chk1-phosphorylated residues on $p21^{Waf1}$ map on the region of interaction with PCNA, a role for PCNA in $p21^{Waf1}$ degradation after DSBs is predictable, as already suggested.[34]

The observations that the $p21^{Waf1\text{-}T145A/S146A}$ is slightly degraded in a Chk1-independent way, and that $p21^{Waf1}$ is partially decreased in $p53^{-/-}$ cells treated with a Chk1 inhibitor and BLM, suggest that besides Chk1 activity, other mechanisms can promote $p21^{Waf1}$ downregulation. Since the ATR-GSK3β pathway mediates the degradation of $p21^{Waf1}$ in response to UV irradiation,[12,35] we inhibited GSK3β but no differences in $p21^{Waf1}$ degradation were detected. Notably, UCN-01 and PF-477736 not only do not inhibit GSK3β but, in some conditions, they indirectly activate this kinase.[39] In some cell types, $p21^{Waf1}$ is induced by DNA damage but rapidly cleaved by caspases.[40] Inhibition of caspases in our cells did not affect the $p21^{Waf1}$ response to BLM. Finally, we found that REG-γ, an activator of the 20S proteasome, was able to reduce $p21^{Waf1}$ protein levels in undamaged cells,[27] but no after BLM treatment.

Collectively, our data suggest that the ATM-Chk2-p53 and the ATR-Chk1 pathways contribute to modulate the response of human cells to different yields of DNA breaks. Chk1 is particularly active in response to high doses of BLM, while p53 accumulates independently by the amount of damage. At the same time, p53 protein increases with the time up to 24 hrs, while Chk1 at 24 hrs is downregulated. These 2 observations could explain the dose dependence and transient nature of Chk1 mediated $p21^{Waf1}$ downregulation. Therefore, the presence of DSBs generally favors the activation of the ATM-Chk2-p53 pathway, resulting in $p21^{Waf1}$ mRNA and protein accumulation. Yet, when the number of DSBs is high and/or in conditions where the ATM-Chk2-p53 pathway is partially impaired, as frequently seen in cancer cells, the ATR-Chk1 pathway could be transiently prominent. The transient nature of $p21^{Waf1}$ degradation could reflect the necessity to preserve cells that are able to repair the majority of DNA lesions. However, we cannot exclude the role of a factor acting as Chk1 activity regulator toward $p21^{Waf1}$.

The G1/S and G2/M checkpoints were activated by low and high doses of BLM, thus excluding an essential role for $p21^{Waf1}$ downregulation in activating these checkpoints. In contrast, apoptosis, was repressed by overexpression of $p21^{Waf1}$ and even more of $p21^{Waf1\text{-}T145A\text{-}S146A}$, and enhanced by $p21^{Waf1}$ knockdown. A transient $p21^{Waf1}$ downregulation, together with other molecular signals, could be important to start an apoptotic program in presence of irreparable DSBs. Probably, it is not a coincidence that cells with an intact ATM-Chk2 pathway and resistant to apoptosis, like BJ-hTERT fibroblasts, showed no $p21^{Waf1}$ degradation in response to any dose of DSBs-inducing agents. However, $p21^{Waf1}$ should not be considered a general apoptosis inhibitor, since U2OS cells treated with etoposide are prone to cell death although they start to accumulate $p21^{Waf1}$ early after drug exposure and $p21^{Waf1}$ silencing at low dose of damage is sufficient to significantly, but not extensively, increase apoptosis.

Therefore, it is also important to underline that the biological effect of $p21^{Waf1}$ degradation could derive from a decrease under the basal level of this protein, which is known to be active, for example on cell cycle control, also in unstressed conditions. On the other side, downstream events could derive from a time window where $p21^{Waf1}$ does not accumulate and that correlate with the intensity of the damage.

Collectively, our work highlights, i) The presence of a balance between the ATM-Chk2 and ATR-Chk1 pathways during DDR, influenced by the doses and nature of DNA damaging agents, as well as by the cellular background and presence of specific mutations (i.e., in ATM or p53). ii) The role of Chk1 in DSBs response, not limited to S phase. iii) The complexity of $p21^{Waf1}$ regulation at transcriptional and translational level, with mechanisms that could be relevant at different doses and types of damage and at different times during DDR. iiii) The importance of the basal levels of $p21^{Waf1}$.

These observations prompt to reconsider the role of some DDR components during tumorigenesis and cancer treatment, and may have implications for the use of radiation or chemotherapeutic agents inducing DSBs in combination with Chk1 inhibitors. Indeed, Chk1 inhibitors, which are now in clinical trials, could affect not only cell cycle arrest, but also apoptosis and DNA repair, depending on DNA lesion and cellular background.

Materials and Methods

Cell culture and treatments

Osteosarcoma cells (U2OS and Saos-2), ovarian carcinoma (IGROV-1), colon carcinoma (HCT116), neuroblastoma (SH-SY5Y), breast cancer (T47D) and cervix adenocarcinoma (HeLa) cells were cultured in DMEM (BioWhittaker) plus 10% FCS. The EBV-immortalized normal lymphoblastoid cell line (LCL) was cultured in RPMI1640 plus 15% FCS. hTERT-immortalized normal human foreskin fibroblasts BJ-hTERT were cultured in DMEM plus M199 (4:1 ratio) with 10% FCS and 10 μg/ml hygromycin B. Irradiations were performed with a 137^{Cs} source providing 675 cGy/min. KU-55933 (R&D Chemicals) and VRX0466617[20] was used at 10 μM. BLM (Nippon Kayaku) was dissolved in PBS at a concentration of 10 mg/ml (equivalent to 10 U/ml or 7 M), stored at −20°C and used by 1 month. Etoposide (TEVA)

treatments were performed using a final 10 μM concentration unless otherwise indicated. MG132 (Sigma) and CHX (Sigma) were used respectively at 10 μM and 100 μg/ml to evaluate the role of proteasome and mRNA translation in $p21^{waf1}$ protein decrease. To test $p21^{waf1}$ half-life, we treated U2OS cells with 40 μg/ml CHX 20 minutes before the addition of BLM. UCN-01 (Sigma-Aldrich), PF-477736 (Sigma-Aldrich) and VE-821 (SelleckChem) were added 1 hr before BLM exposure, at a concentration of 300 nM, 400 nM and 1 μM, respectively.

Plasmids and site-directed mutagenesis

$p21^{waf1}$ gene cloned in pCEP plasmid was a kind gift of S. Soddu. T145 and S146 mutants to Alanine were obtained using the QuikChange II XL Site Directed Mutagenesis Kit (StrataGene, Agilent Technologies). Chk2 cloning in pCDNA3-HA vector was previously described.[41]

Cells transfection and siRNAs

Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the protocol and lysed in Laemmli buffer for western blot analysis. siRNAs against $p21^{Waf1}$ were Flexi Tube siRNA (Qiagen). siRNA against luciferase (siLUC) was custom made by Thermo Scientific Dharmacon.

Western blots and antibodies

Immunoblots were performed on total cell extracts as described[42] and the antibodies used were: p21 (H164, Santa Cruz), p21-pS146 (Santa Cruz), Phospho-Akt Substrate (RXXS*/T*) (110B7E, Cell Signaling Technology, Beverly, MA) Chk2-pT68 (2661; Cell Signaling Technology), ATM-pS1981 (AF1655, R&D Systems), Smc1-pS966 (ab1276, Abcam), Chk2,[43] p53 (DO7, Zymed), cyclin B1 (BD-Biosciences), γ-H2AX (Upstate, Millipore), cleaved PARP-1 (Cell Signaling Technology), β-actin (Sigma), Chk1-pS345 and Chk1-pS296 (Cell Signaling Technology), hemagglutinin (HA) tag (12CA5; Roche, Mannheim, Germany). Densitometric analyses of protein gel blot signals were done with ImageQuant TL software (GE Healthcare Life Sciences).

Immunofluorescence and flow cytometry analysis

To stain $p21^{Waf1}$ in U2OS, cells were grown on coverslips, rinsed with PBS and fixed with paraformaldehyde. Then cells were permeabilized with 10 mM Hepes pH 7.5, 100 mM NaCl, 1 mM MgCl2, 1 mM EGTA, 0.32 M sucrose, 0.5% Triton X-100, blocked with 3% BSA and incubated at RT for 1 hr with primary antibodies. Following extensive washing, coverslips were incubated with AlexaFluor488-conjugated anti-rabbit antibodies for 1 hr at RT. DNA was stained with 0.2 μg/ml DAPI. Images were captured with a Nikon Eclipse E1000 microscope equipped with a DXM1200F digital camera and signal intensity evaluated by ImageJ software. Click-iT-Edu Alexa Fluor 488 (Life Technologies) staining was performed as suggested by the manufacturer's instruction, with the addition of an immunostaining step to reveal $p21^{Waf1}$, using an Alexa Fluor 555-conjugated anti-rabbit as secondary antibody. Obtained signals were quantified with ImageJ software. Flow cytometry analyses to evaluate DNA content were conducted as previously described.[41]

Semi-quantitative PCR

After treatment, cells were collected and subjected to the simultaneous extraction of RNA and proteins by the Illustra^{TM} triplePrep Kit (GE Healthcare, USA), following the manufacturer's instructions. One μg of RNA was reverse-transcribed into cDNA by the Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen, USA), according to the manufacturer's instruction. PCR was carried out under conditions of linear phase amplification, using primers specific for $p21^{Waf1}$ and for the housekeeping gene β-actin (p21-FW 5′-GCGCTAATGGCGGGCTGCAT-3′ and p21-REV 5′-GCCGGCGTTTGGAGTGGTAG-3′; β-actin-FW 5′-GCTCGTCGTCGACAACGGCTC-3′ and β-actin-REV 5′-CAAACATGATCTGGGTCATCTTCTC-3′). Thirty cycles at 94°C × 15″ 64°C × 15″ and 72°C × 30″ for $p21^{Waf1}$ and 29 cycles at 94°C × 15″ 64°C × 15″ and 72°C × 30″ for β-actin were performed. The PCR products (364 bp for $p21^{Waf1}$ and 352 bp for β-actin) were run on a 2% agarose gel stained with ethidium bromide and photographed. Finally, densitometry analysis was performed and the ratio $p21^{Waf1}$/β-actin calculated.

Kinase assays

In vitro Chk2 and Chk1 kinase assays were performed as previously described[44] using Cdc25C fragment and full length $p21^{Waf1}$ and Chk2 cloned in pGEX-4T-1 vector and produced in *E. coli*. Chk1 was purchased from Millipore.

G1/S and G2/M transition analysis

To test G1/S transition DNA replication was measured using a Click-it EdU assay kit (Invitrogen). To evaluate G2/M transition, nocodazole (100 ng/ml) was added 30 min before bleomycin exposure to trap checkpoint defective cells. Cells were successively fixed and stained with an Alexa Fluor 488-conjugated anti-phospho-Histone H3 (Ser10) to reveal mitotic cells.

Disclosure of Potential Conflicts of Interest

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Supplemental Materials

Supplemental data for this article can be accessed on the publisher's website.

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