The Cancer-associated K351N Mutation Affects the Ubiquitination and the Translocation to Mitochondria of p53 Protein*§

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Background: A new mutant (K351N) of p53 in the tetramerization domain impairs cisplatin-mediated apoptosis in a cisplatin-resistant ovarian carcinoma cell line.

Results: Defects in monoubiquitination of the p53 K351N mutant cause its nuclear accumulation.

Conclusion: K351N mutation impairs p53 targeting to mitochondria and transcription-independent apoptosis.

Significance: K351N mutation of p53 is critical in contributing to and maintaining the resistance to cisplatin.

Stress-induced monoubiquitination of p53 is a crucial event for the nuclear-cyttoplasm-mitochondria trafficking and transcription-independent pro-apoptotic functions of p53. Although an intact ubiquitination pathway and a functional nuclear export sequence are required for p53 nuclear export, the role of specific residues within this region in regulating both processes remains largely unknown. Here we characterize the mechanisms accounting for the nuclear accumulation of a new point mutation (Lys–351 to Asn) in the nuclear export sequence of p53 identified in a cisplatin-resistant ovarian carcinoma cell line (A2780 CIS). We found that K351N substitution abrogates the monoubiquitination of p53 induced by both Mdm2 and MSL2 E3-ligases. As a consequence, cells expressing p53 K351N mutant showed defects in cisplatin-induced translocation of p53 to mitochondria, Bax oligomerization, and mitochondrial membrane depolarization. These data identify K351N as a critical mutation of p53 that contributes to the development and maintenance of resistance to cisplatin.

The tumor suppressor p53 represents a crucial node of a complex signaling pathway that evolved to sense a great variety of cytotoxic and genotoxic stresses. Once activated, p53 may mediate a range of cellular outcomes that vary from cell cycle arrest to DNA repair, senescence, and apoptosis (1).

In normal conditions, p53 is maintained at low levels by continuous ubiquitination and subsequent degradation by the 26 S proteasome (2, 3). Upon stimulation by stress signals, such as DNA damage, hypoxia, and activation of oncogenes, p53 is rapidly stabilized and accumulates inducing cell cycle arrest to allow DNA repair and/or triggers cell death by apoptosis. Indeed, p53 was first characterized as a transcription factor that, in response to several cellular stresses, regulates the expression of genes involved in cell cycle arrest (p21waf1 and Mdm2), DNA repair (GADD45), and apoptosis (Bax, PLMA, Noxa, and p53AIP1). More recent studies evidenced a significant contribution of transcription-independent functions of p53 to apoptosis in response to genotoxic stress (4).

Most of the transcription-independent anti-cancer activities of p53 have been associated to its ability to induce mitochondrial-dependent apoptosis by regulating the function of several Bcl-2 family proteins (5). The Bcl-2 family comprises multidomain proteins with anti-apoptotic (Bcl-2, Bcl-xL, and Bfl-1) and pro-apoptotic functions (i.e. Bax and Bak) and a subset of pro-apoptotic members known as BH3-only proteins with regulatory functions. The BH3-only proteins (i.e. Bim, Noxa, and Puma) function upstream of the pro-apoptotic proteins Bax and Bak and transduce the death stimuli to Bax and/or Bak favoring their oligomerization, the release of cytochrome c from the mitochondrial membrane, and the activation of the caspase-9 death pathway (5). Several controversial mechanisms have been described in various cell types and under different genotoxic stimuli for accounting the transcription-independent pro-apoptotic activities of p53 at the mitochondria. Indeed, p53 has been shown to mediate mitochondrial cell death by interacting with mitochondria and directly triggering the permeabilization of the outer mitochondrial membrane (6, 7) or by leading to either Bax (8, 9) or Bak activation and oligomerization (10–12). Independent of the pathways, the hallmark of these transcription-independent activities of p53 is its accumulation in the cytosol or mitochondria. Thus, the identification of both the mechanisms and specific residues and/or domains of p53...
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involved in its nuclear export are actually the object of intense studies.

Three distinct functional domains can be identified in p53: the activation domain (residues 1–45), the DNA binding domain (residues 100–300), and the tetramerization domain (TD, residues 324–356). The majority of cancer-associated p53 mutations (>90%) involve missense mutations of its DNA binding domain (13). These alterations affect the ability of p53 to trans-activate or repress specific target genes involved in growth arrest, drug resistance, and apoptosis (1, 14). 13.6% of p53 mutations occur outside exons 5–8 (13), and only 3% occur in the TD, which consists of nearly 8% of the entire protein (15). The TD of p53 is essential for efficient p53 transcriptional activity and affects both the affinity and conformation of the interaction with DNA (16). Moreover, the TD domain of p53 contains a putative nuclear export signal (residues 340–351) important for the cellular relocalization of p53 and its cytosolic functions independent of transcription (17). The characterization of mutants in the TD shows that most of them do not oligomerize, are unable to bind to DNA, are inefficient to stimulate transcription of p53 target genes, and do not inhibit growth of tumor cells in colony formation assays (16). Thus, the low frequency of mutation in the TD has been explained by the consideration that mutations in this domain are less advantageous to cancer progression, because such mutants are unable to oligomerize with the wild-type proteins leaving them free to suppress carcinogenic events (16). Furthermore, most of the mutants of p53 in the TD may retain their transcription-independent pro-apoptotic activities (9, 18).

Our research group has recently identified a point mutation in the p53 TD (K351N) in a cisplatin-resistant sub-clone of the A2780 ovarian carcinoma cell line (A2780 CIS). This mutation was generated by the substitution AAG/AAT in heterozygosis. Although K351N mutation did not affect p53 expression, it was associated to the acquisition of resistance to apoptosis in A2780 CIS, particularly to defects in both cis-dichlorodiammine platinum (CDDP)-induced Bax expression and associated mitochondrial membrane depolarization, cytochrome c release, and activation of caspase-3 (19). K351N substitution significantly reduced the thermodynamic stability of p53 tetramers without affecting the overall half-life of the protein. Moreover, p53 K351N exhibited a reduced ability to bind DNA and to trans-activate its specific target gene promoters (i.e. Bax, p53AIP1, and ΔNp73) when overexpressed in A2780 or Jurkat cells. Most interestingly, data obtained from the analysis of p53 subcellular localization revealed that K351N mutation impaired the nuclear export of p53 induced by cisplatin treatment (20).

In the present work, we analyzed the relevance and mechanisms accounting for the nuclear accumulation of p53 K351N mutant. Several studies have revealed a crucial role of stress-induced monoubiquitination for the nuclear–cytoplasm–mitochondria trafficking and transcription-independent pro-apoptotic function of p53 (21). Mdm2 (murine double minute) (22, 23) and MSL2 (male specific lethal 2) E3 ligases (24) have been both implicated in the monoubiquitination of p53. We found that K351N mutation impairs both Mdm2- and MSL2-mediated monoubiquitination of p53. As a consequence, the p53 K351N mutant loses the ability to translocate to the mitochondria and to induce Bax oligomerization and mitochondria membrane depolarization. Thus, the nuclear retention of transcriptional inactive p53 K351N specifically confers resistance to CDDP-induced apoptosis by impairing p53 targeting to mitochondria and contributing to the development and maintenance of resistance to cisplatin.

EXPERIMENTAL PROCEDURES

Cell Lines, Antibodies, and Reagents—The human primary osteogenic sarcoma, p53<sup>−/−</sup> Saos-2, H1299 (ATCC HTB85), and the human ovarian carcinoma cell line A2780 WT and its cisplatin-resistant sub-line A2780 CIS (European Collection of Cell Cultures, London, UK) were cultured in DMEM supplemented with 10% FCS, t-glutamine, penicillin, and streptomycin (Invitrogen). To retain cisplatin resistance, 1 μM cisplatin was added to the culture medium of A2780 CIS every two passages (25). The Jurkat T cell line (JCH7C17) was maintained in RPMI 1640 supplemented with 10% FCS, t-glutamine, penicillin, and streptomycin.

CDDP was obtained from Pfizer. Doxorubicin and paclitaxel were purchased from Sigma. MG132 was from Calbiochem (Merck Millipore). Anti-p53 (DO-1), anti-p53 (FL-393), anti-Bax (N-20), anti-PCNA (PC-10), and anti-GAPDH (FL-335) Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-MDM2 (Ab-1) was from Calbiochem (Merck Millipore). Anti-OxPhos Complex II 70-kDa subunit (A-11142) was from Molecular Probes (Invitrogen).

Plasmids, Cell Transfection, and Luciferase Assays—FLAG-p53 WT and FLAG-p53 K351N constructs were generated by PCR from the cDNA of A2780 WT cells, as previously described (20). pCMV-Mdm2 expression vector has been previously described (26). The pRES-F-HA-ML2 construct was generated as described before (24).

Bax-luciferase reporter construct was obtained by subcloning the PCR-generated fragment (−715 to −317 bp) from the bax gene promoter into BglII-HindIII sites of the pGL3-luciferase Enhancer vector (Promega) (19). The HA-tagged ubiquitin (HA-Ub) expression vector, carrying a fusion protein formed by an epitope of the HA and the entire open reading frame of the ubiquitin between the CMV enhancer and the SV40 polyadenylation signal, has been previously described (27).

pESC-p53WT and pESC-K351N were generated by PCR from FLAG-p53 WT and FLAG-p53 K351N constructs. Briefly, p53 WT or K351N were amplified using oligonucleotides containing N-terminal BamHI and C-terminal XhoI restriction sites for cloning into plasmid p-ESC-His under the regulated promoter Gal1–10 (Stratagene).

Saos-2 cells were transiently transfected with the indicated expression vectors by using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instruction. JCH7C17 cells were
transfected by electroporation using 30 μg of total DNA in 400 μl of RPMI 1640 supplemented with 20% FCS. Electroporation was performed in 0.45-cm electroporation cuvettes (Gene Pulser, Bio-Rad) at 960 microfarads and 250 V.

At the indicated times, luciferase activity was measured according to the manufacturer’s instruction (Promega). Transfection efficiency was tested by co-expressing a plasmid encoding an enhanced green fluorescent protein (eGFP, Clontech). Luciferase activity determined in triplicate was expressed either as fold induction over the basal activity of cells transfected with empty vectors or as arbitrary luciferase units after normalization to GFP values.

siRNA Transfection—p53 sMART pool siRNA oligonucleotides and scrambled siRNA (control, ctr) were purchased from Dharmacon (Chicago, IL). Cationic lipid complexes, prepared by incubating 100 pmol of indicated siRNA with 2 ml of Lipofectamine 2000 in 200 μl of Opti-MEM (Invitrogen) for 20 min, were added to adherent A2780 WT cells in a final volume of 2 μl. After overnight incubation, cells were washed and cultured in the presence or absence of 30 μM CDDP, 2 μM doxorubicin or 1 μM paclitaxel for an additional 48 h. At the end of incubation apoptosis was evaluated by PI staining.

Protein Extracts Preparation, Mitochondria Purification, Immunoprecipitation, and Immunoblotting—Total protein extracts were obtained by lysing cells for 30 min at 4 °C in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 1% SDS) in the presence of protease and phosphatase inhibitors.

Mitochondrial fractions were obtained by resuspending cells in a hypotonic solution (10 mM Tris-HCl, pH 7.5, 10 mM KCl, 2 mM MgCl₂, 1 mM sodium orthovanadate). The suspension was added with extraction buffer twice (20 mM HEPES, pH 7.5, 0.4 M mannitol, 140 mM sucrose, 2 mM EGTA) and homogenized with a loose fitted homogenizer (pottet). Intact cells and nuclei were centrifuged for 10 min at 750 × g. The supernatant was spun at 10,000 × g for 10 min. The pellet was lysed for 30 min at 4 °C in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM EGTA, 1 mM MgCl₂) in the presence of protease and phosphatase inhibitors and represented the mitochondrial fraction.

Proteins were resolved by SDS-PAGE and blotted onto nitrocellulose membranes. Blots were incubated with the indicated primary antibodies, extensively washed, and, after incubation with horseradish peroxidase (HRP)-labeled goat anti-rabbit or goat anti-mouse Abs (Amersham Biosciences), developed with horseradish peroxidase (HRP)-labeled goat anti-rabbit or goat anti-mouse Abs (Amersham Biosciences), developed with the enhanced chemiluminescence detection system (ECL, Amersham Biosciences).

For p53 immunoprecipitation, cytosolic extracts were precleared for 1 h with protein A-Sepharose (Amersham Biosciences) and then immunoprecipitated for 2 h with anti-p53 (DO-1) Ab pre-adsorbed on protein A/G-Sepharose beads. Proteins were resolved onto nitrocellulose membrane, and blots were incubated with the indicated primary antibodies.

Analysis of Bax Oligomerization—To detect Bax oligomerization, mitochondria-enriched fractions were incubated in the presence 0.05% glutaraldehyde for 30 min at room temperature before resolving in non-reducing 12% SDS-PAGE followed by anti-Bax Western blot analysis. The bands representing monomers and oligomers of Bax were quantified with ImageJ, and the data were expressed as the mean of oligomer/monomer ratio ± S.D. of three independent experiments.

In Vivo Ubiquitination Assays—Jurkat cells were transfected with 10 μg of HA-Ub together with 10 μg of p53 WT or p53 K351N alone or in combination with 5 μg of Mdm2 or 5 μg of MSL2. 3 h after transfection, 10 μM MG132 was added, where indicated. After 6 h, cells were resuspended in 1% SDS in TBS and boiled twice for 5 min each time. TBS containing Triton X-100 was added to each lysate to obtain a final concentration of 0.3% SDS and 1% Triton X-100. After preclearing with Protein A-Sepharose, p53 was immunoprecipitated with anti-p53 (DO-1) Ab. Immunoprecipitated proteins were resolved by SDS-PAGE and analyzed by Western blotting anti-HA. The in vivo ubiquitination of p53 in CDDP-treated A2780 cell lines was performed as described above on A2780 WT or CIS cells treated for 6 h with CDDP (30 μM).

Apoptosis Analysis by PI and Annexin V Staining—Cells, treated as indicated, were carefully resuspended in PBS containing 0.1% Triton X-100 (Sigma) and 100 units/ml RNase A (Sigma), were stained with 50 μg/ml PI (Sigma) and incubated at 37 °C for 15 min. Apoptosis was analyzed by using a FACSCalibur (BD Biosciences), by a biparametric analysis of FL2-H versus SSC-H graphs. Cells showing a content of less than 2C DNA (hypodiploid cells) and high SSC-H (granular, highly condensed cells) were regarded as apoptotic. The mean frequencies of apoptotic cells were calculated at least from three independent experiments and statistically analyzed using Student’s t test. The percentage of specific apoptosis was calculated as follows: % specific apoptosis = 100 × [(% induced apoptotic cells – % spontaneous apoptotic cells)/(100 – % spontaneous apoptotic cells)]. Apoptosis was also evaluated by staining cells with Annexin V conjugates by using a commercially available kit (Molecular Probes Inc., Invitrogen, UK).

Analysis of Variation in ΔΨm.—Variation in Mitochondrial Membrane Potential (ΔΨm) at the single mitochondrial level was detected by using tetramethyl rhodamine methyl ester. 5 × 10⁵ cells were incubated for 20 min at 37 °C in 10 mM Hepes, 135 mM NaCl, 5 mM CaCl₂, with 200 nm tetramethyl rhodamine methyl ester. Samples were analyzed by using FACSCalibur flow cytometer.

Yeast Plasmids, Strains, and Growth Conditions—In this study, we used the Saccharomyces cerevisiae strain BY4741 (MAT α, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0) (28) for heterologous expression of native or mutant p53 and the yeast strain W301-1B (MATα, ade2–1, ura3–1, his3–11,15, leu2–3112, trp1–1, can1–100) transformed with the yeast multicopy YepDP1–8 vector expressing mutant p53 deleted in the nuclear localization signal (p53ΔNLSI) (29). Yeast strains were grown on rich medium YP (1% yeast extract, 2% peptone) supplemented with 2% glucose (YPD) and minimal medium SC (0.67% yeast nitrogen base) containing 2% glucose (SD) or 2% galactose (SGal) supplemented with 10 μg/ml of the appropriate nutritional requirements according to the genotype of the strains. Agar (2%) was added for solid plates. Transformation of yeast cells with p-ESC-His or pESC-p53WT or pESC-K351N was performed by the lithium acetate procedure, as described by Gietz et al. (30). Individual clones were grown overnight in SD.
medium (Ga1–10 repressing conditions) until exponential
growth phase. To induce p53 expression, cells were washed
three times with water and resuspended in SC medium with
galactose (2%, w/v).

**Fluorescence Microscopy**—For analysis of mitochondria mor-
nphology, we used plasmid pYX232-mtGFP (31), which targets
GFP into mitochondrial matrix. For image acquisition of the
cells containing this plasmid, we used an Axioskop2 fluores-
cence microscope (Carl Zeiss, Jena, Germany) equipped with a
digital camera (micro-charge-coupled device).

**RESULTS**

**K351N Mutation Impairs Ubiquitination of p53**—Recently,
we have identified a point mutation in the p53 TD (K351N) in
the cisplatin-resistant derivative subclone (CIS) of the A2780
ovarian carcinoma cell line (25). This mutation conferred a spe-
cific resistance to apoptosis induced by CDDP but not by other
anti-cancer drugs, such as paclitaxel or doxorubicin (Fig. 1,
A and B). These data are consistent with our findings that, in
contrast to CDDP (20), paclitaxel- and doxorubicin-mediated
apoptosis in A2780 cells was p53-independent, as evidenced by
p53 silencing with specific siRNA (supplemental Fig. 1S
A and B). Consistently with our previous data (20), the resistance to
CDDP-induced apoptosis of A2780 CIS was associated to
reduced mitochondria membrane depolarization (Fig. 1C). We
further demonstrated that p53 K351N exhibits a reduced tran-
scriptional activity (at least 50%) on pro-apoptotic target genes
(i.e. bax) (Fig. 1D), and, most importantly, K351N mutation also
affected p53 nuclear export (20).

To identify the mechanisms accounting for the nuclear accumu-
lation of p53 K351N mutant, we specifically looked at p53
ubiquitination. Previous studies have demonstrated that
Mdm2-mediated monoubiquitination of p53 is sufficient for its
nuclear export. Thus, we analyzed the effect of K351N muta-
tion on the ubiquitination of p53 by Mdm2. To do that, we
performed in vivo ubiquitination assays (32). We transfected
Jurkat cells (JCH7) with expression vectors encoding
HA-tagged ubiquitin, p53 WT and Mdm2 (ratio 2:1) in the
presence or absence of the proteasome inhibitor MG132 to pre-
vent p53 degradation. 6 h after transfection, p53 was immuno-
precipitated, and its ubiquitination status was analyzed by
anti-HA Western blotting. The data reported in Fig. 2
A, evidenced a typical ladder of high molecular weight bands,
corresponding to polyubiquitinated and monoubiquitinated p53
(Fig. 2A, lane 5 versus lane 4). To ensure that the high molecular
bands observed in the anti-p53 IP corresponded to polyubiquitinated
and monoubiquitinated p53 and not to other
co-precipitating proteins, we also performed anti-p53 Western
blotting of each cell extracts from the
in vivo ubiquitination
assays. A similar ladder was observed, thus indicating that the
high molecular bands observed in anti-p53 IP were, indeed,
ubiquitinated p53 (Fig. 2B and supplemental Fig. 1S). Con-
versely, K351N mutation strongly impaired the extent of Mdm2-induced ubiquitination of p53 (Fig. 2B, lane 5 versus lane 3).

Recently, MSL2 has been identified as a novel E3 ligase that promotes the monoubiquitination of p53 and regulates its cytoplasmic localization (24). Consistently with these data, we found that MSL2 induced a significant monoubiquitination of p53 WT (10 μg) or p53 K351N and CMV-Mdm2 (5 μg) expression vectors. After 3 h, 10 μM MG132 was added, where indicated. Cells were lysed under denaturing conditions, and the ubiquitination of p53 was analyzed by Western blotting using anti-p53 DO-1. A sample of each lysate was analyzed by ant-GAPDH Western blotting (middle panel). The overexpression of Mdm2 was analyzed by anti-Mdm2 Abs (lower panel). The expression of MSL2 was analyzed in total lysates by anti-HA Western blotting. A–C, polyubiquitinated and monoubiquitinated p53 forms are indicated on the right. Molecular weight markers (MW) are indicated on the left.

Defective CDDP-induced Monoubiquitination of p53 Affects Its Mitochondrial Translocation in Resistant A2780 CIS Cells—Monoubiquitination has been described to promote p53 translocation to mitochondria, where it can directly launch apoptosis. Indeed, bypassing the nucleus by directly targeting p53 to mitochondria is sufficient to activate the apoptotic program (9, 18).

Firstly, we analyzed the p53 ubiquitination status in both A2780 WT and CIS cells treated with CDDP. Interestingly, we found that 6 h after CDDP treatment, p53 was primarily monoubiquitinated in A2780 WT cells (Fig. 3A, lane 2). On the contrary, CDDP treatment failed to induce a similar ubiquitination status in A2780 CIS cells (Fig. 3A, lane 4 versus lane 2). We next analyzed if the loss of CDDP-induced p53 monoubiquitination in A2780 CIS cells was associated with impaired translocation of p53 to mitochondria (9, 18). The treatment of sensitive A2780 WT cells with CDDP induced a strong and persistent (6–24 h) translocation of p53 to mitochondria (Fig. 3B, lanes 1–3). By contrast, the mitochondrial accumulation of p53 in the resistant A2780 CIS cells was markedly reduced also.

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![Figure 2](image-url)
24 h after CDDP treatment (Fig. 3B, lanes 4–6). Consistently with previous data in the p53-deficient cell line (9, 18), the analysis of mitochondrial localization of p53 WT or K351N expressed in Saos-2 cells revealed that p53 WT rapidly translocates and accumulates to mitochondria in the absence of additional DNA damage (Fig. 3C, lane 2). K351N mutation significantly compromised the mitochondria localization of p53, as evidenced by the strong reduction (>60%) of p53 K351N levels (Fig. 3C, lane 3). The purity of mitochondrial fractions was verified by analyzing both the expression of complex II p70 protein, a subunit of the succinate dehydrogenase complex and anti-PCNA. Thus, defective CDDP-induced monoubiquitination of p53 was associated to impaired mitochondrial localization of p53 in cisplatin-resistant A2780 CIS cells.

The Impaired Mitochondrial Translocation of p53 K351N Compromises Bax Oligomerization and p53-dependent Mitochondria Damage—The translocation of p53 to mitochondria promotes the oligomerization and activation of Bax (8). Therefore, we analyzed the oligomeric status of Bax in response to CDDP treatment in either A2780 WT or CIS cells. Cells were treated with CDDP for the indicated times, after cross-linking with 0.05% glutaraldehyde, proteins were resolved by a non-reducing SDS-PAGE and Bax oligomerization was analyzed by Western blotting. CDDP treatment induced a strong increase of Bax oligomers in A2780 WT after 6 h of CDDP treatment (Fig. 4A). The increase of Bax oligomerization correlated with the translocation of p53 to the mitochondria (Fig. 3B). CDDP-treatment of A2780 CIS cells induced a significant lower percentage of oligomers, and monomeric Bax was largely present after 24 h (Fig. 4A). Consequently, the ratio between oligomeric and monomeric Bax fraction was highly reduced in A2780 CIS cells (Fig. 4B). Although a physical association of p53 and Bax was reported in some cell types (33, 34), we did not find a direct association of p53 and Bax. Indeed, any co-immunoprecipitation of Bax with p53 was observed neither in A2780 WT cells (Fig. 4C) nor in Saos-2 cells transfected with p53 WT or K351N constructs (supplemental Fig. S2). These data are consistent with data observed in other cell systems (8). The reduced mitochondrial localization of p53 (Fig. 3B) and the impaired Bax oligomerization observed in A2780 CIS were also accompanied by the inhibition of mitochondrial membrane depolarization when compared with the parental A2780 WT line (18% on A2780 CIS versus 41% on A2780 WT cells) (Fig. 4D). Altogether these data strongly suggest that CDDP-induced apoptosis is dependent on p53 translocation to mitochondria, where it promotes Bax oligomerization and mitochondria dysfunctions.

To demonstrate a direct correlation between the translocation of p53 to mitochondria and mitochondria damage, we used the unicellular S. cerevisiae model system. In the last years, it has been accepted to use yeast as a model for the study of apo-

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**FIGURE 3.** Impaired p53 monoubiquitination and mitochondria membrane localization of p53 in CDDP-treated A26780 CIS cells. A, A2780 WT and A2780 CIS cells were treated for 6 h with 30 μM CDDP. p53 immunoprecipitations (IP) were performed under denaturing conditions using anti-p53 DO-1 Ab. Precipitated p53 was analyzed by Western blotting using anti-p53 FL-393 Ab (upper panel). A sample of each lysate was analyzed for GAPDH expression (lower panel). The positions of Ig heavy chain (IgH) and mono- or polyubiquitinated p53 are indicated on the right. Molecular weights (MW) are reported on the left. B, A2780 WT and A2780 CIS cells were treated for 6 and 24 h with 30 μM CDDP. Mitochondria were isolated and p53, OxPhos Complex II 70-kDa subunit (p70), and PCNA expressions were analyzed by Western blotting. Data were quantified and expressed as -fold of induction (F.I.) over the basal level after normalization to p70 expression. The lower panel refers to total p53 expression. C, Saos-2 cells were transfected with empty vector (Vec), or p53 WT or K351N, and both mitochondrial extracts (upper panels) and total lysates (lower panels) were analyzed as in B. B and C, anti-complex II p70 Ab and anti-PCNA Western blotting were performed for both equal loading of proteins and purity of mitochondrial fractions.
ptosis. Indeed, the basal machinery of the apoptotic process is evolutionary conserved, because in yeast we can observe all phenotypic markers of apoptosis such as chromatin condensation, mitochondria fragmentation, and cytochrome c release. In particular, *S. cerevisiae* has been successfully used to study the regulation of apoptosis by either WT or mutated p53 (35). More recent data also revealed the conservation in yeast of functional transcription-independent p53 apoptotic functions and p53-induced mitochondria fragmentation (29, 36).

Recombinant BY4741/pEsc-HIS yeast strains, expressing the empty vector (Vec) or p53 WT or p53 K351N under the Gal1/Gal10-inducible promoter, were transformed together with mitochondria-targeted green fluorescent protein (mito-GFP). This construct has been efficiently used to analyze the mitochondrial morphology and to correlate abnormal mitochondria changes with defective functions (31). All strain cells were initially grown on glucose-containing minimal medium (SD) to keep the Gal1/Gal10 promoter in a repressed state and then cultured for 21 h with 2% galactose-containing minimal medium (SGal) to induce p53 expression. As shown in Fig. 5, p53 WT expression strongly induced mitochondria fragmentation in yeast cells. By contrast, the mitochondria damage was markedly reduced in cells expressing p53 K351N, as evidenced by the reduced percentage of cells with fragmented mitochondria (Fig. 5B). As a control, we used the yeast strain W303/p53/H9004NLSI expressing a p53 mutant deleted of the NLSI (residues 305–322) that has been previously described to fail to localize to mitochondria and to induce mitochondria fragmentation (29). Therefore, K351N mutation confers resistance to mitochondria-dependent apoptosis by affecting p53 accumulation to mitochondria.

**DISCUSSION**

The pro-apoptotic functions of p53 in response to several genotoxic stresses were originally linked to its ability to activate mitochondrial cell death by regulating the expression of several pro-apoptotic members of the Bcl-2 family, in particular Bax and Puma. More recently, an extensive area of research evidenced an important additional role of p53 in triggering apoptosis independently of its transcriptional functions and iden-
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FIGURE 5. K351N mutation affects p53-induced fragmentation of mitochondria in S. cerevisiae. The BY4741 S. cerevisiae strain cells expressing pEsc-His (Vec), or pESC-p53WT, or pESC-K351N, and the W303–1B strain expressing p53ΔNLSI mutant (W303/p53ΔNLSI) were transformed with mito-GFP, which targets GFP into the mitochondrial matrix, were grown in glucose (SD), and then shifted for 21 h in galactose containing minimal medium (SGal). The mitochondria morphology (A) was evaluated by analyzing the GFP with an Axioskop2 fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with a digital camera (micro-charge-coupled device). The quantification of cell population showing fragmented mitochondria is reported in B. Data represent the mean ± S.D. of three independent experiments.

K351N mutation affected p53-induced nuclear export of p53, thus affecting also the transcription-independent pro-apoptotic activities of p53. Several apoptotic stimuli in different experimental systems evidenced the mitochondrial membrane as a critical site for the transcription-independent pro-apoptotic functions of p53 (7, 9, 10, 18). In initial experiments, p53-dependent cytochrome c release from isolated mitochondria was abrogated when Bax was immuno-depleted from the reaction mix, indicating that the apoptotic potential of p53-containing cytoplasts required Bax (43). Other studies reported that p53 can mediate mitochondrial cell death by interacting with mitochondria and directly triggering the permeabilization of the outer mitochondrial membrane (7) or through the direct activation of Bak (10) or Bax (8). Cisplatin treatment of sensitive A2780 WT cells efficiently induces p53 translocation to mitochondria, Bax oligomerization, mitochondrial membrane depolarization, and apoptosis. All these events are strongly impaired in the resistant A2780 CIS cells expressing mutant p53 K351N. Moreover, the data from the ectopic expression of p53 in either p53-negative Saos-2 cells and in the yeast system, plainly demonstrate that p53 K351N mutant exhibits a reduced capability to accumulate at mitochondria (Fig. 3C) and to induce mitochondria fragmentation (Fig. 5), respectively. In addition to mitochondrial-targeted p53 actions, a parallel transcription-independent p53 death pathway was proposed to take place in the cytosol. Accumulation of p53 in the cytosol following genotoxic stresses (i.e. UV and γ-irradiation) was described to play a key role in the activation of Bax, and a physical association of p53 and Bax was reported to occur in stressed melanocytes (33, 34). However, these data were not confirmed in other cell types, and a “hit and run” model was proposed to explain the functional interaction between p53 and Bax (8). Consistently with these observations, we did not detect any direct interaction between cytosolic p53 WT and Bax in neither CDDP-treated A2780 WT cells (Fig. 4C) nor Saos-2 cells expressing ectopic p53 (supplemental Fig. S2). Altogether, these data suggest that K351N mutation impairs p53-dependent Bax oligomerization and loss of mitochondrial membrane potential by affecting the molecular modifications necessary for the nuclear export and the translocation of p53 to mitochondria.

p53 protein translocation to mitochondria in response to hypoxic and oxidative stresses has been recently described to depend on its ubiquitination by Mdm2 (23). Initially p53-Mdm2 interaction was described to repress p53 activity by promoting p53 ubiquitination and its degradation by proteasome (2, 3). It is now clear that the ubiquitination of p53 by Mdm2 may serve not only to promote p53 degradation but also to regulate p53 sub-cellular localization and activity. Early studies demonstrated that the type of p53 ubiquitination by Mdm2 controls the fate of p53. Polyubiquitination of p53 induced by Mdm2 mediates either nuclear or cytoplasmic proteasomal degradation (44–46). By contrast, Mdm2-mediated monoubiquitination stabilizes p53, thus promoting its nuclear export...
and translocation to mitochondria (22, 23). As previously shown, a cluster of six C-terminal lysine residues within p53 is required for Mdm2-mediated ubiquitination and nuclear export (47, 48). These evidences were recently extended by the findings that Mdm2-mediated ubiquitination of these C-terminal lysine residues contributes to the exposure of the C-terminal nuclear export signal within the TD of p53 and its nuclear export (32, 49). Although, an intact C-terminal nuclear export signal sequence and a functional ubiquitination pathway are required for p53 nuclear export, the role of specific residues within this region in both Mdm2-mediated ubiquitination and nuclear export of p53 has not been extensively investigated. Our data demonstrate that K351N mutation affects Mdm2-mediated ubiquitination of p53 in vitro (Fig. 2) as well as in response to CDDP treatment (Fig. 3A).

Mdm2 binding to p53 is essential to induce the conformational change required for ubiquitination and p53 nuclear export. However, mutation of C-terminal lysine residues (p53 6KR mutant) in p53 was reported to inhibit its Mdm2-mediated nuclear export without affecting Mdm2-p53 interaction (47, 48). Further data from Carter et al. (32) showed that a stable interaction between Mdm2 and p53 6KR mutant might sequester p53 in the nucleus. Consistently, we found that p53 WT and K351N binds Mdm2 at a similar extent. Furthermore, CDDP-induced Mdm2/p53 association is comparable in both sensitive (WT) and cisplatin-resistant (CIS) A2780 cells (data not shown). The cluster of C-terminal lysine residues has been described as the major site for Mdm2-mediated nuclear export of p53, and simultaneous substitution of all six lysine residues is required for ubiquitination by Mdm2 (32, 47–49). What emerges from our findings is that mutation of the single Lys-351 to Asn abrogates ubiquitination induced by both Mdm2 and MSL2 E3-ligases, thus indicating that Lys-351 is clearly a key clue involved in p53 ubiquitination, nuclear export, and mitochondria translocation.

Interestingly, recent data from the Cancer Genome Atlas project evidenced somatic mutations, almost missense mutations, of p53 in the 96% high grade serous ovarian carcinoma from patients, 31% of whom developed a cisplatin resistance (50). These data, together with the observation that the K351N mutation has been selected in a cisplatin-resistant cancer cell line by chronic exposure to CDDP, may identify K351N as a prognostic marker to predict the acquisition of cisplatin-based chemotherapy resistance.

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REFERENCES
1. Vogelstein, B., Lane, D., and Levine, A. J. (2000) Nature 408, 307–310
2. Haurop, Y., Maya, R., Kazaz, A., and Oren, M. (1997) Nature 387, 296–299
3. Kubbutat, M. H., Jones, S. N., and Vousden, K. H. (1997) Nature 387, 299–303
4. Moll, U. M., Wolff, S., Speidel, D., and Deppert, W. (2005) Current Opinion in Cell Biology 17, 631–636
5. Adams, J. M., and Cory, S. (2007) Oncogene 26, 1324–1337
6. Palacios, G., Crawford, H. C., Vaseva, A., and Moll, U. M. (2008) Cell Cycle 7, 2584–2590
7. Wolff, S., Erster, S., Palacios, G., and Moll, U. M. (2008) Cell Res. 18, 733–744
8. Chipuk, J. E., Kuwana, T., Boucher-Hayes, L., Droni, N. M., Newmeyer, D. D., Schuler, M., and Green, D. R. (2004) Science 303, 1010–1014
9. Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P., and Moll, U. M. (2003) Mol. Cell 11, 577–590
10. Leu, J. I., Dumont, P., Hafey, M., Murphy, M. E., and George, D. L. (2004) Nat. Cell Biol. 6, 443–450
11. Pietsch, E. C., Leu, J. I., Frank, A., Dumont, P., George, D. L., and Murphy, M. E. (2007) Cancer Biol. Ther. 6, 1576–1583
12. Pietsch, E. C., Perchiniak, E., Canutescu, A. A., Wang, G., Dunbrack, R. L., and Murphy, M. E. (2008) J. Biol. Chem. 283, 21294–21304
13. Sousi, T. (2005) in 25 Years of p53 (Wisman, K., and Hainaut, P., eds) pp. 225–292, Springer, Berlin
14. Weiss, L., Oren, M., and Rotter, V. (2007) Oncogene 26, 2202–2211
15. Olivier, M., Eales, R., Hollestein, M., Khan, M. A., Harris, C. C., and Hainaut, P. (2002) Hum. Mutat. 19, 607–614
16. Chêne, P. (2001) Oncogene 20, 2611–2617
17. Stommel, J. M., Marchenko, N. D., Jimenez, G. S., Moll, U. M., Hope, T. J., and Wahl, G. M. (1999) EMBO J. 18, 1660–1872
18. Marchenko, N. D., Zaika, A., and Moll, U. M. (2000) J. Biol. Chem. 275, 16022–160212
19. Muscoli, M., Cianfrocca, R., Sajeva, A., Mozzetti, S., Ferrandina, G., Costanzo, A., and Tuosto, L. (2008) Mol. Cancer Ther. 7, 1410–1419
20. Muscoli, M., Montagni, E., Caristi, S., Nomura, T., Kamada, R., Di Agostino, S., Corazzari, M., Piacentini, M., Blandino, G., Costanzo, A., Sakauchi, K., and Tuosto, L. (2009) Cell Cycle 8, 3396–3405
21. Marchenko, N. D., and Moll, U. M. (2007) Cell Cycle 6, 1718–1723
22. Li, M., Brooks, C. L., Wu-Baer, F., Chen, D., Baer, R., and Gu, W. (2003) Science 302, 1972–1975
23. Marchenko, N. D., Wolff, S., Erster, S., Becker, K., and Moll, U. M. (2007) EMBO J. 26, 923–934
24. Kruse, J. P., and Gu, W. (2009) J. Biol. Chem. 284, 3250–3263
25. Behrens, B. C., Hamilton, T. C., Masuda, H., Grottzing, K. R., Whang-Peng, J., Louie, K. G., Knutsen, T., McKoy, W. M., Young, R. C., and Ozols, R. F. (1987) Cancer Res. 47, 414–418
26. Di Stefano, V., Blandino, G., Sacchi, A., D’Orazi, S., and D’Orazi, G. (2004) Oncogene 23, 5185–5192
27. Lapi, E., Di Agostino, S., Donzelli, S., Gal, H., Domany, E., Rechavi, G., Pandolfi, P. P., Givol, D., Strano, S., Lu, X., and Blandino, G. (2008) Mol. Cell 32, 803–814
28. Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P., and Boeke, J. D. (1998) Yeast 14, 115–132
29. Abdelmoula-Souissi, S., Delahodde, A., Bolotin-Fukuhara, M., Gargouri, A., and Mokdad-Gargouri, R. (2011) Apoptosis 16, 746–756
30. Gietz, D., St Jean, A., Woods, R. A., and Schiestl, R. H. (1992) Nucleic Acids Res. 20, 1425
31. Westermann, B., and Neupert, W. (2000) Yeast 16, 1421–1427
32. Carter, S., Bischof, O., Dejean, A., and Voussen, K. H. (2007) Nat. Cell Biol. 9, 428–435
33. Raffo, A. J., Kim, A. L., and Fine, R. L. (2000) Oncogene 19, 6216–6228
34. Wäster, P. K., and Ollinger, K. M. (2005) J. Invest. Dermatol. 129, 1769–1781
35. Hadj Amor, I. Y., Smaoui, K., Chaabène, I., Mabrouk, I., Djemal, L., El-Leuch, H., Allouche, M., Mokdad-Gargouri, R., and Gargouri, A. (2008) FEMS Yeast Res. 8, 1254–1262
36. Coutinho, I., Pereira, C., Pereira, G., Gonçalves, J., Córte-Real, M., and Saraiva, L. (2011) Exp. Cell Res. 317, 1147–1158
37. Speidel, D. (2010) Trends Cell Biol. 20, 14–24
38. Chipuk, J. E., Maurer, U., Green, D. R., and Schuler, M. (2003) Cancer Cell 4, 371–381
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39. Speidel, D., Helmbold, H., and Deppert, W. (2006) Oncogene 25, 940–953
40. Goldstein, I., and Rotter, V. (2009) Cell Cycle 8, 3259–3260
41. Lwin, T. Z., Durant, J. J., and Bashford, D. (2007) J. Mol. Biol. 373, 1334–1347
42. Schlereth, K., Beinoraviciute-Kellner, R., Zeitlinger, M. K., Bretz, A. C., Sauer, M., Charles, J. P., Vogiatzi, F., Leich, E., Samans, B., Eilers, M., Kisker, C., Rosenwald, A., and Stiewe, T. (2010) Mol. Cell 38, 356–368
43. Schuler, M., Bossy-Wetzel, E., Goldstein, J. C., Fitzgerald, P., and Green, D. R. (2000) J. Biol. Chem. 275, 7337–7342
44. Tao, W., and Levine, A. J. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 3077–3080
45. Xirodimas, D. P., Stephen, C. W., and Lane, D. P. (2001) Exp. Cell Res. 270, 66–77
46. Yu, Z. K., Geyer, R. K., and Maki, C. G. (2000) Oncogene 19, 5892–5897
47. Gu, J., Nie, L., Wiederschlain, D., and Yuan, Z. M. (2001) Mol. Cell. Biol. 21, 8533–8546
48. Lohrum, M. A., Woods, D. B., Ludwig, R. L., Bálint, E., and Vousden, K. H. (2001) Mol. Cell. Biol. 21, 8521–8532
49. Nie, L., Sasaki, M., and Maki, C. G. (2007) J. Biol. Chem. 282, 14616–14625
50. The Cancer Genome Atlas Research Network (2011) Nature 474, 609–615