CellCycle

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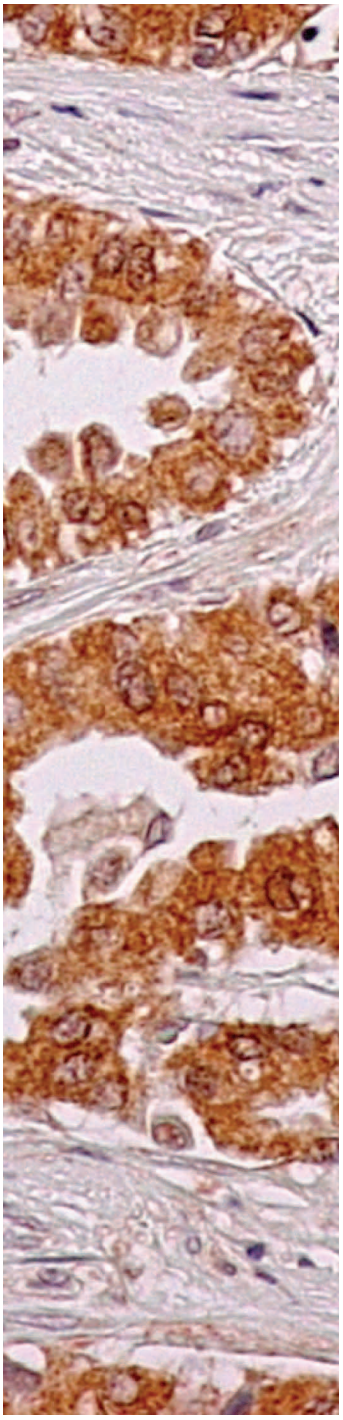

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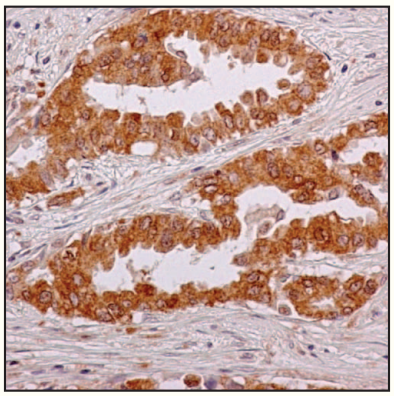

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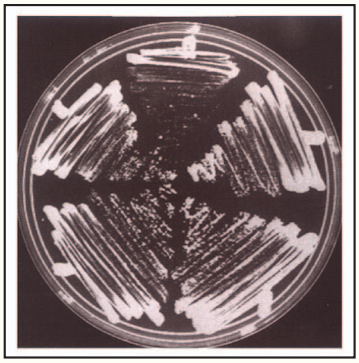

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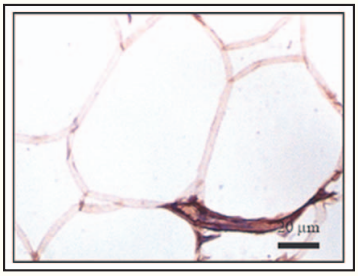

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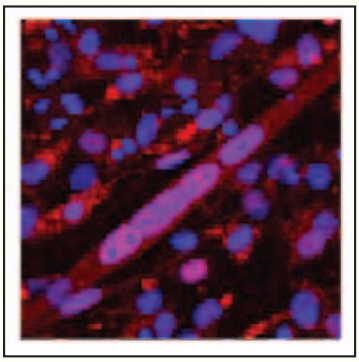

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Restoring p53 active conformation with zinc increases the response of mutant p53 tumor cells to anticancer drugs

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Key words: mutant p53 reactivation, apoptosis, zinc, p53 transcriptional activity, cancer therapy

Absence of p53 expression or expression of mutant p53 (mtp53) are common in human cancers and are associated with increased cancer resistance to chemo- and radiotherapy. Therefore, significant efforts towards pharmaceutical reactivation of defective p53 pathways are underway. We previously reported that, in HIPK2 knockdown background, p53 undergoes misfolding with inhibition of DNA binding and transcriptional activities that correlate with increased chemoresistance, and that zinc rescues wild-type p53 activity. Zinc has a crucial role in the biology of p53, in that p53 binds to DNA through a structurally complex domain stabilized by zinc atom. In this study, we explored the role of zinc in p53 reactivation in mutant p53-expressing cancer cells. We found that zinc re-established chemosensitivity in breast cancer SKBR3 (expressing R175H mutation) and glioblastoma U373MG (expressing R273H mutation) cell lines. Biochemical studies showed that zinc partly induced the transition of mutant p53 protein (reactive to conformation-sensitive PAb240 antibody for mutant conformation) into a functional conformation (reactive to conformation-sensitive PAb1620 antibody for wild-type conformation). Zinc-mediated p53 reactivation also reduced the mtp53/p73 interaction restoring both wtp53 and p73 binding to target gene promoters by ChIP assay with in vivo induction of wtp53 target gene expression, which rendered mutant p53 cells more prone to drug killing in vitro. Finally, zinc administration in U373MG tumor xenografts increased drug-induced tumor regression in vivo, which correlated with increased wild-type p53 protein conformation. These results show that the use of zinc might restore drug sensitivity and inhibit tumor growth by reactivating mutant p53.

Introduction

The tumor suppressor p53 is a transcription factor that is activated in response to DNA damage to trigger different cellular outcomes, such as cell cycle arrest, senescence and apoptosis.[1] As such, it is the major hurdle on the way to tumor formation, and therefore, it is the most frequently inactivated protein in cancer cells; thus, it undergoes point mutations in at least 50% (www.iarc.fr/p53) of human tumors and deregulation of regulatory proteins in the remaining 50%.[2] The p53 protein is commonly divided into three functional domains: the acidic amino-terminal domain that is required for transcriptional activation (TAD, residues 1–94); the central core, which is the sequence-specific DNA-binding domain (DBD, residues 94–292), and the carboxy-terminal tetramerization domain and C terminus (TAT, CT, residues 292–393).[3] Most tumor-associated p53 mutations (about 95%) cluster in the DBD, hampering the ability of p53 to recognize the sequence-specific response elements (SRE) within target promoters[4] and therefore inhibiting its tumor suppressor activity. Those p53 mutations are classified as either contact (e.g., R273H and R273C) or structural mutations (e.g., R175H, V143A, Y220C, G245S, R249S, F270L, R282W), which destabilize DBD and may change its conformation, both resulting in diminished DNA binding.[4] The mutant p53 can be distinguished from wild-type by two monoclonal antibodies, PAb1620 and PAb240, that recognize active (folded) and denatured (unfolded) p53 conformation, respectively.[5,6] The important feature of these mtp53 conformations is their flexibility and the reversibility of the conformational changes. The best example is the temperature-sensitive p53 mutations that can keep a wtp53 conformation and activity at low temperature and a mutant conformation at body temperature. The loss of the 1620 epitope by raise of temperature was correlated with reduction in binding to target DNA and was inversely correlated with the gain of the 240 epitope, indicating a transition between two alternative conformers.[7] Close to 10% of the p53 mutations were found to be temperature-sensitive,[8] and in some of those that were tested, conformation-dependent restoration of p53 function was

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obtained with temperature change. The p53 structure includes one zinc ion as an important cofactor that also stabilizes the second and third loops of the DBD domain and, therefore, is needed for wtp53 function.[3,9] Interestingly, mutant p53 proteins are prone to loss of the Zn^{2+} atom that is bound to the wild-type core, promoting aggregation of mutant p53 and therefore unfolding.[10] It was shown that p53 mutations in residues involved in the binding of zinc (e.g., C176F, H179R, C238S, C242S and perhaps also R175H) are common and result in the loss of DNA binding.[3] Furthermore the removal of zinc from p53 by chelating agents resulted in change of conformation and loss of function that can be reversed by adding zinc.[11] This does not imply that only mutations of the zinc-coordinating residues mentioned above may affect zinc binding. On the contrary, many other mutations at the DBD are known to change the DBD conformation and affect the affinity for zinc, thereby destabilizing DBD and disrupting DNA binding.[12] Hence, the attempt to overcome the effect of mutation by change of p53 conformation became an important challenge even for mutants that are not temperature-sensitive.

Numerous studies demonstrated the association between mutant p53 expression and poor response to conventional chemo- and radiotherapies, and recently, it has been proposed that mtp53 might also have pro-oncogenic activity leading to chemoresistance and tumor progression.[13] In this regard, Bossi and co-workers showed that abrogation of mutant p53 expression reduced tumor malignancy of human cancer cell lines, attempting to inhibit one of the pro-oncogenic mutant p53 function.[14] Another mtp53 pro-oncogenic characteristic is the protein complexes formation between mtp53 and the family member p73, interfering with p73 ability to induce apoptosis.[15,16] Thus, the disruption of the protein complex mtp53/p73 by small peptides has been shown to selectively increase the response of tumor cells to anticancer drugs.[17] Therefore, mutant p53 is a promising target for novel antitumor therapies, as p53 reactivation should restore drug-induced apoptosis through several mechanisms, p53-dependent or -independent, resulting in efficient removal of tumor cells.

We recently reported that depletion of homeodomain-interacting protein kinase 2 (HIPK2), the p53 apoptotic activator,[18] leads to wtp53 misfolding with acquisition of a misfolded "mutant-like" conformation recognized by specific antibodies.[19] The p53 misfolding in HIPK2-knockdown background depends on deregulation of metallothioneins, a group of proteins with high zinc-binding capacity;[20] thus, zinc supplementation to HIPK2-depleted cells restores wtp53 conformation as well as DNA-binding and transcriptional activities.[19,20] These findings prompted us to evaluate whether zinc could also affect mutant p53 and improve the sensitivity of mutant p53-expressing cells to drugs for antitumor response.

Results

Zinc improves drug-induced cell death in mutant p53-expressing cells. To evaluate whether zinc could affect mutant p53 function, we first analyzed the effect of zinc on long-term survival of SKBR3 breast cancer (expressing R175H mutation) and U373MG glioblastoma (expressing R273H mutation) cells in response to drugs. Zinc supplementation to a pulse of Adriamycin (ADR) or cisplatin (cispl) markedly increased cell death in both cell lines compared to drug treatment alone (**Fig. 1,** upper parts). Quantification of the colony assays showed significant reduction of long-term cell survival following combination treatments, compared to drug treatment alone in both cell lines (**Fig. 1,** lower parts). These findings suggest that supplemental zinc might strongly improve the sensitivity of mutant p53-expressing cells to anti-tumor drugs.

Zinc restores wtp53 activity in H1299-H175 cells. Does the improvement of drug response in mtp53 cells depend on restoration of wild-type p53 activities? To answer this question, we started examining the effect of zinc on the conformational stability of p53 in living cells. We first used the p53-null H1299 lung carcinoma cells that were transfected with p53H175 mutant expression vector and p53 protein conformation analyzed with immunoprecipitation technique using PAb1620 and PAb240 antibodies. We observed that zinc increased the PAb1620-reactive (folded) phenotype and reduced the PAb240-reactive (unfolded) phenotype (**Fig. 2A**), suggesting the transition, at least partly, of the protein into a functional conformation. To demonstrate this hypothesis, the zinc treatment of H175-transfected cells should result in (1) recruitment of p53 onto canonical target gene promoters, (2) induction of p53 transcriptional activity, (3) in vivo activation of wtp53 target genes and (4) reduction of cell survival. To this aim, H1299 cells were transfected with p53H175 expression vector, and in vivo p53-DNA binding was analyzed using chromatin immunoprecipitation (ChIP) technique. Cells, treated with cisplatin alone or in combination with zinc, were crosslinked with formaldehyde, and p53 was immunoprecipitated with monoclonal DO1 antibody, which does not discriminate between the active and the inactive conformations of p53, and the amount of co-precipitated p53-bound elements was determined by PCR. The results showed that, in basal condition and after drug treatment alone, p53 was not recruited onto canonical target promoters, such as *p21*^{Waf1} and N-myc Downstream-Regulated Gene 1 (*NDRG1*),[21,22] while it was recruited onto Multi-Drug Resistance 1 (*MDR1*) promoter, a known target of mtp53,[23] (**Fig. 2B**). Interestingly, zinc supplementation to the drug treatment strongly reverted the p53-DNA binding activity, restoring the wtp53 binding to canonical target promoters to the detriment of mtp53-activated promoter (**Fig. 2B**).

We next addressed the question of whether zinc could restore the transcriptional transactivation function to mtp53 in living cells, as measured by promoter-reporter activation. H1299 cells were co-transfected with p53H175 expression vector and with p53-inducible reporter genes. A significant induction of the p21-luciferase activity was seen only after the combination of zinc with drug treatment (**Fig. 2C**). Similar results were obtained with the synthetic PG13-luc reporter or the natural Noxa-luc promoter (not shown). Total p53 levels were unchanged as measured on protein immunoblot with mAbDO1 (**Fig. 2C,** lower part), corroborating the evidence seen in **Figure 2A** that zinc is indeed affecting p53 conformation.

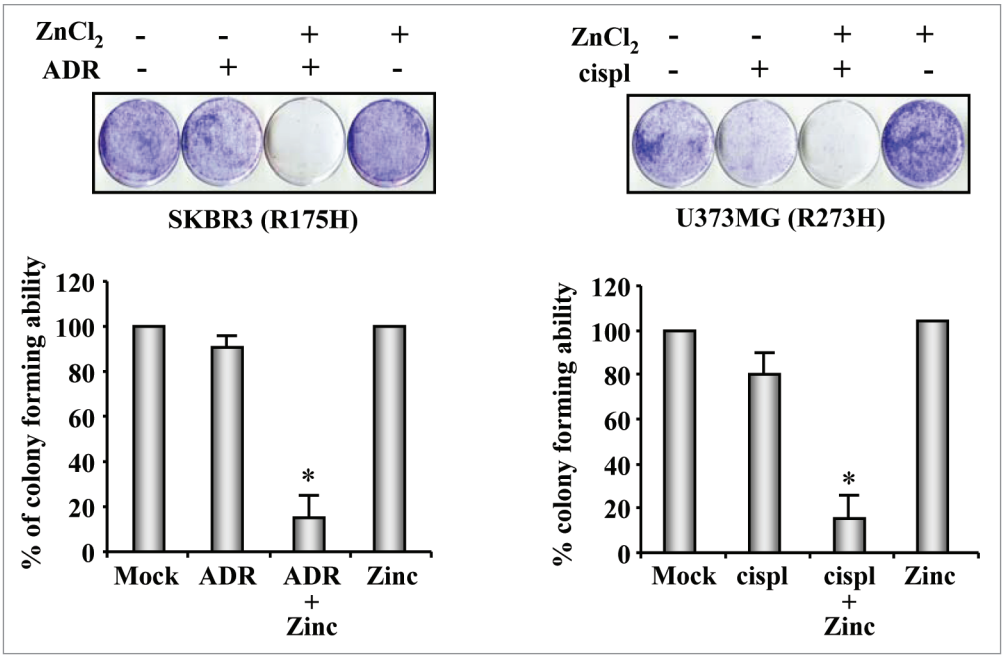

Figure 1. Cell survival after zinc and drug administration. SKBR3 (expressing R175H mutation) and U373MG (expressing R273H mutation) cells (4 x 10^4) were plated in 60 mm dish and 24 h later treated with $ZnCl_2$ (100 μM) for 16 h before adding ADR (1.5 μg/ml) or cisplatin (cispl) (3 μg/ml) for 2 h. Cells were then washed with PBS and changed with fresh medium. $ZnCl_2$ was added to culture medium every two days. Death-resistant colonies were stained with crystal violet 14 days later and counted for quantification ±SD (lower parts). *p = 0.001.

To determine if mutant p53 could be functionally restored in vivo, the transcription of endogenous p53 target genes was examined by measuring RNA expression. H1299 cells were transfected with wtp53 or p53H175 expression vectors and treated with cisplatin alone or in combination with zinc. The results showed that, while wtp53 overexpression induced the apoptotic target genes, p53H175 overexpression did not, neither alone or after cisplatin treatment (**Fig. 2D**). On the contrary, wtp53 pro-apoptotic target genes were induced in p53H175-transfected cells only after zinc supplementation to cisplatin treatment (**Fig. 2D**), suggesting that conformationally stabilized p53 can now activate relevant downstream targets in mtp53 cells to the same extent as wtp53-expressing cells. This finding was further corroborated by cDNA microarray analysis of H1299 cells stably transfected with p53H175 expression vector, showing that zinc supplementation to cisplatin treatment restored the wtp53 transcriptional transactivation compared to the cisplatin treatment alone (not shown). Finally, H1299 control cells and H1299 cells stable transfected with H175 vector were plated for long-term survival colony assay. The results showed that cisplatin treatment markedly reduced long-term survival of H1299 control cells compared to the H175-expressing cells (**Fig. 2E**). Interestingly, the drug resistance of H175-expressing cells was reverted by zinc supplementation to drug treatment that significantly reduced long-term cell survival (**Fig. 2E**), as evidenced by the quantification of the colony assay (**Fig. 2F**).

Reactivation of wtp53 conformation and activity in mtp53 expressing SKBR3 and U373MG cells. The physiological relevance of the above results was challenged in cancer cells with endogenous p53 mutations. We observed that zinc increased the PAb1620-reactive (folded) phenotype and reduced the PAb240-reactive (unfolded) phenotype in both SKBR3 and U373MG cell lines (**Fig. 3A**). Then, ChIP assay showed that, in basal condition or after drug treatment alone, p53 was not recruited onto canonical target promoters, while it was recruited onto *MDR1* promoter. As with H1299-H175 cells, zinc supplementation to drug-treated SKBR3 and U373MG cells strongly reverted the p53-DNA binding activity, restoring the wtp53 binding to canonical target promoters, to the detriment of mtp53-activated promoter (**Fig. 3B**). In agreement, significant induction of Bax-luciferase activity was seen only after combination of zinc with drugs in both SKBR3 and U373MG cells (**Fig. 3C**). Similar results were obtained with the synthetic p53-inducible PG13-luc reporter or the natural Noxa-luc promoters (not shown). Finally, in vivo transcription of endogenous p53 target genes was examined by RT-PCR. The results showed that zinc supplementation to drug treatment strongly induced the expression of endogenous p53 target genes, including *p21*, *Bax* and *Noxa*, while it reduced the expression of the mtp53 target *MDR1* gene (**Fig. 3D**), suggesting restoration of wtp53 activity in vivo.

Zinc impairs the p53/p73 protein complex in mtp53 expressing cells. As *MDR1* is a target of mtp53,[23] the above reported findings showing abolishment of p53 recruitment onto *MDR1* promoter and inhibition of *MDR1* expression following zinc and drug combination, raising the question whether zinc could also affect the mtp53 pro-oncogenic activity. One of the mtp53 oncogenic characteristics is the protein complexes formation between mtp53 and the family member p73, interfering with p73 ability to induce apoptosis.[15,16] Thus, the disruption of the protein complex mtp53/p73 by small peptides has been shown

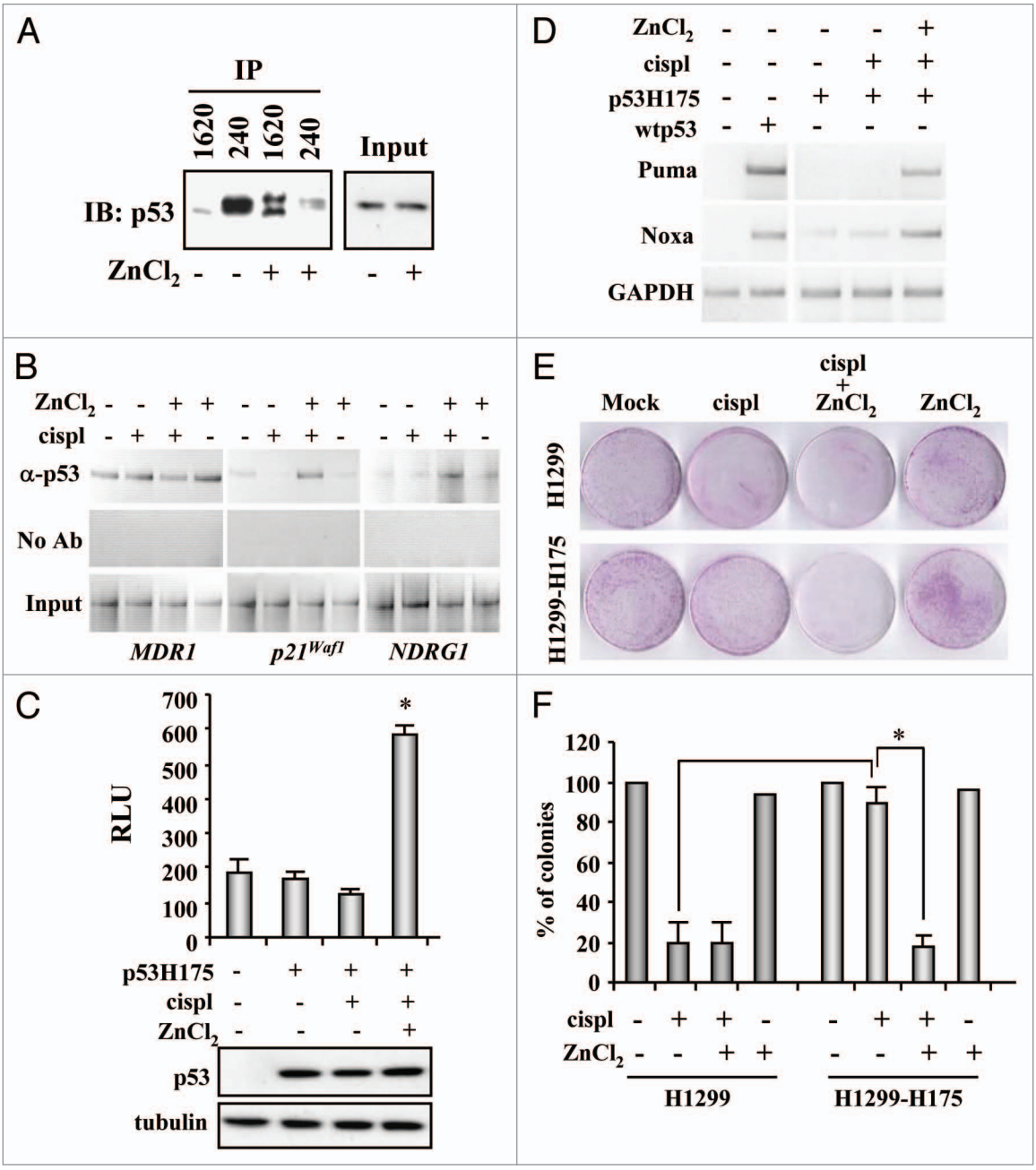

Figure 2. Zinc induces the transition of mutant p53 protein into a functional conformation. (A) H1299 cells were transiently transfected with p53H175 mutant expression vector and 24 h later treated with $ZnCl_2$ (100 μM) for 24 h. Total cell extracts were immunoprecipitated (IP) with conformation-specific antibodies, PAb1620 (for wild-type, folded conformation) and PAb240 (for unfolded conformation), and then immunoblotted (IB) with anti-p53 DO1 antibody. Inputs represent 1/10 of total cell extracts used for IP. (B) H1299 cells were transiently transfected with p53H175 mutant expression vector and 24 h later treated with $ZnCl_2$ (100 μM) and cisplatin (cispl, 3 μg/ml) for, respectively, 24 and 16 h before being assayed for chromatin immunoprecipitation (ChIP) analysis with anti-p53 antibody. PCR analyses were performed on the immunoprecipitated DNA samples using specific primers for wtp53 target promoters (*NDRG1* and *p21*^{Waf1}) or mtp53 target promoter (*MDR1*). A sample representing linear amplification of the total input chromatin (Input) was included as control. Additional controls included immunoprecipitation performed with non-specific immunoglobulins (No Ab). (C) H1299 cells were co-transfected with p21-luc promoter (2 μg) and p53H175 expression vector (0.1 μg). Twenty-four hours after transfection, cells were treated as in (B) before measuring luciferase activity. Results, normalized to β-galactosidase activity, are the mean ± SD of three independent experiments performed in duplicate. *p = 0.001. RLU, relative luciferase units. Below is shown the western immunoblotting of total cell extracts. Anti-tubulin is used as protein loading control. (D) H1299 cells were transiently transfected with wtp53 (0.1 μg) or p53H175 (0.1 μg) mutant expression vector and treated as in (B). Wild-type p53 target genes were determined by RT-PCR. GAPDH was used as loading control. (E) Colony-forming ability of H1299 control cells or stable transfected with p53H175 mutant expression vector. 4 x 10^4 cells were plated and 24 h later pre-treated with $ZnCl_2$ (100 μM for 16 h) and then a pulse (2 h) of cisplatin (cispl, 2.5 μg/ml). Cells were then washed and fresh culture medium added. Death-resistant colonies were stained with crystal violet 14 days later and (F) counted for quantification ±SD (lower parts). *p = 0.001.

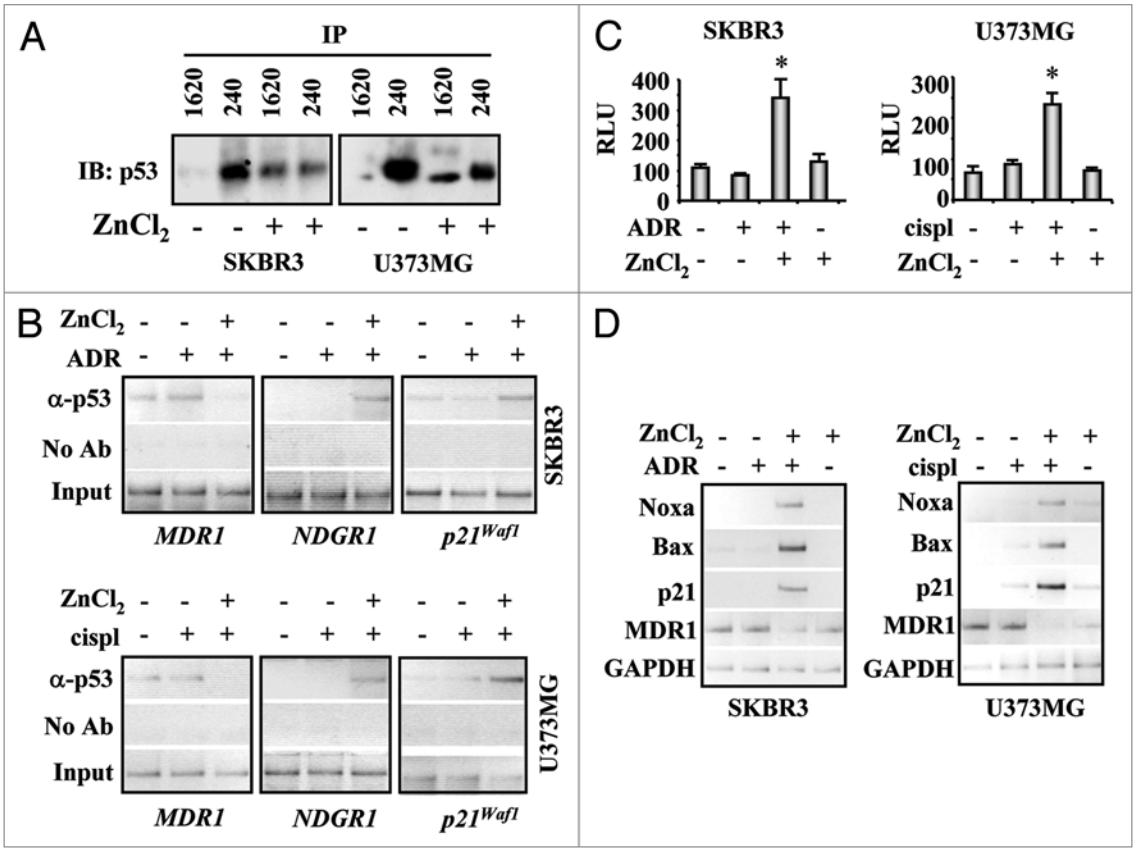

Figure 3. Reactivation of wtp53 conformation in mtp53 cells is required for p53 apoptotic transcriptional activity. (A) SKBR3 and U373MG cells were left untreated or treated with $ZnCl_2$ (100 μM) for 24 h. Total cell extracts were immunoprecipitated (IP) with conformation-specific antibodies PAb1620 and PAb240 and then immunoblotted (IB) with anti-p53 DO1 antibody. (B) SKBR3 and U373MG cells were treated with ADR (1.5 μg/ml for 16 h) and cisplatin (cispl, 2.5 μg/ml for 16 h) and $ZnCl_2$ (100 μM for 24 h) before being assayed for chromatin immunoprecipitation (ChIP) analysis with anti-p53 antibody. PCR analyses were performed on the immunoprecipitated DNA samples as in **Figure 2B**. A sample representing linear amplification of the total input chromatin (Input) was included as control. Additional controls included immunoprecipitation performed with non-specific immunoglobulins (No Ab). (C) SKBR3 and U373MG cells were transfected with the Bax-luc reporter gene and 16 h after transfection treated with drugs and $ZnCl_2$ as in (B) before measuring luciferase activity. Results, normalized to β-galactosidase activity, are the mean ± SD of three independent experiments performed in duplicate. *p = 0.001. RLU, relative luciferase units. (D) SKBR3 and U373MG cells, treated as in (B), were assayed for RT-PCR analysis of p53 target genes. GAPDH was used as loading control.

to selectively increase the response of tumor cells to anticancer drugs.[17] Co-immunoprecipitation assays revealed that the amount of mtp53 bound to p73 was diminished, although to a different extent, upon zinc treatment in both SKBR3 and U373MG cell lines (**Fig. 4A**), which correlated with increase of conformationally stabilized p53 (**Fig. 3A**). The analysis of the in vivo occupancy of the regulatory regions of *p21*, *MDR1* and *NDRG1* promoters revealed that the p73 recruitment mirrored that of reversible mutant/wild-type p53. Thus, p73 was recruited onto *MDR1* promoter in basal condition and after drug treatment alone, whereas it was not recruited onto the target promoters *NDRG1* and $p21^{Waf1}$ (**Fig. 4B, compare with 3B**). Zinc supplementation to drug treatment completely reverted the p73-DNA binding activity as a result of dissociating the mtp53/p73 complex; moreover, the increased amount of bound p73 to DNA paired with a more pronounced H4 histone acetylation (**Fig. 4B**), thus providing evidence for active p73 transcriptional activity. These findings indicate that zinc, by reactivating p53, was able to disrupt the protein complex mtp53/p73, re-gaining both wtp53 function and likely also p73-mediated antitumor effects, as shown by the inhibition of cell growth following zinc and drug administration (**Fig. 1**). This finding was also evidenced in the HIPK2-knockdown model, where p53 undergoes protein misfolding with loss of DNA binding and transcriptional activities, and the tumors acquire chemoresistance that can be reverted by zinc supplementation.[19] We found that the mtp53/p73 interacting complexes, clearly observed in HIPK2 depleted cells (HIPK2i) in basal condition, were completely abolished by zinc supplementation (**Sup. Fig. 1a**). Therefore both p53 and p73 recruitment onto target promoters was evaluated by ChIP assay. The results showed that p53 was recruited onto *MDR1* promoter after HIPK2 depletion (siHIPK2) compared to the siRNA control cells (si-C), and that this binding, also present after drug treatment, was abolished by zinc supplementation to ADR (**Sup. Fig. 1b**), indicative of wtp53 restoration, as previously reported in reference 19. In a similar manner, p73 was recruited onto apoptotic *Puma* promoter in si-RNA control cells after ADR treatment (**Sup. Fig. 1b**); this recruitment was abolished

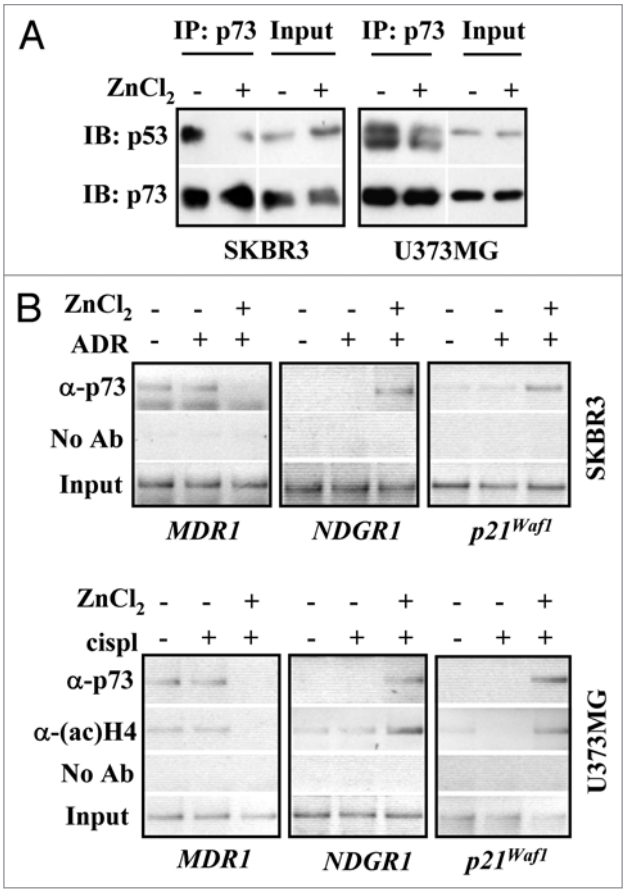

Figure 4. Zinc impairs the mtp53/p73 protein complex. (A) Total cell lysated (1 mg) derived from SKBR3 and U373MG cells treated with $ZnCl_2$ (100 μM) for 24 h were immunoprecipitated with anti-p73 antibody. The immunoprecipitates and an aliquot (50 μg) of total cell extracts were loaded onto 12% acrylamide gel. The resulting blots were probed with the indicated antibodies. (B) SKBR3 and U373MG cells were treated with ADR (1.5 μg/ml for 16 h) and cisplatin (cispl, 2.5 μg/ml for 16 h), respectively, and $ZnCl_2$ (100 μM for 24 h) before assayed for chromatin immunoprecipitation (ChIP) analysis with anti-p73 or anti-ac-H4 antibodies. PCR analyses were performed on the immunoprecipitated DNA samples using specific primers as in **Figure 3B** and the occupancy of p73 and H4 histones acetylation of the amplified promoter regions are shown. A sample representing linear amplification of the total input chromatin (Input) was included as control. Additional controls included immunoprecipitation performed with non-specific immunoglobulins (No Ab).

following HIPK2 depletion even in the presence of ADR, but it was re-established by zinc in combination with ADR (**Sup. Fig. 1b**), in agreement with our finding of zinc reactivation of mutant p53 protein and likely inhibition of mtp53 oncogenic function, for antitumor response.

Zinc supplementation in combination with chemotherapy improves drug-induced apoptosis. Next, we evaluated the biological outcome of mutant p53 activation. We transfected H1299 cells with low amount of wtp53 (to avoid massive apoptosis before cisplatin treatment) or mtp53H175 expression vectors and treated with cisplatin and zinc to monitor cell death. The results show that, as expected, wtp53 overexpression induced cell death that was only slightly increased by cisplatin (**Fig. 5A**); on the contrary, the overexpression of the H175 mutant did not induce cell death, notwithstanding cisplatin treatment, unless in combination with zinc (**Fig. 5A**). Similar results were obtained with both SKBR3 and U373MG cell lines that underwent significant cell death only after zinc supplementation to drug treatment (**Fig. 5B**), suggesting that p53 reactivation is involved in the zinc-induced cell death. This hypothesis was confirmed at the protein level by western immunoblotting, where zinc supplementation to drug treatment induced Bax expression (**Fig. 5B,** lower part).

We also attempted to compare the sensitivity to drugs obtained after zinc supplementation with that obtained after mtp53 depletion by RNA interference, as it has been shown that mtp53 depletion restores chemosensitivity.[14] To this aim, SKBR3 and U373MG cells were transiently transfected with pSuper-p53 vector, to deplete endogenous mtp53 protein (si-p53), or with pSuper control vector (si-RNA) and subsequently treated as above (see **Fig. 5B**) for western immunoblotting of total cell extracts. The results show that drug sensitivity in mtp53 cells, evaluated as cleavage of the apoptotic marker poly(ADP-ribose)polymerase (PARP), was obtained either after zinc supplementation to drugs (**Fig. 5C,** compare lanes 3 with lanes 2) or after depletion of mtp53 (si-p53) (**Fig. 5C,** compare lanes lanes 5 with lanes 2); in the latter, zinc supplementation did not further enhance the drug-induced PARP cleavage (**Fig. 5C,** compare lanes 6 with lanes 5). These findings suggest that reactivation of wtp53 or depletion of mtp53 proteins might both end in reduction of cell resistance to anticancer drugs, although the molecular mechanisms underlying this effect may be different in both conditions.

Zinc administration in U373MG xenografts improves drug-induced tumor regression. To finally test whether zinc increased tumor response to drugs in vivo, we generated tumor xenografts in athymic nude mice using U373MG cells. We observed a significant decrease in the volume of U373MG xenografts after 14 days of zinc supplementation to drug treatment, compared to drug treatment alone (**Fig. 6A**), while zinc administration did not show any toxic effect by itself (not shown). After 14 days of zinc administration, the average tumor volume was 823 mm^3 in control mice compared to 625 mm^3 in cisplatin$^-$ and 436 mm^3 in cisplatin + zinc-treated mice. The differences in tumor volumes were statistically significant in cisplatin + zinc vs. control (-47%, p = 0.0339), and cisplatin + zinc vs. cisplatin (-30%, p = 0.0339) while cisplatin alone showed only 24% tumor growth reduction (p = 0.0946) (**Fig. 6A**). The growth of tumors in a comparable way resumed upon cessation of zinc treatment, confirming the adjuvant activity of zinc in improving cisplatin treatment. Tumors were harvested and p53 conformation analyzed by immunoprecipitation and western blotting. The results showed that zinc supplementation increased the PAb1620-reactive phenotype (**Fig. 6B**), suggesting the transition of the p53 protein into a functional conformation also in vivo. This hypothesis was confirmed by RT-PCR analysis that showed that the p53 target genes were induced in U373MG-derived tumors only after zinc supplementation to cisplatin (**Fig. 6C**). Altogether, these data indicate that zinc increased tumor response to drug in vivo by reactivating p53 oncosuppressor activity.

Discussion

In this study, we provide evidence that zinc modified the equilibrium between p53 mutant and wild-type conformation, positively reactivating some of the most frequently p53 mutated residues, such as Arg175 and Arg273. The partial shift toward wild-type p53 conformation led to reactivation of wtp53 functions such as DNA binding and transactivation of target genes in response to DNA damage and in inhibition of some "pro-oncogenic" functions, such as interaction with family member p73 and inhibition of mutant p53 target *MDR1*. This resulted in restoring drug sensitivity and inhibition of tumor growth.

The p53 tumor suppressor pathway blocks tumor progression by triggering apoptosis or cellular senescence in response to genotoxic stress, and the restoration of wtp53 activity is extremely helpful for eradicating established tumors, as shown by several genetic studies in vivo.[24-26] Thus, the reactivation of the wtp53 pathway has the potential to reduce tumor growth either in combination with other therapies or alone. The p53 tumor suppressor activity may be inhibited by mutation of the *p53* gene that occurs in a significant fraction of human tumors, although at variable frequency in different tumor types. Most tumor-associated p53 mutations (about 95%) cluster in the DBD, hampering the ability of p53 to recognize the sequence-specific response elements (SRE) within target promoters[4] and activate target genes in response to DNA damage, therefore inhibiting its tumor suppressor activity. Given that numerous studies demonstrated the association between mutant p53 expression and poor response to conventional chemo- and radiotherapies,[13] various strategies have been designed to restore function to mtp53 and to re-establish specific DNA binding and/or stabilize the folding of the protein. The observation that many p53 mutants share a common property, that is, reduced thermostability and misfolding or partial unfolding, raises the possibility of designing drugs that stabilize the wild-type conformation and thus restore wild-type function.[27-30] Here, we took advantage of our previous findings showing that p53 acquires a misfolded, "mutant-like" conformation in HIPK2-depleted cell, due to deregulation of metallothionein and zinc that can be reverted by zinc supplementation,[19,20] to evaluate the effect of zinc on conformation and activity of mutant p53 proteins. We found that also in mutant p53, zinc modified the equilibrium between p53 mutant and wild-type conformation toward wild-type conformation, restoring sequence-specific DNA binding of both His175 and His273 mutant p53, enhancing the recruitment of p53 to target promoters and inducing expression of p53 target genes in response to genotoxic stress. In our hands, we obtained p53 reactivation only after zinc supplementation to drugs, raising the question whether zinc is directly acting on mutant p53 or likely modifying additional indirect pathways useful for restoration of drug sensitivity. In previous studies, we found that zinc supplementation to drug restored

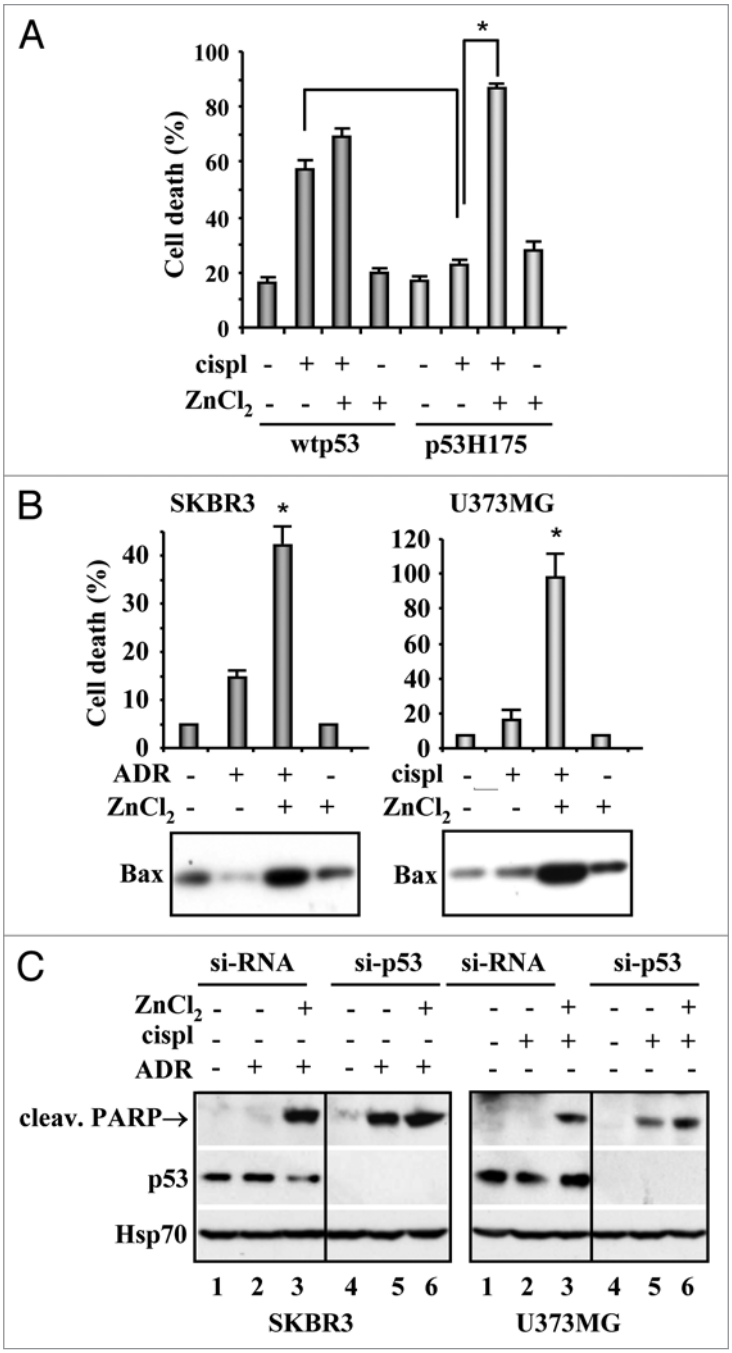

Figure 5. Reactivation of wtp53 conformation in mtp53 cells. (A) H1299 cells were transiently transfected with wtp53 (0.1 μg) or p53H175 (0.1 μg) mutant expression vector and treated $ZnCl_2$ (100 μM) and cispl (3 μg/ml) for, respectively, 36 and 24 h. Cell death measurements were assayed by Tunel assay. The results are the mean of three independent experiments performed in triplicate ± SD. *p = 0.001. (B) SKBR3 and U373MG cells were treated with ADR (1.5 μg/ml for 24 h) and cisplatin (cispl, 2.5 μg/ml for 24 h), respectively, and $ZnCl_2$ (100 μM for 36 h) and cell death measured as in (A) ± SD. *p = 0.001. Below is shown the western immunoblotting of total cell extracts for Bax expression. (C) SKBR3 and U373MG cells were transiently transfected with p-Super control (si-C) or pSuper-p53 (si-p53) vector for p53 depletion. Twenty-four hours after transfection cells were treated with ADR (1.5 μg/ml for 16 h) and cisplatin (cispl, 2.5 μg/ml for 16 h), respectively and $ZnCl_2$ (100 μM for 24 h) before assayed for western immunoblotting of total cell extracts for the indicated antibodies. Anti-Hsp70 is used as protein loading control.

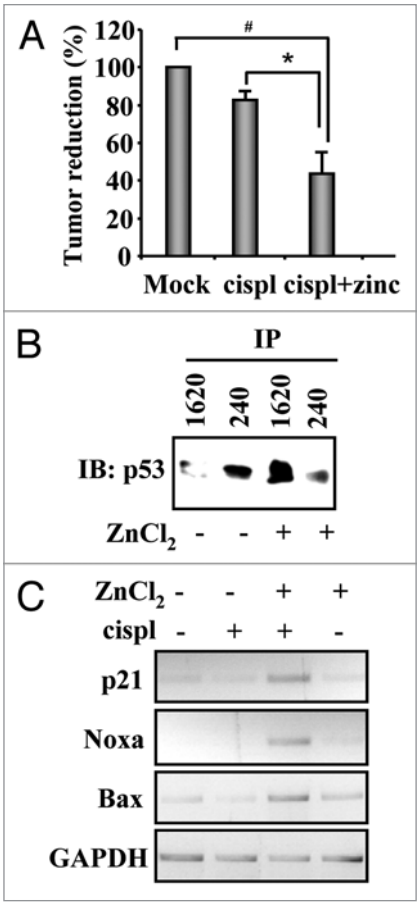

Figure 6. Zinc administration increases tumor cells chemosensitivity, cell death and tumor regression. (A) Growth inhibition of U373MG-derived tumors by combination treatment with cisplatin and zinc. Established xenografts were harvested after 14 days of zinc administration to cisplatin treatment. The differences in tumor volumes were -47% in cisplatin + zinc vs. control ($^{\#}p = 0.0339$) and -30% cisplatin + zinc vs. cisplatin ($^{*}p = 0.0339$), while cisplatin alone showed only 24% tumor growth reduction ($p = 0.0946$). Standard deviation is shown. (B) Untreated and zinc-treated (for 7 days) tumors were harvested, lysated and total cell extracts were immunoprecipitated (IP) with conformation-specific antibodies PAb1620 (for wild-type, folded conformation) and PAb240 (for unfolded conformation) and then immunoblotted (IB) with anti-p53 DO1 antibody. (C) RNA samples from explanted tumors at day 14 after cell injection, treated with cisplatin and zinc, alone or in combination, were used for RT-PCR analyses of wtp53 target genes. The mRNA levels were normalized to GAPDH expression.

misfolded p53 function in the HIPK2-knockdown background, although, zinc supplementation itself to HIPK2-depleted cells induces p53 binding to *p21* promoter and p21 transcription.[19] Similarly to zinc supplementation, metallothionein (MT2A) depletion strongly increases the PAb1620-reactive (folded) phenotype and reduces the PAb240-reactive (unfolded) phenotype, allowing wtp53 to be activated by genotoxic damage,[20] suggesting that zinc might modify the equilibrium between p53 mutant and wild-type conformation by acting on additional molecules involved in maintaining intracellular zinc buffering for wtp53 conformation. Moreover, zinc could also affect the redox state of p53,[31] as a reducing environment promotes correct p53 folding and DNA binding, impairing aggregation. Another hypothesis is that zinc might affect binding to chaperone proteins such as Hsp40, Hsp70 and Hsp90, as binding to Hsp proteins may stabilize p53 in an unfolded conformation, and therefore, disruption of Hsp binding to mutant p53 should rescue p53 conformation.[32] Yet the question remains as to how zinc affects mutant p53 and why we need genotoxic stress to fully activate p53 apoptotic function. One possible explanation may be that, only after the zinc-induced modification of the equilibrium between p53 mutant and wild-type conformation, p53 might be prone to drug-induced activation. A final answer to this question should come from structural studies by X-ray crystallography and/or NMR.

The mutant p53 reactivation by zinc restored chemosensitivity in mutant p53 cells by enhancing the recruitment of p53 to target promoters and inducing expression of p53 target genes in response to genotoxic stress. However, we also found that zinc strongly reduced the mtp53/p73 interaction, which is thought to inhibit p73-dependent apoptosis[33] and is considered one of the p53 "pro-oncogenic" function; reduction of mtp53/p73 interaction led to recruitment of p73 onto target gene promoters, suggesting that additional mechanisms other than wtp53 reactivation might contribute to restoration of chemosensitivity in mutant p53 cells treated with zinc. The disruption of the protein complex mutantp53/p73 increases selectively the response of tumor cells to anticancer drugs,[17] thus, our findings suggest that zinc might act on mtp53 pro-oncogenic activities, such as mtp53/p73 interaction, unleashing a p73 response that, along with downregulation of MDR expression, could contribute to drug-induced tumor cell death. The inhibition of mtp53 pro-oncogenic activities by zinc is in agreement with recent findings showing that depletion of mutant p53 reduces chemosensitivity and tumorigenicity of cancer cells by suppressing mtp53 gain of function.[14,34] Therefore, both p53 reactivation by zinc and mtp53 depletion by siRNA, might end in reduction of cell resistance to anticancer drugs, although the molecular mechanisms underlying this effect may be different in both conditions and needing further evaluation.

Based on the data presented here, together with our previous studies on p53 misfolding and wild-type p53 reactivation by zinc,[18-20] we emphasize the potential therapeutic efficacy of zinc in p53 restoration in human cancer treatment. Zinc has a crucial role in the biology of p53 in that p53 binds to DNA through structurally complex domain stabilized by zinc atom.[12] In addition, zinc can modulate the function of molecules involved in tumor progression and p53 function, such as HIF-1, HIPK2 and MDM2 in vitro and in vivo,[35-37] without toxic effects, making it an attractive adjuvant molecule whose combination warrants further investigation for effective anticancer therapies. Combination therapy is a key strategy against the development of drug resistance. As in the clinic the functional status of p53 has been associated with the prognosis, progression and the therapeutic response of tumors, our findings support a logical step forward to use supplemental zinc to modify of the equilibrium between p53 mutant and wild-type conformation and restore p53- and likely p73-dependent apoptotic response to drugs.

Materials and Methods

Cell culture and treatments. The human lung cancer H1299 (p53-null), the p53H175 stable transfected H1299 (H1299-H175, kindly provided by M. Oren, The Weizmann Institute of Science, Rehovot, Israel), the human colon cancer RKO control and HIPK2 depleted (HIPK2i)[38] and the glioblastoma cell line U373MG (expressing R273H p53 mutation) were maintained in RPMI-1640 (Life Technology-Invitrogen), while human breast cancer SKBR3 (carrying R175H p53 mutation) was maintained in DMEM (Life Technology-Invitrogen), all supplemented with 10% heat-inactivated fetal bovine serum plus glutamine and antibiotics.

Subconfluent cells were treated with adriamycin (ADR) diluted into the medium to a final concentration of 1.5 μg/ml, cisplatin (cispl) diluted into the medium to a final concentration of 2.5 μg/ml and zinc chloride ($ZnCl_2$) diluted into the medium to a final concentration of 100 μM, for the indicated period of time.

Viability, colony and tunel assays. Equal cell numbers were plated in triplicate in 60 mm Petri dishes and 24 h later treated with adriamycin (ADR, 1.5 μg/ml) or cisplatin (cispl, 2.5 μg/ml) for 24 h and zinc chloride ($ZnCl_2$, 100 μM) for 36 h. Both floating and adherent cells were collected, and cell viability was determined by Trypan blue exclusion by direct counting with a hemocytometer, as reported in reference 38.

For long-term cell survival, cells were pre-treated with $ZnCl_2$, (100 μM) for 16 h before adding 2 h pulse of chemotherapeutics. After treatments, cells were washed, trypsinized, counted and equal cell number re-plated in duplicate with fresh medium, in 60 mm Petri dishes. Death-resistant colonies were stained with crystal violet 14 days later.

For TUNEL assay, 4×10^4 cells were spun on a slide by cyto-centrifugation and subsequently fixed in 4% paraformaldheyde for 30 min at room temperature. After rinsing with PBS, the samples were permeabilized in a solution of 0.01% Triton X-100 in sodium citrate for 2 min. Samples, washed with PBS, were then incubated in the TUNEL reaction mix for 1 h at 37°C according to the manufacture's instructions (Roche, Germany). Cells were counter-stained with Hoechst 33342 before analysis with a fluorescent microscope (Zeiss).

Western blotting, p53 conformational immunoprecipitation and mtp53/p73 co-immunoprecipitation. Total cell extracts were prepared by incubation in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 150 mM KCl, 1 mM dithiothreitol, 1% Nonidet P-40) and a mix of protease inhibitors (SIGMA) and resolved by SDS-polyacrilamide gel electrophoresis. Proteins were transferred to a polyvinylidene difluoride membrane (PVDF, Millipore) and incubated with the primary antibodies, followed by an anti-immunoglobulin-G-horseradish peroxidase antibody (BioRad). Immunoblotting was performed with the following antibodies: monoclonal anti-poly(ADP-ribose) polymerase (PARP, BD Pharmingen, CA), monoclonal anti-p53 (Ab-DO1) and polyclonal anti-Bax (both from Santa Cruz Biotechnology), anti-tubulin (Immunological Sciences) and monoclonal anti-Hsp70 (Stressgene, BC Canada).

For p53 immunoprecipitation cells or tumors were lysed in immunoprecipitation buffer (10 mM Tris, pH 7.6; 140 mM NaCl; 0.5% NP40 and protease inhibitors) for 20 min on ice and cleared by centrifugation. Pre-cleared supernatants (200 mg) were immunoprecipitated overnight at 4°C with the conformation-specific monoclonal antibodies Pab1620 (wild-type specific) and PAb240 (mutant specific) (Calbiochem) pre-adsorbed to protein G-agarose (Pierce). Immunocomplexes were collected by centrifugation, separated by 9% SDS-PAGE and blotted onto PVDF membrane (Millipore). Immunoblotting was performed with rabbit polyclonal anti-p53 (FL393) or mouse monoclonal Ab-DO1 antibodies (both form Santa Cruz Biotechnology).

For mtp53/p73 interaction, cells were lysed in immunoprecipitation buffer, for 20 min on ice and cleared by centrifugation. Pre-cleared supernatants (1 mg) were immunoprecipitated for 2 h at 4°C with affinity purified rabbit anti-p73 antibody A300-126A (lot A300-126A-2, Bethyl Laboratories, Inc.,) pre-adsorbed to protein G-agarose (Pierce). Immunocomplexes were collected by centrifugation, separated by 9% SDS-PAGE and blotted onto PVDF membrane (Millipore). Immunoblotting was performed with rabbit polyclonal anti-p73 (H79, Santa Cruz Biotechnology). Immunoreactivity was detected with the Advanced-ECL chemoluminescence reaction kit (Amersham., IL).

RNA extraction and reverse transcription (RT)-PCR analysis. Cells or tumors were harvested in TRIzol Reagent (Invitrogen), and total RNA was isolated following the manufacturer's instructions essentially as described in reference 19. PCR was performed by using genes specific oligonucleotides under conditions of linear amplification. PCR products were run on a 2% agarose gel and visualized by ethidium bromide staining using UV light. The housekeeping GAPDH mRNA was used as internal control.

Chromatin immunoprecipitation (ChIP) assay. Chromatin Immunoprecipitation (ChIP) analysis was carried out essentially as described in reference 19. Protein complexes were cross-linked to DNA in living cells by adding formaldehyde directly to the cell culture medium at 1% final concentration. Chromatin extracts containing DNA fragments with an average size of 500 bp were incubated overnight at 4°C with milk shaking using monoclonal anti-p53 antibody (DO1, Santa Cruz Biotechnology) and affinity purified rabbit anti-p73 antibody A300-126A (lot A300-126A-2, Bethyl Laboratories, Inc.). Before use, protein G (Pierce) was blocked with 1 μg/μL sheared herring sperm DNA and 1 μg/μL BSA for 3 h at 4°C and then incubated with chromatin and antibodies for 2 h at 4°C. PCR was performed with HOT-MASTER Taq (Eppendorf) using 2 μL of immuniprecipitated DNA and promoter-specific primers for human *MDR1*, *p21*^{Waf1}, *Puma* and *NDRG1* promoters. Immunoprecipitation with non-specific immunoglobulins (IgG; Santa Cruz Biotechnology) was performed as negative control. The amount of precipitated chromatin measured in each PCR was normalized with the amount of chromatin present in the input of each immunoprecipitation. PCR products were run on a 2% agarose gel and visualized by ethidium bromide staining using UV light.

Transfection, plasmids and transactivation assay. Cells (SKBR3, U373MG) were transiently transfected with the

cationic polymer LipofectaminePlus method (Invitrogen) according to manufacturers' instructions or (H1299 and the stable H1299-H175) with the the N,N-bis-(2-hydroxyethyl)-2-amino-ethanesulphonic acid-buffered saline (BBS) version of the calcium phosphate procedure.[39] For transient transfection, the p53H175 expression vector (kindly provided by G. Piaggio, National Cancer Institute "Regina Elena", Rome, Italy) was also used. The plasmid reporters used were the luciferase reporter gene driven by the p53-dependent synthetic PG13-luc reporter (kindly provided by M. Oren, Weizmann Institute, Rehovot, Israel), the p53-dependent natural promoters Noxa-luc (kindly provided by T. Taniguchi, University of Tokyo, Japan), Bax-luc and $p21^{Waf1}$-luc. The amount of plasmid DNA in each sample was equalized by supplementing with empty vector. Transfection efficiency was normalized with the use of a co-transfected β-galactosidase (β-gal) plasmid. Luciferase activity was assayed on whole cell extract, and the luciferase values were normalized to β-galactosidase activity and protein content and expressed as relative luciferase unit (RLU).

siRNA interference. Cells were plated at semiconfluence in 35 mm dishes the day before transfection. Control pSuper and pSuper-p53 (kindly provided by S. Soddu, National Cancer Institute "Regina Elena", Rome, Italy),[40] vectors were transfected overnight using LipofectaminePlus reagent (Invitrogen) and 24 h later cells were trypsinized and replated for the indicated experiments.

Tumorigenicity in nude mice. Six-week-old CD-1 athymic nude (nu/nu) mice (Charles River Laboratories) were used for in vivo studies. 5×10^6 viable U373MG cells were inoculated into the flanks of nude mice and allowed to develop into 300 mm^3 tumor nodules (in about 7 days) at the injection site. Mice were then randomized in four groups (5–6 mice/group) as follows: (1) $ZnCl_2$ (10 mg zinc/kg body weight) alone; (2) cisplatin alone (3.3 mg/kg body weight) injected i.p. at day one and two more times at days 2 and 3; (3) cisplatin + zinc combination pre-treatment with $ZnCl_2$ and after 6 h cisplatin treatment as in group 2 and (4) PBS control. $ZnCl_2$ was administrated once daily by oral administration, over the course of two weeks. Tumor dimensions were measured every other day, and their volumes were calculated from caliper measurements of two orthogonal diameters (x and y, larger and smaller diameters, respectively) by using the formula volume = $xy^2/2$, as previously described in reference 19. The antitumor effect of the combination treatment, zinc + cispl, was evaluated by comparing the relative tumor size with tumors treated with cisplatin only or zinc only after 14 days of zinc administration to cisplatin treatment. All mouse procedures were carried out in accordance with tnstitutional standard guidelines.

Statistics. All experiment, unless indicated, were performed at least three times. All experimental results were expressed as the arithmetic mean and standard deviation (SD) of measurements was shown. Student's t-test was used for statistical significance of the differences between treatment groups. Statistical analysis was performed using analysis of variance at 5% ($p < 0.05$) or 1% ($p < 0.01$).

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Note

Supplemental materials can be found at:
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