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Zinc supplementation augments *in vivo* antitumor effect of chemotherapy by restoring p53 function

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Activated p53 is necessary for tumor suppression. Homeodomain-interacting protein kinase-2 (HIPK2) is a positive regulator of functional p53. HIPK2 modulates wild-type p53 activity toward proapoptotic transcription and tumor suppression by the phosphorylation of serine 46. Knock-down of HIPK2 interferes with tumor suppression and sensitivity to chemotherapy. Combined administration of adriamycin and zinc restores activity of misfolded p53 and enables the induction of its proapoptotic and tumor suppressor functions *in vitro* and *in vivo*. We therefore looked for a cancer model where HIPK2 expression is low. MMTV-*neu* transgenic mice overexpressing HER2/*neu*, develop mammary tumors at puberty with a long latency, showing very low expression of HIPK2. Here we show that whereas these tumors are resistant to adriamycin treatment, a combination of adriamycin and zinc suppresses tumor growth *in vivo* in these mice, an effect evidenced by the histological features of the mammary tumors. The combined treatment of adriamycin and zinc also restores wild-type p53 conformation and induces proapoptotic transcription activity. These findings may open up new possibilities for the treatment of human cancers *via* the combination of zinc with chemotherapeutic agents, for a selected group of patients expressing low levels of HIPK2, with an intact p53. In addition, HIPK2 may serve as a new biomarker for tumor aggressiveness.

TP53 (also called p53) is a key tumor-suppressor gene that is mutated or lost in ~50% of all human cancer cases.[1] Moreover, downstream targets or upstream regulators of p53, such as p14ARF and MDM2, are altered in many tumors with an intact p53. p53 is activated in response to a variety of cellular and genotoxic stress conditions, leading to induction of apoptosis, senescence, growth arrest, DNA repair, antioxidant defense and metabolic homeostasis.[2] Induction of p53 in response to stress occurs essentially by posttranslational modifications resulting in protein stabilization and conformational changes, that increase the affinity of p53 protein for its specific DNA-binding site.[3] p53 may also exert its various functions by interacting with heterologous factors to regulate transcription.[4] p53 may lose its transcriptional activity due to low affinity for its DNA-binding site, which may stem from either mutations in the gene in the majority of cases[5] or from conformational changes of the protein.[6] An emerging field of research is the quest for drugs exploiting the vast knowledge accumulated regarding the p53 pathway, among which is the approach to develop drugs reactivating wild-type p53.[7]

Among the molecules involved in p53 posttranslational regulation is homeodomain- interacting protein kinase-2,[8] a serine/threonine kinase that specifically phosphorylates p53 at serine 46 (Ser46) in response to severe DNA damage, regulating p53-induced apoptosis by enhancing the p53-mediated transcriptional activation of apoptotic target genes.[9,10] Thus, HIPK2 depletion by siRNA impairs p53 oncosuppressor function, induces chemoresistance, and increases *in vivo* tumor growth.[11] HIPK2 plays an important role in maintaining wild-type p53 native conformation, as assessed by immunoprecipitation studies with conformation-specific antibodies.[12] HIPK2 depletion by siRNA induces p53 misfolding,[12] also due to upregulation of metallothioneins (MT), a group of proteins with high zinc binding capacity, as

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the removal of zinc causes p53 to adopt a mutant conformation.[13–15] p53 protein misfolding in HIPK2 knockdown background can be reverted by either zinc supplementation or MT2A depletion, which restores wild-type p53 conformation, as well as DNA-binding and transcriptional activity in response to drugs.[12,16] Therefore, an intact HIPK2 function is important for p53 activity and tumor response to drugs.[17,18]

Looking for an animal cancer model to further establish the ability to restore p53 functionality by zinc supplementation in the presence of low HIPK2 levels and wild-type p53, we searched the GEO database and found that the transgenic HER2/*neu* mouse model, that develops mammary tumors spontaneously,[19] expresses low HIPK2 levels in the tumors. Therefore, it is reasonable to hypothesize that these tumors would present misfolded p53 protein with abolishment of its tumor suppressor function, leading to chemoresistance. This study investigated the antitumor effect of zinc supplementation to chemotherapy in the HER2/*neu* mouse model, aimed to reestablish wild-type p53 function and overcome drug-resistance. The positive results of this study, hereby shown, suggest a new therapeutic approach for cancer patients with low HIPK2 levels and intact p53 in the tumor, by combining zinc and chemotherapeutic agents.

Material and Methods

Mouse model

Transgenic mice (FVB/N-Tg(MMTV-*neu*)202Mul) containing the rat proto-oncogene c-*neu* transgene targeted to mammary epithelium by the MMTV-LTR promoter[20] were purchased from Jackson Laboratories (Bar Harbor, ME). In the experiment comparing the effect of adriamycin *vs.* that of the combination of adriamycin and $ZnCl_2$ (Fig. 2), spontaneous breast tumors were allowed to grow for 5 days after they were first palpated, and palpations were performed on a weekly basis. Twenty-three mice were then randomized to three groups and treated with adriamycin (10 mg/kg body weight), combination of adriamycin and $ZnCl_2$ (10 mg zinc/kg body weight), or PBS. Adriamycin was injected i.p. once at Day 1. In the experiment assessing the effect of $ZnCl_2$ alone treatment (Fig. 2*a*, insert), spontaneous breast tumors were allowed to grow for 1–3 weeks after they were first palpated. Mice were then randomized to two groups (three mice per group) and treated with either $ZnCl_2$ (10 mg zinc/kg body weight), or PBS. For both experiments, $ZnCl_2$ was administered once daily by oral administration (gavage), over the course of the 12 days of the experiment. Tumor dimensions were measured every other day and their volumes were calculated from caliper measurements of two orthogonal diameters (x and y, larger (x) and smaller (y) diameters, respectively) by using the formula: volume $= xy^2/2$, as previously described.[21] The effect of each treatment is displayed by comparing tumor size throughout the 12 days' experiment compared to its size at Day 1. All mouse procedures were carried out in accordance with institutional standard guidelines.

Pathology

Mammary tumors were paraffin-embedded, cut into 4-μm sections, and then stained with hematoxylin and eosin, Masson's trichrome staining or caspase 3, for light microscopy. Masson's Trichrome staining was performed with a one-step green/red kit according to the manufacturer's protocol (American Master Tech, #KTTRGPT). Caspase 3 staining was performed using the cleaved caspase-3 (Asp175) rabbit mAb (Cell Signaling Technology, cat no. #9664S).

Immunoprecipitation of p53 protein

For p53 conformational studies, 30 mg of tumor tissue, each taken from a single mouse, were homogenized on ice in lysis buffer (50 mM Tris–HCl pH 7.5; 50 mM NaCl; 5 mM EDTA, 150 mM KCl, 1 mM fresh DTT, 1% NP40; plus protease and phosphatase inhibitors). Protein was extracted using the RNA/Protein Purification Kit (NORGEN, Canada). Precleared supernatants (250 μg) were immunoprecipitated overnight at 4°C with the conformation-specific monoclonal antibodies PAb1620 or PAb240 (wild-type conformation specific and mutant conformation specific, respectively; generous gift of Prof. Moshe Oren, Weizmann Institute, Rehovot, Israel) followed by 1-hr incubation at 4°C with anti-mouse IgG sepharose beads (Sigma). Beads containing the immunocomplexes were collected by centrifugation, washed five times with PBS, separated by 10% SDS-PAGE, and blotted onto nitro-cellulose membrane. Immunoblotting was performed with mouse monoclonal anti-p53 antibody (DO-1, Santa Cruz Biotechnology) and revealed by enhanced chemiluminescence system (ECL, Amersham, IL), according to the manufacturer's protocol.

Quantitative real-time PCR analysis

Quantitative real-time PCR was performed using the EvaGreen® dye (Biotium), according to the manufacturer's protocol, with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems).

Samples were normalized to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers used were as follows (5' to 3'): Bax forward, GGAGCAGCTTGGGAGCG; reverse, AAAAGGCCCCTGTCTTCATGA; Noxa forward CCACCTG AGTTCGCAGCTCAA; reverse, GTTGAGCACACTCGTCCT TCAA; PUMA forward, ATGGCGGACGACCTCAAC, reverse AGTCCCATGAAGAGATTGTACATGAC; GAPDH forward, CCAGTATGACTCCACTCACG; reverse, GACTCC ACGACATACTCAGC.

Statistical analysis

ANOVA was performed with repeated measures. The within subject factor was time and the between subject factor was group. Post-hoc analysis was Tukey's Method for Multiple comparisons.

Results

MMTV-*neu* mice express low HIPK2 levels

Following our previous work detailing the interaction between p53 and HIPK2, mainly the ability to restore the

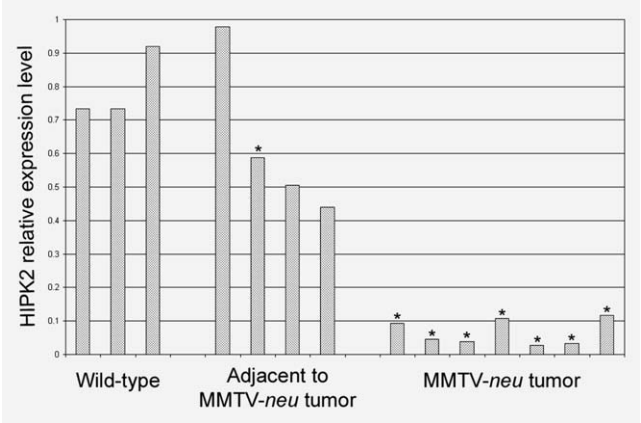

Figure 1. MMTV-*neu* mice express low HIPK2 levels in their mammary tumors. Microarray analysis of mammary tumors and adjacent tissue from MMTV-*neu* transgenic mice, and wild-type mammary glands, using Affymetrix microarrays (http://www.ncbi.nlm.nih.gov/geo/, dataset record GDS1222, probeset ID 103833_at). Each bar in the wild-type group represents analysis of RNA pooled from five different mice; all other bars represent RNA from a single mouse each. Results show low HIPK2 levels in the mammary tumors of MMTV-*neu* mice, compared to intermediate levels in the adjacent tissue and to high levels in normal mammary tissue of wild-type mice. *, defined as "absent" by the microarray data analysis software. For our analysis data was taken from Ref. 19, with kind permission from Dr. Ruth A. Keri.

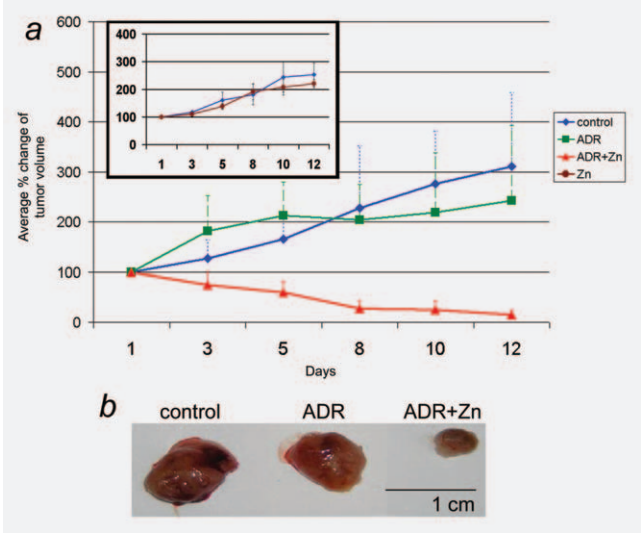

Figure 2. Inhibition of tumor growth in transgenic MMTV-*neu* murine breast cancer model by combined adriamycin and zinc therapy. (*a*) Tumor progression was monitored in MMTV-*neu* mice receiving either adriamycin alone (ADR, eight mice), a combination of adriamycin and zinc (ADR+Zn, seven mice), or PBS (control, eight mice). Tumors of the control group and those receiving adriamycin alone increased significantly in their size, whereas tumors receiving combined treatment with adriamycin and zinc were reduced by ~ 85%. ANOVA and Tukey's test showed a statistically significant difference between the combined adriamycin and zinc group versus the control and adriamycin alone groups ($p < 0.05$), no statistically significant difference was found between the control and adriamycin alone groups. Insert: Tumor progression was monitored in MMTV-*neu* mice receiving either zinc alone (Zn, three mice), or PBS (control, three mice). Zinc alone treatment had no effect on tumor size. (*b*) Representative pictures of mammary tumors from the groups detailed above, harvested at Day 12.

p53 proapoptotic pathway *via* a combination therapy of adriamycin and zinc,[12,16,22] we looked for a mouse model where HIPK2 levels are low in the tumor. Searching the gene expression omnibus (GEO) database we discovered that the MMTV-*neu* transgenic mice, which represent a mouse model of mammary tumor in humans,[20] indeed express low levels of HIPK2 in the mammary tumors, as opposed to intermediate and high levels of HIPK2 in the adjacent preneoplastic tissue of MMTV-*neu* mice and normal mammary tissue from wild-type control mice, respectively (Fig. 1). In this model, inactivated *neu* (HER2/ErbB2) is under the transcriptional control of the mouse mammary tumor virus promoter/enhancer, resulting in the development of focal mammary tumors after long latency, with many of the tumor-bearing transgenic mice developing metastases in the lungs.[20] This period of latency is affected by parity and luteinizing hormone levels.[23]

Inhibition of tumor growth in MMTV-*neu* mice by combined adriamycin and zinc therapy

We next evaluated the antitumor effect of a combined adriamycin and zinc therapy. Spontaneous breast tumors were allowed to grow for 5 days after first palpated. Mice were then randomized to three groups and treated with adriamycin alone or in combination with zinc, or with PBS as control. The antitumor effect of the various treatments was evaluated by calculating the relative tumor size compared with its size at Day 1. As can be seen in Figure 2, the combination of adriamycin and zinc resulted in an average decrease of 85% in tumor size, whereas adriamycin alone was associated with a 2.4-fold increase in tumor size, an effect similar to that seen in the control group, which was by a 3.1-fold. As can be seen in Figure 2*a* insert, treatment with zinc alone had no effect on tumor size, which is in accordance with previous results,[12,16,24] showing that zinc alone treatment does not have any effect either on activation of p53 or on tumor reduction.

H&E staining of the tumors harvested after 12 days revealed that tumors from mice treated with combination of adriamycin and zinc, showed vast areas of necrosis, with adjacent areas of fibrosis, confirmed by Masson staining (Fig. 3). Signs of necrosis are already evident after 48 hr (Fig. 3, left column), also in the adriamycin alone group. Smaller areas of spontaneous necrosis were also seen in the control group, at both time points (data not shown). Staining with an antibody against cleaved caspase 3, a critical player in the apoptotic pathway, showed positive staining in the necrotic area of mice treated with a combination of adriamycin and zinc (Fig. 3, right column), compared with no positive staining in the control or adriamycin alone treatments.

These results support the hypothesis that the addition of zinc to adriamycin treatment induces tumor growth

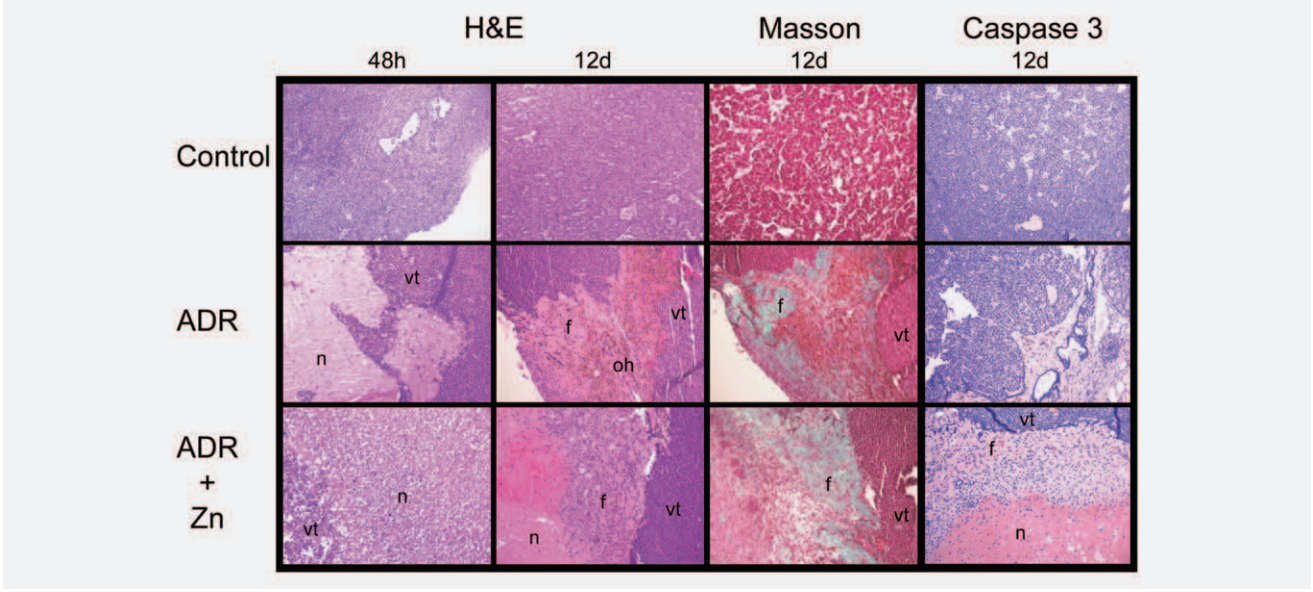

Figure 3. Mammary tumors of MMTV-*neu* mice respond to the combined treatment of adriamycin and zinc. Mammary tissues of mice treated with adriamycin alone (ADR) or with a combination of adriamycin and zinc (ADR+Zn) were harvested at 48 hr and at Day 12, and subjected to H&E, Masson (turquoise) and caspase 3 (red-brown) staining. Mammary tumors taken from mice treated with the combination of adriamycin and zinc, demonstrate vast area of necrosis, which at Day 12 are partially replaced by fibrosis. These necrotic areas of mice treated with the combination of adriamycin and zinc are positive for caspase 3, while no positive staining was found in the other groups. f, fibrosis; n, necrosis; oh, old hemorrhage (hemosiderin laden macrophages); vt, vital tumor. Original magnifications, ×20.

inhibition and cell death *in vivo* in the MMTV-*neu* breast cancer mouse model.

Restoration of misfolded p53 and activation of its down-stream proapoptotic pathway via the combined adriamycin and zinc therapy

To evaluate whether p53 function was negatively affected in the MMTV-*neu* breast cancer mouse model, we first analyzed p53 conformation. The partial and inadequate activity of the p53 protein, in the setting of low HIPK2 levels, is mainly due to misfolding of the p53 protein,[12,16,22] although its DNA sequence is wild-type. Tumors were harvested 48 hr after the beginning of treatments and p53 protein conformation was analyzed by immunoprecipitation technique, using the conformation-specific antibodies, PAb1620 and PAb240, which identify folded (wild-type conformation) and misfolded (mutant conformation) isoforms, respectively,[25,26] followed by immunoblotting with an anti-p53 antibody (DO-1), recognizing both conformations. In untreated control tumors, most of the p53 protein was in mutant conformation, with a wild-type/mutant conformation ratio of 0.21 (Fig. 4). Treatment with adriamycin alone did not significantly change this ratio (0.45). On the other hand, the combination of adriamycin and zinc resulted in a marked shift towards the wild-type conformation, with a ratio of 4.48 (Fig. 4). Similar results were obtained using the FL-393 anti-p53 antibodies (data not shown).

Finally, since HIPK2 modulates p53 activity mainly towards proapoptotic transcription,[9,11,22] we tested using quantitative real-time PCR the activation of several proapoptotic p53 targets in the tumors, 48 hr after the beginning of treatments. The results show a more prominent activation in response to the combined adriamycin and zinc therapy, compared with adriamycin alone (Fig. 5).

Taken together, these findings highlight, for the first time, the presence of a misfolded p53 protein in MMTV-*neu* breast cancers *in vivo*, that correlates with low HIPK2 expression, and could be reverted into wild-type p53 conformation by zinc supplementation, resulting in restoration of p53 proapoptotic transcriptional activity in response to drug.

Discussion

HIPK2 knockdown has been shown to induce p53 misfolding and inactivation of its tumor suppressor functions, that can be restored by zinc supplementation.[17] In the present study, we looked for a cancer model that showed physiological low HIPK2 expression, to evaluate whether zinc supplementation to adriamycin treatment can inhibit tumor growth by reactivating p53 oncosuppressor function. We found that the MMTV-*neu* breast cancer murine model, that overexpresses HER2/*neu* and develops mammary tumors at puberty with a long latency,[20] expresses low levels of HIPK2 as opposed to intermediate and high levels of HIPK2 in the adjacent preneoplastic tissue of MMTV-*neu* mice and normal mammary tissue from wild-type control mice, respectively.[19] In the low HIPK2-expressing tumors, the balance between wild-type and mutant conformations of the p53 protein is in favor of the mutant conformation at baseline (Fig. 4), suggesting p53 inactivation in response to drug. Indeed, p53 proapoptotic gene transcription was markedly induced only in tumors treated with combined adriamycin and zinc therapy (Fig. 5), which

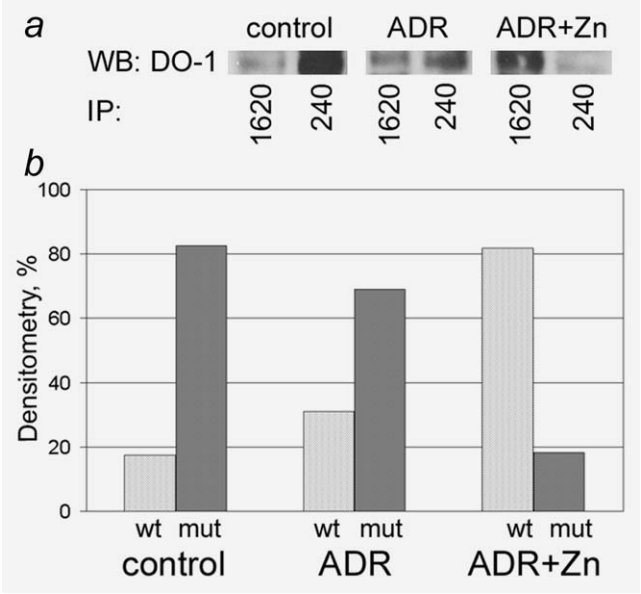

Figure 4. Restoration of misfolded p53 by combined adriamycin and zinc therapy. (*a*) Western blot analysis of immunoprecipitated p53 in mammary tumors. Protein lysates were prepared from tumors of untreated (control), adriamycin alone (ADR) treated and combination of adriamycin and zinc (ADR+Zn) treated mice. Equal amounts of protein (250 μg) were taken to p53 immunoprecipitation using the PAb1620 (wild-type conformation) and PAb240 (mutant conformation) antibodies, followed by western blot analysis using anti-p53 antibody (DO-1), recognizing both conformations. (*b*) Quantification of western blot bands using EZ-Quant Gel software. Results are shown as relative fractions of the total amount of p53 protein. Combination of adriamycin and zinc resulted in a marked shift in favor of the wild-type conformation, as opposed to the control or adriamycin alone treatment, in which the mutant conformation is more abundant.

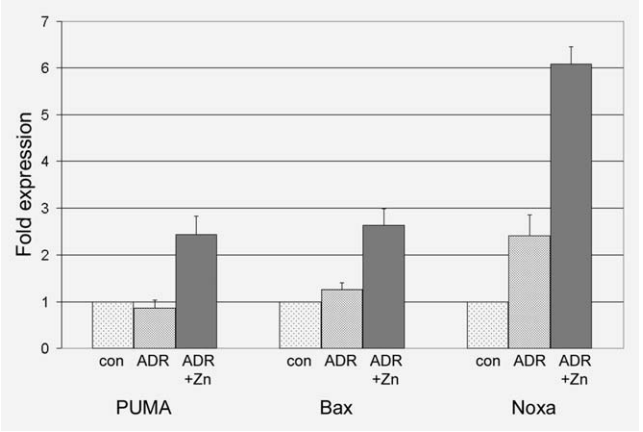

Figure 5. p53 apoptotic target genes are upregulated in response to the combined adriamycin and zinc therapy. Mammary tumors were taken from MMTV-*neu* mice treated with either adriamycin alone (ADR), a combination of adriamycin and zinc (ADR+Zn), or PBS as control (con). Tumors were harvested after 48 hr from the beginning of treatments. mRNA levels were determined by quantitative real-time PCR using the ABI PRISM® 7900HT system, and normalized to GAPDH. PUMA, Bax and Noxa expression was upregulated more prominently in response to the combined treatment of adriamycin and zinc, compared with adriamycin alone. Each group included three mice. Results display mean ± SD of three experiments, each done in triplicate.

correlated with a change of the wild-type/mutant conformation ratio and therefore with a shift in favor of the wild-type p53 protein conformation (Fig. 4). Reactivation of wild-type p53 function was presumably responsible for the dramatic effect on tumor size seen in Figure 2. Importantly, the restoration of wild-type p53 activity is extremely helpful for eradicating established tumors, as shown by several *in vivo* studies,[27–29] in accordance with the "oncogene addiction" theory.[30]

A major determinant of successful cancer therapy is the ability of cancer cells to activate apoptotic cell death, mainly due to functional p53 activity. Although mutations in the p53 gene are detected in about 50% of human cancers, indirect mechanisms also lead to p53 inactivation.[31] Among these mechanisms is the knockdown of the p53 regulator HIPK2, which induces loss of wild-type p53 function, reduces apoptotic drug-response and increases tumor progression.[17] Here, for the first time it is shown that HIPK2 undergoes mRNA downregulation during tumor development in the MMTV-*neu* breast cancer murine model (Fig. 1), in agreement with previous observation of HIPK2 downregulation in human breast, thyroid and colorectal[21,32] carcinoma tissues. However, the molecular mechanisms underlying HIPK2 downregulation still need to be elucidated.

It would be intriguing to evaluate the role of HIPK2 and the effect of combining zinc with chemotherapeutic agents regarding the formation of pulmonary metastases in the MMTV-*neu* murine model, since many of the tumor-bearing transgenic mice develop metastases in the lungs.[20]

It was recently shown that changes in the expression levels of HIPK2 between the normal and tumor tissue of each colorectal carcinoma patient have a positive effect on survival, irrespective of p53 function, raising the possibility of a p53-independent apoptotic modulation.[33] However, this study found no significant difference in the range of expression level in the tumors compared with the normal mucosa or in a subgroup of tumors.[33]

It should be noted that zinc is known to play a role in a wide range of functions in the organism, including DNA synthesis, cell division and protein synthesis. Zinc is a cofactor of over 250 enzymes and also plays a structural role in a large number of zinc finger proteins. These include growth factors, cytokines, receptors, enzymes and transcription factors.[34,35] This diversity of effects raises the possibility that the antitumor effect shown in this study may be also unrelated to the HIPK2-p53 pathway.

As the major side effect of adriamycin is chronic cardiotixicity,[36] we used H&E staining to examine and compare the hearts of mice treated with adriamycin alone with those treated with the combination of adriamycin and zinc. There was no evidence of cardiotoxicity in either group (data not shown). The lack of any pathology can be explained by the fact that chronic cardiotoxicity is dose-related, with a steep

increase in risk above a cumulative dose of 550 mg/m^2,[37] which is much higher than that administered in our experiment (10 mg/kg body weight, administered once).

Our findings clearly show that the combined adriamycin and zinc therapy resulted in a dramatic effect on tumor size in a murine model of mammary breast cancer. We hypothesize that the combination of adriamycin and zinc is superior to either adriamycin or zinc alone due to the effect of zinc on p53 functionality, either by direct effect on p53 conformation[13–15] or by stabilization of HIPK2,[38] which prevents MT2A upregulation[16] and allows phosphorylation of p53 at the Ser46 residue[16,22] and Lys382 acetylation at the C-terminus,[22] combined with the well-known activation of the p53 pathway by adriamycin. As both chemotherapy and zinc are affecting various cellular processes, one can not rule out that the combined therapies synergize in additional cell death mechanisms.

In conclusion, these results further reinforce the rationale for offering the combined treatment of chemotherapy and zinc to cancer patients whose tumors are characterized by wild-type p53 and low expression of HIPK2.

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