Tumor suppressor WWOX binds to ΔNp63α and sensitizes cancer cells to chemotherapy

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The WWOX tumor suppressor is a WW domain-containing protein. Its function in the cell has been shown to be mediated, in part, by interacting with its partners through its first WW (WW1) domain. Here, we demonstrated that WWOX via WW1 domain interacts with p53 homolog, ΔNp63α. This protein–protein interaction stabilizes ΔNp63α, through antagonizing function of the E3 ubiquitin ligase ITCH, inhibits nuclear translocation of ΔNp63α into the nucleus and suppresses ΔNp63α transactivation function. Additionally, we found that this functional crosstalk reverses cancer cells resistance to cisplatin, mediated by ΔNp63α, and consequently renders these cells more sensitive to undergo apoptosis. These findings suggest a functional crosstalk between WWOX and ΔNp63α in tumorigenesis.

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The WW domain-containing oxidoreductase (WWOX) gene encodes a 46-kDa tumor suppressor. WWOX contains two N-terminal WW domains and a central short dehydrogenase reductase domain. Through its first WW (WW1) domain, WWOX interacts with a growing list of partners, and thus involved in different signaling pathways ranging from growth suppression, differentiation, and transcription modulation. WWOX binds the proline-rich motif (PPxY) found in a number of proteins. Among these partners are p73, Ap2, and Runx2.1,2

WWOX binds to ΔNp63α and sensitizes cancer cells to chemotherapy.
**Results**

**WWOX–ΔNp63 physical interaction.** In previous work, we reported that WWOX physically and functionally interacts with its WW1 domain with the p53 homolog, p73 through its PPxY motif. Thus, we hypothesized that WWOX, through the same mechanism, might bind to other members of the p53 family, mainly p63 which contains a PPxY motif. To test our hypothesis, we cotransfected HEK293 cells with either the expression vectors encoding Myc–WWOX and HA–TAp63 or HA–ΔNp63x. Cells lysates were immunoprecipitated (IP) with anti-HA or anti-Myc antibodies followed by immunoblotting (IB) with HRP-conjugated antibody to HA or Myc. The results revealed that WWOX binds to ΔNp63x as determined by immunoprecipitation with anti-Myc and IB with anti-HA antibody (Figure 1a, upper panel, lane 7), while it failed to do so with TAp63x (Figure 1a, upper panel, lane 4). As a control, there were no detectable complexes in anti-IgG immunoprecipitates (Figure 1a, lanes 3 and 6). Of note and due to unknown reasons, we were unable to see the interaction in reverse (Figure 1a, lower panel).

To further confirm ΔNp63x–WWOX interaction, we utilized another cell system in which HA–TAp63x or HA–ΔNp63x are stably expressed in previously described tet-On-inducible SaOS2 cells. SaOS2 cells were transduced with low MOI of Ad-ΔNp63x. Cells lysates were IP with anti-HA or anti-ΔNp63x antibodies followed by IB with HRP-conjugated antibody to HA and anti-ΔNp63x. As shown in Figure 1b, only ΔNp63x was able to interact with WWOX (lane 6 versus 3). To ultimately prove the selective interaction of WWOX with ΔNp63x rather than with TAp63x, we performed GST-pulldown assay using bacterial GST–WWOX fusions on cell lysates extracted from HEK293T cells transfected with either HA–TAp63x or HA–ΔNp63x. Also in this experimental system, we confirmed physical association between WWOX and ΔNp63x (Figure 1c, lane 6 versus 3).

Since we were unable to see specific co-IP between Myc–WWOX and HA–ΔNp63x in reverse using anti-HA and IB with anti-Myc antibody (Figure 1a, lower panel), we repeated the experiment as in Figure 1a but used antibodies against WWOX and ΔNp63x for IB. Using this approach, we were able to see specific interaction between WWOX and ΔNp63x in both co-IP directions (Figure 1d). Taken together, these results suggest that WWOX specifically binds ΔNp63x.

**Mapping of WWOX–ΔNp63x interaction.** To map the region in WWOX responsible for binding to ΔNp63x, we did the same IP as mentioned above using WWOX-Y33R in which tyrosine (Y) was replaced with arginine (R) (a point mutation in WW1 domain that was previously shown to abrogate WWOX binding ability to its partners). While a physical interaction was revealed between WWOX and ΔNp63x, WWOX–Y33R abolished this interaction (Figure 2a, lane 4 versus 7), indicating that WWOX interacts with ΔNp63x via its WW1 domain. Results from Figure 1c (lane 4 versus 7) also confirm this finding. To further confirm that WWOX interacts with ΔNp63x via its WW1 domain, we cotransfected HEK293 cells with expression vectors encoding HA–ΔNp63x and different mammalian GST–WWOX domains (GST–WW1, GST–WW2, GST–WW1,2, GST–SDR). Cells lysates were pulled down using GST beads followed by IB with anti-HA–HRP-conjugated antibodies. As shown in Figure 2b, only WW1 domain of WWOX was able to bind to ΔNp63x.

We next examined whether PPxY motif within ΔNp63x is responsible for WWOX–ΔNp63x association. Using site-directed mutagenesis, we generated point mutations in the PPxY motif by replacing the two prolines and tyrosine with alanine generating ΔNp63x–AAxA and determined the ability of this mutant to bind WWOX by GST-pulldown assay. Unexpectedly, ΔNp63x–AAxA was still able to bind WWOX similar to intact ΔNp63x (Figure 2c), suggesting that WW1 domain of WWOX binds to a different motif, rather than PPxY, within ΔNp63x.

**WWOX inhibits ΔNp63x ubiquitination and degradation mediated by ITCH.** ΔNp63x ubiquitination and degradation is mediated by the ubiquitin E3-ligase ITCH. Since this effect on ΔNp63x is dependent on ITCH WW domains and our results here show that ΔNp63x interacts with WW1 domain of WWOX, we next set to examine whether WWOX affects ΔNp63x ubiquitination mediated by ITCH. To this end, HEK293 were cotransfected with HA–UB and Myc–ΔNp63x alone or Myc–ΔNp63x and Flag–ITCH, or Myc–ΔNp63x, Flag–ITCH, and WWOX. At 24 h, cells were treated with the proteasome inhibitor MG132 for an additional 4 h. Lysates were subjected to immunoprecipitation using anti-Myc antibody followed by IB with anti-HA–HRP antibody. We found that while expression of ITCH increases ubiquitination of ΔNp63x (Figure 3a, middle lane), coexpression of WWOX abrogated this ubiquitination event (Figure 3a, right lane).

To prove that WWOX affects ΔNp63x ubiquitination by competing on the interaction between ΔNp63x and ITCH, we performed coimmunoprecipitation assay between ΔNp63x and ITCH in the presence of either WWOX or mutant WWOX–Y33R. To this end, we cotransfected HEK293 cells with HA–ΔNp63x, Flag–ITCH, and Myc–WWOX or Myc–WWOX–Y33R. At 24 h, cells were treated with the proteasome inhibitor MG132 for an additional 2 h. Lysates were subjected to immunoprecipitation using anti-HA, IgG, anti-Flag, and anti-Myc antibodies followed by IB with HRP-conjugated antibody to HA, Flag, or Myc. We found that while WWOX expression reduced the interaction between ΔNp63x and ITCH, WWOX–Y33R was unable to do so (Figure 3b, upper panel, lane 5 versus 10), suggesting that the presence of mutant WWOX rescues ITCH–ΔNp63x association. This reduced interaction between ΔNp63x and ITCH was most likely due to association of ΔNp63x and WWOX, but not WWOX–Y33R (Figure 3b, upper panel, lane 4 versus 9 and middle panel, lane 2 versus 7). Notably, no change was observed when using anti-Flag antibodies (Figure 3b, lower panel, lane 2 versus 7).

To examine whether WWOX effect on ΔNp63x ubiquitination affects ΔNp63x protein levels, we analyzed the half-life of ΔNp63x in the presence or absence of WWOX using the protein synthesis inhibitor, cycloheximide (CHX). Whereas WWOX led to increased half-life of ΔNp63x, WWOX–Y33R mutant that does not interact with ΔNp63x (Figure 2), had little effect if at all, on ΔNp63x half-life (Figure 3c). These data were also validated using the inducible ΔNp63x-expressing SaOS2
cells. While control untransduced, Ad-GFP-transduced, and Ad-WWOX-Y33R-transduced cells (Figure 3d) showed no effect on ΔNp63α stability, cells transduced with Ad-WWOX displayed increased ΔNp63α protein levels (Figure 3d). To further confirm the importance of WWOX in controlling the protein level of ΔNp63α, we generated stable HaCaT cells clones expressing shRNA constructs specifically targeting the human WWOX mRNA and analyzed consequences on ΔNp63α levels. As shown in Figure 3e, WWOX-depleted HaCaT (KD) cells displayed lower ΔNp63α levels compared with control shRNA-expressing (EV) cells.

To further show that WWOX stabilizes ΔNp63α by specifically inhibiting its degradation via the proteasome, we did the same experiment shown in Figure 3d, except for the use of the proteasome inhibitor MG132 prior to cell lysis. As shown in Figure 3f (lane 2), WWOX overexpression was associated with stabilization of ΔNp63α. Since WWOX did not bind TAp63α, we examined whether it indeed does not affect its half-life. To this end, levels of TAp63α in the presence or absence of WWOX and CHX was examined. We found that neither WWOX nor WWOX-Y33R were able to affect TAp63α stability (data not shown). Altogether, these results suggest that WWOX antagonizes ITCH effect on ΔNp63α and stabilizes its protein levels.

**WWOX sequesters ΔNp63α in the cytoplasm.** The results obtained above led us to question the significance of the interaction between WWOX and ΔNp63α. ΔNp63α is a transcription factor that localizes in the nucleus, where it binds DNA and transactivates target genes such as K14, and BPAG-1. In contrast, WWOX is predominantly known as a cytoplasmic protein, though some reports demonstrate nuclear localization under certain conditions.24 Thus, we asked the question about the possibility of whether WWOX can affect ΔNp63α localization or vice versa. To answer this, we studied the localization of ΔNp63α by subcellular fractionation. We transfected HEK293 cells with ΔNp63α in the presence or absence of WWOX. After 24 h, we prepared lysates from both the nuclear and cytoplasmic fractions. Successful fractionation was confirmed by the exclusive presence of GAPDH and lamin in the cytoplasmic and nuclear fractions, respectively. We found that, although ΔNp63α alone localizes mainly in the nuclear fraction, coexpression of ΔNp63α and WWOX was associated with increased ΔNp63α presence in the cytoplasm concomitant with less nuclear ΔNp63α levels (Figure 4a). To further confirm this finding, we tested the distribution of ΔNp63α using immunofluorescence and confocal microscopy. HeLa cells were transiently cotransfected with GFP–WWOX and HA–ΔNp63α. Localization of the HA- or GFP-tagged proteins was then determined by immunofluorescent staining using the appropriate antibodies. As shown in Figure 4b, when present alone ΔNp63α is mainly localized in the nucleus while WWOX is predominantly cytoplasmic. However, when WWOX is coexpressed with ΔNp63α, it is sequestered and colocalizes with WWOX in the cytoplasm (Figure 4b, arrow heads).

To further confirm these results, we used SaOS2 cells overexpressing both WWOX and ΔNp63α. Also, in these cells WWOX colocalization with ΔNp63α lead to its sequestration in the cytoplasm (Figure 4c, arrow heads). Interestingly, cells displaying nuclear ΔNp63α have significantly reduced expression of WWOX (Figure 4b and c), which might explain this partial sequestration of ΔNp63α. However, we cannot exclude that there might be other factor(s) regulating ΔNp63α sequestration into the cytoplasm. Altogether, our results
suggest that WWOX binds ΔNp63α in the cytoplasm and prevents its translocation to the nucleus.

**WWOX suppresses ΔNp63α transactivation ability.** Since our above results demonstrate that WWOX sequesters ΔNp63α in the cytoplasm, we next set to determine whether WWOX might affect its transactivation function. To test this hypothesis, we transfected HEK293T cells with constructs containing the luciferase gene driven by K14 or BPAG-1 promoters that contain ΔNp63α response elements. At 24 h, cells were lysed and luciferase activity was assessed. As expected, expression of WWOX alone has no effect on luciferase activity of these promoters while ΔNp63α had significant transactivation (Figure 5a). By contrast, coexpression of WWOX with ΔNp63α significantly suppressed ΔNp63α transactivation function in a dose-dependent manner (Figure 5a). This effect was significantly attenuated when expressing WWOX-Y33R (Figure 5b and c). Cumulatively, our findings suggest that WWOX sequesters ΔNp63α in the cytoplasm, and this is associated with its reduced transactivation function.

**WWOX antagonizes ΔNp63α-induced chemoresistance.** ΔNp63α was shown to play a crucial role in determining cellular chemosensitivity, while WWOX was shown to promote apoptosis in certain contexts. Considering these findings, we hypothesized that WWOX affects ΔNp63α function in chemosensitivity, that is, upon treatment of cisplatin. To test this, inducible ΔNp63α SaOS2 cells were stably transduced with either WWOX or empty lentiviral vectors. Successful expression of WWOX and ΔNp63α, following doxycycline (Dox) treatment, is shown in Figure 6a. SaOS2 cells expressing ΔNp63α, WWOX or both together were next treated with cisplatin for 48 h. Cells were next collected and percentage of dead cells, assessed by trypan blue exclusion, and apoptotic cells, as assessed by propidium iodide using FACS analysis, was determined. We found that treatment of cells with cisplatin induced cell death by fourfolds (Figure 6b). Upon treatment of ΔNp63α-expressing SaOS2 cells (Dox) with cisplatin, no significant change was observed. Importantly, expression of WWOX and cisplatin treatment significantly increased cell death by eightfold (Figure 6b). Intriguingly, coexpression of ΔNp63α and WWOX significantly sensitized cisplatin-treated cells to undergo cell death/apoptosis (∼14-folds) as compared with SaOS2 expressing ΔNp63α alone (Figure 6b). Similar results were obtained by examining percentage of sub-G1 population (Figure 6c). Although these observations suggest that WWOX antagonizes ΔNp63α-induced chemoresistance, it does not explain the fact that WWOX expression alone exhibited less percentage of cell death and apoptosis (Figure 6b and c). To address this issue, we examined whether Dox treatment can affect cisplatin-induced cell growth/death. In fact, several reports have shown that Dox by itself can induce growth arrest and apoptosis and could enhance cisplatin effect in cancer cells. To test this in our settings, we utilized control SaOS2 cells to examine their sensitivity upon treatment of Dox and cisplatin. We observed that treatment of Dox alone induces cell death by 2.6-fold as compared with untreated cells (Figure 6d), consistent with previously published data. Of note, while cisplatin treatment increased cell death by sevenfold, this effect was increased to 9.3-fold upon treatment with both Dox and cisplatin. These results suggest that both Dox and cisplatin have a synergistic effect on cell death in agreement with previously published data. Taken together, our findings suggest that WWOX attenuates ΔNp63α-mediated cisplatin chemoresistance.

**Discussion**

The p53 family that includes in addition to p53, p63, and p73 proteins have both common and distinct functions. This family
of proteins play very important roles in cell differentiation, stemness and plasticity, in immune response regulation, in tumorigenesis and tumor suppression, in development and reproduction, DNA damage, and apoptosis and cell-cycle regulation. In addition to regulating each other’s function, the WW domain-containing proteins, including WWOX, YAP, and ITCH, were shown to regulate p73 and p63 functional outcome. Altogether, these findings argue that WW domain proteins could compete with other WW domain-containing proteins, like YAP and ITCH, for binding common target proteins, such as ErbB4 and p73, hence determining functional outcomes. Further research would be necessary to decipher and characterize these motifs.

Figure 3 WWOX inhibits ITCH-mediated ubiquitination of ΔNp63α and increases its half-life. (a) HEK293 cells were transfected with the indicated plasmids. After 24 h, cells were treated with 20 μM MG132 for 4 h. Lysate was prepared and IP with anti-Myc (ΔNp63α) and detected with anti-HA antibodies (UB). (b) HEK293 cells were cotransfected with plasmids encoding HA–ΔNp63α and Flag–ITCH and Myc–WWOX or Myc–WWOX-Y33R. After 24 h, cells were lysed and immunoprecipitation was performed as follows: lanes 2 and 7: anti-HA; lanes 3 and 8: anti-IgG; lanes 4 and 9: anti-Myc; and lanes 5 and 10: anti-Flag antibodies. Immunoblotting was done using the indicated antibodies. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (f) Tet-On-inducible SaOS2 cells were used as in (d) but instead of CHX, cells were treated with the proteasome inhibitor MG132.
sequestering TAp63 in inactive hetero-tetramers (ΔNp63α-TAp63 tetramers). Therefore, it is possible that WWOX might disrupt the formation of these inactive hetero-tetramers, and by this, inhibits ΔNp63α functions and relieves the inhibitory effect on TAp63. Of note, our data also indicate that WWOX selectively binds ΔNp63α, but not TAp63α, perhaps due to conformational elements that are not yet resolved.

Recent evidence shows that p63 plays an important role in conferring either chemoresistance or chemosensitivity. While TAp63 correlates with and induces chemosensitivity, ΔNp63α expression directly correlates with a poor clinical response to cisplatin in HNSCC and leads to chemoresistance by different mechanisms. Consequently, to fully understand the role of p53 family members in a particular context, the integration of the activities of all the isoforms, their modulators, and their partners must be assessed in a context-specific manner.

In summary, we provide evidence that ΔNp63α and WWOX physically interact, and that this interaction results in an increased chemosensitivity to cisplatin and increased rate of cell death. Additional genetic and biochemical approaches will elucidate the biological consequences of this association in normal and cancer cells.

Materials and Methods

Cell culture and transient transfection. HEK293, and HaCaT cells were grown in DMEM, ΔNp63α-tet-ON SaOS2 in RPMI. All cells were supplemented with 10% FBS (Gibco, Grand Island, NY, USA), glutamine, and
penicillin/streptomycin (Biological Industries, Beit-Haemek, Israel). To induce the expression of $\text{\textit{DNp63}}^a$, SaOS2 cells were treated with 2 mg/ml doxycyclin (Sigma-Aldrich, St. Louis, MO, USA) for 48 h. All expression vectors used were previously reported in 21, 23. Transient transfections were achieved using Mirus TransLTi (Mirus Bio LLC, Madison, WI, USA). In all cell lines used in this article, p53 function is lost by different mechanisms, including mutation and loss of expression and function.

GST-pulldown, immunoprecipitation, and immunoblot analysis. Cells were lysed by using Nonidet P-40 lysis buffer containing 50 mmol/l Tris (pH 7.5), 150 mmol/l NaCl, 10% glycerol, 0.5% Nonidet P-40, and protease inhibitors (Sigma-Aldrich). In GST-pulldown, lysates were mixed with glutathione-sepharose 4B (GST beads) (GE Healthcare, Waukesha, WI, USA) and rocked for 2 h at 4°C. Thereafter, the beads were washed four times with the same buffer containing 0.1% Nonidet P-40. For immunoprecipitation, lysates were pre-cleared with mouse anti-IgG (Zymed, Carlsbad, CA, USA) immunoprecipitations were carried out in the same buffer, and lysates were washed four times with the same buffer containing 0.1% Nonidet P-40. Western blotting was conducted under standard conditions. Antibodies used were monoclonal anti-HA (Covance, Princeton, NJ, USA), monoclonal anti-p63 (4A4), monoclonal anti-Myc–HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA), monoclonal anti-Flag, anti-Flag–HRP and anti-Vinculin (Sigma-Aldrich), anti-HA–HRP (Roche Applied Science, Indianapolis, IN, USA) and monoclonal anti-WWOX antibodies.  

Luciferase assay. HEK293 cells seeded in 12-well plates were cotransfected with the relevant plasmids together with different plasmids containing different $\text{\textit{DNp63}}^a$ responsive elements of various $\text{\textit{DNp63}}^a$ target genes. Renilla luciferase was used as an internal control. Cells were collected 24 h later and Firefly and Renilla luciferase activities were assayed with Dual-Luciferase Assay System (Promega, Madison, WI, USA). Firefly luciferase activity was normalized to Renilla luciferase activity. All experiments were done at least thrice.

Subcellular fractionation. Nuclear and cytoplasmic extracts were prepared as follows. First, cells were scraped in PBS, and after centrifugation, the cell pellet was resuspended in a hypotonic lysis buffer [10 mmol/l HEPES (pH 7.9), 10 mmol/l KCl, 0.1 mmol/l EDTA] supplemented with 1 mmol/l DTT and a broad-spectrum cocktail of protease inhibitors (Sigma-Aldrich). The cells were allowed to swell on ice for 15 min, then NP40 was added, and cells were lysed by vortex. After centrifugation, the cytoplasmic fraction was collected. Afterwards, nuclear extracts were obtained by incubating nuclei in a hypertonic nuclear extraction buffer (20 mmol/l HEPES (pH 7.9), 0.42 mol/l KCl, 1 mmol/l EDTA) supplemented with 1 mmol/l DTT for 15 min at 4°C. The nuclear fraction was collected after centrifugation.

In vivo ubiquitination assay. HEK293 cells were cotransfected with HA–UB, Myc–$\text{\textit{DNp63}}^a$ with or without Flag–ITCH or WWOX as indicated in Figure 3a. After 24 h, cells were treated with MG-132 (Sigma; 20 μmol/l) for 4 h. Lysates were IP using anti-Myc antibody, washed 4 times, and immunoblotted with anti-HA–HRP.

Measurement of steady-state and half-life of $\text{\textit{DNp63}}^a$ protein level. HEK293T cells were transfected with $\text{\textit{DNp63}}^a$ with or without $\Delta$WWOX suppresses $\text{\textit{DNp63}}^a$ transactivation function. HEK293 cells were transiently cotransfected with the luciferase reporter construct carrying the $\text{\textit{DNp63}}^a$ responsive element derived from the promoters of K-14 and BPAG-1 in addition to either WWOX alone, $\text{\textit{DNp63}}^a$ alone, or with increasing amount of WWOX. In all experiments, empty vector was cotransfected to normalize plasmid concentration where required. At 24 h after transfection, cells were lysed and luciferase activity was determined. Results are shown as fold induction of the luciferase activity compared with control cells transfected with empty vector alone and are the average of three experiments. Bars represent STDV. (b, c) HEK293 cells were treated as in (a) though fixed amount of K14-Luc (b) or BPAG-1-Luc (c) and either WWOX or WWOX-Y33R (0.5 μg) and 0.1 μg $\text{\textit{DNp63}}^a$. Cells were analyzed as in (a).
Coexpression of WWOX and ΔNp63α suppresses ΔNp63-induced resistance to chemotherapy. (a) SaOS2-ΔNp63α-tetOn cells were transduced with Lentivirus expressing WWOX or Lenti-EV expression vector. Stable cells were generated. Treatment of these cells with 2 μg/ml doxycyclin (Dox) for 48 h was performed to induce ΔNp63α expression. Immunoblot analysis revealed expression of WWOX and ΔNp63α using anti-WWOX and anti-HA HRP, respectively. (b, c) Cells from (a) were treated with 2 μg/ml Dox for 48 h followed by treatment with 40 μM Cisplatin (Cis) for an additional 48 h. (b) Columns represent the relative percentage of dead cells determined by trypan blue. Error bars represent STDV. (c) Columns represent the relative percentage of sub-G1 population as assessed by flow cytometry and propidium iodide. Error bars represent STDV. (d) Control SaOS2 cells were treated with 2 μg/ml Dox for 48 h followed by treatment with 40 μM Cisplatin (Cis) for an additional 48 h. Columns represent the relative percentage of dead cells determined by trypan blue.

**Conflict of Interest**

The authors declare no conflict of interest.

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