

# Tumor suppressor WWOX binds to $\Delta$ Np63 $\alpha$ 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,  $\Delta$ Np63 $\alpha$ . This protein–protein interaction stabilizes  $\Delta$ Np63 $\alpha$ , through antagonizing function of the E3 ubiquitin ligase ITCH, inhibits nuclear translocation of  $\Delta$ Np63 $\alpha$  into the nucleus and suppresses  $\Delta$ Np63 $\alpha$  transactivation function. Additionally, we found that this functional crosstalk reverses cancer cells resistance to cisplatin, mediated by  $\Delta$ Np63 $\alpha$ , and consequently renders these cells more sensitive to undergo apoptosis. These findings suggest a functional crosstalk between WWOX and  $\Delta$ Np63 $\alpha$  in tumorigenesis.

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**Subject Category:** Cancer

The WW domain-containing oxidoreductase (WWOX) gene encodes a 46-kDa tumor suppressor.<sup>1,2</sup> 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 $\alpha$ , Ap2 $\gamma$ , ErbB4, Jun, and Runx2.<sup>1,2</sup>

The gene spans the fragile site *FRA16D* that includes a genomic region involved in chromosome translocation in multiple myelomas and in hemi- and homozygous deletions in cancers and cancer-derived cell lines; in addition, the WWOX promoter region is frequently hypermethylated in cancers.<sup>3,4</sup> Wwox-knockout mice demonstrated that WWOX functions as a *bona fide* tumor suppressor. Spontaneous osteosarcomas in juvenile Wwox-knockout and lung papillary and mammary carcinomas in adult Wwox-heterozygous mice were observed. Additionally, Wwox-heterozygous mice significantly develop more ethyl nitrosourea-induced lung tumors and B-cell lymphomas and more *N*-nitrosomethylbenzylamine-induced forestomach tumors in comparison with WT littermates.<sup>5–8</sup>

p63 is a member of the p53 family that includes p53, p63, and p73 proteins.<sup>9,10</sup> It is a family of transcription factors that are highly homologous with very distinct functions. In general, all members of the family have three structural domains that are essential for their function: a DNA-binding domain (DBD),

an oligomerization domain (OD) (all function as tetramers), and a transactivation domain (TA). The use of a second promoter generates N-terminal truncated isoforms that in p73 and p63 lack the TA domain ( $\Delta$ N isoforms).

The most common isoform of p63 is a truncated gene product termed  $\Delta$ Np63. Whereas the TAp63 proteins are capable of transactivation, the  $\Delta$ Np63 forms can also act in a dominant-negative fashion to counteract the transcriptional activity of the TAp63 isoforms and p53.<sup>9,10</sup> This isoform is highly expressed in basal or reserve cells, immature squamous epithelium, and epithelial stem cells. Both  $\Delta$ Np63 and TAp63 can be subdivided further into three unique C-terminal sequence variants, conveniently designated as  $\alpha$ ,  $\beta$ , and  $\gamma$ , yielding a total of six discrete p63 gene products. The major functioning isoform is  $\Delta$ Np63 $\alpha$ .  $\Delta$ Np63 $\alpha$  is essential to the formation of the epidermis and its appendages, such as hairs and sebaceous glands since it regulates epithelial development and differentiation.<sup>11–13</sup> Most tumors (> 80% of primary head and neck squamous cell carcinomas (HNSCCs), as well as other squamous cell epithelial malignancies and non-small cell lung cancer) retain p63 expression, where it is often overexpressed and occasionally amplified. Of note,  $\Delta$ Np63 $\alpha$  is the predominant isoform at the protein level.<sup>14–17</sup> In addition,  $\Delta$ Np63 $\alpha$  expression leads to chemotherapeutic reagent resistance by different mechanisms.<sup>18–20</sup>

In this work, we show that WWOX binds  $\Delta$ Np63 $\alpha$ , changes its cellular localization, inhibits its transcriptional activity, and counteracts its chemoresistance-induced phenotype.

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**Abbreviations:** WWOX, WW domain-containing oxidoreductase; UB, Ubiquitin; OD, oligomerization domain; TA, transactivation domain; HNSCCs, head and neck squamous cell carcinomas; IP, immunoprecipitation; IB, immunoblotting; Ad, adenovirus; MOI, Multiplicity of infection; CHX, cycloheximide; KD, knockdown; EV, empty virus; Dox, doxycycline; WT, wild type; PY, proline-tyrosine rich motif

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## Results

**WWOX– $\Delta$ Np63 physical interaction.** In previous work,<sup>21</sup> we reported that WWOX physically and functionally interacts via 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 $\alpha$  or HA– $\Delta$ Np63 $\alpha$ . 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  $\Delta$ Np63 $\alpha$  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 TAp63 $\alpha$  (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  $\Delta$ Np63 $\alpha$ –WWOX interaction, we utilized another cell system in which HA–TAp63 $\alpha$  or HA– $\Delta$ Np63 $\alpha$  are stably expressed in previously described tet-On-inducible SaOS2 cells.<sup>22</sup> SaOS2 cells were transduced with low MOI of Ad-WWOX. Cells lysates were IP with anti-HA or anti-WWOX antibodies followed by IB with HRP-conjugated antibody to HA and anti-WWOX. As shown in Figure 1b, only  $\Delta$ Np63 $\alpha$  was able to interact with WWOX (lane 6 *versus* 3). To ultimately prove the selective interaction of WWOX with  $\Delta$ Np63 $\alpha$  rather than with TAp63 $\alpha$ , we performed GST-pulldown assay using bacterial GST–WWOX fusions on cell lysates extracted from HEK293T cells transfected with either HA–TAp63 $\alpha$  or HA– $\Delta$ Np63 $\alpha$ . Also in this experimental system, we confirmed physical association between WWOX and  $\Delta$ Np63 $\alpha$  (Figure 1c, lane 6 *versus* 3).

Since we were unable to see specific co-IP between Myc–WWOX and HA– $\Delta$ Np63 $\alpha$  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  $\Delta$ Np63 $\alpha$  for IB. Using this approach, we were able to see specific interaction between WWOX and  $\Delta$ Np63 $\alpha$  in both co-IP directions (Figure 1d). Taken together, these results suggest that WWOX specifically binds  $\Delta$ Np63 $\alpha$ .

**Mapping of WWOX– $\Delta$ Np63 $\alpha$  interaction.** To map the region in WWOX responsible for binding to  $\Delta$ Np63 $\alpha$ , 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<sup>21</sup>). While a physical interaction was revealed between WWOX and  $\Delta$ Np63 $\alpha$ , WWOX-Y33R abolished this interaction (Figure 2a, lane 4 *versus* 7), indicating that WWOX interacts with  $\Delta$ Np63 $\alpha$  via its WW1 domain. Results from Figure 1c (lane 4 *versus* 7) also confirm this finding. To further confirm that WWOX interacts with  $\Delta$ Np63 $\alpha$  via its WW1 domain, we cotransfected HEK293 cells with expression vectors encoding HA– $\Delta$ Np63 $\alpha$  and different mammalian GST–WWOX domains (GST–WW1, GST–WW2, GST–WW1,2, GST–

SDR). Cell 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  $\Delta$ Np63 $\alpha$ .

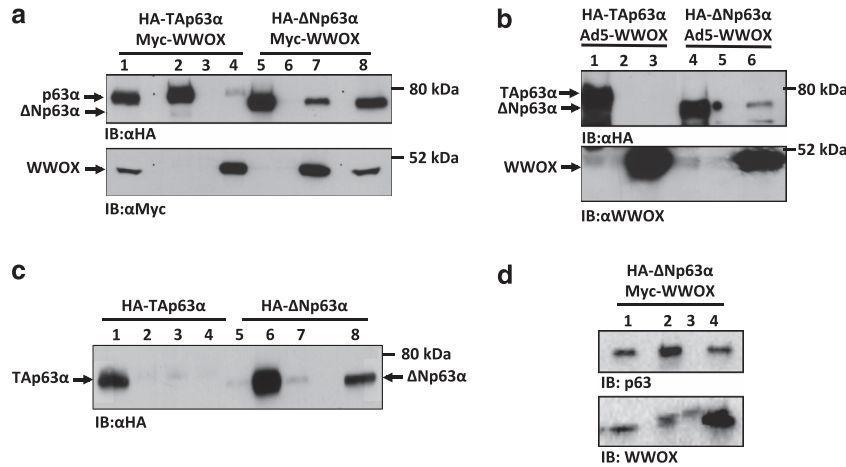
We next examined whether PPxY motif within  $\Delta$ Np63 $\alpha$  is responsible for WWOX– $\Delta$ Np63 $\alpha$  association. Using site-directed mutagenesis, we generated point mutations in the PPxY motif by replacing the two prolines and tyrosine with alanine generating  $\Delta$ Np63 $\alpha$ -AAxA and determined the ability of this mutant to bind WWOX by GST-pulldown assay. Unexpectedly,  $\Delta$ Np63 $\alpha$ -AAxA was still able to bind WWOX similar to intact  $\Delta$ Np63 $\alpha$  (Figure 2c), suggesting that WW1 domain of WWOX binds to a different motif, rather than PPxY, within  $\Delta$ Np63 $\alpha$ .

## WWOX inhibits $\Delta$ Np63 $\alpha$ ubiquitination and degradation mediated by ITCH.

$\Delta$ Np63 $\alpha$  ubiquitination and degradation is mediated by the ubiquitin E3-ligase ITCH.<sup>23</sup> Since this effect on  $\Delta$ Np63 $\alpha$  is dependent on ITCH WW domains and our results here show that  $\Delta$ Np63 $\alpha$  interacts with WW1 domain of WWOX, we next set to examine whether WWOX affect  $\Delta$ Np63 $\alpha$  ubiquitination mediated by ITCH. To this end, HEK293 were cotransfected with HA–UB and Myc– $\Delta$ Np63 $\alpha$  alone or Myc– $\Delta$ Np63 $\alpha$  and Flag–ITCH, or Myc– $\Delta$ Np63 $\alpha$ , 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  $\Delta$ Np63 $\alpha$  (Figure 3a, middle lane), coexpression of WWOX abrogated this ubiquitination event (Figure 3a, right lane).

To prove that WWOX affects  $\Delta$ Np63 $\alpha$  ubiquitination by competing on the interaction between  $\Delta$ Np63 $\alpha$  and ITCH, we performed coimmunoprecipitation assay between  $\Delta$ Np63 $\alpha$  and ITCH in the presence of either WWOX or mutant WWOX-Y33R. To this end, we cotransfected HEK293 cells with HA– $\Delta$ Np63 $\alpha$ , 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  $\Delta$ Np63 $\alpha$  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– $\Delta$ Np63 $\alpha$  association. This reduced interaction between  $\Delta$ Np63 $\alpha$  and ITCH was most likely due to association of  $\Delta$ Np63 $\alpha$  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  $\Delta$ Np63 $\alpha$  ubiquitination affects  $\Delta$ Np63 $\alpha$  protein levels, we analyzed the half-life of  $\Delta$ Np63 $\alpha$  in the presence or absence of WWOX using the protein synthesis inhibitor, cycloheximide (CHX). Whereas WWOX led to increased half-life of  $\Delta$ Np63 $\alpha$ , WWOX-Y33R mutant that does not interact with  $\Delta$ Np63 $\alpha$  (Figure 2), had little effect if at all, on  $\Delta$ Np63 $\alpha$  half-life (Figure 3c). These data were also validated using the inducible  $\Delta$ Np63 $\alpha$ -expressing SaOS2



**Figure 1** WWOX physically interacts with  $\Delta$ Np63 $\alpha$  but not TAp63 $\alpha$ . (a) HEK293 cells were cotransfected with plasmids encoding Myc-WWOX and HA-TAp63 $\alpha$  or HA- $\Delta$ Np63 $\alpha$ . After 24 h, cells were lysed and immunoprecipitation was performed as follows: lanes 2 and 5: anti-HA; lanes 3 and 6: anti-IgG; and lanes 4 and 7: anti-Myc antibodies. Immunoblotting was done using anti-HA-HRP or anti-Myc-HRP antibodies. Lane 1 shows input of TAp63 $\alpha$  and lane 8 shows input of  $\Delta$ Np63 $\alpha$ . (b) SaOS-2 cells, stably transfected with a doxycycline-inducible vector containing the HA-TAp63 $\alpha$  or the HA- $\Delta$ Np63 $\alpha$  gene, were infected with 10 MOI of Ad5-WWOX for 24 h then induced with doxycycline for an additional 24 h. Subsequently, cells were lysed and immunoprecipitation was performed as follows: lanes 1 and 4: anti-HA; lanes 2 and 5: anti-IgG; and lanes 3 and 6: anti-WWOX antibodies. Immunoblotting was done using anti-HA-HRP and anti-WWOX antibodies. (c) HEK293 cells were transfected with plasmids encoding HA-TAp63 $\alpha$  or HA- $\Delta$ Np63 $\alpha$ . After 24 h, cells were lysed and GST-pulldown was performed using GST alone (lanes 2 and 5) or GST-WWOX (lanes 3 and 6) or GST-WWOX-Y33R (lanes 4 and 7). Lanes 1 and 8 show 2.5% of input of each lysate. (d) HEK293 cells were cotransfected with plasmids encoding HA- $\Delta$ Np63 $\alpha$  and Myc-WWOX. After 24 h, cells were lysed and immunoprecipitation was performed as follows: lane 2: anti-HA; lane 3: anti-IgG; and lane 4: anti-Myc antibodies. Immunoblotting was done using anti-p63 and anti-WWOX antibodies. Lane 1 shows 2.5% of input of lysate. A higher band in the IgG (lane #2) and Myc (lane #3) is observed in the anti-WWOX blot (lower) likely due to antibody non-specificity

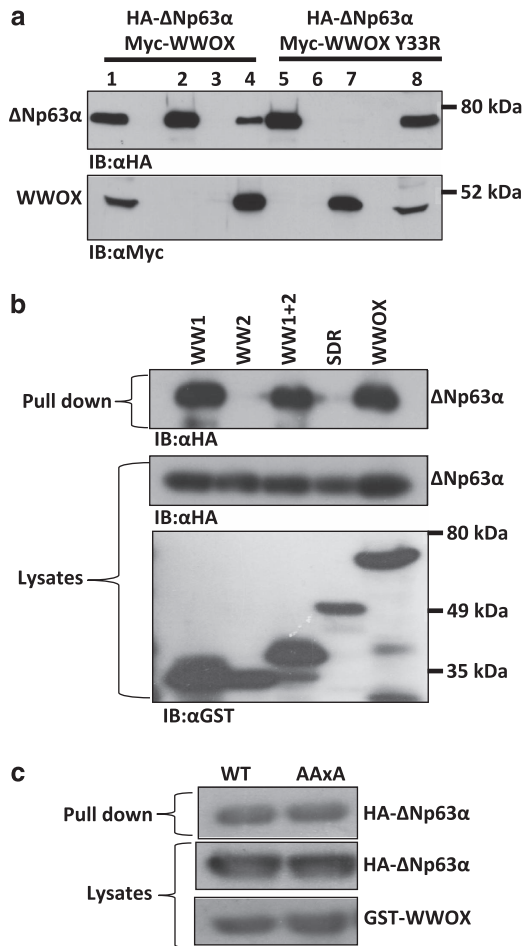
cells. While control untransduced, Ad-GFP-transduced, and Ad-WWOX-Y33R- transduced cells (Figure 3d) showed no effect on  $\Delta$ Np63 $\alpha$  stability, cells transduced with Ad-WWOX displayed increased  $\Delta$ Np63 $\alpha$  protein levels (Figure 3d). To further confirm the importance of WWOX in controlling the protein level of  $\Delta$ Np63 $\alpha$ , we generated stable HaCaT cells clones expressing shRNA constructs specifically targeting the human WWOX mRNA and analyzed consequences on  $\Delta$ Np63 $\alpha$  levels. As shown in Figure 3e, WWOX-depleted HaCaT (KD) cells displayed lower  $\Delta$ Np63 $\alpha$  levels compared with control shRNA-expressing (EV) cells.

To further show that WWOX stabilizes  $\Delta$ Np63 $\alpha$  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  $\Delta$ Np63 $\alpha$ . Since WWOX did not bind TAp63 $\alpha$ , we examined whether it indeed does not affect its half-life. To this end, levels of TAp63 $\alpha$  in the presence or absence of WWOX and CHX was examined. We found that neither WWOX nor WWOX-Y33R were able to affect TAp63 $\alpha$  stability (data not shown). Altogether, these results suggest that WWOX antagonizes ITCH effect on  $\Delta$ Np63 $\alpha$  and stabilizes its protein levels.

**WWOX sequesters  $\Delta$ Np63 $\alpha$  in the cytoplasm.** The results obtained above led us to question the significance of the interaction between WWOX and  $\Delta$ Np63 $\alpha$ .  $\Delta$ Np63 $\alpha$  is a transcription factor that localizes in the nucleus, where it binds DNA and transactivates target genes such as K14, and BPAG-1.<sup>10</sup> In contrast, WWOX is predominantly known as a cytoplasmic protein,<sup>1,2</sup> though some reports demonstrate nuclear localization under certain conditions.<sup>24</sup> Thus,

we asked the question about the possibility of whether WWOX can affect  $\Delta$ Np63 $\alpha$  localization or vice versa. To answer this, we studied the localization of  $\Delta$ Np63 $\alpha$  by subcellular fractionation. We transfected HEK293 cells with  $\Delta$ Np63 $\alpha$  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  $\Delta$ Np63 $\alpha$  alone localizes mainly in the nuclear fraction, coexpression of  $\Delta$ Np63 $\alpha$  and WWOX was associated with increased  $\Delta$ Np63 $\alpha$  presence in the cytoplasm concomitant with less nuclear  $\Delta$ Np63 $\alpha$  levels (Figure 4a). To further confirm this finding, we tested the distribution of  $\Delta$ Np63 $\alpha$  using immunofluorescence and confocal microscopy. HeLa cells were transiently cotransfected with GFP-WWOX and HA- $\Delta$ Np63 $\alpha$ . 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  $\Delta$ Np63 $\alpha$  is mainly localized in the nucleus while WWOX is predominantly cytoplasmic. However, when WWOX is coexpressed with  $\Delta$ Np63 $\alpha$ , 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  $\Delta$ Np63 $\alpha$ . Also, in these cells WWOX colocalization with  $\Delta$ Np63 $\alpha$  lead to its sequestration in the cytoplasm (Figure 4c, arrow heads). Interestingly, cells displaying nuclear  $\Delta$ Np63 $\alpha$  have significantly reduced expression of WWOX (Figure 4b and c), which might explain this partial sequestration of  $\Delta$ Np63 $\alpha$ . However, we cannot exclude that there might be other factor(s) regulating  $\Delta$ Np63 $\alpha$  sequestration into the cytoplasm. Altogether, our results



**Figure 2** WWOX via its WW1 domain interacts with  $\Delta$ Np63 $\alpha$ . **(a)** HEK293 cells were cotransfected with plasmids encoding HA- $\Delta$ Np63 $\alpha$  and Myc-WWOX or Myc-WWOX-Y33R. After 24 h, cells were lysed and immunoprecipitation was performed as follows: lanes 2 and 5: anti-HA; lanes 3 and 6: anti-IgG; and lanes 4 and 7: anti-Myc antibodies. Immunoblotting was done using anti-HA-HRP or Myc-HRP antibodies. Lanes 1 and 8 show 2.5% of input of each lysate. **(b)** HEK293T cells were transiently cotransfected with the expression plasmid encoding HA- $\Delta$ Np63 and mammalian GST-WW1 or GST-WW2 or GST-WW1+2 or GST-SDR or GST-WWOX expression vectors. At 24 h after transfection, whole-cell lysates were prepared and complexes were captured with glutathione-sepharose, and bound protein was detected by HA immunoblot. **(c)** HEK293T cells were transiently cotransfected with the expression plasmid encoding GST-WWOX and wt  $\Delta$ Np63 $\alpha$  or PY mutant HA- $\Delta$ Np63 $\alpha$  (AAxA). At 24 h after transfection, whole-cell lysates were prepared and complexes were captured with glutathione-sepharose, and bound protein was detected by HA immunoblot

suggest that WWOX binds  $\Delta$ Np63 $\alpha$  in the cytoplasm and prevents its translocation to the nucleus.

#### WWOX suppresses $\Delta$ Np63 $\alpha$ transactivation ability.

Since our above results demonstrate that WWOX sequesters  $\Delta$ Np63 $\alpha$  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  $\Delta$ Np63 $\alpha$  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  $\Delta$ Np63 $\alpha$  had

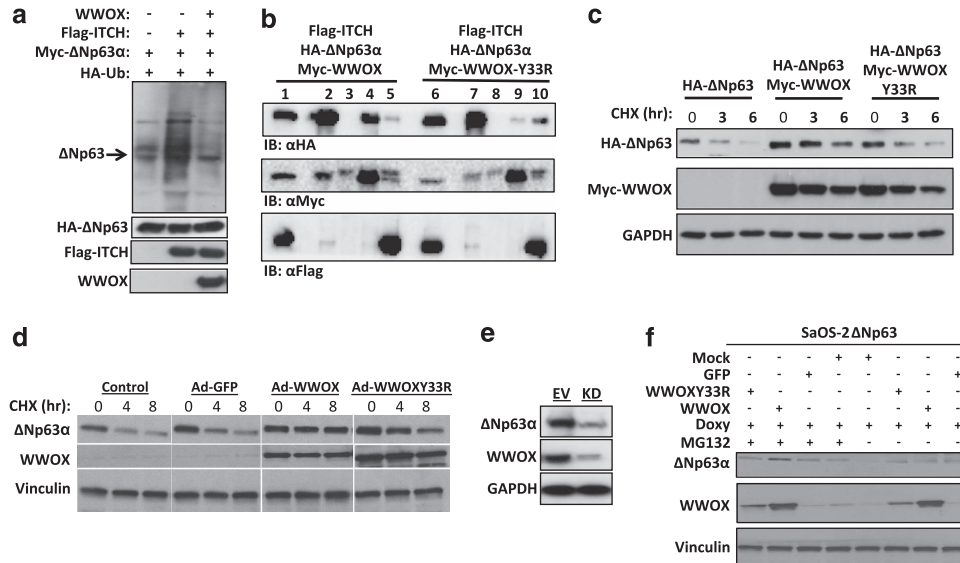
significant transactivation (Figure 5a). By contrast, coexpression of WWOX with  $\Delta$ Np63 $\alpha$  significantly suppressed  $\Delta$ Np63 $\alpha$  transactivation function in a dose-dependent manner (Figure 5a). This effect was significantly attenuated when expressing WWOX-Y33R (Figure 5b and c). Cumulatively, our findings suggest that WWOX sequesters  $\Delta$ Np63 $\alpha$  in the cytoplasm, and this is associated with its reduced transactivation function.

#### WWOX antagonizes $\Delta$ Np63 $\alpha$ -induced chemoresistance.

$\Delta$ Np63 $\alpha$  was shown to play a crucial role in determining cellular chemosensitivity,<sup>18,20,25</sup> while WWOX was shown to promote apoptosis in certain contexts.<sup>1,2</sup> Considering these findings, our results here prompted us to examine whether WWOX affects  $\Delta$ Np63 $\alpha$  function in chemosensitivity, that is, upon treatment of cisplatin. To test this, inducible- $\Delta$ Np63 $\alpha$  SaOS2 cells were stably transduced with either WWOX or empty lentiviral vectors. Successful expression of WWOX and  $\Delta$ Np63 $\alpha$ , following doxycycline (Dox) treatment, is shown in Figure 6a. SaOS2 cells expressing  $\Delta$ Np63 $\alpha$ , 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  $\Delta$ Np63 $\alpha$ -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  $\Delta$ Np63 $\alpha$  and WWOX significantly sensitized cisplatin-treated cells to undergo cell death/apoptosis (~14-folds) as compared with SaOS2-expressing  $\Delta$ Np63 $\alpha$  alone (Figure 6b). Similar results were obtained by examining percentage of sub-G1 population (Figure 6c). Although these observations suggest that WWOX antagonizes  $\Delta$ Np63 $\alpha$ -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<sup>26-28</sup> and could enhance cisplatin effect in cancer cells.<sup>26-29</sup> 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.<sup>26-28</sup> 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 has a synergistic effect on cell death in agreement with previously published data.<sup>26-29</sup> Taken together, our findings suggest that WWOX attenuates  $\Delta$ Np63 $\alpha$ -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



**Figure 3** WWOX inhibits ITCH-mediated ubiquitination of  $\Delta$ Np63 and increases its half-life. (a) HEK293 cells were transfected with the indicated plasmids. After 24 h, cells were treated with 20  $\mu$ M MG132 for 4 h. Lysate was prepared and IP with anti-Myc ( $\Delta$ Np63 $\alpha$ ) and detected with anti-HA antibodies (UB). (b) HEK293 cells were cotransfected with plasmids encoding HA- $\Delta$ Np63 $\alpha$  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 anti-HA-HRP, anti-Myc\_HRP, or anti-Flag-HRP antibodies. Lanes 1 and 6 show 2.5% of input of each lysate. A higher band in the IgG (lanes #3 and 8) and Flag (lanes #5 and 10) is observed in the anti-Myc blot (middle) likely due to antibody non-specificity. (c) HEK293 cells were cotransfected with WWOX and  $\Delta$ Np63 $\alpha$ . After 24 h, cells were treated with 20  $\mu$ g/ml CHX at the indicated time points and analyzed as shown. (d) Tet-On-inducible SaOS2 cells were infected with 10 MOI of Ad5-WWOX for 24 h then induced with doxycycline for additional 24. Cells were treated with CHX as indicated and blotted with anti-HA or anti-WWOX. Vinculin was used as loading control. (e) HaCaT cells were transduced with lentiviral-vector of WWOX (KD) or scramble shRNA (EV) constructs. Lysates were analyzed 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

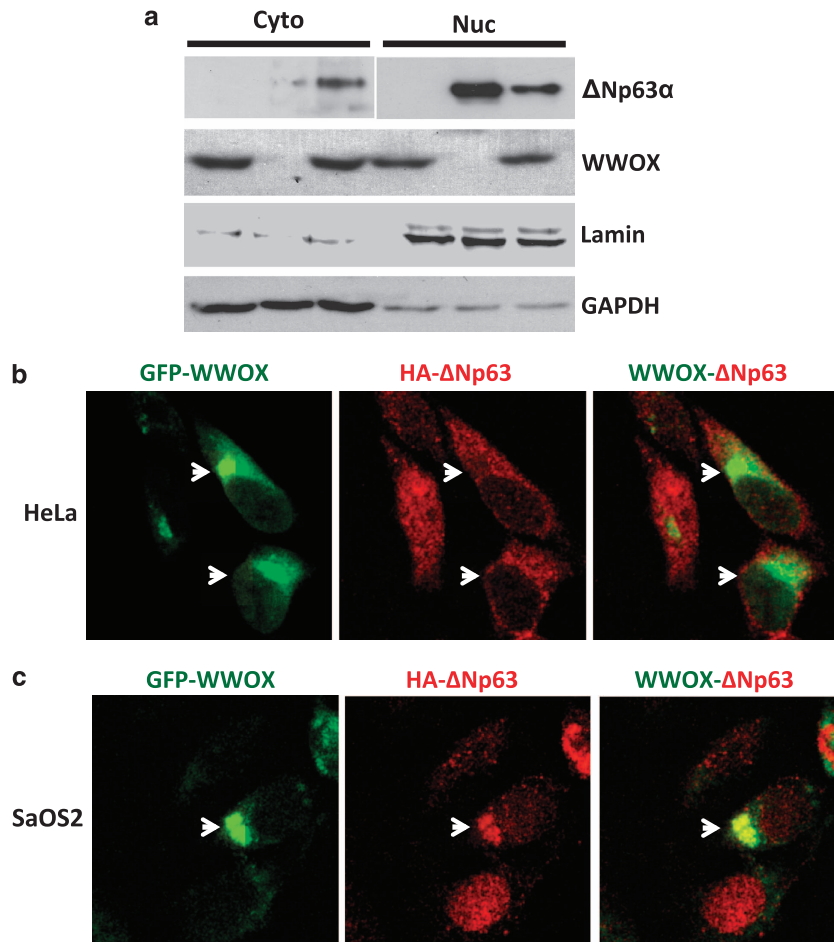
of proteins play very important roles in cell differentiation stemness and plasticity,<sup>30–33</sup> in immune response regulation,<sup>34</sup> in tumorigenesis and tumor suppression,<sup>33,35–38</sup> in development and reproduction,<sup>39,40</sup> DNA damage,<sup>41</sup> and apoptosis and cell-cycle regulation.<sup>33,42,43</sup> In addition to regulating each other's function,<sup>37,44,45</sup> the functional outcome of these proteins are regulated by different mechanisms, including miRNAs, post-translational modifications, and protein–protein interactions.<sup>43,46–48</sup> Of particular interest, the WW domain-containing proteins, including WWOX, YAP, and ITCH, were shown to regulate p73 and p63 functional outcome.<sup>21,23,49–52</sup> Here, we further show that WWOX, via its WW1 domain, associates with  $\Delta$ Np63 $\alpha$  and antagonizes the ubiquitin E3 ligase ITCH-mediated ubiquitination and degradation of  $\Delta$ Np63 $\alpha$ . Importantly, we found that WWOX suppresses  $\Delta$ Np63 $\alpha$  transactivation function perhaps through sequestering it in the cytoplasm. Consistent with these findings, WWOX enhances chemosensitivity to cisplatin in SaOS2 cells expressing  $\Delta$ Np63 $\alpha$ . Together, these findings argue for an unforeseen functional crosstalk between WWOX and  $\Delta$ Np63 $\alpha$ .

Previous characterization of WWOX partners revealed its WW domains interaction with PPxY-containing proteins.<sup>1,2</sup> Nevertheless, other non-PPxY members were also reported including Hyaluronidase,<sup>53</sup> Jnk1,<sup>54</sup> Tau,<sup>55,56</sup> and Mdm2.<sup>24</sup> Although WW1 domain of WWOX mediates this interaction, our results demonstrate that this is not mediated by the PPxY motif of  $\Delta$ Np63 $\alpha$ . These data might suggest that WW1 domain of WWOX might interact with other proline-rich motifs rather than the canonical PPxY (PY) motif. In fact, it was shown

recently that classical WW domains, known to interact with canonical PY motifs, could also bind non-canonical pSP or pTP motifs highlighting the plasticity of WW domains interactions.<sup>57</sup> Further research would be necessary to decipher and characterize these motifs.

The E3 ubiquitin ligase ITCH binds, ubiquitinates, and promotes the degradation of  $\Delta$ Np63 $\alpha$ , and a single amino-acid substitution in the PY domain of  $\Delta$ Np63 (Y449F) was shown to be sufficient to reduce its ability to interact with ITCH WW domains. Here, we show that WWOX competes with ITCH on binding to  $\Delta$ Np63 $\alpha$  and inhibits  $\Delta$ Np63 $\alpha$  ubiquitination mediated by ITCH and thus increases  $\Delta$ Np63 $\alpha$  protein stability. In support of this, expression of point mutant WWOX-Y33R resulted in significant rescue of ITCH- $\Delta$ Np63 $\alpha$  interaction. In fact, we have previously shown that WWOX can 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.<sup>49</sup> Altogether, these findings argue that WW domain proteins could compete with each others to determine functional outcome of their common PY-containing targets.

For a transcription factor to be functional, it is not enough to be expressed, but it has to have the optimal localization. Thus, although WWOX stabilizes  $\Delta$ Np63 $\alpha$  protein, it inhibits its transcriptional transactivation function, in part, by sequestering it in the cytoplasm. Another possibility by which WWOX might antagonize  $\Delta$ Np63 $\alpha$  is by inhibiting its dominant-negative effect on the proapoptotic p63 isoform TAp63.  $\Delta$ Np63 $\alpha$  inhibits the transcriptional activity of TAp63 by competition for the same responsive elements or by



**Figure 4** WWOX overexpression sequesters  $\Delta$ Np63 $\alpha$ . (a) HEK293 cells were transfected with either WWOX alone (left lane),  $\Delta$ Np63 $\alpha$  alone (middle lane), or WWOX and  $\Delta$ Np63 $\alpha$  together (right lane). After 24 h, cells were lysed into cytoplasmic and nuclear fractions as indicated. Lamin and GAPDH were used as nuclear and cytoplasmic fraction markers, respectively. (b) HeLa cells were transfected with GFP-WWOX and HA- $\Delta$ Np63 $\alpha$ . After 24 h, cells were fixed and stained using anti HA antibody. Immunofluorescence staining was analyzed using confocal microscopy. (c) SaOS2 cells were transfected with GFP-WWOX and HA- $\Delta$ Np63 $\alpha$ . After 24 h, cells were fixed and stained using anti HA antibody. Immunofluorescence staining was analyzed using confocal microscopy

sequestering TAp63 in inactive hetero-tetramers ( $\Delta$ Np63 $\alpha$ -TAp63 tetramers).<sup>58</sup> Therefore, it is possible that WWOX might disrupt the formation of these inactive hetero-tetramers, and by this, inhibits  $\Delta$ Np63 $\alpha$  functions and relieves the inhibitory effect on TAp63. Of note, our data also indicate that WWOX selectively binds  $\Delta$ Np63 $\alpha$ , but not TAp63 $\alpha$ , perhaps due to conformational elements that are not yet resolved.

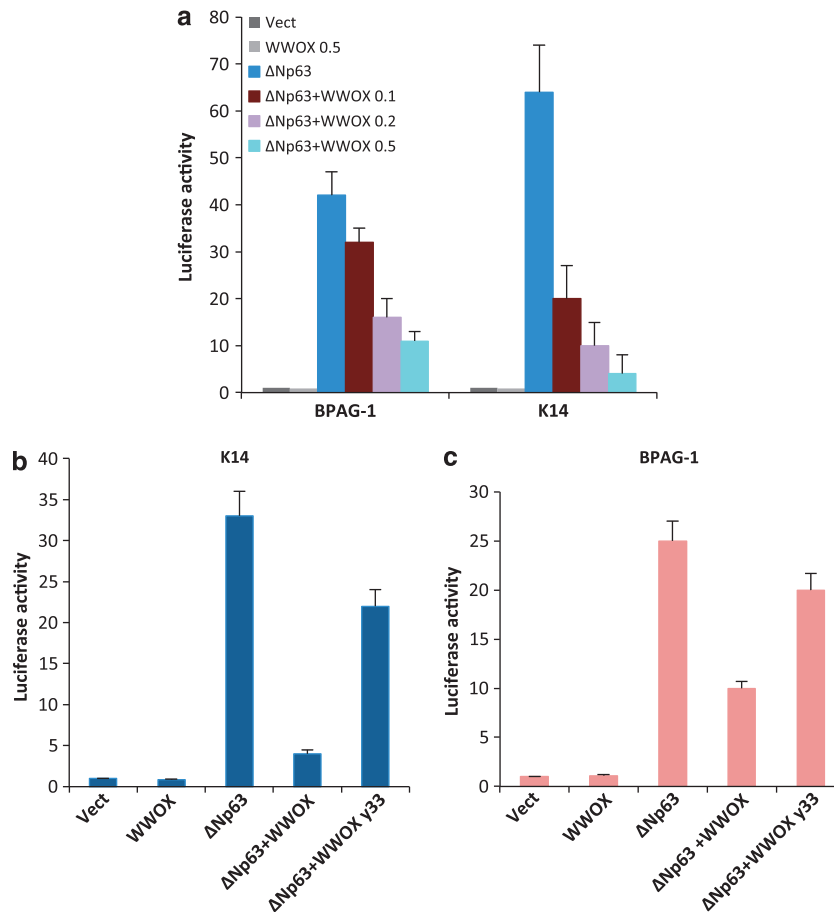
Recent evidence shows that p63 plays an important role in conferring either chemoresistance or chemosensitivity. While TAp63 correlates with and induces chemosensitivity,<sup>59–61</sup>  $\Delta$ Np63 $\alpha$  expression directly correlates with a poor clinical response to cisplatin in HNSCC<sup>18</sup> and leads to chemoresistance by different mechanisms.<sup>19,20</sup> Recently, it has been shown that the chemotherapeutic agents cisplatin, by inducing c-Abl, increases  $\Delta$ Np63 $\alpha$  phosphorylation and interaction with YAP leading to  $\Delta$ Np63 $\alpha$  stabilization and resistance to death.<sup>25</sup> We show here that WWOX also binds to  $\Delta$ Np63 $\alpha$ , stabilizes its protein, however, inhibits its function and leads to more cisplatin sensitivity. These data might suggest that WW domain interactions might regulate  $\Delta$ Np63 $\alpha$  functional outcome.

The role of  $\Delta$ Np63 in cancer seems to be controversial. While some evidence suggests that  $\Delta$ Np63 acts as a guardian against tumor migration and metastasis,<sup>37,62,63</sup> others suggest that  $\Delta$ Np63 correlates with more proliferative and stem cell like cancer cell phenotypes, acts as a pro-inflammatory factor, and correlates with poor survival.<sup>63–66</sup> 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  $\Delta$ Np63 $\alpha$  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,  $\Delta$ Np63 $\alpha$ -tet-ON SaOS2 in RPMI. All cells were supplemented with 10% FBS (Gibco, Grand Island, NY, USA), glutamine, and



**Figure 5** WWOX suppresses  $\Delta$ Np63 transactivation function. (a) HEK293T cells were transiently cotransfected with the luciferase reporter construct carrying the  $\Delta$ Np63 $\alpha$  responsive element derived from the promoters of K-14 and BPAG-1 in addition to either WWOX alone,  $\Delta$ Np63 $\alpha$  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  $\mu$ g) and 0.1  $\mu$ g  $\Delta$ Np63 $\alpha$ . Cells were analyzed as in (a)

penicillin/streptomycin (Biological Industries, Beit-Haemek, Israel). To induce the expression of  $\Delta$ Np63 $\alpha$ , SaOS2 cells were treated with 2  $\mu$ g/ml doxycycline (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.<sup>21</sup>

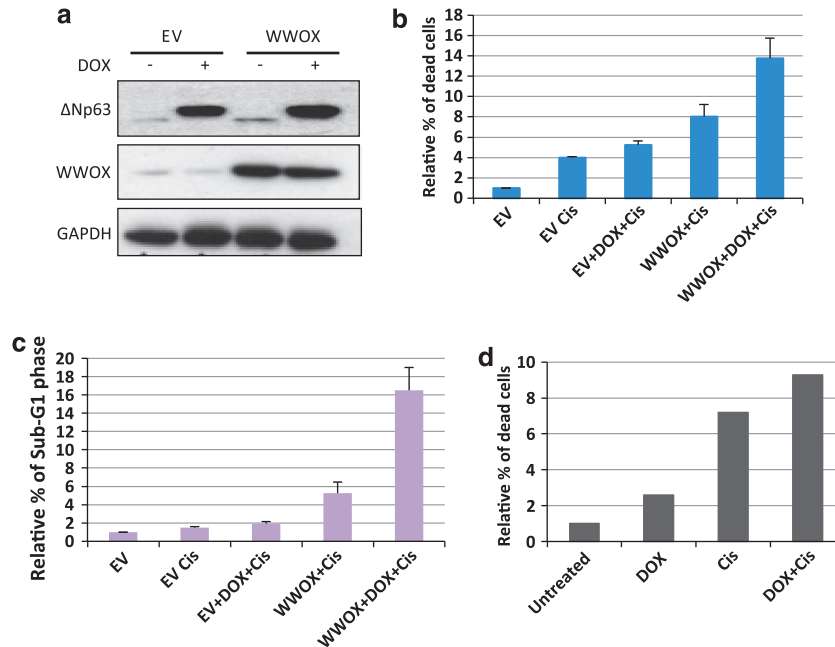
**Luciferase assay.** HEK293 cells seeded in 12-well plates were cotransfected with the relevant plasmids together with different plasmids containing different

$\Delta$ Np63 $\alpha$  responsive elements of various  $\Delta$ Np63 $\alpha$  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 reconstituted 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- $\Delta$ Np63 $\alpha$  with or without Flag-ITCH or WWOX as indicated in Figure 3a. After 24 h, cells were treated with MG-132 (Sigma; 20  $\mu$ mol/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  $\Delta$ Np63 protein level.** HEK293T cells were transfected with  $\Delta$ Np63 $\alpha$  with or without



**Figure 6** Coexpression of WWOX and  $\Delta$ Np63 $\alpha$  suppresses  $\Delta$ Np63-induced resistance to chemotherapy. (a) SaOS2- $\Delta$ Np63 $\alpha$ -tetOn cells were transduced with Lenti-WWOX or Lenti-EV expression vector and stable cells were generated. Treatment of these cells with 2  $\mu$ g/ml doxycyclin (Dox) for 48 h was performed to induce  $\Delta$ Np63 $\alpha$  expression. Immunoblot analysis revealed expression of WWOX and  $\Delta$ Np63 $\alpha$  using anti-WWOX and anti-HA HRP, respectively. (b, c) Cells from (a) were treated with 2  $\mu$ g/ml Dox for 48 h followed by treatment with 40  $\mu$ M 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  $\mu$ g/ml Dox for 48 h followed by treatment with 40  $\mu$ M Cisplatin (Cis) for an additional 48 h. Columns represent the relative percentage of dead cells determined by trypan blue

Myc-WWOX. At 24 h post-transfection, cells were lysed or treated with the protein synthesis inhibitor CHX (Sigma-Aldrich; 100  $\mu$ g/ml) for 3 and 6 h. Cell lysates were subjected to IB as indicated.

**Immunofluorescence.** Cells were seeded on round slide-cover slips in 12-well plates. After 24 h, cells were transfected with the expression plasmids. At 24 h post-transfection, cells were fixed in 3.7% PBS-buffered formaldehyde, permeabilized with 0.05% Triton X-100 at room temperature. Cells were then incubated for 1 h in 10% goat serum (Invitrogen, Carlsbad, CA, USA), with primary antibody for 1 h and for 1 h with secondary antibody. Anti-mouse Texas red-conjugated antibody-647 (Molecular Probes, Carlsbad, CA, USA) was used to detect  $\Delta$ Np63 $\alpha$ . Cells were examined by confocal microscopy (Olympus, Tokyo, Japan) under 60 $\times$  magnification.

### Conflict of Interest

The authors declare no conflict of interest.

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