A novel regulator of the p53-mediated mitochondrial apoptotic pathway

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Running Title: Tid1 is a novel regulator of p53-mediated mitochondrial apoptotic pathway

Abstract

The p53 tumor suppressor protein induces apoptosis in response to genotoxic and environmental stress. Recent studies have revealed the existence of a transcription-independent mitochondrial p53 apoptosis pathway, however the mechanism regulating p53 translocation to mitochondria and subsequent initiation of apoptosis was not known. Here, we show that Tid1, also known as mtHsp40 or Dnaja3, interacts with p53 and directs its translocation to mitochondria in cells exposed to hypoxia. Overexpression of Tid1 in tumor cells promoted mitochondrial localization of both wildtype and mutant forms of p53 and was able to restore the pro-apoptotic activity of mutant p53 proteins that were otherwise unable to induce apoptosis. Tid1's mitochondrial signal sequence and DnaJ domain were both required for the movement of the p53-Tid1 complex from the cytosol to the mitochondria. Our findings establish Tid1 as a novel regulator of p53 localization and apoptotic function.

Apoptosis is an intrinsic cellular response to specific environmental stresses and occurs via p53-dependent and p53-independent mechanisms[1,2]. Cells respond to hypoxia, DNA damage and oncogene deregulation by activating the p53-mediated cell death pathway. These stress stimuli rapidly lead to elevated cellular levels of p53 as p53 dissociates from MDM2, which normally targets its degradation[3-5]. While some p53 protein remains in the nucleus, a fraction also accumulates in the mitochondria, where it directly initiates an apoptotic program[6-8]. At the mitochondria, p53 forms complexes with members of the Bcl-2 protein family, which regulate the integrity of the mitochondrial

membrane[9-11]. Mitochondrial p53 shifts the balance between anti-apoptotic and pro-apoptotic Bcl-2 family members, leading to release of cytochrome *c* into the cytoplasm, and initiation of the caspase proteolytic cascade, eventually resulting in cell death[12, 13]. p53's ability to induce apoptosis at the mitochondria is independent of its transcriptional activity, as mutant p53 that lacks DNA binding capacity still retains the ability to induce apoptosis[14, 15]. Additionally, transfection of p53-null cells with mitochondrially targeted p53 proteins has been shown to induce apoptosis[16, 17]. Functional analysis of two common polymorphic variants of human p53, Arg72 and Pro72, has shown that the Arg72 variant not only induces apoptosis much more efficiently than the Pro72 variant, but is also localized to the mitochondria with greater efficiency[18].

Although these findings clearly demonstrate that p53-mediated cell death occurs when p53 is localized in the mitochondria, the mechanisms involved in regulating the intracellular trafficking of p53 between the cytosol, the nucleus, and the mitochondria remain unclear. Chaperone proteins are suggested to stabilize and assist in the transport of p53 to the mitochondria. Mitochondrial Hsp40 (mtHsp40), also known as Tid1, contains a mitochondrial signal sequence in its amino-terminus and resides mainly in the mitochondria[19]. Prior to translocation into the mitochondria, Tid1 is retained in the cytosol, allowing for its interaction with cytosolic proteins. Since Tid1 also associates with Hsp70 and Hsp90[20], which are known to form complexes with p53[21], it is possible that Tid1 interacts directly or indirectly with p53. Observations that Tid1 functions both inside and outside of the mitochondria prompted us to address the possibility that Tid1 is involved in p53 trafficking. We have previously shown in breast cancer cell lines that Tid1 functions as a cochaperone with Hsp70, and that this interaction is essential for

increasing proteasomal degradation of ErbB2 via the ubiquitination pathway[22]. Here, we hypothesized that by transporting p53 to the mitochondria, Tid1 acts as a critical regulator of p53-mediated apoptosis in response to environmental stresses. We found that Tid1 interacts with p53 and Hsp70 to form a multi-subunit complex that is responsible for guiding both mutant and wildtype p53 to mitochondria during initiation of the mitochondrial apoptotic pathway.

Results

Hypoxia induces p53 translocation to the mitochondria where it interacts with Tid1 and mtHsp70

To determine whether p53 and Tid1 interact in the mitochondria under cellular stress, we subjected MCF-7 human breast cancer cells, which express wildtype p53, to oxidative stress and performed immunostaining to look for evidence of colocalization. We chose to mimic hypoxia in these experiments, as hypoxic conditions frequently exist within solid tumors[23], and thus apoptosis induced by hypoxic stress reflects the biology of solid tumors *in vivo.* Desferroxamine (DFX) is an iron chelator that mimics hypoxia by enhancing the accumulation of hypoxia-inducible factor 1 α (HIF1 α), which stimulates the transcription of hypoxia associated genes[24]. DFX upregulates p53 expression and induces rapid translocation of p53 into the mitochondria[10, 25]. Without DFX treatment, colocalization of p53 and Tid1 was rarely visualized due to the low levels of p53 in MCF-7 cells (Fig. 1a, upper panel). However, when cells were treated with DFX, p53 levels increased (Fig. 1b), and p53 co-localized with both Tid1 and Mitotracker (Fig. 1a, bottom panel). Similar results were observed with cells exposed to hypoxic stress using

a gas pack pouch system, and cells treated with camptothecin to cause genotoxic stress (Suppl. Fig. 1). In contrast to altered p53 protein levels, immunoblotting of whole cell lysates demonstrated that DFX treatment had no effect on the protein levels of Tid1 and mtHsp70 (Fig. 1b). The two bands observed in the Tid1 immunoblot represent alternatively spliced isoforms of Tid1 expressed in human cells: the long form: $Tid1_L$ (43 kDa), and the short form, $Tid1_S$ (40 kDa) [19, 26].

To confirm the interaction of p53 and Tid1 using a biochemical approach, MCF-7 cells subjected to hypoxic stress were lysed and p53, Tid1, and mtHsp70 were immunoprecipitated from separate aliquots of whole cell lysates. Immunoblotting was performed using the indicated antibodies (Fig. 1c). Consistent with Figure 1a, these experiments confirmed the presence of an interaction between Tid1 and p53, as Tid1 reciprocally co-immunoprecipitated with p53 in both the presence and absence of DFX. Also consistent with previous reports describing an interaction between p53, Tid1, and mtHsp70[20, 21], p53 and Tid1 were present in anti-mtHsp70 immunoprecipitates. In addition, untreated and DFX-treated MCF-7 cells were separated into nuclear, cytoplasmic and mitochondrial fractions to determine the subcellular distribution of p53 and Tid1. In untreated cells, p53 levels were undetectable by immunoblotting, whereas Tid1 was detected only in mitochondrial fractions. Following DFX treatment, p53 levels increased and predominantly localized to mitochondria, although p53 was also found in the cytoplasm and nucleus in smaller amounts. Likewise, in DFX-treated cells, a majority of the Tid1 was found in the mitochondria, although smaller amounts of Tid1 protein were detected in cytosolic fractions (Fig. 1d, two upper panels). In addition to the two lower molecular weight bands attributable to the processed $Tid1_L$ and $Tid1_S$

isoforms, a third higher molecular weight band was detected in the cytosolic fraction representing the full-length, unprocessed precursor form of the Tid1 proteins (Tid1-UP). The precursor form of Tid1 contains an amino-terminal presequence that directs its post-translational import into the mitochondrial matrix, where the targeting sequence is cleaved by the mitochondrial processing peptidase, generating the mature Tid1 protein (Tid1-P)[19, 27]. Under normal conditions, the precursor form of Tid1 is undetectable in total cell extracts by immunoblotting due to the small amount of precursor form, but when Tid1 is overexpressed or purified from the cytosolic fraction, this form becomes detectable. Immunoprecipitation of p53 from the different cellular fractions revealed that Tid1 formed a complex with p53 in both the cytosol and mitochondria (Fig. 1d). Collectively these data demonstrate that p53 interacts with Tid1 in the cytoplasm and mitochondria in response to hypoxic stress.

Tid1 regulates p53 translocation into the mitochondria in response to cellular stress

Phosphorylation/acetylation modifications of p53 do not appear to be involved in its mitochondrial targeting, and p53 itself lacks a mitochondrial translocation motif[28, 29]. We hypothesized that a mitochondrial trafficking factor that associates with p53 must be responsible for its translocation. Because Tid1 interacts with p53 in both the cytoplasm and mitochondria, we decided to investigate whether Tid1 directly modulates p53 translocation to the mitochondria under hypoxic stress. To do this, we examined the effect of overexpressing both isoforms of Tid1 on p53 localization in DFX-treated MCF-7 cells through immunostaining. MCF-7 cells were infected with adenoviral Tid1

constructs to overexpress Tid-1_S (Ad-Tid1_S) or Tid-1_L (Ad-Tid1_L). Infected cells (yellow arrows) were differentiated from uninfected cells (white arrows) on the basis of GFP expression, as the Tid1 expression vectors used were bicistronic and also contained an expression cassette for GFP (Suppl. Fig. 2). In infected cells overexpressing either Tid1_L or Tid1_S, p53 predominantly localized to the mitochondria (Fig. 2c). In contrast, in uninfected, mock and Ad-Scramble infected cells, p53 localized to the mitochondria, cytoplasm and nucleus. These results suggest that Tid1 promotes mitochondrial trafficking of p53.

To further assess the functional role of Tid1 in hypoxia-induced translocation of p53 to the mitochondria, we employed a Tid1 shRNA construct, Ad-shTid1, to suppress the expression of Tid1 (Fig. 2d). Infection with Ad-shTid1 resulted in over 90% suppression of Tid1 expression in MCF-7 cells, compared to Ad-Scramble infected cells (Fig. 2e). Loss of Tid1 inhibited mitochondrial localization of p53 and surprisingly, led instead to an accumulation of p53 in the nucleus (Fig. 2f bottom row of panels). However, no difference in the transcriptional activity of p53 in control versus Tid1 knockdown cells was observed (Suppl. Fig. 3). At present, it is unclear why p53 localizes to the nucleus in Tid1 knockdown cells rather than remaining in the cytoplasm, and we are actively investigating possible mechanisms for this observation. Overall, the observation that Tid1 interacts with p53 (Fig. 1), and that changes in Tid1 expression levels significantly alter the subcellular distribution of p53 (Fig. 2) strongly suggest that Tid1 functions as a mitochondrial trafficking factor, or at least as a cytosolic retention factor for p53.

Overexpression of Tid1 enhances p53-dependent apoptosis

To elucidate the functional significance of Tid1-mediated trafficking of p53 to the mitochondria, we investigated whether Tid1 knockdown or overexpression directly inhibited or promoted mitochondrial apoptosis in response to hypoxia. As shown in Fig. 3a, knocking down Tid1 expression significantly reduced apoptosis (~10% apoptotic cells) compared to Ad-Scramble infected cells (~28% apoptotic cells), confirming that Tid1 expression is required for mitochondrial apoptosis to proceed following DFX treatment. Moreover, cells overexpressing either Ad-Tid1$_S$ or Ad-Tid1$_L$ consistently exhibited higher levels of apoptosis (~55% apoptotic cells; Fig. 3b) than control cells. This data is in agreement with the immunostaining data (Fig 2) illustrating the dependence of p53 mitochondrial localization on Tid1 expression, and strongly suggests that localization of p53 to mitochondria is required for hypoxia-induced apoptosis, and that this process is dependent on Tid1.

While hypoxic stress initiates apoptosis at the level of the mitochondria via the intrinsic pathway, mitochondrial cell death can also be stimulated by external stimuli, such as TNF or the Fas ligand[30]. Activation of the extrinsic pathway signals cell death through activation of caspase 8 and is p53-independent; in contrast, the intrinsic pathway is initiated at the mitochondria by the release of cytochrome *c*, which binds to caspase 9 to form the apoptosome[8]. Formation of the apoptosome leads to autoproteolytic processing and activation of caspase 9, and eventually other downstream caspases such as caspase 3, 6 and 7[31, 32]. Recent studies have demonstrated that MCF-7 lacks procaspase 3, however other caspases, such as caspase 6 or 7, can compensate in its absence[33, 34], thus, MCF-7 cells can still undergo

apoptosis through the intrinsic pathway. To verify that Tid1 regulated hypoxia-induced cell death through the intrinsic p53-mediated apoptotic pathway rather than through the p53-independent extrinsic pathway, two different apoptotic inhibitors, a caspase-8 inhibitor (Z-IETD-FMK)[35] and Bax-inhibitor peptide (BIP)[36] (which inhibit the extrinsic and intrinsic apoptosis pathways respectively) were tested to confirm which cell death pathway was activated in response to hypoxia. As summarized in Fig. 3c, pre-treatment of cells with BIP led to a significant decrease in amount of apoptosis observed in DFX treated MCF-7 cells in both the presence and absence of Tid1 overexpression. In contrast, pre-treatment of cells with the caspase-8 inhibitor did not have any appreciable effects on the degree of apoptosis observed following DFX treatment in any of the cell lines examined. Overall, these results demonstrate that Tid1 regulates hypoxia-induced apoptosis through its effects on the p53-dependent intrinsic mitochondrial cell death pathway.

Overexpression of Tid1 rescues the apoptotic activity of mutant p53

Tid1 itself is a tumor suppressor protein, and may exert its tumor suppressor activity by functioning as a cell death regulator[28, 37, 38]. To examine the physiological significance of Tid1 in p53-mediated apoptosis induced by hypoxic stress, we examined the effects of Tid1 overexpression on hypoxia-induced apoptosis in tumor cells lacking p53, and in cells expressing mutant forms of p53. HCT116 colon carcinoma cells that either lacked p53 ($p53^{-/-}$) or that expressed wildtype ($p53^{wt}$) were infected with either Ad-$Tid1_S$ or Ad-Scramble. As expected, no endogenous p53 in HCT116 $p53^{-/-}$ cells was detectable before or after DFX treatment, whereas the level of p53 in HCT116 $p53^{wt}$

cells strikingly increased following DFX treatment (Fig. 4a). Next, we examined the effect of overexpressing Tid1 on mitochondrial p53-mediated apoptosis. Ad-Scramble or Ad-Tid1$_S$-infected HCT116 p53^{wt} cells exhibited apoptosis following DFX treatment (Fig. 4b). In contrast, no p53 protein or increase in apoptosis were observed even after DFX treatment in HCT116 p53$^{-/-}$ cells. These results are consistent with the data from MCF-7 cells (Fig 1, 2) and confirm that Tid1 alone is insufficient to induce cell death and that p53 is required for this process. Furthermore, these results indicate that Tid1-mediated regulation of the p53-dependent mitochondrial apoptotic pathway is a previously unidentified mechanism of Tid1 tumor suppression.

The question of whether or not mutant forms of p53 can induce apoptosis remains controversial. One study reported that p53 mutants, unlike wildtype p53, were unable to form complexes with Bcl-2 in human cancer cells, were compromised in mediating outer mitochondrial membrane permeabilization and failed to undergo apoptosis[39]. In contrast, others have found that cells harboring the R273H, Gln22, Ser23, or various other p53 mutants can still undergo apoptosis[40, 41]. It is possible that mutant p53 can mediate apoptosis at the mitochondria through additional Bcl-2-independent mechanisms; for example, mutant p53 also directly binds with other pro-apoptotic proteins such as BAX in response to cellular stress[42]. We investigated whether or not Tid1 was still able to interact with mutant p53 and direct its translocation to the mitochondria by co-infecting HCT116 p53$^{-/-}$ cells with Ad-p53^{wt} or Ad-p53$^{mt\ -121F}$ and Ad-Tid1 or Ad-Scramble. p53$^{mt-121F}$ contains a mutated DNA binding domain, and has been used in previous studies of p53-mediated apoptosis[43]. When the extent of hypoxia-induced apoptosis was assessed in these cells, we found that p53-mediated

apoptosis was significantly rescued in $p53^{mt\text{-}121F}$ infected cells overexpressing Tid1 (Fig. 4c), with similar levels of apoptosis as cells expressing $p53^{wt}$. In addition, the levels of mutant or wildtype p53 and Tid1 that colocalized in the mitochondria correlated positively with the amount of apoptosis observed in the cells. As shown in Fig. 4d, in sharp contrast to Ad-Scramble infected cells, in Ad-Tid1-infected cells both wildtype and mutant $p53^{mt\text{-}121F}$ were located in the mitochondria. Taken together, these results indicate that overexpression of Tid1 can restore the apoptotic function of mutant p53 by facilitating its translocation to the mitochondria during hypoxia.

Overexpression of Tid1 in p53 mutant cancer cell lines restores the pro-apoptotic ability of p53 mutants

To further confirm the functional role of Tid1 in p53-mediated apoptosis, we tested whether overexpression of Tid1 modulates the subcellular localization of p53 in other tumor cell lines, including the breast cancer cell lines, T47D ($p53^{mt\text{-}L194F}$), SK-BR-3 ($p53^{mt\text{-}R175H}$), and BT474 ($p53^{mt\text{-}E285K}$)[44], and the glioma cell lines, U87 ($p53^{wt}$), and U373 ($p53^{mt\text{-}R273H}$)[45]. As indicated in Fig. 5a, the breast cancer and glioma cells expressed high levels of several different forms of mutant p53, which have been shown to lack transcriptional activity[44, 45]. We observed that Tid1 protein levels in p53 mutant cancer cell lines were substantially lower than the protein levels observed in p53 wildtype cancer cells (Fig. 5a), correlating with the lower levels of apoptosis observed in response to hypoxia. To evaluate the effect of Tid1 expression levels, each cell line was infected with Ad-$Tid1_S$ (Fig. 5b). As shown in Fig.5c, cancer cells with mutant p53 that expressed only endogenous levels of Tid1 (white arrows) showed nuclear localization of

p53, while overexpression of Tid1 in these cells (yellow arrows) caused mutant p53 to move to the mitochondria in the vast majority of infected cells. Furthermore, cells expressing mutant p53 underwent spontaneous apoptosis when Tid1 was overexpressed (Fig. 5d), but did not exhibit significant levels of apoptosis in the absence of Tid1 overexpression or DFX treatment (Suppl. Fig.4). Overall, these results suggest that Tid1 overexpression can restore the p53-mediated apoptotic response in cancer cells with mutant p53.

Cytosolic retention of mutant Tid1 can inhibit p53-mediated apoptosis

Tid1 is comprised of several distinct functional domains: an amino-terminal signal sequence (NT), a DnaJ-domain, a glycine- and phenylalanine rich region (G/F), a cysteine-rich domain (CXXCXGXG), and a carboxy-terminal domain (CT) (Fig. 6a)[21]. We predicted that several of these domains would be required for successful translocation of p53 to mitochondria and induction of apoptosis in response to hypoxia. Like most mitochondrial matrix proteins encoded by nuclear DNA, both $Tid1_L$ and $Tid1_S$ have a predicted mitochondrial processing sequence (LRP-GV) in the NT domain[21], resulting in cleavage at amino acid 66 upon entry into the mitochondria. Hence, loss of the NT domain would be expected to inhibit mitochondrial translocation and apoptosis downstream of p53. In addition, Tid1 is known to interact with mtHsp70 through its conserved DnaJ domain[20], and mtHsp70 interacts with p53 (Fig. 1)Thus, we also predicted that the DnaJ domain of Tid1 would be required for p53 binding and subsequent translocation to the mitochondria during hypoxia. To test these predictions, we used adenoviral constructs to overexpress wildtype or mutant Tid1 (Fig. 6a) in MCF-

7 cells, and examined p53 translocation and apoptosis in response to hypoxia. The expression of wildtype or mutant Tid1 in MCF-7 cells was verified by immunoblotting (Fig. 6b). As expected, both p53 and Tid1 were retained in the cytosol following DFX treatment of cells expressing either the amino-terminal deletion mutant (Ad-ΔNT) or the DnaJ domain mutant (Ad-HQ). In contrast, mitochondrial translocation of p53 occurred as expected in control cells infected with Ad-Scramble or overexpressing wildtype Tid1. In accordance with these results, immunostaining with p53 demonstrated that little, if any, overexpressed Tid1-ΔNT or Tid1-HQ co-localized with Mitotracker (Fig. 6c). Moreover, the retention of p53 in the cytosol significantly decreased the amount of apoptosis observed (compare Ad-Tid1s *vs.* Ad- ΔNT or Ad-HQ in Fig. 6d), confirming that p53 localization to the mitochondria is required for hypoxia-induced apoptosis. In summary, ablation of the mitochondrial targeting sequence or mutation of the DnaJ domain of Tid1 restricted p53 localization to the cytosol, although it had no effect on the expression levels of p53 (Fig. 6b, 6c). These data indicate that both the NT and the DnaJ domain of Tid1 are required for p53-mediated apoptosis and strongly suggest that Tid1 is a key factor in p53 mitochondrial translocation and cell death signaling.

Discussion

Many tumors contain p53 mutations, but whether or not tumor cells harboring mutant p53 have an impaired p53-mediated apoptosis pathway remains controversial. In the nucleus, p53 mediates its cellular functions through its ability to regulate the transcription of multiple target genes, including many that promote either cell cycle arrest or apoptosis[1,2, 32]. However, a growing amount of evidence points to a direct role

for p53 in a transcription-independent mitochondrial apoptosis pathway. For example, several other groups have reported that $p53^{Gln22Ser23}$, which is completely deficient in the ability to activate or repress transcription, can still induce apoptosis[6]. Our studies provide experimental evidence that cancer cells expressing several different p53 mutants have an intact mitochondrial p53-mediated apoptotic pathway, and that successful p53 translocation to the mitochondria is sufficient for apoptosis to occur. By mediating this translocation event, Tid1 expression is able to promote hypoxia-induced apoptosis in cancer cells expressing both mutant and wildtype p53.

In conclusion, we have shown that Tid1 is a novel regulator of p53-mediated apoptosis. Under normal conditions, p53 forms a complex with MDM2 and is rapidly degraded by polyubiquitylation. However, when cells are subjected to genotoxic or environmental stresses such as hypoxia, p53 dissociates from MDM2, leading to the up-regulation of p53 protein levels. When this occurs, p53 is then free to form a complex with Tid1 and mtHsp70, which is directed to the mitochondria where p53 initiates apoptosis (Fig. 7). On the other hand, in many cancer cells p53 mutations decrease p53 degradation, leading to elevated basal levels of p53 that, nevertheless, lack apoptotic potential. This is likely due, at least in part, to the fact that cells with mutant p53 often also express smaller amounts of Tid1 protein, which prevents efficient localization of p53 to the mitochondria and induction of apoptosis in these cells. This model is supported by our observation that overexpressing Tid1 in cells with mutant p53 was sufficient to rescue both the mitochondrial localization of p53 and the ability of the cells to undergo apoptosis. Overall, we have clearly shown that Tid1 is a novel regulator of the p53-mediated apoptosis pathway activated in the early stages of the stress

response. This finding has direct clinical relevance as it suggests that cancer therapeutics that increase Tid1 expression levels in cancer cells may be a novel means of promoting apoptosis in tumors that express either wildtype or mutant forms of p53.

Methods

Cell lines and Treatments

The human breast cancer cell lines, MCF-7 ($p53^{wt}$), T47D ($p53^{mt\text{-}L194F}$), SK-BR-3 ($p53^{mt\text{-}R175H}$), BT474 ($p53^{mt\text{-}E285K}$), and the human glioma cell lines, U87 ($p53^{wt}$), U373 ($p53^{mt\text{-}R273H}$), were grown in DMEM supplemented with 10% fetal bovine serum. The human colon carcinoma cell lines HCT116 (expressing wildtype *p53*) and HCT116 (*p53*-null), were kindly provided by Dr. Karl Riabowol (University of Calgary, Calgary, AB) and were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum. To mimic hypoxia, cells were incubated in medium containing 125 μM desferroxamine mesylate (DFX, Sigma) for the time periods indicated in the Figure legends. Bax inhibitor peptide (BIP) and a corresponding negative control peptide were obtained from Calbiochem and used at a final concentration of 200 μmol/L. BIP is a cell-permeable synthetic peptide that inhibits Bax translocation to mitochondria and the initiation of the intrinsic mitochondrial cell death pathway. BIP's sequence was based on the Bax-inhibiting domain of Ku-70, a protein that suppresses the mitochondrial translocation of Bax and inhibits Bax-mediated apoptosis[46]. The caspase-8 inhibitor, Z-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethylketone (Z-IETD-FMK) was purchased from Calbiochem and stock solutions were prepared in DMSO. Aliquots of this stock solution were diluted in culture medium to obtain a final concentration of 200 μmol/L Z-IETD-FMK. The final

concentration of DMSO was 0.05%, which did not influence the extent of apoptosis in MCF-7 cells. BIP or its negative control peptide, and Z-IETD-FMK or DMSO alone was added to the culture medium 3 hrs before the addition of DFX. Cells were then cultured in the presence of DFX and each inhibitor or negative control for an additional 6 hours.

Plasmids

Full-length cDNAs encoding human $Tid1_S$, $Tid1_L$, the $Tid1_S$ NH2-terminal deletion mutant (Ad-ΔNT) or the $Tid1_s$ DnaJ domain mutant (Ad-HQ) were cloned into the *Bgl*II and *Hin*dIII sites of pAdTrack-CMV adenovirus shuttle vector (Q-Biogene). Breast cancer cell lines in 10 cm plates (1×10^5 cells/plate) were infected with adenoviruses containing the various constructs for 6 hrs. Thereafter, the growth medium was replaced every 2 days. As pAdTrack-CMV vectors contain a separate enhanced green fluorescent protein (GFP) expression cassette, infection efficiency was monitored by immunofluorescence. We determined that infecting cells with a multiplicity of infection of 5×10^5 produced a 100% infection rate. To generate Ad-shTid1, a Tid1-specific sequence containing a hairpin loop (5'-GATCCCCAGCTACGGCTACGGAGACTTCAAGAGAGTCTCCGTAGCCGTAGCTGTTTTTGGAAA-3') was cloned into the pSuper vector, and then moved to the pShuttle vector (Q-Biogene) at the *Xba*I and *Hin*dIII sites. Adenoviruses expressing $p53^{wt}$ or $p53^{mt\text{-}121F}$ (kindly provided by Dr. Riabowol) were subcloned into pAdTrack-CMV, and recombined with pAdEasy-1 in *Escherichia coli* BJ5183.

Subcellular Fractionation, Immunoprecipitation and Immunoblotting

Cells were lysed with RIPA buffer (20 mM Tris-HCl [pH 7.4], 150 mMNaCl, 0.5% [v/v] NP-40, protease inhibitor cocktail (Roche)). Nuclear, cytoplasmic and mitochondrial fractions were prepared using a Qproteome mitochondria extraction kit (Qiagen) according to the manufacturers' protocol. The purity of each fraction was determined by Western blotting with anti-mtHsp70 (Transduction Labs) and anti-actin (Santa Cruz Biotechnology) antibodies.

Immunoprecipitation was performed by incubating whole cell lysates or individual fractions with the indicated antibody in the presence of Protein A/G beads (Santa Cruz Biotechnology) for 2 hrs. Immunoprecipitates were boiled with 2X SDS sample buffer, resolved on a 10% SDS-PAGE gel, and resulting immunoblots were probed with anti-actin (C-11), anti-p53 (FL393) and anti-Tid1 (RS11) antibodies purchased from Santa Cruz Biotechnology.

Confocal Microscopy

Cells were infected with different combinations of mammalian expression vectors harboring $Tid1_s$ or $Tid1_L$, $p53^{wt}$ or $p53^{mt\text{-}121F}$ and grown in the 16-well Lab-Tek chamber slide system (Nalgene Nunc). Forty eight hours following infection, cells were incubated in the presence or absence of DFX as described above. Cells were stained with 50 nM Mitotracker (Invitrogen) at 37°C for 45 minutes and then fixed in 4% formaldehyde at room temperature for 1 hour. Fixed cells were blocked with 2% (w/v) bovine serum albumin in phosphate-buffered saline. The anti-p53 (FL393) polyclonal antibody and anti-Tid1 (RS11) monoclonal antibody were used in combination with Alexa Fluor-568

anti-rabbit, Alexa Fluor-488 anti-mouse, or Alexa Fluor-633 anti-mouse antibody (Invitrogen) in colocalization studies. Nuclei were stained with TO-PRO iodide (Invitrogen). Stained cells were examined by immunofluorescence microscopy using a Zeiss LSM 510 Laser Scanning Confocal Microscope.

Apoptosis assays

Apoptosis was induced by treating cells grown in 16-well Lab-Tek chamber slides with DFX for 6 hrs. Apoptotic cells were detected by TUNEL assay using the *In Situ* Cell Death Detection Kit AP (Roche Diagnostics) according to the manufacturer's protocol and quantitated by counting the number of TUNEL-positive relative to TUNEL-negative cells. A minimum of 100 cells were counted for 4 different fields for each experimental condition and represented as a percentage of cells experiencing apoptosis.

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Figure 1. Hypoxia induces p53 translocation to the mitochondria, where it interacts with Tid1 and mtHsp70. (**a**) Hypoxic stress causes p53 to translocate to mitochondria, where it co-localizes with Tid1. MCF-7 breast cancer cells expressing wildtype p53 were incubated in the presence or absence of 125 μM desferroxamine (DFX) for 6 hours and then labeled with Mitotracker (green) to stain mitochondria, as well as anti-Tid1 antibody (red) and anti-p53 antibody (blue). The cellular localization of each protein was visualized by confocal microscopy. (**b**) Western blotting was performed to assess expression levels of p53, Tid1 and mtHsp70 in 20 μg of cell lysates obtained from MCF-7 cells incubated in the presence or absence of 125 μM DFX for 6 hourrs. (**c**) Cell lysates (500 μg) from MCF-7 cells were immunoprecipitated using 2 μg of anti-p53, anti-Tid1, anti-mtHsp70 or control antibody. Immunocomplexes were resolved on a 10% SDS-PAGE gel, blotted to nitrocellulose membranes, and probed with anti-p53 or anti-Tid1 or anti-mtHsp70 antibodies. C* indicates control samples where immunoprecipitation was performed using control IgG. (**d**) Subcellular fractionation of MCF7 cell lysates was performed using the Qproteome Cell Compartment Kit (w, whole cell lysates; n, nucleus; c, cytoplasm; m, mitochondria). The purity of each cell fraction was monitored by immunoblotting with cytoplasmic (actin), and mitochondrial (mtHsp70) markers. To compare protein expression levels within each cell fractions, the same volume (20 μl) of fractionated cell lysates were loaded in each well and samples were analyzed using Western blotting and immunoprecipitation as described above. (C* control IgG).

Figure 2. Tid1 regulates the subcellular localization of p53. MCF-7 cells were infected with adenoviral Tid1 constructs to overexpress Tid-1_S (Ad-Tid1_S) or Tid-1_L (Ad-Tid1_L) or to knock down expression of Tid1 (Ad-shTid1). Control cells were mock-infected, or infected with Ad-Scramble control virus. Forty-eight hours following infection, cells were treated with DFX (125 μM) for 6 hours. (**a**) Proteins in whole cell lysates were resolved by SDS–PAGE and analyzed by Western blotting with anti-Tid1 or anti-actin antibodies, confirming overexpression of Tid1 isoforms. (**b**) DFX-treated MCF-7 cells were stained with Alexa-Fluor-633 anti-Tid1 or Alexa-Fluor-568 anti-p53 antibody to show colocalization of p53 and Tid1 isoforms when Tid1 is overexpressed under conditions of hypoxic stress. (White arrows – uninfected cells; Yellow arrows – infected cells) (**c**) Ad-shTid1 effectively and specifically knocks down Tid1 expression in MCF-7 cells, as shown by Western blotting of whole cell lysates for Tid1. (**d**) Colocalization of Tid1 and p53 in the mitochondria of hypoxic cells is inhibited when Tid1 expression is knocked down. Cells were stained with antibodies against Tid1 (green) or p53 (red). Propidium iodide (PI, blue) was used for nuclear staining.

Figure 3. Overexpression of Tid1 enhances p53-dependent apoptosis in response to hypoxia. (**a**) MCF-7 cells were mock-infected or infected with Ad-Scramble or Ad-shTid1. Twenty-four hours following infection, cells were treated with 125 μM DFX for 6 hours. Cells were co-stained with TO-PRO iodide (blue) and an *in situ* TUNEL assay (red) to detect apoptotic cells. The graph summarizes three separate experiments and shows the percentage of apoptotic cells (mean ± S.E.M.; % apoptosis calculated as the number of red cells compared to the total number of cells.) (**b**) Overexpression of Tid1

isoforms increases the extent of apoptosis observed in breast cancer cells subjected to hypoxic stress. MCF-7 cells were mock-infected or infected with Ad-Scramble, Ad-Tid1$_S$ or Tid1$_L$. Twenty-four hours after infection, cells were treated with 125 μM DFX for 6 hours, and then stained using the TUNEL assay. (**c**) Tid1-mediated apoptosis occurs through the intrinsic mitochondrial cell death pathway. MCF-7 cells were mock infected or infected with Ad-Scramble or Ad-Tid1$_S$. Then, cells were treated with Bax-inhibitor peptide (BIP, 200 μmol/L), Caspase-8 inhibitor (Z-IETD-FMK, 200 μmol/L) or the corresponding vehicle controls 3 hours prior to the addition of DFX (125 μM). Cells were cultured in the presence of DFX and either the inhibitors or vehicle controls for an additional 6 hours. Apoptotic cells were detected by the *in situ* TUNEL assay. Results are representative of three independent experiments.

Figure 4. Overexpression of Tid1 rescues the pro-apoptotic activity of mutant p53 in colon cancer cells. (**a**) Whole cell lysates from HCT116 p53^{wt} and HCT116 p53$^{-/-}$ colon cancer cells treated with and without DFX were subjected to immunoblotting for p53 and Tid1. (**b**) Following infection with Ad-Tid1$_s$, HCT116 p53^{wt} and HCT116 p53$^{-/-}$ cells were treated with 125 μM DFX for 6 hours. Apoptosis was measured using the *in situ* TUNEL assay. (**c**) HCT116 p53$^{-/-}$ cells were co-infected with Ad-Tid1$_s$ and Ad-p53^{wt} or Ad-p53$^{mt\text{-}121F}$. Forty-eight hours following infection, cells were treated with 125 μM DFX for 6 hours, and then apoptosis was measured using the *in situ* TUNEL assay. Results are representative of three independent experiments. (**d**) HCT116 p53$^{-/-}$ cells co-infected with the indicated viruses were treated with DFX, and then stained with anti-p53 and anti-Tid1 to determine subcellular localization.

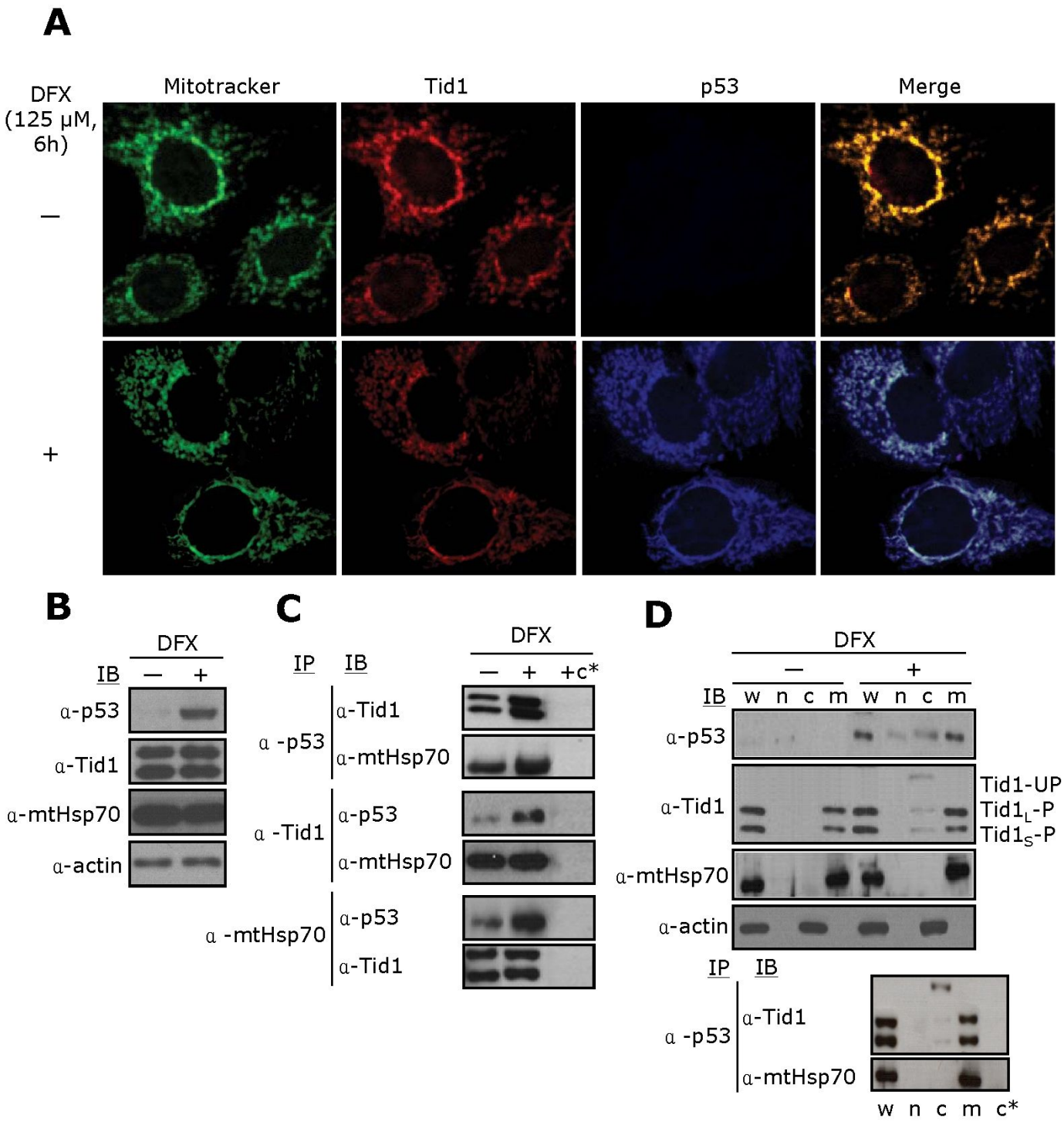
Figure 5. The pro-apoptotic activity of tumor-derived p53 mutants is restored by overexpressing Tid1 in breast cancer and glioma cell lines. (**a**) Immunoblotting with anti-p53, anti-Tid1, and anti-actin was performed on whole cell lysates from five breast cancer lines and two glioma cell lines. In contrast to wild type p53, protein levels of mutant p53 are constitutively stabilized. (**b**) Each cancer cell line was either mock-infected or infected with Ad-Tid1$_S$. Forty-eight hours following infection, expression levels of p53 and Tid1 were assessed by Western blotting of whole cell lysates. Results are representative of three independent experiments. (**c**) Confocal microscopy images of a panel of cancer cell lines stained with anti-p53 and anti-Tid1 antibodies following infection with Ad-Tid1$_S$ and treatment with DFX. (White arrows – uninfected cells; Yellow arrows – cells infected with Ad-Tid1$_S$). (**d**) Each cancer cell line was either mock-infected or infected with Ad-Tid1$_S$. Forty-eight hours following infection, cells were treated with 125 μM DFX for 6 hours. Cells were stained with TO-PRO iodide (blue) and the *in situ* TUNEL assay to assess apoptosis. Results are representative of three independent experiments.

Figure 6. Cytosolic retention of mutant Tid1 inhibits p53-mediated apoptosis induced by hypoxic stress. (**a**) Diagram of the Tid1 deletion mutant constructs (N – N-terminal; DNA J – DnaJ domain; G/F - Glycine/phenylalanine-rich domain; CXXC – Cysteine-rich domain; CT – C-terminal domain). (**b**) MCF-7 cells were infected with Ad-Scramble, Ad-Tid1$_S$, the Tid1 amino-terminal deletion mutant (Ad-ΔNT) or the Tid1 DnaJ domain mutant (Ad-HQ). Forty-eight hours following infection, cells were treated

with 125 μM DFX for 6 hours. Subcellular fractionation of MCF-7 cell lysates was performed using the Qproteome Cell Compartment Kit (w - whole cell lysates; n - nucleus; c - cytoplasm; m - mitochondria). Fractions were resolved by SDS-PAGE and Western blotting and immunoprecipitations were performed as described previously. (**c**) Cells infected with wildtype and mutant Tid1 constructs were treated with DFX and then stained with anti-p53 and anti-Tid1 antibodies. (**d**) Cells were stained with TO-PRO iodide (blue) and the *in situ* TUNEL assay (red) to assess the extent of apoptosis observed in the presence or absence of DFX. Results are representative of three independent experiments.

Figure 7. Proposed model of Tid1's role in regulating the translocation of p53 to mitochondria. In the absence of stress, wild type p53 is normally degraded following polyubiquitylation by MDM2. Under conditions of stress such as hypoxia, the p53–MDM2 complex is disrupted and protein levels of p53 are rapidly stabilized, allowing p53 to interact with Tid1, which results in mitochondrial translocation of p53 and the formation of a complex with mtHsp70.

S. -W. Kim Figure-1



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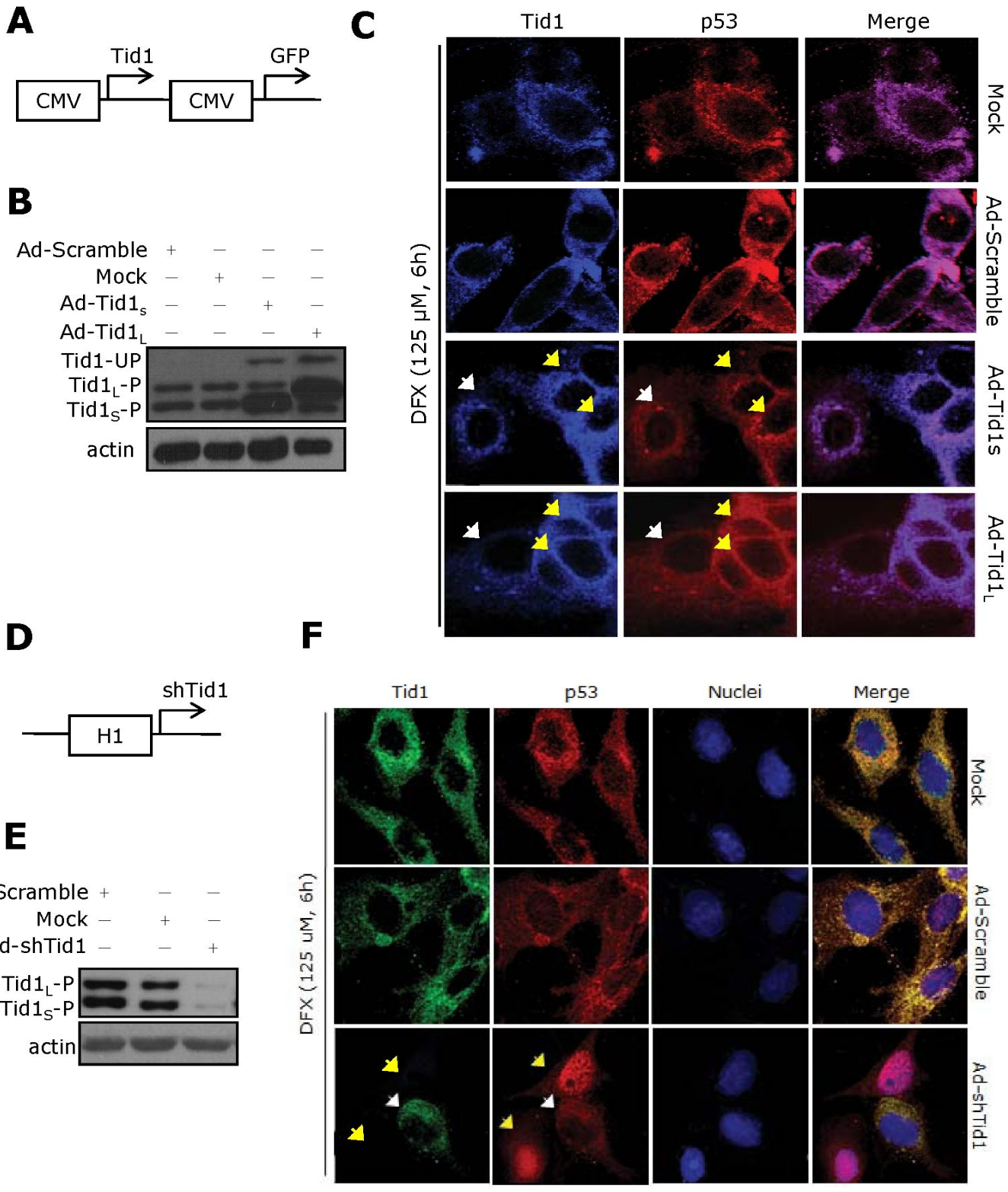

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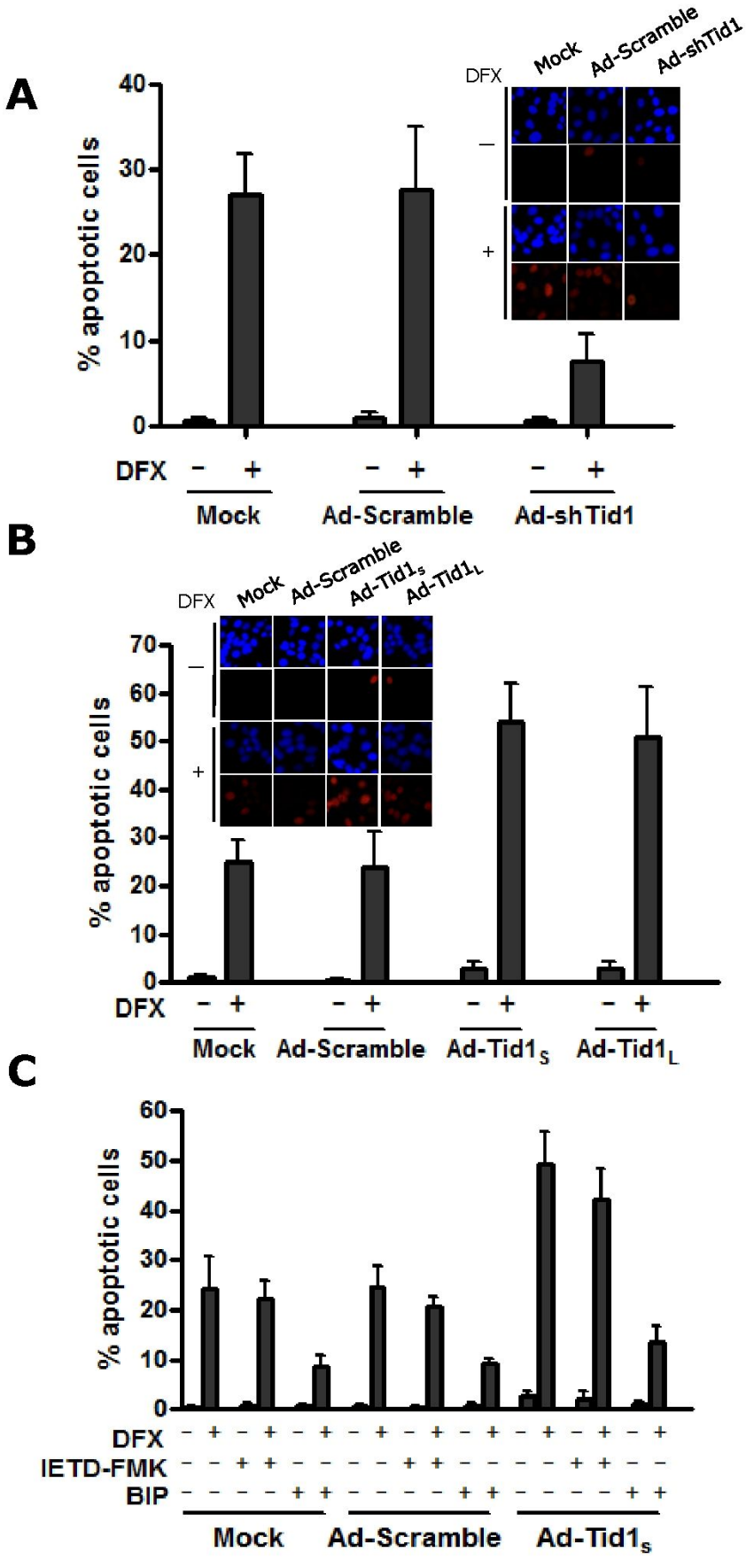

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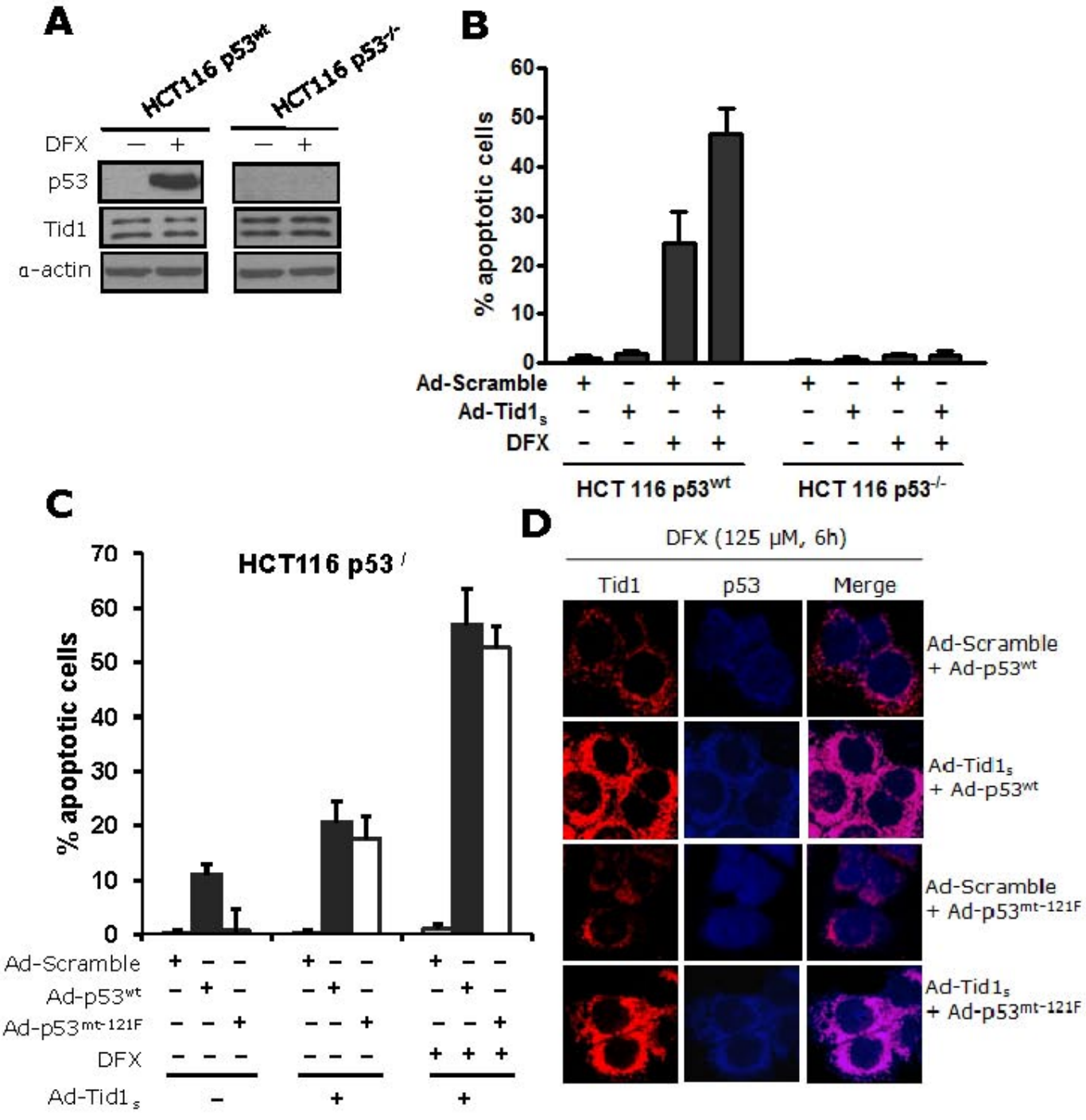

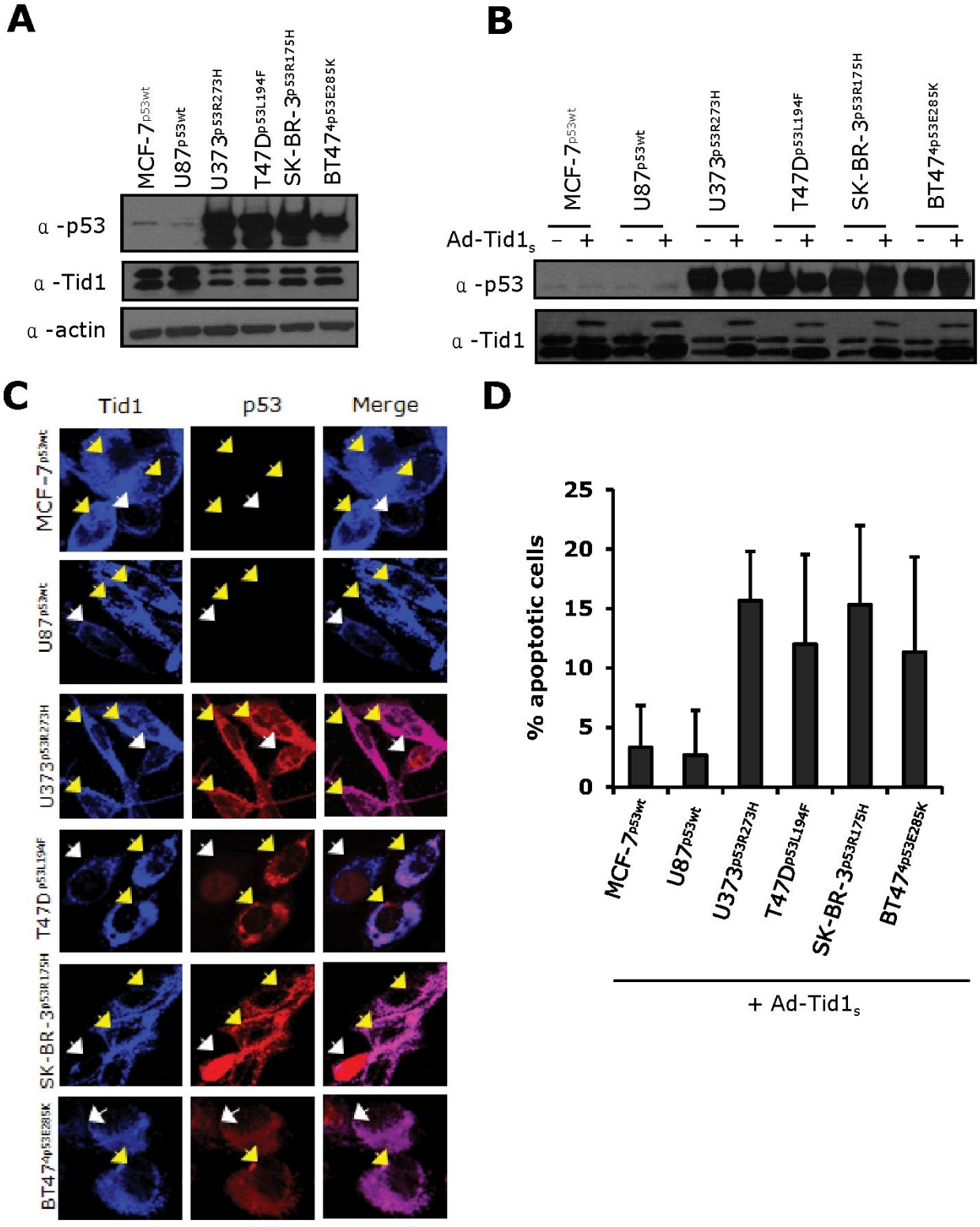
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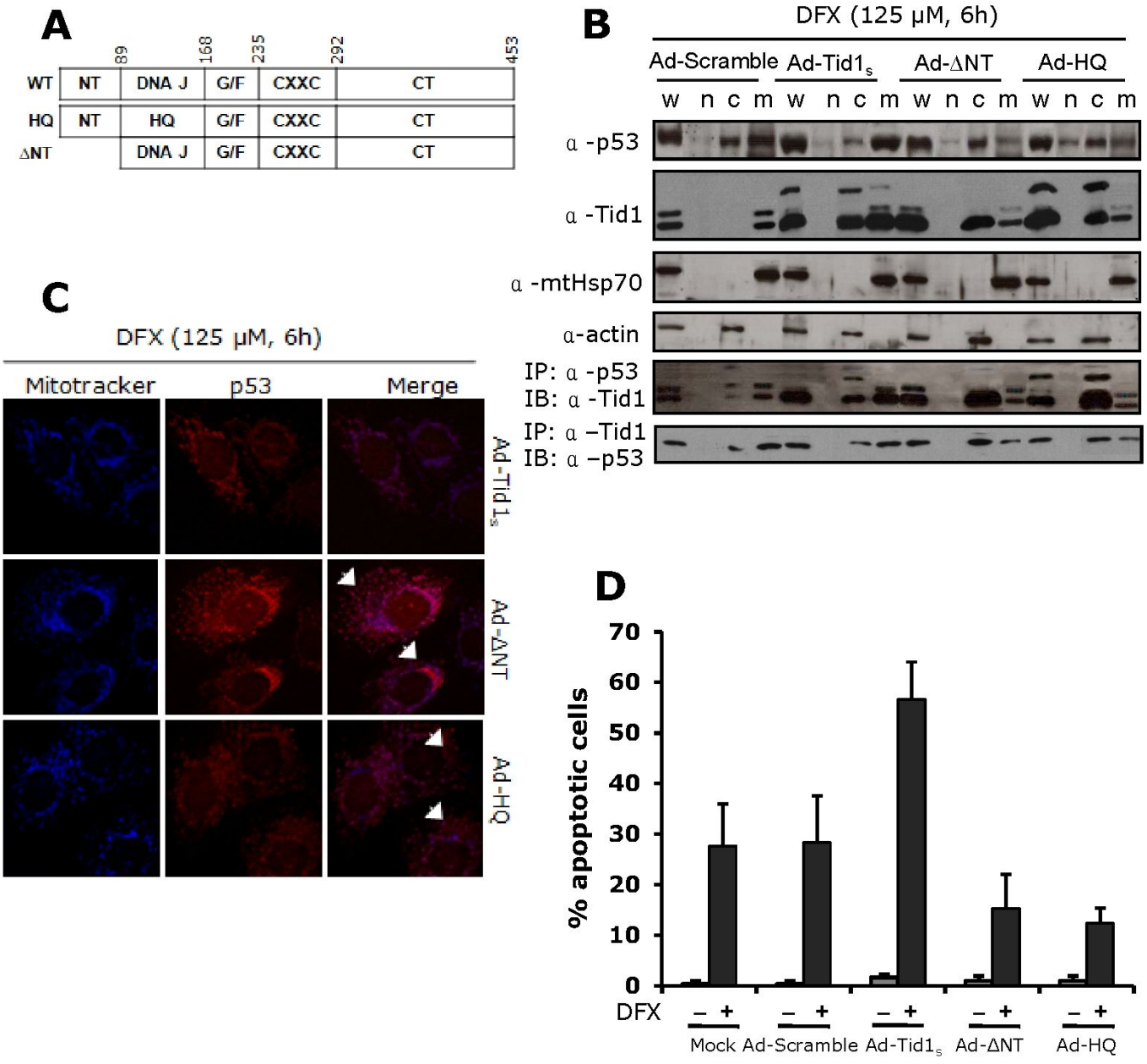
α -Tid1
α -actin
B
Ad-Tid1s
α -p53
α -Tid1
C
Tid1
p53
Merge
D
% apoptotic cells
25
20
15
10
5
0
+ Ad-Tid1s

S. -W. Kim Figure-6



S. -W. Kim Figure-7