

# Induction of p53-independent apoptosis by the BH3-only protein ITM2Bs

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**Abstract** The p53 tumor suppressor protein is critically involved in cell cycle regulation and programmed cell death. Here we show that expression of the BH3-only protein ITM2Bs is able to induce apoptotic cell death in p53+/+, as well as in p53−/− cell lines. This cell death involves neither subcellular redistribution of p53 nor transcriptional regulation of p53 target genes such as Bax, Ras, Puma or Bcl-2. Together, our data provide evidence for a p53-independent apoptotic role of ITM2Bs.

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**Key words:** ITM2Bs; p53; Apoptosis; Interleukin-2

## 1. Introduction

The Bcl-2 family proteins are central regulators of apoptosis because they integrate survival and death signals that are generated outside and inside the cell [1,2]. Mutual interaction between pro- and anti-apoptotic members determines whether a cell will die or not. Thus, Bcl-2 members act like checkpoints through which survival and death signals pass before they determine the cell fate. The family is subdivided into two classes: anti-apoptotic members such as Bcl-2 and Bcl-x<sub>L</sub>, which protect cells from apoptosis, and pro-apoptotic members, including multi-BH-containing proteins, as well as the large group of BH3-only death proteins [3,4] to which ITM2Bs can be assigned [5,6].

Integral membrane protein 2B (ITM2B) is a type II integral membrane protein that is associated with chondrogenic differentiation [7]. ITM2B belongs to a multiple gene family containing, at least, two other members, ITM2A and ITM2C. The three isoforms share 50% identity at the amino acid level, with most of the similarity in the COOH-terminal domain. Alternative splicing of ITM2B generates the long and short forms of ITM2B. The short form, ITM2Bs, which contains the BH3 domain of Bcl-2 family members, induces apoptosis in interleukin (IL)-2-, but not in IL-4-stimulated cells [5,6]. Biochemical and confocal studies have demonstrated that association of ITM2Bs with mitochondria correlates with loss of mitochondrial membrane potential, release of cytochrome *c* and, as a final consequence, induction of apoptosis. More-

over, the apoptosis-inducing activity of ITM2Bs correlates with caspase-9 and caspase-3 activation [6].

Despite the central role in cell death, the mechanism of p53-mediated apoptosis after cellular stress remains unclear. Current evidence indicates that p53 induces cell death by a mode of action involving transactivation of target genes and direct signaling events that are transcription-independent [8,9]. Concerning the transactivation mode, a diverse list of p53-regulated genes are candidates for participating in p53-apoptotic function. Evidence for an additional transcription-independent pathway for p53-mediated apoptosis is accumulating. In some cell types, p53-dependent apoptosis occurs in the absence of gene transcription or protein synthesis [10–12].

Mitochondria are central integrators and transducers of pro-apoptotic signals. A major reason for the central role of mitochondria lies in their ability to store critical apoptotic activators and effectors of cell death in their intermembrane space. These include cytochrome *c*, Smac/Diablo, EndoG and finally apoptosis-inducing factor, which activates nuclear endonucleases and procaspase-2 and -9 [13–18]. Permeabilization of mitochondrial membrane causes the release of these activators and triggers the killing of the cell. It has recently been shown that a fraction of induced p53 protein directly translocates to mitochondria in response to death signals from anticancer drugs [19,20]. This translocation is specific for p53-dependent apoptosis, but not for p53-independent apoptosis [19]. In this manuscript we describe that ITM2Bs induces apoptosis independently of p53.

## 2. Materials and methods

### 2.1. Cells, lymphokines, antibodies and reagents

The murine T cell line TS1αβ, positive for p53 expression, can be propagated independently in IL-2, IL-4 or IL-9 [21]. Cells were cultured in RPMI 1640 supplemented with 5% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 10 mM HEPES, 0.55 mM arginine, 0.24 mM asparagine, 0.05 mM 2-mercaptoethanol (2-ME) and 5 ng/ml of IL-2. HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FCS, 10 mM HEPES, 2 mM glutamine and 1 mM sodium pyruvate. K562 cells were cultured in DMEM supplemented with 10% heat-inactivated FCS and 2 mM glutamine. THP1 cells were cultured in RPMI supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES and 0.05 mM 2-ME.

K562 is a p53-deficient cell line derived from chronic myelogenous leukemia and highly undifferentiated [22,23]. THP1 is an acute monocytic leukemia cell line that has a 26-base deletion beginning at codon 174 of the p53 coding sequence thus lacking p53 [24]. HeLa is a cervical adenocarcinoma cell line that expresses undetectable or very low levels of p53 [25]. These cell lines were purchased from the ATCC Biosource Center. Anti-p53, anti-Ras, anti-Bcl-2, anti-Bax and anti-

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Tim23 antibodies were purchased from Transduction Laboratories. Anti-Puma antibody was from ProSci. Anti-tubulin antibody was from Santa Cruz and anti-histone antibody from Sigma. Anti-ITM2B serum has been previously described [5]. Lipofectamine was from Invitrogen. DEAE dextran and ECL reagent were from Pharmacia. Wild type (wt) and dominant negative p53 (DN, mutation at amino acid 249) mutant were a kind gift from D. May, Pasteur Institute, France.

## 2.2. Estimation of apoptosis by propidium iodide staining

Estimation of apoptosis was performed as previously described [26].

## 2.3. Transient transfection

Transfections were performed using DEAE dextran or lipofectamine reagent. Cells in exponential growth were washed in TS buffer (25 mM Tris-HCl pH 7.4, 137 mM NaCl, 5 mM KCl, 0.7 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, and 0.6 mM PO<sub>4</sub>HNa<sub>2</sub>). A total of 5 µg of DNA, 750 µl of TS buffer and 750 µl of freshly prepared DEAE dextran (1 mg/ml) in TS buffer were mixed successively with cells and incubated at 37°C for 1 h, centrifuged and resuspended in 12 ml of RPMI 1640/5% FCS supplemented with 5 ng/ml of IL-2. Cells were analyzed 20 h after transient transfection. Lipofectamine transfection was done according to the manufacturer's protocol.

## 2.4. Western blot

Western blot was performed as previously described [27].

## 2.5. Nuclear extracts

Cells were resuspended for 2 min in buffer A (50 mM NaCl, 10 mM HEPES pH 8, 0.5 mM sucrose, 1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine and 0.2% Triton X-100). Lysates were centrifuged (4500×g, 3 min, 4°C). Nuclei were resuspended in 1 ml buffer B (50 mM NaCl, 10 mM HEPES pH 8, 25% glycerol, 0.1 mM EDTA, 0.5 mM spermidine and 0.15 mM spermine) and centrifuged (4500×g, 3 min, 4°C). Nuclear proteins were extracted at 4°C for 30 min in 60 µl of buffer C (350 mM NaCl, 10 mM HEPES pH 8, 25% glycerol, 0.1 mM EDTA, 0.5 mM spermidine and 0.15 mM spermine). Supernatants were cleared by centrifugation (30 min, 4°C, 4500×g) and stored at -80°C. All buffers were supplemented with protease inhibitors (Sigma cocktail).

## 2.6. Isolation of mitochondria and cytosolic (S-100) fraction

Mitochondria were isolated using the protocol described by Fleischer et al. [5].

## 3. Results

### 3.1. ITM2Bs induces apoptosis independently of p53

We have previously shown the pro-apoptotic activity of ITM2Bs via loss of mitochondrial membrane potential and caspase activation [5,6]. Recently, some reports have described BH3-only members as p53-independent or -dependent mediators of apoptosis. For this reason, we explore the impact of the BH3-only protein ITM2Bs on p53-independent cell death. ITM2Bs, p53wt and p53DN mutant were overexpressed in IL-2-stimulated TS1αβ cells. As shown in Fig. 1A, expression of ITM2Bs in IL-2-stimulated cells results in a strong induction of apoptosis, compared with the low level of apoptosis observed in cells transfected with the empty vector (mock transfectants). Expression of p53wt moderately induces apoptosis in IL-2-stimulated cells, while co-expression of both ITM2Bs and p53wt does not have a synergistic effect. Moreover, co-expression of p53DN and ITM2Bs does not result in a blockage of the apoptotic activity of ITM2Bs. Expression of p53DN alone does not induce apoptosis in IL-2-stimulated cells (Fig. 1A). Expression of transfected ITM2Bs and p53 was confirmed by Western blot (Fig. 1B). The transcriptional activity of transfected p53 was estimated by the induction of Ras expression. This result suggests a p53-independent apoptotic activity of ITM2Bs in TS1αβ cells.

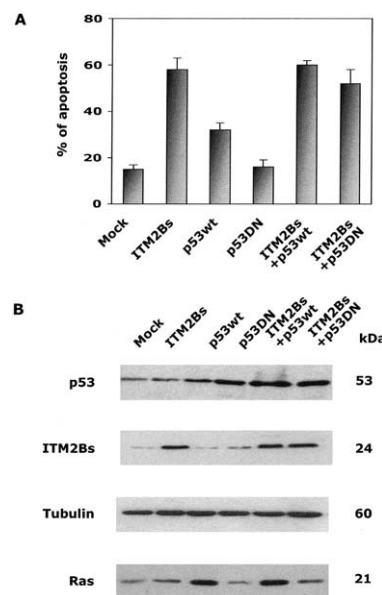


Fig. 1. p53-independent apoptotic activity of ITM2Bs in TS1αβ cells. A: Cells were transfected with ITM2Bs, p53wt, p53DN or the empty vector (mock) using the DEAE dextran method and maintained after transfection in IL-2 for 20 h. Cells were washed, permeabilized and stained with propidium iodide. Samples were analyzed by flow cytometry. Standard deviation is shown where  $n=4$ . B: Expression of the transiently transfected ITM2Bs and p53 was confirmed by direct comparison of protein level in mock and transfected cells. The blot was probed with anti-tubulin as internal control of protein loading. Molecular weight of the corresponding proteins is shown.

To confirm the ability of ITM2Bs to promote a p53-independent apoptotic pathway, ITM2Bs, p53wt and Bad were overexpressed in different cell lines negative for the expression of p53. The HeLa cell line contains very low to undetectable levels of p53wt [25], the THP1 cell line is negative for the expression of p53 [24] and K562 represents a p53-deficient cell line caused by frameshift mutation [22]. The absence of p53 transcript was confirmed in THP1 by reverse transcription polymerase chain reaction (data not shown). Expression of ITM2Bs in HeLa cells results in a strong induction of apoptosis, compared with the low level of apoptosis observed in mock transfectants (Fig. 2A). Expression of p53wt moderately induces apoptosis while co-expression of p53wt with ITM2Bs does not increase the level of apoptosis induced by ITM2Bs alone, suggesting that they do not act synergistically (Fig. 2A). Bad expression was used as positive control of apoptosis induction. Expression of transiently transfected proteins was confirmed by direct comparison of protein level in transfected and mock controls. Similarly, expression of ITM2Bs in THP1 cells results in a significant induction of apoptosis, which is not increased by the co-expression of p53wt and ITM2Bs (Fig. 2B). A modest induction of apoptosis was detected upon expression of p53wt alone (Fig. 3A). Expression of transfected proteins was examined by Western blot.

Expression of ITM2Bs was also able to induce a high level of apoptosis in p53 null K562 cells (Fig. 2C), compared with the low level of apoptosis detected in mock transfectants. This apoptotic response is not accelerated upon overexpression of ITM2Bs and p53wt. Finally, and as previously described, a low level of apoptosis was detected after expression of p53wt [23]. Interestingly, almost no induction of apoptosis was de-

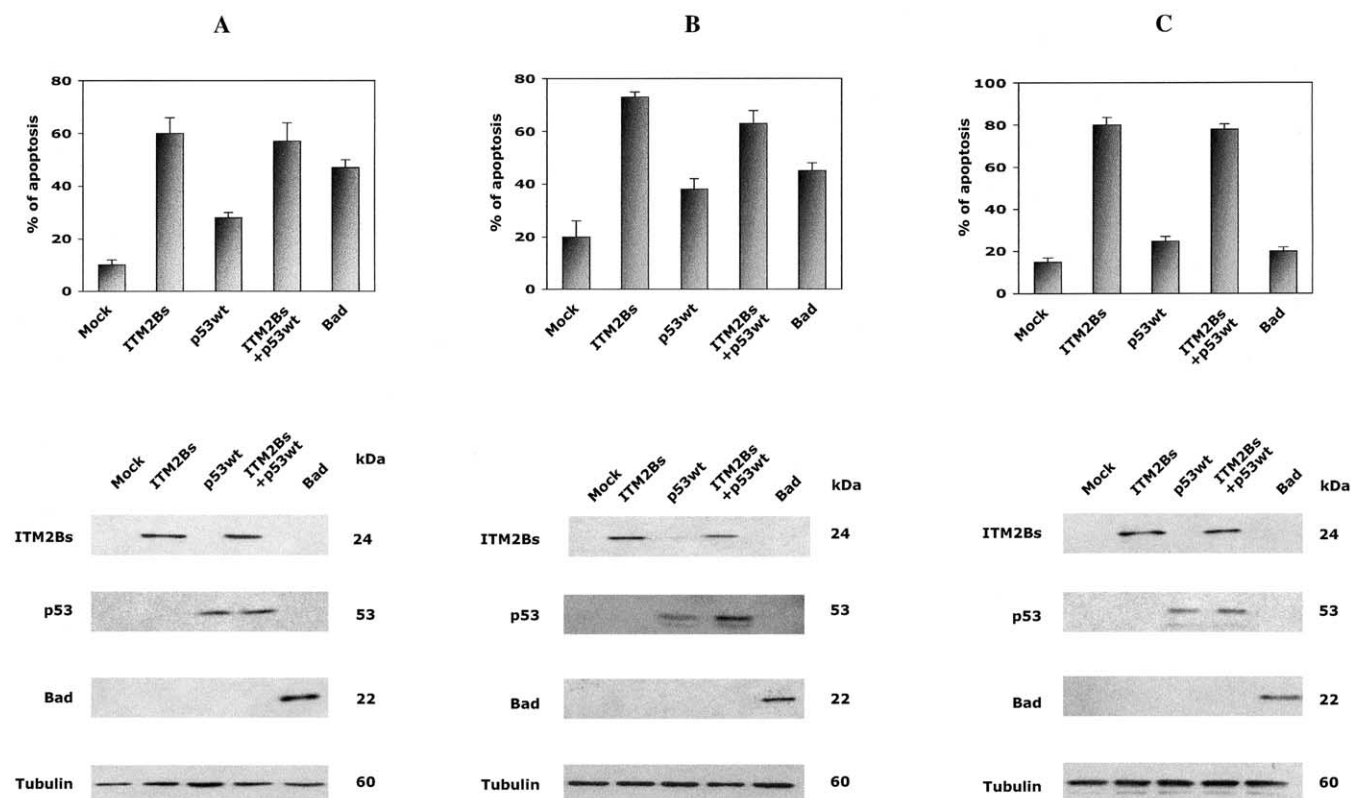


Fig. 2. Expression of ITM2Bs induces apoptosis in HeLa, THP1 and K562 cells. A: HeLa cells were transfected or co-transfected with ITM2Bs, p53wt, Bad or the empty vector (mock) using the lipofectamine method and maintained in culture for 20 h after transfection. Apoptosis positive cells were analyzed as in Fig. 1. Standard deviation is shown where  $n=3$ . Expression of the transiently transfected proteins was confirmed by Western blot. Molecular weight of the corresponding proteins is shown. B: THP1 cells were transiently transfected or co-transfected with ITM2Bs, p53wt, Bad or the empty vector (mock) using DEAE dextran and maintained after transfection for 20 h in culture medium. Cells were washed, permeabilized and stained with propidium iodide. Standard deviation is shown where  $n=3$ . The blot was probed with anti-tubulin as internal control of protein loading. C: K562 cells were transiently transfected or co-transfected with ITM2Bs, p53wt, Bad or the empty vector (mock) using lipofectamine and maintained after transfection in culture medium for 20 h. Cells were analyzed as in Fig. 1. Standard deviation is shown where  $n=3$ .

ected upon overexpression of Bad (Fig. 2C). Expression of transfected proteins was confirmed by Western blot. Taking together, these results suggest a p53-independent cell death pathway triggered by ITM2Bs in different p53 negative and positive cellular models.

### 3.2. Relationship between ITM2Bs expression and p53 cellular localization

Following IL-2 starvation, TS1 $\alpha$  cells cease to proliferate and undergo apoptosis, showing around 40% of apoptotic cells upon 24 h of growth factor deprivation [5]. To study the effect of IL-2 deprivation on p53 expression, TS1 $\alpha$  cells were IL-2-stimulated or -deprived for different time periods and total p53 expression was analyzed by Western blot. p53 was observed in control IL-2-stimulated cells and was similar throughout the deprivation period analyzed, even upon 24 h of IL-2 deprivation (Fig. 3). To determine the subcellular localization of p53 upon ITM2Bs expression in TS1 $\alpha$  cells, we isolated nuclear, mitochondrial and cytosolic (S-100) proteins from IL-2-stimulated mock or ITM2Bs transfected cells. Similar levels of p53 were detected in mitochondrial extracts of mock or ITM2Bs-transfected cells (Fig. 3). In the same direction, comparable levels of p53 were found in cytosolic (S-100) or nuclear extracts of mock or ITM2Bs-expressing cells (Fig. 3), showing the highest amount of p53 in the nu-

cleus. As internal control of proper protein fractionation procedure, membrane was probed with Tim23 (mitochondrial marker), caspase-3 (cytosolic marker) and histones (nuclear marker).

### 3.3. Transcriptional regulation via p53 is not involved in ITM2Bs-induced apoptosis

The pro-apoptotic Bcl-2 family members Bax and Puma are reported to be transcriptionally regulated by p53, as well as Ras and Bcl-2, which have also been described as targets of p53. In concordance, an increase of Bax, Puma and Ras protein expression was found in TS1 $\alpha$  cells following p53 overexpression (Fig. 4A). In contrast to recent publications [28], Bcl-2 expression was not modified upon p53 expression in TS1 $\alpha$  cells. On the contrary, similar levels of Bax, Bcl-2 and Ras expression were detected in mock or ITM2Bs-expressing TS1 $\alpha$  cells (Fig. 4A) while Puma expression could not be detected in both conditions. In the p53 null K562 cell line, ITM2Bs could not induce Bax expression (Fig. 4B) and no changes in Bcl-2 and Ras expression were observed following p53 or ITM2Bs overexpression. Finally, we analyzed whether ITM2Bs itself could be a p53-inducible gene. Fig. 4C shows that overexpression of p53 does not transcriptionally activate ITM2Bs expression in IL-2-stimulated TS1 $\alpha$  or in THP1 cells. As a positive control, IL-2-deprivation of

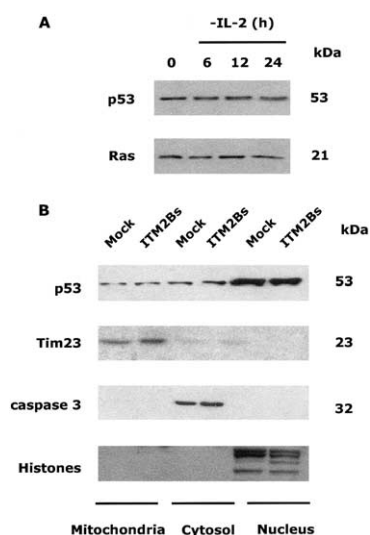


Fig. 3. Expression of ITM2Bs does not modify the expression and subcellular localization of p53. A: TS1αβ cells were IL-2-stimulated or -deprived for the indicated times and then lysed. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to nitrocellulose, probed with anti-p53 antibody and revealed using the ECL system. The blot was re-probed with pan-Ras antibody as internal control of protein loading. Similar results were obtained in three independent experiments. Molecular weights of the corresponding proteins are shown. B: Nuclear, mitochondrial and cytosolic (S-100) proteins were isolated from mock or ITM2Bs-transfected cells, separated by SDS–PAGE, transferred to nitrocellulose and immunoblotted with anti-p53, anti-Tim23 (mitochondrial marker), anti-caspase-3 (cytosolic marker) and anti-histones (nuclear marker). Similar results were obtained in three independent experiments. Molecular weight of the corresponding proteins is shown.

TS1αβ cells induces ITM2Bs expression (Fig. 4D). Taken together, these results strongly suggest that ITM2Bs induces apoptosis in a p53-independent manner since the expression of several p53 target genes is not modified by ITM2Bs and vice versa p53 itself seems not to be involved in the regulation of ITM2Bs expression.

#### 4. Discussion

The pathways governing apoptosis in mammalian cells are complex and the pro- and anti-apoptotic variations regulating cell viability change according to cell type and also between normal and tumor cells [2,29,30]. We have identified ITM2Bs as a novel p53-independent regulator of cell death. Expression of the BH3-only protein ITM2Bs induces apoptosis in the IL-2-stimulated murine T cell line TS1αβ. ITM2Bs is also able to trigger apoptosis in several p53 negative cell lines. We observed a dramatic cell death induced by ITM2Bs in THP1, HeLa and K562 cell lines, which clearly illustrates that the p53 status is not a prerequisite for ITM2Bs-induced apoptosis. We also found that ITM2Bs promotes apoptosis in these cells irrespective of Bcl-2. Expression of p53wt induces cell death in the p53 positive TS1αβ, as well as in the p53 negative background THP1 and HeLa cells, but not in the K562 p53 null cell line. Our results show that expression of p53wt protein is compatible with continuous proliferation of the K562 cell line. The growth characteristics of p53wt-expressing K562 cells did not differ from that of mock cells, which is in agreement with previous results [23].

It is believed that p53 protein with mutations at certain amino acid residues loses its transactivation function [31]. Interestingly, other p53 mutants have gain of function in some p53 null cell lines [32–34]. However, our studies propose that the dominant negative p53 mutant R249S does not activate an apoptotic pathway in IL-2-stimulated TS1αβ cells.

Recently, it has been shown that a fraction of p53 translocates to mitochondria in response to death signals from anticancer drugs [19,20]. This translocation seems to be specific for p53-dependent apoptosis, but not for p53-independent apoptosis. In agreement, we were not able to detect translocation of p53 to the mitochondria upon ITM2Bs expression, confirming that ITM2Bs triggers apoptosis via a p53-independent mechanism.

Many proteins have been shown to induce apoptosis independently of p53, including p19Arf [35], the transcription factor E2F-1 [36], the papillomavirus E2 [37], c-Myc [38], the adenovirus E4orf4 [39], the simian virus 40 small t antigen [40] and Bcl-2 antisense oligonucleotides [41]. According to

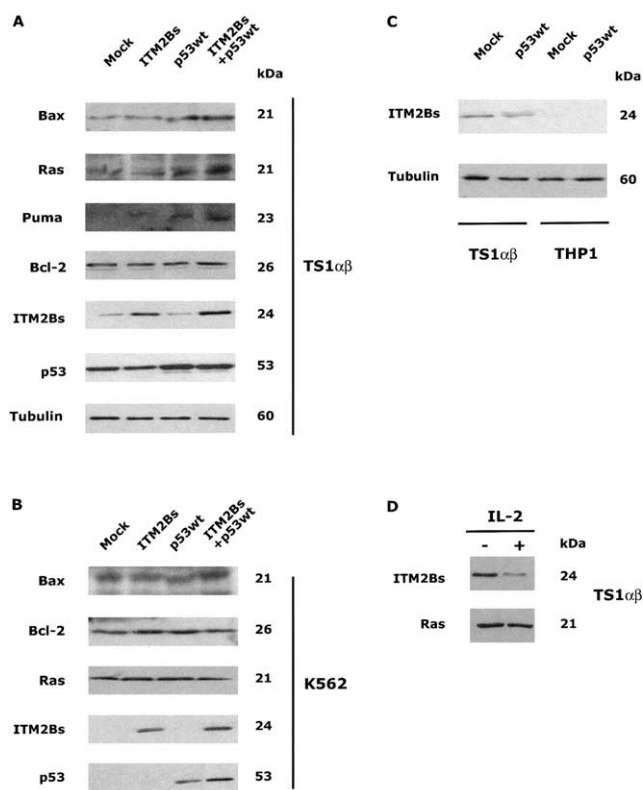


Fig. 4. ITM2Bs does not modify expression of p53 target genes and is not a direct transcriptional target of p53. TS1αβ (A) and K562 (B) cells were transiently transfected or co-transfected with ITM2Bs, p53wt or the empty vector (mock). Proteins were separated by SDS–PAGE and blotted with anti-Bax, anti-ITM2Bs, anti-Bcl-2, anti-Ras, anti-Puma, anti-tubulin and anti-p53. Protein bands were detected using the ECL system. Similar results were obtained in two independent experiments. Molecular weight of the corresponding proteins is shown. C: TS1αβ cells were transiently transfected with p53wt or the empty vector (mock transfectants). Proteins were separated by SDS–PAGE and blotted with anti-ITM2Bs. The blot was probed with anti-tubulin as internal control of protein loading. Similar results were obtained in three independent experiments. Molecular weight of the corresponding proteins is shown. D: Proteins from IL-2-stimulated or -deprived cells were separated by SDS–PAGE and blotted with anti-ITM2Bs and anti-Ras. Proteins were detected as above. Similar results were obtained in three independent experiments.



our results, ITM2Bs may be included in this group of proteins.

Depending on the cell type, p53-induced apoptosis either requires transcriptional activation [42] or not [10,11]. The presence of mutants that transactivate p53, but are defective for apoptosis, suggests that p53 induces apoptosis through transactivation-dependent and -independent mechanisms. The pro-apoptotic Bcl-2 member Bax was the first apoptotic factor to be identified as a target for p53 transactivation [43]. Interestingly, p53 also represses the expression of Bcl-2 and other genes, contributing to the induction of apoptosis by blocking survival signals [28]. The death receptor Fas was shown to be induced by p53, suggesting that p53 may also induce caspase activation through death receptor signaling [44]. In contrast, overexpression of p53 in the murine T cell line TS1 $\alpha$  $\beta$  is not able to induce ITM2Bs expression. In addition, no consensus binding sites for p53 were observed in the ITM2Bs promoter, suggesting that ITM2Bs is not a direct transcriptional target for p53. Moreover, we were not able to detect activation of p53 target genes such as Bax, Ras or Bcl-2 repression in ITM2Bs transfected TS1 $\alpha$  $\beta$  cells. Our results allow us to propose that a p53-independent apoptotic pathway is involved in the cell death activity of ITM2Bs.

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