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CROSSTALK BETWEEN TAp73 AND TGF- β IN FIBROBLAST REGULATES iNOS EXPRESSION AND Nrf2-DEPENDENT GENE TRANSCRIPTION

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Abstract

Inducible nitric oxide synthase (iNOS) activity produces anti-tumor and anti-microbial effects but also promotes carcinogenesis through mutagenic, immunosuppressive and pro-angiogenic mechanisms. The tumor suppressor p53 contributes to iNOS downregulation by repressing induction of the *NOS2* gene encoding iNOS, thereby limiting NO-mediated DNA damages. This study focuses on the role of the p53 homologue TAp73 in the regulation of iNOS expression. Induction of iNOS by immunological stimuli was upregulated in immortalized MEFs from TAp73^{-/-} mice, compared to TAp73^{+/+} fibroblasts. This overexpression resulted both from increased levels of *NOS2* transcripts, and from an increased stability of the protein. Limitation of iNOS expression by TAp73 in wild-type cells is alleviated by TGF- β receptor I inhibitors, suggesting a cooperation between TAp73 and TGF- β in suppression of iNOS expression. Accordingly, downregulation of iNOS expression by exogenous TGF- β 1 was impaired in TAp73^{-/-} fibroblasts. Increased NO production in these cells resulted in a stronger, NO-dependent induction of Nrf2 target genes, indicating that the Nrf2-dependent adaptive response to nitrosative stress in fibroblasts is proportional to iNOS activity. NO-dependent induction of two HIF-1 target genes was also stronger in TAp73-deficient cells. Finally, the antimicrobial action of NO against *Trypanosoma musculi* parasites was enhanced in TAp73^{-/-} fibroblasts. Our data indicate that tumor suppressive TAp73 isoforms cooperate with TGF- β to control iNOS expression, NO-dependent adaptive responses to stress, and pathogen proliferation.

Keywords : inducible nitric oxide synthase, nitric oxide, transforming growth factor beta, nuclear factor erythroid 2-related factor 2.

1. Introduction

In mammals, nitric oxide is produced from L-arginine by NO synthase enzymes (NOS). NO and other reactive nitrogen species (RNS) derived from NO have multiple physiological actions, such as in the cardiovascular, nervous and immune systems. In particular, inducible NOS (iNOS) encoded by *NOS2* gene has been identified early on as an important component of antimicrobial and antitumor immune defenses (reviewed in [1, 2]). However, prolonged NO exposure, such as at inflammatory and infectious sites, can be also detrimental to host cells since RNS can cause cellular injury and DNA damages by multiple direct and indirect mechanisms [3]. The tumor suppressor p53 is a transcriptional factor of major importance in cellular response to DNA damage by driving the expression of a large set of target genes involved in apoptosis, cell cycle arrest, senescence and DNA repair. Transrepression of the *NOS2* gene by p53 is a feedback mechanism that limits NO-mediated DNA damages triggering p53 activation [3]. However, in an inflammatory microenvironment, immune and non-immune cells produce higher concentrations of NO, which may favor p53 mutations and activate oncogenic pathways in tumor and stromal cells, promoting tumorigenesis [3].

The p53 protein belongs to a structural family including p63 and p73, which perform tumor suppressive and tumor promoting functions [4, 5]. Each family member features a N-terminal transactivation domain (TA), a central DNA-binding domain (DBD) and a C-terminal oligomerization domain. Significant homology in the DBD of the three homologues allows recognition of and competition for p53-responsive elements in common target genes such as *CDKN1A*, *BAX* and *PUMA*. Multiple promoter usage and alternative splicing generate several variants for each p53 family member. TAp73 isoforms featuring a full-length TA domain are transcriptionally active whereas DNp73 polypeptides generated from the same P1 promoter (Δ Ex2p73, Δ Ex2/3p73, Δ N'p73) or from a P2 cryptic one (Δ Np73) lack a complete TA domain and are therefore mostly transactivation-deficient. Alternative splicing at the 3'-end of p73 messengers produces an additional diversity of variants (α , β , γ , δ , ...) [4].

Knockout mouse models of all p73 isoforms revealed important functions of p73 in the central nervous system, reproduction, inflammation, and sexual behavior [6]. Distinct neurological defects were also found in isoform-specific TAp73^{-/-} and Δ Np73^{-/-} animals [5, 7]. Mice lacking TAp73 are tumor-prone and exhibit genomic instability, confirming the tumor-suppressive action of these isoforms. By contrast, DNA damage response and p53 activation are enhanced in Δ Np73 knockout mice. TAp73 is required for proper macrophage

polarization and the resolution of inflammatory responses [8]. Both TAp73 and DNp73 are overexpressed in a variety of human cancers and this upregulation is associated with poor prognosis [4, 9]. Although dominant-negative effects of DNp73 against the tumor suppressors p53 and TAp73 might explain overexpression of DNp73 isoforms in neoplasms, benefits to tumor cells provided by increased TAp73 levels are not completely understood. Among the proposed hypotheses, participation of TAp73 in redox maintenance has recently received attention, since reprogrammed metabolism in cancer cells increases reactive oxygen species (ROS) production and generates mild oxidative stress. TAp73-dependent induction of glucose-6-phosphate dehydrogenase and glutaminase 2, as well as control of mitochondrial ROS production by TAp73-mediated regulation of cytochrome c oxidase 4 isoform expression, might help tumor cells to cope with increased ROS production [10]. Much less is known regarding the interplay between p73 and RNS. In a recent study using p53-deficient leukemia cells, we observed that TAp73 expression is increased by NO, generated either by chemical NO donors or by iNOS activity [11]. This upregulation raised the possibility of a feedback action of TAp73 on NO production, similar to the repression of *NOS2* promoter by p53, enhancing the key role of p53 family members in NO control. However, to the best of our knowledge, this hypothesis has not yet been explored.

In this study, we used immortalized fibroblasts isolated from TAp73^{+/+} and TAp73^{-/-} mice to investigate the role of TAp73 in the induction of iNOS expression by inflammatory cytokines. We found that TAp73 isoforms exerted negative effects on iNOS expression, at transcriptional and post-translational levels. This action resulted from cooperation between TAp73 and the TGF- β signaling pathway, emphasizing the potential role of this crosstalk in a context-dependent regulation of gene expression [12, 13].

2. Material and methods

2.1. Cell culture and treatment

Mouse embryonic fibroblasts (MEFs) were obtained from wild-type or isoform-specific TAp73 knockout mice already described [5]. Spontaneous immortalization of these cells was achieved according to the 3T3 protocol [14]. Isolated clones were then cultured in DMEM supplemented with glutamine, sodium pyruvate, 55 μ M 2-mercaptoethanol and 5 or 10% FCS. Some experiments were carried out in serum-depleted medium (0.5% FCS). Fibroblasts were plated in culture medium at a density of 2.5×10^4 cells/cm² and allowed to recover for 24 h. Then, the medium was replaced with 0.24 ml/cm² of RPMI 1640 culture medium supplemented with the indicated concentration of FCS. Unless otherwise stated, iNOS expression was induced by a cocktail of three cytokines (cytomix) including IFN- γ (50

U/ml), TNF- α (20 ng/ml) and IL-1 β (20 ng/ml). TGF- β 1 (5 ng/ml) was added 30 min before cytomix treatment. In decay experiments, culture medium was renewed 24h after the beginning of cytomix treatment and cycloheximide (10 μ g/ml) was added. Cells were then harvested at the indicated times for immunoblotting or RT-qPCR analysis.

2.2. Cytokines and chemicals

Human TNF- α , IL1- β and TGF- β 1 were obtained from ImmunoTools, rat IFN- γ , lipopolysaccharide (LPS) from *Salmonella enteritidis*, cycloheximide and aminoguanidine were purchased from Sigma-Aldrich. S-ethyl-isothioureia (SEITU) and MG-132 were from Merck/Calbiochem. The TGF-RI/ALK5 inhibitors SB-525334 and SB-431542 were obtained from Selleckchem.

2.3. Antibodies and Western blot analysis

Antibodies used in this study were anti-iNOS (610328, BD Transduction Laboratories), anti- β -actin and anti- α -tubulin (AC-74 and DM-1A respectively, Sigma-Aldrich), anti-HO-1 (BML-HC3001, Enzo Life Sciences), and anti-phospho-Smad2/3 (8828, Cell Signaling). For immunoblotting, whole cell extracts were prepared in a 50 mM Tris-HCl lysis buffer, pH 7.4, supplemented with 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM EDTA, a protease inhibitor cocktail (Sigma-Aldrich), 1 mM Pefabloc (Merck/Calbiochem), 3 mM Na₃VO₄ and 50 mM NaF. Lysates were separated in 9% SDS-PAGE, transferred to nitrocellulose membrane and probed with the indicated antibodies. The protein bands visualized as infrared fluorescent signals at 680 and 800 nm were recorded and quantified with an Odyssey scanner (LI-COR Biosciences), as previously described [11].

2.4. RNA extraction and RT-qPCR

Total RNA was extracted from fibroblasts using Tri Reagent (Sigma-Aldrich) according to the user's manual. A quantity of 1 μ g RNA was used for cDNA synthesis with the qScript cDNA synthesis kit (Quanta Biosciences). Real-time PCR was performed on a StepOnePlus apparatus (Applied Biosystems). Reactions were prepared with the PerfeCTa SYBR Green SuperMIX (Quanta Biosciences) and run using the following program: 95°C for 3 min followed by 40 cycles of 95°C for 15 s and 60°C for 50 s. A melting curve (65-95°C) was generated at the end of each run. All samples were run in 2-3 technical replicates. Fold changes were calculated using the 2^{- $\Delta\Delta$ Ct} method and normalized to 18S rRNA or *Gusb* RNA levels. Primers used for qPCR are listed in Supplemental Table 1.

2.5. Measurement of nitrite production

The amount of nitrite, a stable oxidation product of nitric oxide, was measured in fibroblast culture supernatants using the Griess reagent, as previously described [15].

2.6. Measurements of TGF- β 1 protein levels by ELISA

Concentrations of TGF- β 1 in culture supernatants and FCS were determined by ELISA using the human/mouse TGF- β 1 ELISA Ready-SET-Go from eBiosciences/Affymetrix Company according to manufacturer's instructions.

2.7. Measurement of antiparasitic activity of fibroblasts

Parasite growth was evaluated in 96-multiwell plates containing 2.5×10^4 fibroblasts/well. The day before infection, culture medium was replaced by a low-serum medium (RPMI-1640 with 0.5% FCS). Fibroblasts were infected with 4×10^4 *Trypanosoma musculi* parasites, in a final volume of 250 μ l/well and stimulated with cytomix, with or without TGF- β 1 (5 ng/ml), SEITU (50 μ M) and SB-525334 (5 μ M). After 72h of co-culture, 50 μ l of the supernatant was withdrawn to measure nitrite production. Finally, the parasites were carefully resuspended, transferred into ice-cold PBS and counted using a Malassez hematimeter.

2.8. *Statistical analysis.* All results are presented as the mean \pm standard deviation of at least three values. Comparison of two means was performed using a two-tailed unpaired Student's *t* test. Other data were analyzed using one-way or two-way analysis of variance (ANOVA) with Statview. The Student-Newman-Keuls post-hoc test was used for all pairwise comparisons of mean responses among the different treatment groups. Differences between groups were considered significant if the *p* value was less than 0.05, and mentioned in the Figure legends.

3. Results

3.1. Loss of TAp73 expression amplifies iNOS induction in mouse fibroblasts

TAp73^{+/+} or TAp73^{-/-} fibroblasts resulting from the spontaneous immortalization of MEFs were tested for iNOS induction. A much stronger iNOS expression was observed in cells lacking TAp73 isoforms (Fig. 1A&B). Similar results were obtained with four independent clones of

TAp73^{+/+} and TAp73^{-/-} fibroblasts (Fig. 1A-C) and two cocktails of stimuli that induced maximal expression of iNOS protein in these cells (50 U/ml IFN- γ , 20 ng/ml TNF- α and 20 ng/ml IL1- β - here referred to as cytomix - or 50 U/ml IFN- γ , 20 ng/ml TNF- α and 1 μ g/ml LPS). In kinetics experiments, increased iNOS expression in TAp73^{-/-} fibroblasts was evidenced at all time points, without significant difference in the kinetics profiles compared to wild-type cells (Fig. 1C&D). Nitrite production, which reflects iNOS activity, was also strongly enhanced in TAp73^{-/-} fibroblasts, suggesting no limitation of NO synthesis by cofactors or substrate restriction (Fig. 1E). Transcript levels of iNOS were also much higher in TAp73^{-/-} fibroblasts (Fig. 1F). These data demonstrate a negative action of TAp73 isoforms on iNOS expression at RNA and protein levels.

3.2. Absence of TAp73 isoforms increases iNOS half-life

Several reports have shown that iNOS is subject to post-translational modifications that modify its sensitivity to proteasome degradation [16, 17]. We therefore investigated whether TAp73 might control iNOS half-life by measuring iNOS decay in the presence of cycloheximide, added 24h after induction of iNOS by cytomix (Fig. 2A&B). Our results show an almost three-fold decrease in iNOS half-life in TAp73^{+/+} fibroblasts (Fig. 2C: 10.1 \pm 2.2 h and 27.0 \pm 4.6 h in TAp73^{+/+} and TAp73^{-/-} cells, respectively, n = 4). The proteasome inhibitor MG-132 increased iNOS stability in TAp73^{+/+} fibroblasts (Fig. 2D&E). Under these conditions, iNOS decay in the presence of cycloheximide became similar to the one measured in TAp73^{-/-} cells, which was only marginally affected by MG-132. Since proteasome-mediated degradation of iNOS requires ubiquitination of the protein, we therefore hypothesized that TAp73 might be somehow involved in the regulation of iNOS ubiquitination (see below).

3.3. Culture conditions influence iNOS responsiveness to TAp73

We fortuitously noticed that TAp73-dependent limitation of iNOS expression was stronger when fibroblasts were maintained several days in culture before iNOS induction by cytomix (Fig. 3A&B). This effect might be mediated by a component produced by the fibroblasts themselves that could accumulate with time in the culture medium. The concentration of serum in the culture medium also had an influence on the sensitivity of fibroblasts to the TAp73 status. Serum depletion greatly increased iNOS induction in TAp73^{+/+} fibroblasts, up to the level observed in TAp73^{-/-} cells (Fig. 3C&D). Therefore, the striking difference between iNOS induction in TAp73^{+/+} and TAp73^{-/-} fibroblasts is linked to the presence of a significant concentration of serum in the culture medium.

3.4. Down-regulation of iNOS expression by TAp73 is dependent on the TGF- β pathway

TGF- β is a pluripotent cytokine that regulates cell growth and differentiation, deposition of extracellular matrix, wound healing and immune homeostasis [18, 19]. The TGF- β 1 isoform is ubiquitously expressed within the body and is notably endowed with essential anti-inflammatory functions, including suppression of iNOS expression in a tissue-dependent manner [19, 20]. We noticed that several properties of TGF- β 1 are in agreement with the characteristics of TAp73-dependent inhibition of iNOS induction described above. Many cell types including fibroblasts produce TGF- β 1. The cytokine is also present at variable concentrations in most FCS batches. More importantly, iNOS protein stability is reduced by TGF- β 1 [21]. Therefore, we investigated whether the downregulation of iNOS by TGF- β could be dependent on TAp73 isoforms. To prevent serum TGF- β from interfering with iNOS expression, fibroblasts were maintained in serum-depleted culture medium (0.5% FCS). Under these conditions and as previously mentioned (Fig. 3C&D), iNOS induction by cytomix was very similar in TAp73^{+/+} and TAp73^{-/-} fibroblasts (Fig. 4A&B). Addition of TGF- β 1 at 5 ng/ml strongly decreased iNOS amounts in wild-type fibroblasts but only slightly downregulated iNOS expression in TAp73-deficient cells (Fig. 4A&B). TGF- β 1 similarly decreased nitrite accumulation in TAp73^{+/+} cell culture medium but was almost ineffective on TAp73^{-/-} cells (Fig. 4C). We measured that TGF- β 1 at 24h diminished by 70% iNOS levels in TAp73^{+/+} fibroblasts but only by 20% in TAp73^{-/-} cells (Fig. 4D). Thus, in serum-depleted cell cultures, addition of exogenous TGF- β 1 was sufficient to restore the negative effect of TAp73 on iNOS expression and activity. These experiments suggested that TAp73 requires TGF- β to limit iNOS induction in fibroblasts. According to this assumption, inhibition of the TGF- β signaling pathway should prevent TAp73 from downregulating iNOS expression. SB-525334 is a potent and selective inhibitor of TGF- β receptor I (TbRI/ALK5). In the presence of 10% FCS in the culture medium and without addition of exogenous TGF- β , SB-525334 strongly upregulated iNOS induction by cytomix in wild-type fibroblasts (Fig. 4E&F), suggesting as suspected that serum TGF- β was involved in TAp73-dependent down-regulation of iNOS induction. SB-525334 also moderately increased iNOS levels in TAp73 knockout fibroblasts, in agreement with a weaker repression of iNOS expression by TGF- β 1 in these cells (Fig. 4D). The TbRI/ALK5 inhibitor also prevented inhibition of iNOS induction by exogenous TGF- β 1 in TAp73^{+/+} fibroblasts. Indeed, in the presence of SB-525334 the difference in iNOS levels induced in wild-type and knockout fibroblasts cultured in a 10% FCS medium was completely abolished (Fig. 4F). There was also a high increase in nitrite concentrations measured in TAp73^{+/+} cell cultures treated with SB-525334 (Fig. 4G). Overall, these data demonstrate a crosstalk between TAp73 and TGF- β signaling in the downregulation of iNOS induction. Genetic deficiency of TAp73 isoforms greatly diminishes the suppression of iNOS

expression by exogenous TGF- β 1 and possibly by endogenous TGF- β present in the cell culture medium.

3.5. Analysis of the Smad-dependent response to TGF- β 1 in TAp73-deficient fibroblasts

We started to explore the mechanisms that could explain how TAp73 isoforms cooperate with the TGF- β pathway to suppress iNOS expression. Signaling events initiated by TGF- β ligands include canonical Smad-dependent and non-canonical Smad-independent pathways. The C-terminal phosphorylation of Smad2 (Ser465/467) and Smad3 (Ser423/425) is an early event in the Smad-dependent cascade activated by TGF- β binding to the T β RI/T β RII receptor complex. The suppressive effect of TGF- β 1 on iNOS expression in macrophages requires both Smad2 and Smad3 [22]. We therefore analyzed levels of phospho-Smad2/3 in fibroblasts after TGF- β 1 treatment. While we could not detect any Smad3 phosphorylation in wild-type fibroblasts, we observed a robust accumulation of phospho-Smad2 in response to exogenous TGF- β 1, which was prevented by SB-525334 treatment (Fig. 5A). Phosphorylation of Smad2 was maximal at 1h and was maintained at a significant level beyond 8 hours of treatment (Fig. 5B). Interestingly, the degree of Smad2 phosphorylation by TGF- β 1 was identical in TAp73^{+/+} and TAp73^{-/-} fibroblasts (Fig. 5C&D). Similarly, Smad4 and T β RI/ALK5 protein levels were not affected by TAp73 genetic deficiency (results not shown). Finally, we measured by ELISA significant amounts of TGF- β 1 (\geq 500 pg/ml) in different batches of serum, but we did not notice any difference between TAp73^{+/+} and TAp73^{-/-} fibroblasts concerning their ability to produce TGF- β 1, with a similar daily production close to 65 pg/ml (Fig. 5E&F). Consequently, our results indicate that increased iNOS induction in TAp73^{-/-} fibroblasts and loss of the suppressive effect of TGF- β 1 on iNOS expression in these cells can neither be due to a deficiency in endogenous TGF- β 1 production nor attributed to alterations upstream of Smad2/3 phosphorylation in the TGF- β pathway. It can therefore be suggested that, most likely, TAp73 deficiency might alter events downstream of Smad phosphorylation or alternative non-canonical TGF- β signaling cascades that could play a role in TGF- β -mediated iNOS suppression in fibroblasts.

In search of an explanation for the reduced degradation of iNOS protein in TAp73^{-/-} fibroblasts, we analyzed the expression of *Spsb1* and *Spsb2* genes in response to TGF- β 1. These genes encode SOCS-box- and SPRY domain-containing proteins that act as iNOS recognition subunits of ECS E3 ubiquitin ligases complexes. In a recent study, expression of *Spsb1* was induced in macrophages by TGF- β associated with endotoxin [22]. Results depicted on Figure 6 show that the expression of *Spsb1*, but not *Spsb2*, is strongly induced by TGF- β 1 in wild-type fibroblasts. Unexpectedly, TAp73 deficiency completely prevented the

upregulation of *Spsb1* by TGF- β 1, suggesting that *Spsb1* could be a master regulator of iNOS lifetime in response to TGF- β 1 stimulation, but only in the presence of TAp73 isoforms.

3.6. Enhanced *Nrf2*-dependent gene expression in TAp73^{-/-} fibroblasts

NO is a powerful cytostatic agent, limiting cell proliferation by diverse mechanisms including dNTPs depletion through ribonucleotide reductase inhibition [15]. Not surprisingly, iNOS induction by cytomix in fibroblasts inhibited cell growth at 24h (Fig. S1). Between 24 and 48h, proliferation of TAp73^{+/+} fibroblasts almost completely stopped when the number of TAp73^{-/-} cells diminished indicating some toxicity. These two effects were NO-dependent because the NOS inhibitor SEITU completely prevented cytostasis of TAp73^{+/+} fibroblasts at 24h and protected TAp73^{-/-} cells from cell death at 48h. The *Nrf2* transcription factor coordinately regulates a large group of genes involved in the protection against oxidative and nitrosative stress and in the metabolism of xenobiotics. Since we did not observe cell death at 24h in TAp73 knockout fibroblasts exhibiting increased NO production compared to TAp73 wild-type ones, we hypothesized an enhanced *Nrf2*-dependent antioxidant adaptive response in TAp73^{-/-} cells. We therefore analyzed the inducible expression of the *Nrf2* target genes *Hmox1*, *Srxn*, *Nqo1* and *Sesn2* implicated in the adaptation to oxidative/nitrosative stress and encoding for heme oxygenase 1 (HO-1), sulfiredoxin, NAD(P)H quinone oxidoreductase 1 and sestrin 2, respectively. Transcript levels were measured in untreated fibroblasts, in cells stimulated with cytomix to induce iNOS expression or treated both with cytomix and the iNOS inhibitor aminoguanidine which completely blocked iNOS activity (Fig. 7A), with no toxicity and more importantly with no effect on iNOS mRNA induction (Fig.7B). The results showed a stronger increase in the induction of the 4 genes by cytomix in TAp73^{-/-} fibroblasts compared to TAp73^{+/+} cells (Fig. 7C-F). The induction of *Hmox1*, *Srxn* and *Nqo1* was totally prevented by aminoguanidine, confirming that increased NO production triggered an enhanced adaptive response to nitrosative stress in TAp73^{-/-} fibroblasts. Induction of sestrin 2 by cytomix was resistant to aminoguanidine treatment, indicating a regulatory mechanism independent of NO production. Further experiments showed that exogenous TGF- β 1 reduces the level of *Nos2* transcripts by 33.9 ± 5.0 % in wild-type cells but had no significant effect in TAp73^{-/-} fibroblasts (Fig.8 A). This decrease in *Nos2* transcripts expression was correlated with a similar reduction in mRNA levels of *Nrf2* target genes induced by NO. However, TGF- β 1 treatment did not significantly modify *Hmox1*, *Srxn* and *Nqo1* mRNA levels in TAp73^{-/-} fibroblasts (Fig.8 B-D). Treatment of TAp73^{+/+} fibroblasts with SB-525334 considerably increased the induction of these transcripts, close to the value measured in TAp73^{-/-} cells. These results were confirmed at the protein level for HO-1 (Fig. 8E).

A few experiments were also carried out using low-passage primary MEFs. As already observed in immortalized fibroblasts, we noticed a decreased induction of *Nos2* mRNA synthesis in TAp73^{+/+} MEFs compared to TAp73^{-/-} ones and a correlative lower induction of Nrf2 target gene transcripts *Hmox1* and *Nqo1* (Fig. S3A-C) that were induced in an NO-dependent manner. Both induction of *Nos2* and Nrf2 target genes in primary wild-type MEFs were strongly potentiated by the TGF-RI/ALK5 inhibitor SB-431542.

Taken together, our data demonstrate that TAp73 isoforms cooperate with TGF- β to repress induction of iNOS expression and to decrease NO production in TAp73^{+/+} fibroblasts. The Nrf2-dependent antioxidant cellular response induced by NO is finely adjusted to NO levels and results in a stronger adaptive Nrf2-dependent gene response in TAp73^{-/-} cells.

3.7. Modified NO-dependent biological functions in TAp73^{-/-} fibroblasts

In an *in vitro* wound healing assay (scratch assay), we observed that cytomix treatment significantly decreased the migration rate of TAp73^{+/+} fibroblasts (Fig. S2). Addition of the iNOS inhibitor SEITU restored the wound healing ability of these cells almost to the control value, showing that NO inhibited wound closure under these conditions. The behavior of TAp73^{-/-} fibroblasts was notably different as their migration rate was not decreased by cytomix nor increased by inhibition of NO production. These results suggested that the wound closure rate of fibroblasts is altered in the absence of TAp73 expression. In particular, inhibition by NO is no longer observed.

Since both NO and TAp73 can modulate angiogenesis through the regulation of hypoxia-inducible factor α (HIF-1 α) in normoxia [1, 23, 24], we also tested the ability of TAp73-proficient and TAp73-deficient fibroblasts to regulate two well recognized HIF-1 target genes, vascular endothelial growth factor A (*Vegf-A*) and the plasminogen activator inhibitor *PAI-1*. We first confirmed the elevated level of *Nos2* mRNA in cytomix-treated TAp73^{-/-} fibroblasts and the large increase in *Nos2* expression in TAp73^{+/+} cells in the presence of the TGF-RI/ALK5 inhibitor SB-431542 (Fig. 9A). As previously described by Dulloo *et al.*, lack of TAp73 expression in untreated cells led to a reduced expression of *Vegf-A* mRNA in normoxia (Fig. 9B). Cytomix treatment upregulated *Vegf-A* levels in TAp73^{+/+} fibroblasts. Unexpectedly, *Vegf-A* levels in TAp73^{-/-} fibroblasts stimulated with cytomix were even higher, indicating the existence of a compensatory mechanism that overrides the decrease caused by TAp73 genetic deficiency. Because of its ability to stabilize HIF-1 α in normoxia [1], NO was a likely candidate. Indeed, we observed that inhibition of iNOS activity by SEITU decreased *Vegf-A* transcript levels close to control values. Moreover, *Vegf-A* expression in TAp73^{+/+} fibroblasts stimulated in the presence SB-431542 strongly increased in parallel with the overexpression of *Nos2* in these cells, whereas neither *Vegf-A* levels nor *Nos2*

expression changed in TAp73^{-/-} upon addition of SB-431542 (Fig 9A&B). These results highlight an alternative mechanism for *Vegf-A* upregulation in TAp73^{-/-} fibroblasts, which is dependent on dysregulation of NO production that counterweights impaired expression of *Vegf-A* caused by TAp73 deficiency. Very similar results were obtained in analysis of *PAI-1* mRNA variations (Fig. 9 C), except that similar levels of *PAI-1* mRNA were found in untreated TAp73^{+/+} and TAp73^{-/-} fibroblasts.

3.8. Enhanced antimicrobial activity of TAp73-deficient fibroblasts

It is recognized that iNOS activity exerts cytotoxic and cytostatic activities against several bacteria and parasites including trypanosomes [2]. Apart from professional phagocytes, non-immune cells such as fibroblasts also exhibit broad-spectrum antimicrobial functions, which can also depend on NO production [25]. *Trypanosoma muscili* is a natural parasite in mice. Murine infection caused by this trypanosome is a helpful animal model to study host-parasite relationship. It is established that iNOS activity efficiently inhibits the extracellular growth of *T. muscili* [26]. Therefore, we analyzed the impact of the cooperation between TAp73 isoforms and TGF- β on the ability of fibroblasts to limit the growth of *T. muscili* in a NO-dependent manner. Parasites were co-cultured with wild-type or TAp73^{-/-} fibroblasts for 3 days, in the presence of various treatments including the iNOS-inducing cytomix, TGF- β 1, the TbRI/ALK5 inhibitor SB-525334 and the NOS inhibitor SEITU. Enumeration of the parasites at the end of the experiment showed that treatment of the fibroblasts with cytomix limited parasite proliferation (Fig. 10A). As expected, this effect was dependent on NO production, as shown by the recovery of parasite growth in the presence of SEITU. Interestingly, the antiproliferative effect of NO was significantly higher with TAp73^{-/-} fibroblasts (Fig. 10A), which exhibited a stronger iNOS activity compared to wild-type cells (Fig. 10B). Furthermore, exogenous TGF- β 1 decreased the NO-dependent antiproliferative effect of wild-type fibroblasts but had no effect on TAp73^{-/-} cells. In parallel, TGF- β 1 also decreased significantly iNOS activity in TAp73^{+/+}, but not in TAp73^{-/-} fibroblasts (Fig. 10B). These data show that TAp73 isoforms and TGF- β equally modulate the trypanostatic activity of iNOS expressed in fibroblasts in response to proinflammatory cytokines.

4. Discussion

4.1. TAp73 contributes to limit iNOS expression

Although iNOS activity plays important roles in redox signaling and in immune host defense against microbes and tumors, pro-inflammatory, mutagenic and pro-carcinogenic actions of a

persistent production of NO have also been reported [1, 3]. Expression of iNOS needs therefore to be tightly controlled to preserve redox homeostasis and limit toxic and detrimental actions of NO and RNS. TGF- β contributes to suppress iNOS expression by a diversity of actions including promoter repression, decrease in mRNA and protein stability as well as inhibition of iNOS translation and enzyme activity [20, 21]. NO-induced p53 accumulation also controls iNOS expression by suppressing *NOS2* promoter activity [3].

In this study, we provide strong evidence that the p53 homologue TAp73 also contributes to the downregulation of iNOS expression. We demonstrate that the genetic ablation of TAp73 isoforms in immortalized MEFs greatly increases iNOS induction by a cocktail of pro-inflammatory cytokines. Knocking out TAp73 has a dual effect as it increases iNOS transcript levels and also augments iNOS half-life by an almost 3-fold factor. This last observation was particularly interesting because only a few negative regulators of iNOS expression including TGF- β 1 are able to decrease the stability of the protein [20, 21].

4.2. Cooperation between TAp73 and TGF- β in the control of iNOS levels

Our data showing an increase of iNOS expression in TAp73^{-/-} fibroblasts by two T_bRI/ALK5 inhibitors, the impaired ability of exogenous TGF- β 1 to downregulate iNOS induction in these cells and the influence on iNOS expression of various culture conditions susceptible to modulate exogenous or endogenous TGF- β concentrations all point to a cooperation between TAp73 and the TGF- β pathway. Our data thus demonstrate that maximal inhibition of cytokine-induced iNOS expression by TGF- β is only achieved in the presence of TAp73 isoforms in mouse fibroblasts. Conversely, the TGF- β signaling pathway is necessary for TAp73 to downregulate pro-inflammatory iNOS expression in these cells. We assume that the co-regulation of iNOS expression by TAp73 in association with TGF- β might indicate a new role of TAp73 in the control of inflammation. A previous study, reporting an exacerbation of macrophage polarization toward the pro-inflammatory M1 phenotype in the absence of TAp73 isoforms, also supports this concept [8].

In different studies, suppression of mouse and rat *NOS2* gene expression by TGF- β 1 has been demonstrated to require either Smad3 or Smad2/3, through interactions with the coactivator p300 and the transcription factor C/EBP, or by interfering with IRF3 and STAT1 signaling [22, 27]. In the present study, TAp73^{+/+} and TAp73^{-/-} fibroblasts did not exhibit any significant difference in the phosphorylation of Smad2 induced by TGF- β 1 or in the production of endogenous TGF- β 1. Apparently, enhancement of TGF- β -mediated iNOS downregulation by TAp73 occurs downstream of the early steps of TGF- β receptors activation and Smad2/3 phosphorylation. Involvement of non-canonical TGF signaling

pathways should be also considered because TGF- β -mediated suppression of *Nos2* induction by PKC- or Erk MAPK-dependent mechanisms have been also described [28, 29].

4.3. Functional interplay between TGF- β and p53 family members

A crosstalk between the three p53 family members and Smad signaling pathway was first revealed in 2003 in early *Xenopus* embryogenesis to regulate TGF- β target genes such as *Mix.2* [13, 30]. In human and mouse cells, p53 or p63 were also shown to cooperate with TGF- β superfamily members such as activin in cell cycle arrest or in the induction of the cell cycle inhibitor p21^{CDKN1A} [13]. This transcriptional cooperation requires the binding of p53 to a p53-responsive element (p53RE) on the promoter of the target gene and physical association with Smad2 or Smad3, but not Smad4. The p73 homologue can also interfere with the TGF- β pathway. In the Cordenonsi's report, TAp73 was shown to synergize with TGF- β or activin in the regulation of the *Mix.2* promoter. Immobilized TAp73 was also found to bind Smad2 and Smad3 [13]. In a mouse model of pancreatic adenocarcinoma, loss of TAp73 expression modulated TGF- β signaling by depressing the canonical Smad-dependent pathway and reinforcing Smad-independent signaling [12]. Another group found that Δ Np73 could enhance promoter activation of a few genes induced by TGF- β [31]. The proposed mechanism differs from p53 and TAp73 action because it did not require Δ Np73 binding to a p53RE. Moreover, Δ Np73 augmented the binding of Smad 3 or Smad 4 to Smad-binding-element (SBE) DNA sequences. Whether the cooperation between TGF- β and TAp73 to downregulate iNOS induction and expression requires binding of TAp73 to Smad 2, 3 or 4 is presently unknown. To the best of our knowledge, no SBE has been until now identified in the iNOS promoter. Therefore, the above-mentioned molecular mechanisms that could explain cooperation between p53 family members and TGF- β are most probably irrelevant to the regulation of iNOS expression by TAp73. Obviously, further studies are warranted to address this important question.

4.4. Regulation of SPSB1 by TGF- β and TAp73

Our findings that *Spsb1* levels are co-regulated by TGF- β and TAp73 provide an interesting clue to explain the increased stability of iNOS protein in TAp73^{-/-} fibroblasts. *Spsb1* and *Spsb2* are adaptor proteins belonging to E3 ubiquitin ligase complexes that ubiquitinate iNOS, leading to its proteasomal degradation. In the presence of proteasome inhibitors, iNOS stability is comparable in TAp73-proficient and TAp73-deficient fibroblasts. In striking contrast with *Spsb2*, *Spsb1* expression is induced by TGF- β [32, 33]. Our results indicate that TAp73 is necessary in this process and suggest that a defective induction of *Spsb1* in

TAp73^{-/-} fibroblasts might stabilize iNOS in these cells. Spsb1 has been proposed to act in a feedback loop to suppress TGF- β signaling by targeting Tbr1 for proteasomal degradation [33]. Therefore, reduced expression of Spsb1 in TAp73^{-/-} fibroblasts should have promoted TGF- β signaling and Smad2/3 phosphorylation. Our results are not in agreement with this hypothesis. We noticed that Liu *et al.* mostly used Spsb1 overexpressing cells in their experiments and that silencing of endogenous Spsb1 had only a minor effect on Smad2 phosphorylation. Probably, in cells expressing physiological concentrations of Spsb1, the weak effect of Spsb1 on the TGF- β /Smad signaling pathway is not sufficient to prevent TGF- β -mediated repression of iNOS in cooperation with TAp73.

4.5. Stronger Nrf2-dependent adaptive response to RNS in TAp73^{-/-} fibroblasts overexpressing iNOS

The transcription factor Nrf2 acts as a major regulator for the cellular adaptive defense against oxidative, nitrosative and electrophilic stresses since it controls the expression of genes encoding antioxidant proteins. Activation of Nrf2 is performed by mechanisms that allow its release from Keap1, a substrate adaptor for a Cullin 3-based ubiquitin ligase driving ubiquitin-dependent degradation of Nrf2. S-nitrosylation of Keap1 by NO has been demonstrated to stabilize Nrf2 and to induce Nrf2-dependent transcriptional up-regulation of protective genes [34, 35]. We found that induction of the Nrf2 target genes *Hmox1*, *Srxn*, and *Nqo1* was NO-dependent and enhanced in TAp73^{-/-} fibroblasts. Induction of a stronger adaptive response to elevated levels of RNS is consistent with absence of overt toxicity at 24h in iNOS-expressing TAp73 knockout fibroblasts, in spite of elevated levels of RNS production. It is also in agreement with a dose-dependent S-nitrosylation of Keap1 by NO, suggesting a mechanism whereby the Nrf2-dependent protective response in fibroblasts can adjust to RNS levels [34]. Unexpectedly, induction of the fourth Nrf2 target gene investigated, namely *Ses2* was almost insensitive to iNOS inhibition. NO-independent mechanisms for *Ses2* up-regulation are thus at work in this case. TNF-dependent ROS production or activation of the AP-1 transcription factor by TNF- α and IL-1 β might have contributed to *Ses2* induction.

4.6. Altered NO-dependent functional properties of TAp73^{-/-} fibroblasts

In an attempt to determine whether overproduction of NO by TAp73^{-/-} fibroblasts might modify important functions of these cells, we tested their wound healing ability *in vitro*, the regulation of proangiogenic genes and their antimicrobial activity against *T. musculi*, a natural parasite in mice. There was a good correlation between iNOS activity and parasite

growth inhibition by fibroblasts. For instance, potent inhibition of iNOS induction by TGF- β in TAp73 wild-type fibroblasts was accompanied by a decrease in their ability to control parasite growth. On the opposite, the refractoriness of iNOS induction to TGF- β inhibition completely preserved the strong trypanostatic effect of TAp73^{-/-} fibroblasts upon treatment by this cytokine. NO-dependent upregulation of the HIF-1 target genes *Vegf-A* and *PAI-1* was also enhanced in TAp73^{-/-} fibroblasts, despite the fact that TAp73 genetic deficiency could decrease *Vegf-A* gene expression in transformed MEFs [24]. The rate of wound closure was significantly inhibited by NO in wild-type cells, a result consistent with a previous study showing that large amounts of NO impair wound healing *in vivo* [36]. Surprisingly, migration rate of cytomix-treated TAp73^{-/-} fibroblasts was insensitive to iNOS inhibition. Production of migration factors such as IL-6 is enhanced in TAp73 knockout fibroblasts, raising the possibility of compensatory mechanisms to preserve cell migration capacity of TAp73^{-/-} fibroblasts [8]. This example indicates that overproduction of NO in TAp73^{-/-} fibroblasts does not necessarily lead to an amplification of NO-dependent effects and a thorough analysis of fibroblasts functions involving iNOS expression should be carried out carefully.

Conclusions

Overall, our data suggest that TAp73 cooperates with TGF- β to downregulate iNOS expression in fibroblasts. Thus, the two related tumor suppressors p53 [3] and its homologue TAp73 (investigated in this study) have the ability to limit NO production by the iNOS enzyme, indicating the importance of a tight regulation of RNS for redox homeostasis, redox signaling and maintenance of DNA integrity. In several human inflammatory diseases such as rheumatoid arthritis and osteoarthritis, iNOS expression has been detected in fibroblasts from diseased joints where increased local production of NO may contribute to the pathogenesis of inflammation [37, 38]. Significant NO production and iNOS activity are also found in rodent and human fibroblasts during wound healing and appears to be essential for optimal collagen synthesis [36]. Thus, NO plays a critical role in host defense by clearing the wound of infectious microbes and maintaining a constant blood flow. Moreover, decreased NO production in human diabetes has been associated with impaired wound healing [36]. Matrix metalloproteinases (MMPs) regulation is an important process in the remodeling of extracellular matrix (ECM), that is required for successful repair of damaged tissues in wounds or after infectious or inflammatory injury. Dysregulation of ECM remodeling is associated to fibrotic diseases and tumor invasion. Elevated MMPs production through iNOS induction has been reported in human lung fibroblasts after poly(I:C) treatment used as a model of viral infection [39]. In addition, NO-dependent up-regulation of MMPs was increased in asthmatic patients. These examples highlight important functions for fibroblast-derived NO

in several human physiological and pathophysiological processes. In most of them, TGF- β is another important player. The functional cooperation between TGF- β and p53 is effective in human cells [13]. Since TGF- β has the ability to downregulate iNOS expression in human cells, it would be interesting to determine whether p73 might cooperate with TGF- β in human fibroblasts, especially in those processes dependent on iNOS activity.

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Declaration of interest

None.

Footnote

Abbreviations

HIF-1 α : hypoxia-inducible factor 1 alpha; iNOS: inducible nitric oxide synthase; Nrf2: nuclear factor erythroid-2-related factor 2; RNS: reactive nitrogen species; SEITU: S-ethyl-isothiourea; TGF- β : transforming growth factor-beta; TbRI and TbRII: TGF- β receptor I and II.

References

- [1] D.A. Wink, H.B. Hines, R.Y. Cheng, C.H. Switzer, W. Flores-Santana, M.P. Vitek, L.A. Ridnour, C.A. Colton, Nitric oxide and redox mechanisms in the immune response, *J. Leukocyte Biol.* 89 (2011) 873-891.
- [2] C. Bogdan, Nitric oxide synthase in innate and adaptive immunity: an update, *Trends Immunol.* 36 (2015) 161-178.
- [3] V. Somasundaram, D. Basudhar, G. Bharadwaj, J.H. No, L.A. Ridnour, R.Y.S. Cheng, M. Fujita, D.D. Thomas, S.K. Anderson, D.W. McVicar, D.A. Wink, Molecular Mechanisms of Nitric Oxide in Cancer Progression, Signal Transduction, and Metabolism, *Antioxid. Redox Signal.* (2018) <https://doi.org/10.1089/ars.2018.7527>.
- [4] M.P. DeYoung, L.W. Ellisen, p63 and p73 in human cancer: defining the network, *Oncogene* 26 (2007) 5169-5183.
- [5] R. Tomasini, K. Tsuchihara, M. Wilhelm, M. Fujitani, A. Rufini, C.C. Cheung, F. Khan, A. Itie Youten, A. Wakeham, M.S. Tsao, J.L. Iovanna, J. Squire, I. Jurisica, D. Kaplan, G. Melino, A. Jurisicova, T.W. Mak, TAp73 knockout shows genomic instability with infertility and tumor suppressor functions, *Genes and Dev.* 22 (2008) 2677-2691.
- [6] A. Yang, N. Walker, R. Bronson, M. Kaghad, M. Oosterwegel, J. Bonnin, C. Vagner, H. Bonnet, P. Dikkes, A. Sharpe, F. McKeon, D. Caput, p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours, *Nature* 404 (2000) 99-103.
- [7] M.T. Wilhelm, A. Rufini, M.K. Wetzel, K. Tsuchihara, S. Inoue, R. Tomasini, A. Itie-Youten, A. Wakeham, M. Arsenian-Henriksson, G. Melino, D.R. Kaplan, F.D. Miller, T.W. Mak, Isoform-specific p73 knockout mice reveal a novel role for delta Np73 in the DNA damage response pathway, *Genes Dev.* 24 (2010) 549-560.
- [8] R. Tomasini, V. Secq, L. Pouyet, A.K. Thakur, M. Wilhelm, J. Nigri, S. Vasseur, P. Berthezene, E. Calvo, G. Melino, T.W. Mak, J.L. Iovanna, TAp73 is required for macrophage-mediated innate immunity and the resolution of inflammatory responses, *Cell Death Differ.* 20 (2013) 293-301.
- [9] D. Engelmann, C. Meier, V. Alla, B.M. Putzer, A balancing act: orchestrating amino-truncated and full-length p73 variants as decisive factors in cancer progression, *Oncogene* 34 (2015) 4287-4299.
- [10] E. Candi, M. Agostini, G. Melino, F. Bernassola, How the TP53 family proteins TP63 and TP73 contribute to tumorigenesis: regulators and effectors, *Hum. Mutat.* 35 (2014) 702-714.
- [11] A. Tebbi, O. Guittet, M.H. Cottet, M.F. Vesin, M. Lepoivre, TAp73 induction by nitric oxide: regulation by checkpoint kinase 1 (CHK1) and protection against apoptosis, *J. Biol. Chem.* 286 (2011) 7873-7884.
- [12] A.K. Thakur, J. Nigri, S. Lac, J. Leca, C. Bressy, P. Berthezene, L. Bartholin, P. Chan, E. Calvo, J.L. Iovanna, S. Vasseur, F. Guillaumond, R. Tomasini, TAp73 loss favors Smad-independent TGF-beta signaling that drives EMT in pancreatic ductal adenocarcinoma, *Cell Death Differ.* 23 (2016) 1358-1370.
- [13] M. Cordenonsi, S. Dupont, S. Maretto, A. Insinga, C. Imbriano, S. Piccolo, Links between tumor suppressors: p53 is required for TGF-beta gene responses by cooperating with Smads, *Cell* 113 (2003) 301-314.
- [14] G.J. Todaro, H. Green, Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines, *J. Cell Biol.* 17 (1963) 299-313.
- [15] M. Lepoivre, J.M. Flaman, Y. Henry, Early loss of the tyrosyl radical in ribonucleotide reductase of adenocarcinoma cells producing nitric oxide, *J. Biol. Chem.* 267 (1992) 22994-23000.
- [16] T. Nishiya, K. Matsumoto, S. Maekawa, E. Kajita, T. Horinouchi, M. Fujimuro, K. Ogasawara, T. Uehara, S. Miwa, Regulation of inducible nitric-oxide synthase by the SPRY domain- and SOCS box-containing proteins, *J. Biol. Chem.* 286 (2011) 9009-9019.

- [17] E.L. Paukkeri, T. Leppanen, O. Sareila, K. Vuolteenaho, H. Kankaanranta, E. Moilanen, PPARalpha agonists inhibit nitric oxide production by enhancing iNOS degradation in LPS-treated macrophages, *Br. J. Pharmacol.* 152 (2007) 1081-1091.
- [18] J. Massague, TGFbeta signalling in context, *Nat. Rev. Mol. Cell. Biol.* 13 (2012) 616-630.
- [19] A. Kelly, S.A. Houston, E. Sherwood, J. Casulli, M.A. Travis, Regulation of innate and adaptive immunity by TGFbeta, *Adv. Immunol.* 134 (2017) 137-233.
- [20] Y. Vodovotz, Control of nitric oxide production by transforming growth factor-beta1: mechanistic insights and potential relevance to human disease, *Nitric Oxide* 1 (1997) 3-17.
- [21] H. Takaki, Y. Minoda, K. Koga, G. Takaesu, A. Yoshimura, T. Kobayashi, TGF-beta1 suppresses IFN-gamma-induced NO production in macrophages by suppressing STAT1 activation and accelerating iNOS protein degradation, *Genes Cells* 11 (2006) 871-882.
- [22] Y. Sugiyama, K. Kakoi, A. Kimura, I. Takada, I. Kashiwagi, Y. Wakabayashi, R. Morita, M. Nomura, A. Yoshimura, Smad2 and Smad3 are redundantly essential for the suppression of iNOS synthesis in macrophages by regulating IRF3 and STAT1 pathways, *Int. Immunol.* 24 (2012) 253-265.
- [23] M. Stantic, H.A. Sakil, H. Zirath, T. Fang, G. Sanz, A. Fernandez-Woodbridge, A. Marin, E. Susanto, T.W. Mak, M. Arsenian Henriksson, M.T. Wilhelm, TAp73 suppresses tumor angiogenesis through repression of proangiogenic cytokines and HIF-1alpha activity, *Proc. Natl. Acad. Sci. U S A* 112 (2015) 220-225.
- [24] I. Dulloo, B.H. Phang, R. Othman, S.Y. Tan, A. Vijayaraghavan, L.K. Goh, M. Martin-Lopez, M.M. Marques, C.W. Li, Y. Wang de, M.C. Marin, W. Xian, F. McKeon, K. Sabapathy, Hypoxia-inducible TAp73 supports tumorigenesis by regulating the angiogenic transcriptome, *Nat. Cell Biol.* 17 (2015) 511-523.
- [25] T. Lieke, C. Steeg, S.E. Graefe, B. Fleischer, T. Jacobs, Interaction of natural killer cells with *Trypanosoma cruzi*-infected fibroblasts, *Clin. Exp. Immunol.* 145 (2006) 357-364.
- [26] P. Vincendeau, S. Daulouede, Macrophage cytostatic effect on *Trypanosoma musculi* involves an L-arginine-dependent mechanism, *J. Immunol.* 146 (1991) 4338-4343.
- [27] M.W. Feinberg, M. Watanabe, M.A. Lebedeva, A.S. Depina, J. Hanai, T. Mammoto, J.P. Frederick, X.F. Wang, V.P. Sukhatme, M.K. Jain, Transforming growth factor-beta1 inhibition of vascular smooth muscle cell activation is mediated via Smad3, *J. Biol. Chem.* 279 (2004) 16388-16393.
- [28] D.T. Berg, A. Gupta, M.A. Richardson, L.A. O'Brien, D. Calnek, B.W. Grinnell, Negative regulation of inducible nitric-oxide synthase expression mediated through transforming growth factor-beta-dependent modulation of transcription factor TCF11, *J. Biol. Chem.* 282 (2007) 36837-36844.
- [29] Y.Q. Xiao, C.G. Freire-de-Lima, W.J. Janssen, K. Morimoto, D. Lyu, D.L. Bratton, P.M. Henson, Oxidants selectively reverse TGF-beta suppression of proinflammatory mediator production, *J. Immunol.* 176 (2006) 1209-1217.
- [30] K. Takebayashi-Suzuki, J. Funami, D. Tokumori, A. Saito, T. Watabe, K. Miyazono, A. Kanda, A. Suzuki, Interplay between the tumor suppressor p53 and TGF beta signaling shapes embryonic body axes in *Xenopus*, *Development* 130 (2003) 3929-3939.
- [31] M. Niemantsverdriet, P. Nagle, R.K. Chiu, J.A. Langendijk, H.H. Kampinga, R.P. Coppes, DeltaNp73 enhances promoter activity of TGF-beta induced genes, *PLoS One* 7 (2012) e50815.
- [32] R.S. Lewis, T.B. Kolesnik, Z. Kuang, A.A. D'Cruz, M.E. Blewitt, S.L. Masters, A. Low, T. Willson, R.S. Norton, S.E. Nicholson, TLR regulation of SPSB1 controls inducible nitric oxide synthase induction, *J. Immunol.* 187 (2011) 3798-3805.
- [33] S. Liu, T. Nheu, R. Luwor, S.E. Nicholson, H.J. Zhu, SPSB1, a novel negative regulator of the transforming growth factor-beta signaling pathway targeting the type II receptor, *J. Biol. Chem.* 290 (2015) 17894-17908.
- [34] B.J. Buckley, S. Li, A.R. Whorton, Keap1 modification and nuclear accumulation in response to S-nitrosocysteine, *Free Radic. Biol. Med.* 44 (2008) 692-698.

- [35] S. Dhakshinamoorthy, A.G. Porter, Nitric oxide-induced transcriptional up-regulation of protective genes by Nrf2 via the antioxidant response element counteracts apoptosis of neuroblastoma cells, *J. Biol. Chem.* 279 (2004) 20096-20107.
- [36] M. Rizk, M.B. Witte, A. Barbul, Nitric oxide and wound healing, *World J. Surg.* 28 (2004) 301-306.
- [37] H. Liu, S.P. Wei, L.Q. Zhi, L.P. Liu, T.P. Cao, S.Z. Wang, Q.P. Chen, D. Liu, Synovial GATA1 mediates rheumatoid arthritis progression via transcriptional activation of NOS2 signaling, *Microbiol. Immunol.* 62 (2018) 594-606.
- [38] I.B. McInnes, B.P. Leung, M. Field, X.Q. Wei, F.P. Huang, R.D. Sturrock, A. Kinninmonth, J. Weidner, R. Mumford, F.Y. Liew, Production of nitric oxide in the synovial membrane of rheumatoid and osteoarthritis patients, *J. Exp. Med.* 184 (1996) 1519-1524.
- [39] T. Ichikawa, H. Sugiura, A. Koarai, Y. Minakata, T. Kikuchi, Y. Morishita, A. Oka, K. Kanai, H. Kawabata, M. Hiramatsu, K. Akamatsu, T. Hirano, M. Nakanishi, K. Matsunaga, N. Yamamoto, M. Ichinose, TLR3 activation augments matrix metalloproteinase production through reactive nitrogen species generation in human lung fibroblasts, *J. Immunol.* 192 (2014) 4977-4988.

Legends for Figures

Fig. 1: *Enhanced induction of iNOS expression in TAp73^{-/-} fibroblasts.* A. Clones of TAp73^{+/+} (wild-type WT-1 and WT-6) and TAp73^{-/-} (knockout KO-4 and KO-5) fibroblasts were stimulated with 50 U/ml IFN- γ , 20 ng/ml TNF- α and either 20 ng/ml IL-1 β (*upper central panel* and *lower panel*) or 1 μ g/ml LPS for 24h (*upper right panel*) or 36h (*lower panel*). Expression of iNOS protein was analyzed by immunoblotting with α -tubulin as a loading control. B. Quantification of iNOS protein expression at 24h. Fibroblasts from independent 4 wild-type and 4 TAp73 KO clones were stimulated with 50 U/ml IFN- γ , 20 ng/ml TNF- α and 20 ng/ml IL-1 β . Mean \pm SD, n = 9, **, $p \leq 0.01$ by Student's *t* test. C and D. Kinetics of iNOS induction in clones WT-1 (TAp73^{+/+}), KO-4 and KO-5 (TAp73^{-/-}) treated as described in B with β -actin as a loading control. D. Mean \pm SD of 3 experiments. E. Time-course of nitrite production by WT-2 TAp73^{+/+} and KO-3 TAp73^{-/-} fibroblasts stimulated as shown in B, in a representative experiment. F. Kinetics of iNOS mRNA levels in TAp73^{+/+} and TAp73^{-/-} fibroblasts treated as described in B. Mean \pm SD of triplicates with SD ≤ 0.1 .

Fig. 2: *Enhanced stability of the iNOS protein in TAp73^{-/-} fibroblasts.* A and B. TAp73^{+/+} (WT) and TAp73^{-/-} (KO) fibroblasts kept in culture medium supplemented with 5% FCS were stimulated with cytomix for 24h before addition of cycloheximide (CHX, 10 μ g/ml). The time-dependent decay of iNOS protein expression was then analyzed by immunoblotting (A) and quantification relative to the protein level at t = 0 (B, mean \pm SD of 4 independent quantifications). C. The half-life of iNOS protein in TAp73^{+/+} and TAp73^{-/-} fibroblasts was determined from data shown in B. The degradation of iNOS protein was fitted according to an exponential decay $E = E_0 \cdot e^{-kt}$ (with E_0 and E being respectively the expression of iNOS protein before and after t hours of CHX treatment, k the decay constant and t the duration of CHX treatment). D and E. Effect of the proteasome inhibitor MG-132 (5 μ M) on iNOS decay in the presence of CHX. After iNOS induction by cytomix for 24 h and a further 8-h cotreatment with CHX in the presence or in the absence of MG-132, iNOS expression was analyzed by immunoblotting (D) and relative quantification of the immunofluorescent signal (E, mean \pm SD, n = 3, **, $p \leq 0.01$).

Fig. 3: *Regulation of iNOS expression by TAp73 is affected by culture conditions.* A and B. TAp73^{+/+} (WT) and TAp73^{-/-} (KO) fibroblasts were maintained for 1, 3 or 5 days in culture medium containing 5% FCS prior to iNOS induction by a cytokine cocktail (cytomix). Expression of iNOS was quantified 24h later by immunoblotting, with α -tubulin and β -actin as loading controls and normalized to expression at day 1. Mean \pm SD, n = 3. C and D. Fibroblasts were cultured for 24h in a culture medium containing 5% or 0.5% FCS before

addition of cytomix to induce iNOS expression. A representative immunoblot (C) and mean \pm SD of the relative quantification of the immunofluorescent signal of 5 independent experiments (D) are presented. *, $p < 0.05$ and **, $p \leq 0.01$.

Fig. 4: *Repression of iNOS expression by TGF- β is less effective in TAp73^{-/-} fibroblasts than in TAp73^{+/+} cells.* A. Kinetics of iNOS induction by cytomix in TAp73^{+/+} (WT) and TAp73^{-/-} (KO) fibroblasts cultured with 0.5% FCS, with or without TGF- β 1 (5 ng/ml). A representative Western blot is depicted. B. Quantitation of iNOS expression shown in A, relative to the α -tubulin control (mean \pm SD of duplicates). C. Time-course of iNOS activity induced by cytomix in TAp73^{+/+} and TAp73^{-/-} fibroblasts, in the presence or in the absence of TGF- β 1 at 5 ng/ml. Mean of triplicates, SD < 2%. D. Relative inhibition by TGF- β 1 of cytomix-induced iNOS expression at 24h in TAp73^{+/+} (WT) and TAp73^{-/-} (KO) fibroblasts (100% indicates complete inhibition). Mean \pm SD of 8 independent values. E. Inhibition of TGF- β 1 signaling by SB-525334 was assessed by immunoblotting on induction of iNOS expression in the presence of 10% FCS. TAp73^{+/+} (WT) and TAp73^{-/-} (KO) fibroblasts were stimulated by the cytomix in the presence or in the absence of TGF- β 1 at 5 ng/ml and/or SB-525334 (5 μ M). F. Quantitation of iNOS expression induced as described in E. Results are mean \pm SD, n = 4. G, iNOS activity measured by nitrite production in TAp73 wild-type and KO fibroblasts stimulated as described in E. Mean \pm SD of triplicates in one representative experiment. *, $p < 0.05$ and **, $p < 0.01$.

Fig. 5: *TAp73 genetic deficiency does not alter Smad2 activation or TGF- β 1 production.* A. Effect of the SB-525334 inhibitor (5 μ M) on Smad2 protein phosphorylation induced in TAp73^{+/+} fibroblasts by TGF- β 1 (TGF) at 5 ng/ml for 1h (*lower panel*: mean \pm SD, n = 3, **, $p \leq 0.01$). B. Kinetics of Smad2 phosphorylation analyzed by immunoblotting in TAp73^{+/+} fibroblasts after treatment with TGF- β 1 (5 ng/ml). C and D. Comparison of Smad2 phosphorylation between wild-type (WT) and TAp73^{-/-} (KO) fibroblasts in response to a treatment with increasing concentrations of TGF- β 1 in a serum-starved medium (n = 3). E and F. Time course and daily production of total TGF- β 1 measured by ELISA in cell culture supernatants of TAp73^{+/+} and TAp73^{-/-} fibroblasts (mean \pm SD, n = 3).

Fig. 6: *TAp73 isoforms are required for Spsb1 induction by TGF- β 1.* A and B. Measure of *Spsb1* (A) and *Spsb2* (B) mRNA expression by RT-qPCR analysis of wild-type and TAp73^{-/-} fibroblasts either untreated (Ctrl) or treated for the indicated time with 5 ng/ml of TGF- β 1 in a serum-starved medium (mean \pm SD, n = 3, **, $p \leq 0.01$, # not significant).

Fig. 7: Increased induction of *Nrf2* target genes in *TAp73*^{-/-} fibroblasts. A. Aminoguanidine (AG) used at 2 mM completely inhibited iNOS activity induced by cytomix treatment (CM) of *TAp73*^{+/+} and *TAp73*^{-/-} fibroblasts. Nitrite production was measured at 24h (n = 3). B-F. mRNA quantification of *Nos2* (B), *Hmox1* (C), *Srxn* (D), *Nqo1* (E), and *Sesn2* (F) by RT-qPCR. Values are expressed relative to wild-type CM-treated (B) or untreated cells (C-F). Data are mean ± SD (n = 4). **, *p* ≤ 0.01 or *, *p* ≤ 0.05.

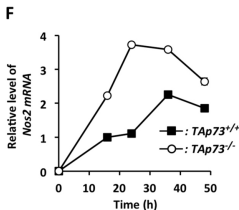
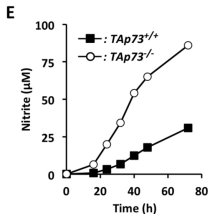
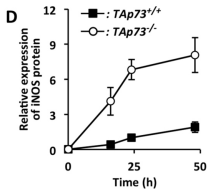
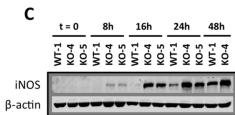
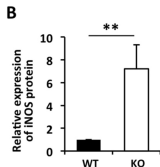
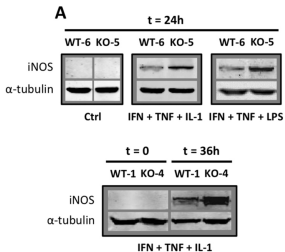
Fig. 8: Inhibition of TGF-β signaling allows similar induction of *Nrf2* target genes in *TAp73*^{+/+} and *TAp73*^{-/-} fibroblasts. A-D. Levels of *Nos2* (A), *Hmox1* (B), *Srxn* (C) and *Nqo1* (D) transcripts were determined by RT-qPCR using wild-type and *TAp73*^{-/-} fibroblasts. A 24-h treatment with cytomix (CM), TGF-β1 (5 ng/ml) (TGF) and 5 μM SB-525334 (SB) was applied as indicated. Values are mean ± SD (n = 4). They are expressed relative to wild-type CM-treated (A) or untreated cells (B-D). **, *p* ≤ 0.01 or *, *p* ≤ 0.05. E. iNOS and HO-1 protein expressions were analyzed by western blot in wild-type (WT) and *TAp73*^{-/-} (KO) fibroblasts treated as indicated in A-D. This immunoblot is representative of 3 independent experiments. Normalized values of HO-1 expression are shown below the protein bands.

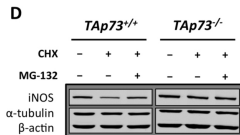
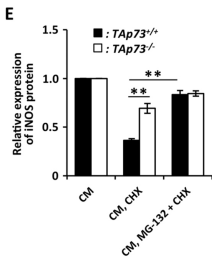
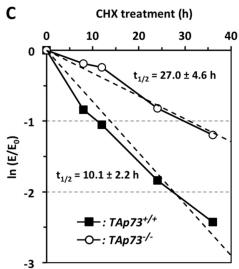
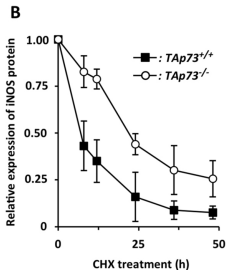
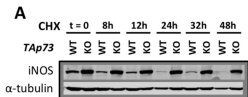
Fig. 9: NO-dependent upregulation of *HIF-1α* target genes in *TAp73*^{-/-} fibroblasts. Quantitation of *Nos2* (A), *Vegf-A* (B), and *PAI-1* (C) transcript levels in *TAp73*^{+/+} and *TAp73*^{-/-} fibroblasts by RT-qPCR. Cells grown in culture medium containing 10% FCS were submitted for 24h to cytomix treatment (CM). The TGF-β pathway was inhibited by SB-431542 at 5 μM (CM + SB). B and C. SEITU (CM + STU) was added at 50 μM to inhibit iNOS activity. Values are expressed relative to wild-type CM-treated (A) or untreated cells (B&C). Data are mean ± SD (n = 3). **, *p* ≤ 0.01.

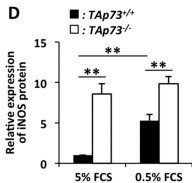
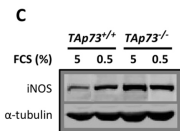
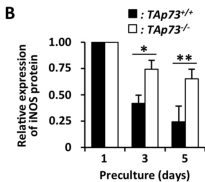
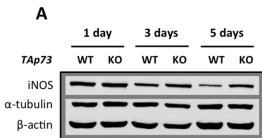
Fig. 10: *TAp73* isoforms and TGF-β1 modulate NO-dependent cytostatic effects during infection of fibroblasts with *Trypanosoma muscili*. A. Effects of iNOS induction and TGF-β1 treatment on *Trypanosoma muscili* proliferation. Parasite growth in co-culture with *TAp73*^{+/+} or *TAp73*^{-/-} fibroblasts was assessed by counting the parasites 3 days after the beginning of the infection with 4x10⁴ parasites. Immediately after infection, TGF-β1 (TGF, 5 ng/ml), iNOS-inducing cytomix (CM), a NOS inhibitor (SEITU, 50 μM) and the ALK5 inhibitor SB-525334 (SB, 5 μM) were added to the wells. All values in histograms are mean ± SD (n = 3), **, *p* ≤ 0.01 or *, *p* ≤ 0.05. B. Nitrite production by wild-type and *TAp73*^{-/-} fibroblasts was measured by Griess assay 24h after the addition of treatments (mean of triplicates).

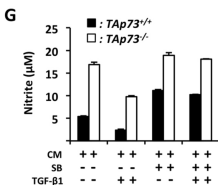
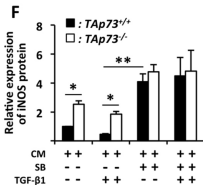
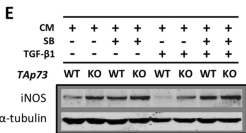
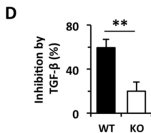
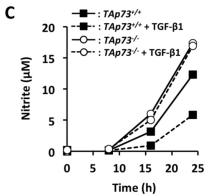
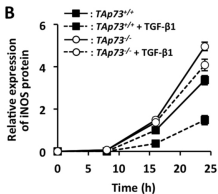
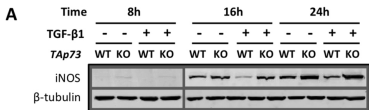
Legend for Scheme 1

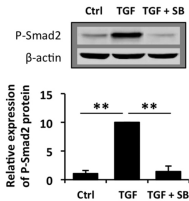
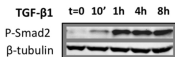
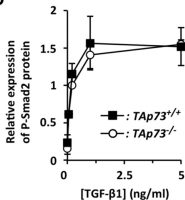
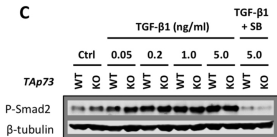
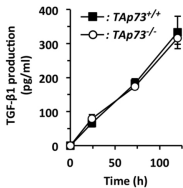
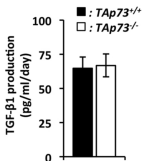
Scheme 1: *TAp73* deficiency weakens the suppression of iNOS expression by TGF- β . Cytokine-induced iNOS expression in TAp73 wild-type fibroblasts is limited by TGF- β acting in cooperation with TAp73 to downregulate iNOS mRNA levels and to reduce iNOS protein half-life. Genetic deficiency of TAp73 isoforms reduces the suppressive action of TGF- β , potentiating iNOS expression. Higher NO production by TAp73^{-/-} fibroblasts results in a stronger Nrf2-dependent adaptive response to nitrosative stress, an increase in NO-dependent induction of HIF-1 target genes and a more pronounced antiparasitic activity against *T. musculi*.

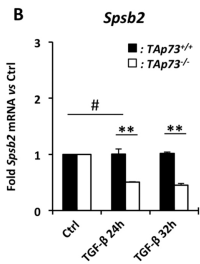
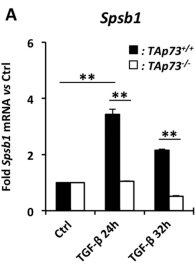


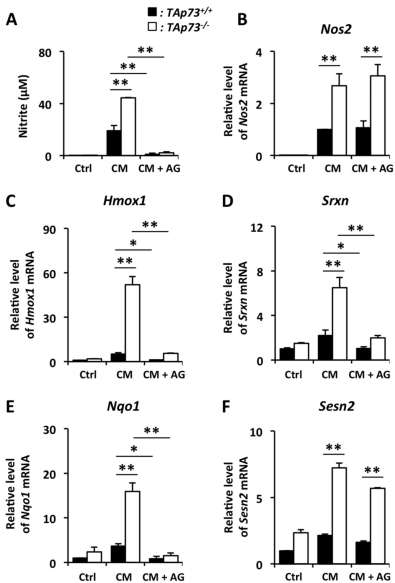


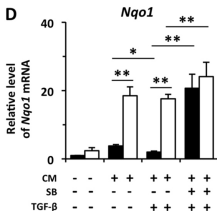
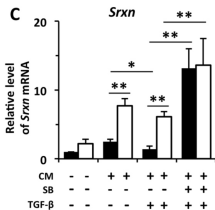
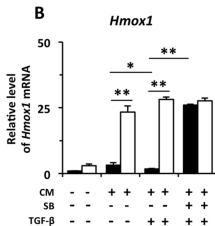
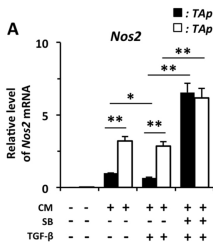




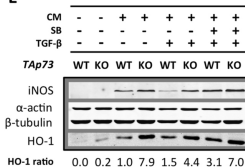
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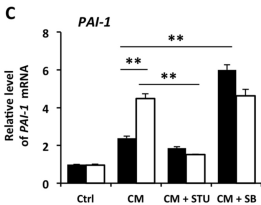
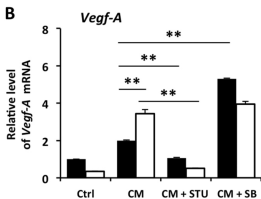
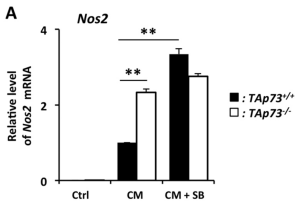


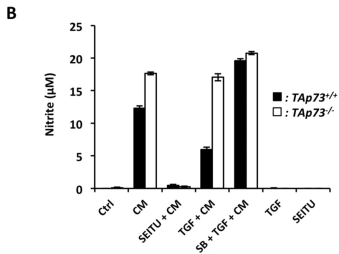
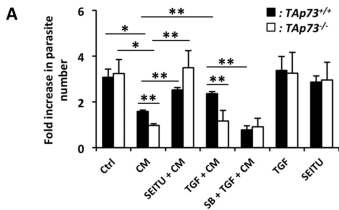




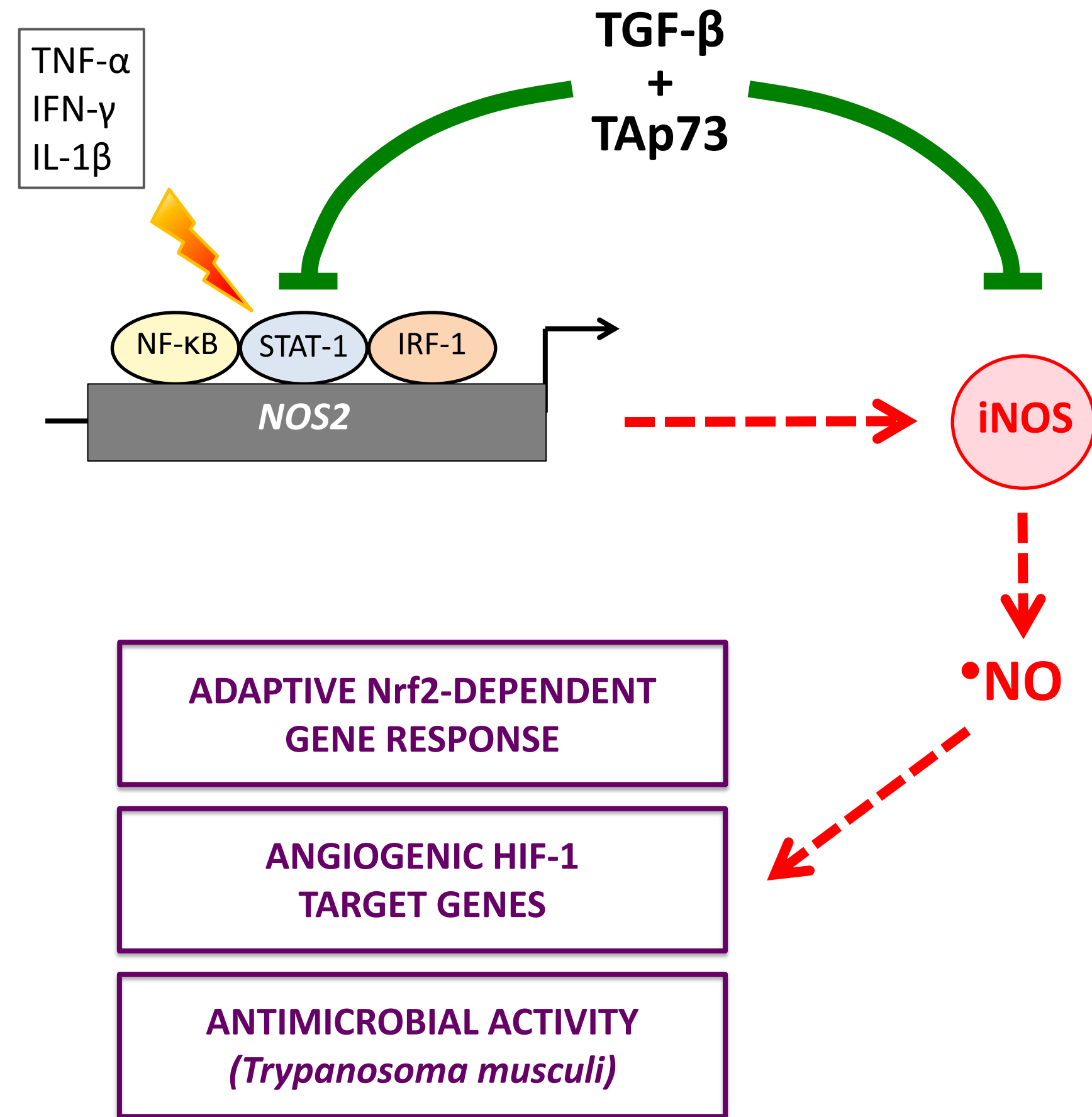
E







TAp73 WT FIBROBLASTS



TAp73 KO FIBROBLASTS

