Δ40p53 isoform up-regulates netrin-1/UNC5B expression and potentiates netrin-1 pro-oncogenic activity

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Netrin-1, a secreted protein recently characterized as a relevant cancer therapeutic target, is the antiapoptotic ligand of the dependence receptors deleted in colorectal carcinoma and members of the UNC5H family. Netrin-1 is overexpressed in several aggressive cancers where it promotes cancer progression by inhibiting cell death induced by its receptors. Interference of its binding to its receptors has been shown, through the development of a monoclonal neutralizing antinetrin-1 antibody (currently in phase II of clinical trial), to actively induce apoptosis and tumor growth inhibition. The transcription factor p53 was shown to positively regulate netrin-1 gene expression. We show here that netrin-1 could be a target gene of the N-terminal p53 isoform Δ40p53, independent of full-length p53 activity. Using stable cell lines, harboring wild-type or null-p53, in which Δ40p53 expression could be finely tuned, we prove that Δ40p53 binds to and activates the netrin-1 promoter. In addition, we show that forcing immortalized human skeletal myoblasts to produce the Δ40p53 isoform, instead of full-length p53, leads to the up-regulation of netrin-1 and its receptor UNC5B and promotes cell survival. Indeed, we demonstrate that netrin-1 interference, in the presence of Δ40p53, triggers apoptosis in cancer and primary cells, leading to tumor growth inhibition in preclinical in vivo models. Finally, we show a positive correlation between netrin-1 and Δ40p53 gene expression in human melanoma and colorectal cancer biopsies. Hence, we propose that inhibition of netrin-1 binding to its receptors should be a promising therapeutic strategy in human tumors expressing high levels of Δ40p53.

apoptosis | netrin-1 | p53 isoform

Several studies have highlighted the crucial role of the secreted protein netrin-1 as an original cancer biomarker and therapeutic target (1–3). Initially discovered as an axon guidance cue during neuronal development, netrin-1 is also involved in cell survival and tumorigenesis (4). Indeed, netrin-1 receptors deleted in colorectal carcinoma (DCC) and UNC5 homolog (UNC5H, i.e., UNC5A-D), belonging to the growing family of dependence receptors (5), induce "positive" signals upon ligand binding leading to differentiation and cell survival, and trigger apoptosis when unbound (6). Hence, cell survival depends on the presence of ligands of dependence receptors, the latter representing ideal tumor-suppressor candidates. Indeed, the current hypothesis is that the expression of such a receptor represents a protective mechanism that limits tumor development. This could be achieved via the induction of apoptosis of tumor cells that would grow in or migrate to regions of limited ligand availability (7). Consequently, cell transformation toward a malignant or metastatic phenotype likely relies either on the loss of dependence receptor expression or on the constitutive inhibition of apoptosis induced by these receptors via ectopic ligand expression. Consistently, netrin-1 up-regulation has been described in several aggressive cancers (8–11), and is associated with tumor progression (8, 11, 12). The identification of netrin-1 as a tumor biomarker led to the development of an innovative personalized anticancer strategy. Indeed, interference of netrin-1 and its receptor binding induces apoptosis and favors tumor growth inhibition in several aggressive cancer models, expressing high netrin-1 levels (1, 8, 10–12). According to these findings, a humanized antinetrin-1 antibody, called NP137, was developed (2) and was assessed in a phase I clinical trial (https://clinicaltrials.gov/ct2/show/NCT02977195) and is now moving to a phase II clinical trial.

Despite the importance of netrin-1 in cell survival and cancer progression, little is known about the regulation of its expression. We formerly demonstrated that the transcription factor p53 was involved in the regulation of netrin-1 gene expression, following chemotherapy (1). p53 is a well-known tumor-suppressor gene, through its ability to regulate the expression of different genes involved in a broad range of cellular responses, including apoptosis, cell cycle arrest, senescence, and metabolism (13). p53 functions mainly depend on its ability to induce or repress transcription of target genes. However, transcription-independent activities of p53

Significance

Netrin-1 is frequently overexpressed in aggressive cancers, acting as the antiapoptotic ligand of the dependence receptors DCC and members of the UNC5H family. Accordingly, netrin-1 inhibition induces apoptosis and tumor growth inhibition. We have previously shown that the transcription factor p53 regulates netrin-1 gene expression in cancer cells treated with conventional chemotherapeutic drugs. We show here that netrin-1 is a target gene of the N-terminal p53 isoform Δ40p53, acting independently of full-length p53 activity, thus promoting cell survival. We show that netrin-1 interference, in presence of Δ40p53, triggers apoptosis in cancer and primary cells and tumor growth inhibition in preclinical in vivo models. We propose that netrin-1 inhibition is a promising therapeutic strategy in tumors expressing high levels of Δ40p53.

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have been described previously (14, 15). The transactivation activity of p53 requires two distinct transcriptional activation domains (TAD), encompassing residues 1 to 40 (TAD1) and 41 to 83 (TAD2) (16). Experiments in mice bearing inactivating point mutations in one or both TADs demonstrated a functional specialization of each TAD, which could regulate different target genes and effector pathways (17, 18). Interestingly, in the last few years, several alternative p53 transcripts were identified (19, 20). These protein isoforms are generated by different mechanisms, such as alternative splicing sites, promoters, and translational initiation sites (21, 22). Some of these p53 isoforms lack TAD1 (Δ40p53) or both transactivation domains (Δ133p53) (20). The Δ40p53 isoform can be produced by alternative splicing, leading to the retention of intron 2 (23–25), or through the use of an alternative internal ribosomal entry sequence (IRES) at codon 40 (24). Conversely, the Δ133p53 isoform is generated from an internal promoter, located between intron 1 and exon 5, controlling the expression of a p53 mRNA sequence starting from intron 4 (19, 26).

Even though we previously showed that the full-length p53 (FLp53) directly binds to and activates the netrin-1 promoter (1), the molecular mechanisms underlying this regulation remain unclear. We reveal here that the regulation of netrin-1 and its main dependence receptor UNC5B requires the transactivation activity of p53 in tumor cells. Notably, netrin-1 transcriptional activation requires only p53 TAD2, unlike the majority of p53 target genes (17). Consequently, we show that Δ40p53, and not Δ133p53, can positively regulate netrin-1 and UNC5B gene expression, in an FLp53-independent manner. Moreover, we demonstrate that the expression of Δ40p53 and netrin-1 are correlated in human tumors. Finally, we speculate that treatment of tumor cells expressing Δ40p53 with neutralizing antinetrin-1 antibody could be an innovative cancer therapeutic strategy.

Results

Netrin-1 Regulation by p53 Requires the Transcriptional Activity of the TAD2 Domain. To unravel p53 regulation of netrin-1 expression, in particular the involvement or not of its transcriptional activity (14, 15), and possibly of its N-terminal TADs (17), we generated stable lung cancer H1299 cells bearing point mutations using sleeping beauty-based vectors. Indeed, site-specific L22Q and W23S mutations in TAD1 significantly inhibit the transcriptional activation of the p53 protein (27), whereas W53Q and F54S mutations inactivate TAD2 (Fig. 1*A*) (16). This methodology enabled us to activate wild-type or transcriptionally inactivated p53 mutants upon treatment with doxycycline of this p53-null cell line. Interestingly, basal protein levels of p53 mutants, in particular TAD1 mutants, were elevated compared to wild-type p53, likely due to the inhibition of Mdm2 ubiquitin ligase binding to p53 following the mutation of residues 22/23 (17, 27, 28). As previously shown (1), overexpression of wild-type p53 strongly induced netrin-1, as well as p21, a p53 target gene (Fig. 1*B*). However, while inactivation of p53 TAD1 ($p53^{22,23}$) did not affect netrin-1 protein expression, overexpression of the TAD2 mutant, alone ($p53^{53,54}$) or in combination with the TAD1 mutant ($p53^{22,23,53,54}$), blocked netrin-1 expression and that of its target gene p21 (Fig. 1*B*). This finding was further confirmed by quantitative RT-PCR (Fig. 1*C*), suggesting that netrin-1 up-regulation requires the transcriptional activity of p53, in particular the TAD2 domain.

Δ40p53, but Not Δ133p53, Regulates Netrin-1 and UNC5B Gene Expression. Some p53 alternative transcripts, generated by 5′ splicing, IRES, or alternative promoter, lack TAD1 (Δ40p53) or both TADs (Δ133p53) (Fig. 2*A*), similarly to the transcriptional mutants generated above. We wondered whether different domains of the p53 N-terminal could regulate netrin-1 gene expression using known p53 isoforms. To this purpose, we generated stable lung cancer A549 lines, harboring wild-type p53, inducible for Δ40p53 or Δ133p53 isoforms, and we analyzed gene expression by quantitative RT-PCR. Upon treatment with doxycycline, Δ40p53 overexpression strongly activated netrin-1 gene expression, whereas Δ133p53 induction had no effect (Fig. 2 *B*, *Left*), confirming results obtained with p53 transcriptional mutants. Interestingly, Δ40p53, but not Δ133p53, also up-regulated the main

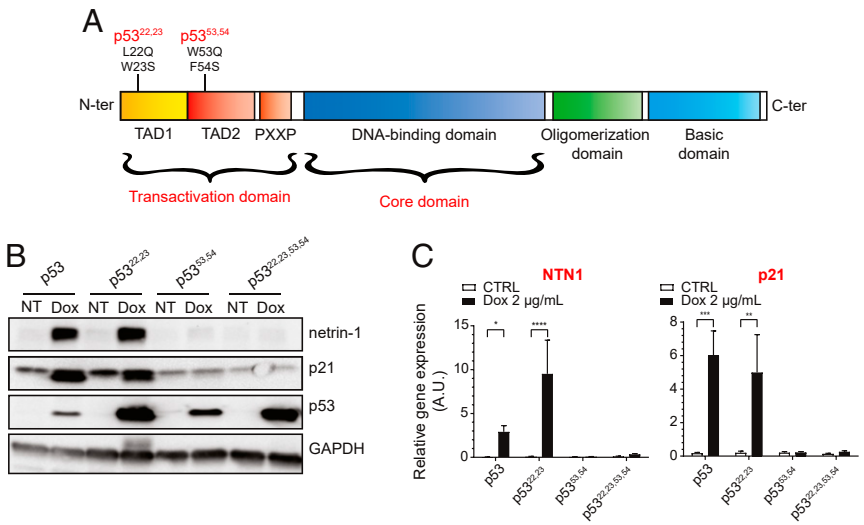

Fig. 1. Netrin-1 regulation by p53 requires the transcriptional activity of the TAD2 domain. (*A*) Schematic representation of the structure of p53 domains. The structure can be divided into three components: an N-terminal domain, encompassing two distinct transactivation domains (TAD1 and TAD2) and a proline-rich domain (PXXP); a core domain with the DNA-binding domain, accounting for binding to responsive elements in a promoter; a C-terminal domain, containing an oligomerization domain, allowing p53 tetramerization, and a basic domain required for protein stability. TAD1- or TAD2-inactivating p53 mutated proteins are indicated in red. (*B* and *C*) TAD2 mutant p53 proteins are unable to trigger netrin-1. Stable H1299 cell lines, inducible for wild-type or mutant p53 proteins, were treated with doxycycline for 24 h and expression of netrin-1, p21, and p53 was analyzed by Western blot (*B*) or quantitative RT-PCR (*C*). The GAPDH protein was used to normalize Western blots, while quantitative RT-PCR was normalized using TATA binding protein (TBP) and β-glucuronidase (GUSB) as housekeeping genes. Immunoblots presented in *B* are representative of three independent experiments. Data in *C* are presented as mean ± SEM ($n = 3$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. A.U., arbitrary units; Dox, doxycycline; NT, not treated; NTN1, netrin-1.

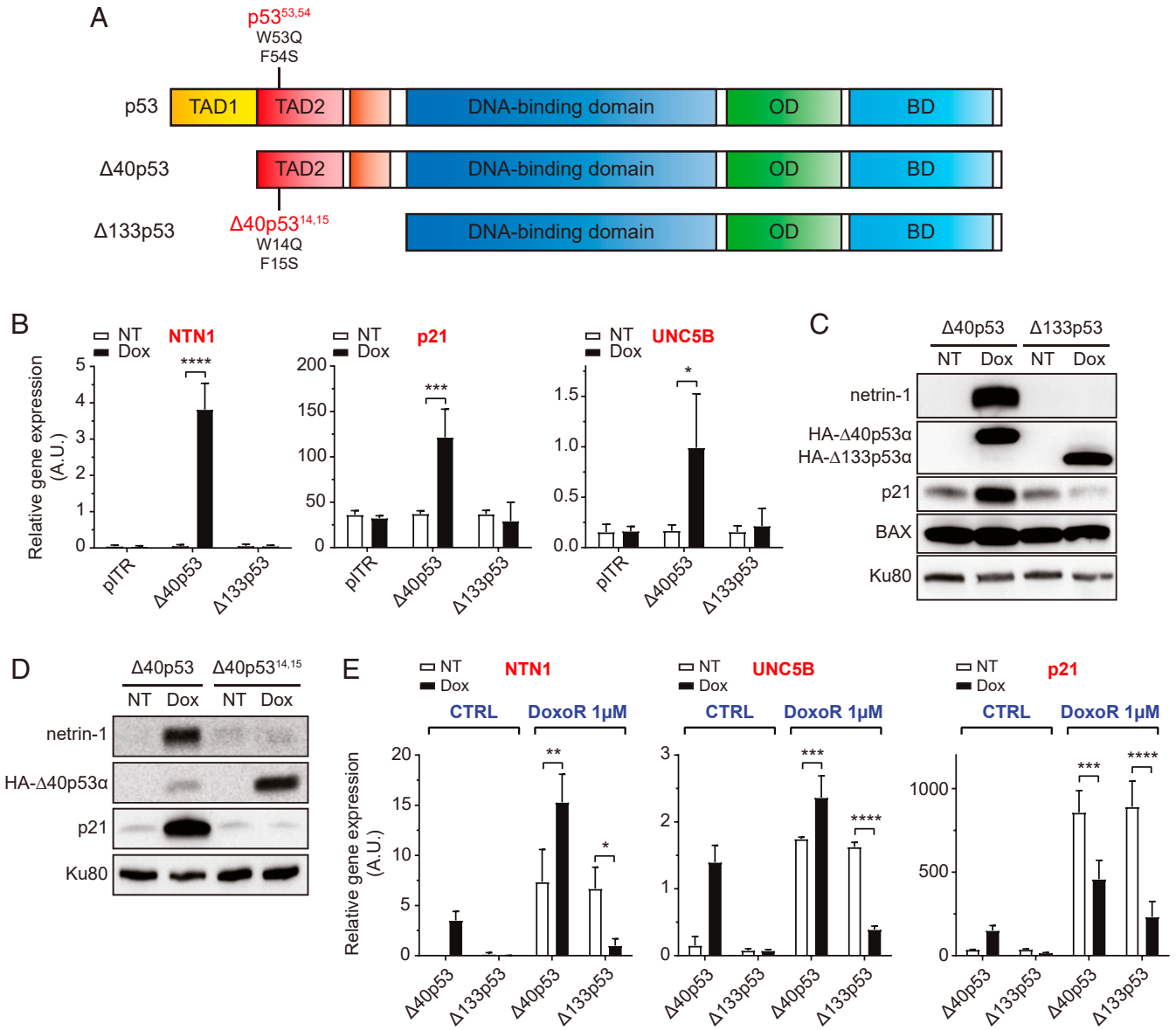

Fig. 2. Δ40p53, but not Δ133p53, regulates netrin-1 and UNC5B expression. (*A*) Schematic representation of the structure of p53 isoform domains. The Δ40p53 isoform lacks TAD1 (in yellow), while Δ133p53 lacks the entire N-terminal domain (including TAD1, TAD2, and PXXP domains) and a small part of DNA-binding domain (in dark blue). (*B* and *C*) Overexpression of Δ40p53 is sufficient to induce netrin-1 gene expression. Stable A549 cell lines, inducible for empty vector (pITR), Δ40p53α-HA, and Δ133p53α-HA, were treated with doxycycline for 24 h. Netrin-1, UNC5B, and p21 gene expression was analyzed by quantitative RT-PCR (*B*), while protein expression of netrin-1, p21, and BAX was evaluated by Western blot (*C*). Expression of HA-tagged p53 isoforms was also assessed by Western blot. (*D*) Δ40p53-dependent netrin-1 induction requires Δ40p53 transcriptional activity. Stable A549 cell lines, inducible for wild-type or TAD-mutated (Δ40p53^{14,15}) Δ40p53 protein, were treated with doxycycline. Netrin-1, p21, and p53 protein expression was evaluated by Western blot. Ku80 protein was used to normalize protein expression. (*E*) Δ40p53 expression, but not Δ133p53, increases doxorubicin-induced netrin-1 up-regulation, while it decreases p21 gene expression upon doxorubicin treatment. Stable A549 cells inducible for Δ40p53 or Δ133p53 were treated with doxycycline or doxorubicin for 24 h. Expression levels of netrin-1 (NTN1), UNC5B, and p21 were evaluated by quantitative RT-PCR. Expression of TBP and GUSB was used as housekeeping genes. Immunoblots in *C* and *D* are representative of three independent experiments. Data in *B* and *E* are presented as mean ± SEM ($n = 3$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. A.U., arbitrary units; BD, basic domain; CTRL, control; Dox, doxycycline; DoxoR, doxorubicin; HA, influenza hemagglutinin; NT, not treated; OD, oligomerization domain.

netrin-1 receptor, UNC5B (Fig. 2 *B*, *Center*) and, despite the fact that Δ40p53 was reported to negatively modulate p21 (29, 30), we observed a positive regulation of p21 by Δ40p53 in our inducible cell lines (Fig. 2 *B*, *Right*). We confirmed the positive regulation of netrin-1 and p21 by Δ40p53 induction at the protein level by Western blot (Fig. 2*C*). It is of interest to note that in this model, Δ40p53 overexpression seemed to rather promote the p53 cell cycle arrest activity, as evidenced by p21 expression, compared to its proapoptotic function, since the BAX protein (Fig. 2*C*), another p53 target gene, remained unaffected.

To confirm that netrin-1 induction by Δ40p53 depends on its transcriptional activity, we conducted point mutations of residues W14 and F15 of Δ40p53 (Δ40p53^{14,15}), equivalent to positions 53 and 54 in FLp53 (Fig. 2*A*). We then generated stable A549 cells inducible for wild-type Δ40p53 and Δ40p53^{14,15} upon treatment with doxycycline, and we assessed protein and transcript levels by Western blot and quantitative RT-PCR analyses. Dysregulation of Δ40p53 transcriptional activity strongly inhibited netrin-1 and p21 protein expression upon doxycycline induction (Fig. 2*D*). Moreover, netrin-1, UNC5B, and p21 gene expression

was very weak following Δ40p53[14,15] overexpression compared to Δ40p53 overexpression (*SI Appendix*, Fig. S1).

Unlike H1299 cells, A549 cells harbor wild-type p53. We thus wondered whether the expression of Δ40p53 and Δ133p53 could affect endogenous FLp53 accumulation and activation upon doxorubicin treatment and in turn affect netrin-1, UNC5B, and p21 gene expression. We treated sleeping-beauty–modified A549 cells with doxycycline (to produce Δ40p53 or Δ133p53) and doxorubicin, and analyzed gene expression by quantitative RT-PCR. In Δ40p53-A549 cells, netrin-1, UNC5B, and p21 gene expression was strongly up-regulated upon treatment with doxycycline and doxorubicin separately (Fig. 2*E*), confirming that Δ40p53 positively regulates these genes, and that endogenous FLp53, induced by doxorubicin, triggers netrin-1 and UNC5B gene expression (1). Interestingly, netrin-1 and UNC5B up-regulation increased further upon induction of Δ40p53, while in Δ133p53-A549 cells doxycycline treatment strongly inhibited netrin-1 and UNC5B induction by doxorubicin (Fig. 2*E*), confirming a dominant-negative role for Δ133p53 against FLp53 (19, 31). Intriguingly, p21 gene expression following doxorubicin treatment was suppressed by doxycycline in both Δ40p53- and Δ133p53-A549 cells (Fig. 2*E*), suggesting that, at least for p21, Δ40p53 may also act as a dominant-negative effector of FLp53.

FLp53 Is Not Necessary for Netrin-1 Regulation by Δ40p53. Even though increasing evidence suggests that Δ40p53 may regulate gene expression independently of FLp53 (32–34), this isoform is also known to function as a FLp53 regulator, since Δ40p53 can oligomerize with FLp53, regulating its activities and functions (24). To assess the involvement of FLp53 in Δ40p53-dependent regulation of netrin-1, we generated stable p53-null H1299 cells, inducible for Δ40p53, FLp53, or both proteins. Gene-expression

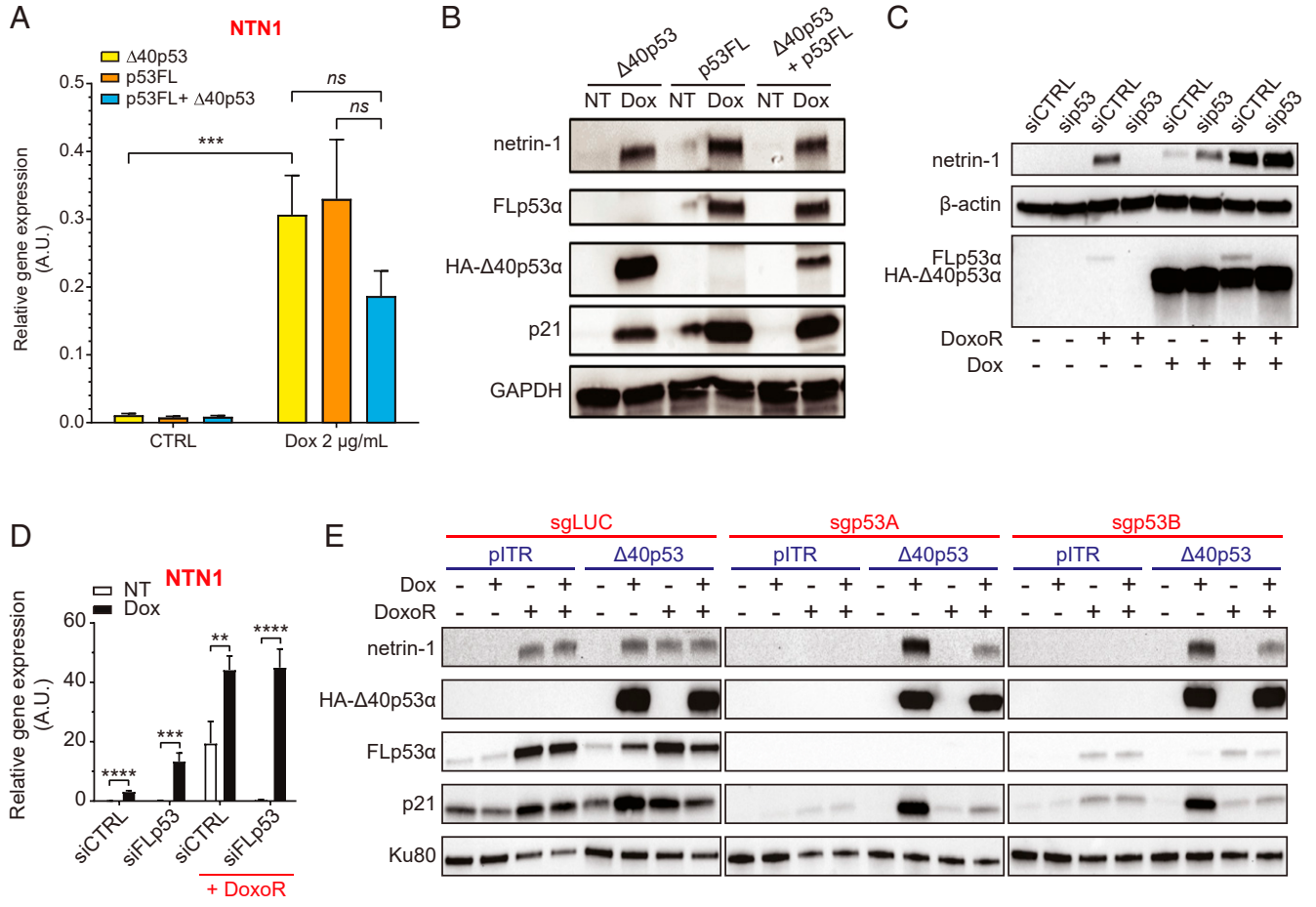

Fig. 3. FLp53 is not necessary for netrin-1 regulation by Δ40p53. (*A* and *B*) Overexpression of FLp53 or Δ40p53 in p53-null cells is sufficient to induce netrin-1 expression. Stable p53-null H1299 cell lines, inducible for full-length p53 (p53FL) or Δ40p53 proteins, alone or in combination (p53FL+Δ40p53), were treated with doxycycline for 24 h. Netrin-1 (NTN1) gene expression was assessed by quantitative RT-PCR ($n = 3$) (*A*). Protein levels of netrin-1 and p21, as well as p53FL and Δ40p53, were evaluated by Western blot (*B*). (*C* and *D*) Silencing endogenous p53 expression does not affect Δ40p53-dependent netrin-1 up-regulation. Stable A549 cell lines, inducible for Δ40p53, were transfected with a scramble siRNA (siCTRL) or with a specific siRNA targeting exon 2 of p53 gene (sip53). Twenty-four hours after transfection, cells were treated with doxorubicin or doxycycline for a further 24 h, and netrin-1 (NTN1) protein and transcript levels were measured by Western blot (*C*) and quantitative RT-PCR ($n = 6$) (*D*), respectively. Expression of endogenous FLp53 and overexpressed Δ40p53α was also evaluated by Western blot, using Pab 1801 p53 antibody (*C*). (*E*) Δ40p53-induced netrin-1 gene expression is not altered by knocking-down endogenous p53. A549 cells were infected using CRISPR/Cas9-modified pLV-hU6-sgRNA-hUbC-dCas9-KRAB-T2a-Puro plasmid, and stable cell lines were generated using firefly luciferase-control (sgLUC) or p53-specific (sgp53A and sgp53B) sgRNAs. Endogenous p53-repressed cell lines were then transfected with empty (pITR) or Δ40p53 sleeping-beauty plasmids. Stable A549 cell lines knockdown for p53 and inducible for Δ40p53 were treated with doxorubicin and doxycycline for 24 h. Efficacy of endogenous p53 repression was evaluated by Western blot, using p53-DO1 antibody, while anti–HA-tagged antibody provided data on Δ40p53 expression. In addition, netrin-1 and p21 protein levels were assessed by Western blot. Immunoblots in *B*, *C*, and *E* are representative of three independent experiments. Quantitative PCR results in *A* and *D* are presented as mean ± SEM; ns, nonsignificant; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. NT, not treated. Dox, doxycycline; DoxoR, doxorubicin; HA, influenza hemagglutinin; sg, small guide; sgLUC, small-guide targeting luciferase.

analysis upon doxycycline treatment revealed a robust induction of netrin-1 gene expression following overexpression of both p53 proteins (Fig. 3*A*). Moreover, coexpression of FLp53 and Δ40p53 did not significantly change netrin-1 expression, compared to separate inductions. We confirmed these results at the protein level by Western blot analysis (Fig. 3*B*).

To ascertain that Δ40p53 regulates netrin-1 per se, regardless of FLp53 expression, we silenced endogenous FLp53 expression in A549 cells inducible for Δ40p53, using a specific small-interfering RNA (siRNA) targeting exon 2 of the p53 gene (i.e., thus avoiding degradation of Δ40p53 mRNA) (Fig. 3*C*). Cells were treated with doxorubicin, in addition to doxycycline, to induce Δ40p53 expression, and FLp53 silencing was then verified by Western blot and quantitative RT-PCR (Fig. 3*C* and *SI Appendix*, Fig. S2*A*). Western blot and quantitative RT-PCR analysis showed a strong accumulation of netrin-1, UNC5B, and p21 upon induction of Δ40p53 or doxorubicin treatment in cells transfected with control siRNA (Fig. 3 *C* and *D* and *SI Appendix*, Fig. S2*A*). Moreover, although FLp53 silencing completely abolished the increase in target gene expression observed in doxorubicin-treated cells, Δ40p53 was able to trigger netrin-1, UNC5B, and p21 gene expression in these cells (Fig. 3 *C* and *D* and *SI Appendix*, Fig. S2*A*).

Finally, to validate the independence of Δ40p53 activity on netrin-1 with respect to FLp53 without relying on siRNA transfections, we stably repressed endogenous FLp53 in A549 cells inducible for Δ40p53, using CRISPR/dCas9-KRAB, a modified CRISPR/Cas9-based system (35). We designed small guide RNAs (sgRNAs), targeting the first canonical p53 promoter, able to direct the binding of a nuclease defective Cas9 (dCas9) enzyme fused to KRAB (Krüppel-associated box) domain of Kox1 (35, 36). The KRAB domain leads to transcription repression of p53 isoforms whose expression is regulated by this promoter—that is, full-length and Δ40p53 isoforms (α, β, and γ)—by mediating local epigenetic reprogramming of histone modifications (37, 38). FLp53 down-regulation was verified by Western blot analysis (Fig. 3*E*) in stably transfected A549 cells treated with doxorubicin. sgp53A completely inhibited basal and drug-induced FLp53 expression, while sgp53B showed a robust repression of FLp53 expression. Accordingly, netrin-1 up-regulation following doxorubicin treatment was completely inhibited in FLp53-repressed cells at protein and transcriptional levels (Fig. 3*E* and *SI Appendix*, Fig. S2*B*), as well as its receptor UNC5B (*SI Appendix*, Fig. S2*C*). However, Δ40p53 induction by doxycycline, not affected by dCas9-KRAB expression since it was regulated by a tetracycline-dependent CMV promoter, was still sufficient to trigger netrin-1 and UNC5B gene expression, even in the absence of FLp53 (Fig. 3*E*). Collectively, these results demonstrate that netrin-1 induction by Δ40p53 is independent of FLp53.

Δ40p53 Directly Binds and Activates the Netrin-1 Promoter. We then assessed whether Δ40p53 regulated netrin-1 via its promoter. Indeed, we previously cloned two different netrin-1 promoters (9, 39), and confirmed the presence of a p53 binding site in the internal netrin-1 promoter (1). Induction of FLp53 or Δ40p53 using doxycycline triggered transcription of firefly luciferase reporter gene placed under the control of the internal netrin-1 promoter or p21 promoter (Fig. 4*A*). However, site-direct mutagenesis of the p53 binding site in the netrin-1 promoter completely abolished promoter transactivation upon induction of both FLp53 and Δ40p53 (Fig. 4*A*).

As Δ40p53, similarly to FLp53, can activate the netrin-1 promoter using the same p53 binding site, we wondered whether Δ40p53 interacted with the netrin-1 promoter in the same region. To ascertain this, we performed a chromatin immunoprecipitation (ChIP) assay, using stable H1299 cells inducible for Δ40p53. PCR results show that, upon IP of overexpressed Δ40p53, using anti–HA-tagged antibody, we amplified a DNA fragment surrounding the p53 binding site in the netrin-1 promoter (Fig. 4*B*). Moreover, we confirmed that Δ40p53 was also able to interact with the p21 promoter (Fig. 4*B*). Altogether, these results demonstrate that Δ40p53 can directly bind the netrin-1 promoter at the same binding site as FLp53, favoring its transcriptional activation.

Endogenous Δ40p53 Regulates Netrin-1 Expression. Having shown that overexpression of Δ40p53 induces the expression of netrin-1 and its receptor UNC5B, we next studied the role of endogenously produced Δ40p53 on netrin-1 regulation. To achieve this, we used immortalized human skeletal myoblasts LHCN-M2,

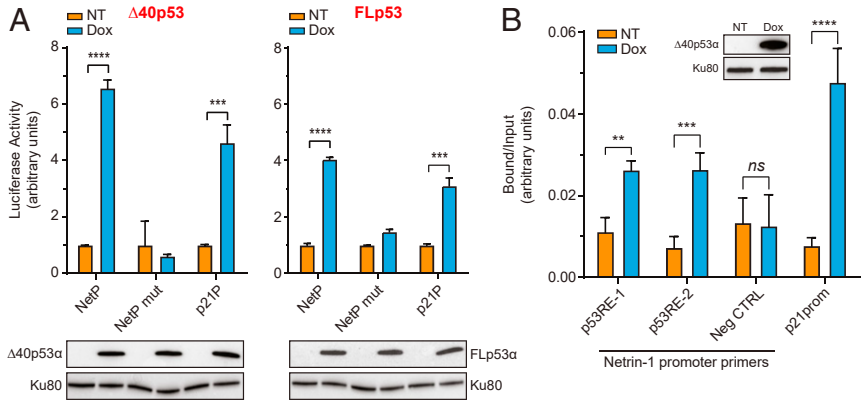

Fig. 4. Δ40p53 directly binds to and activates the netrin-1 promoter. (*A*) Δ40p53 activates the netrin-1 promoter, binding to canonical p53 responsive elements. Stable H1299 cells inducible for Δ40p53 or FLp53 were transfected with wild-type (NetP) or mutated for p53 binding site (NetP mut) promoters, as well as for the p21 promoter (p21P). Twenty-four hours after transfection, cells were treated with 2 μg/mL of doxycycline for a further 24 h and promoter activities were assessed by luciferase assay. Values represent mean ± SEM ($n = 3$). Induction of Δ40p53 and FLp53 was verified by Western blot, using α-HA antibody. (*B*) Δ40p53 directly binds to the netrin-1 promoter. Stable H1299 cells inducible for Δ40p53-HA were treated with 2 μg/mL of doxycycline for 24 h. ChIP was performed using α-HA antibody, as described in *Materials and Methods*. Δ40p53-coprecipitated DNA was analyzed by quantitative PCR, using two different primer couples surrounding the p53 binding site in the netrin-1 promoter (p53RE-1 and -2) (1). A couple of primers, amplifying a not p53-related fragment in the netrin-1 promoter, was used as negative control (Neg CTRL). p21 primers, surrounding the p53 binding site −1,354 contained in the p21 promoter (58), were used to confirm activation and binding of p21 promoter upon Δ40p53 induction. Values represent mean ± SEM ($n = 4$). Western blot in the *Inset*, performed with α-HA antibody, confirmed Δ40p53 expression in IP samples. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; ns, nonsignificant. CTRL, control; Dox, doxycycline; NetP, netrin-1 promoter; p21P, p21 promoter.

harboring wild-type p53. We designed two different sgRNAs, targeting "canonical" ATG in p53 exon 2 (sgFLp53) or the ATG codon 40 in p53 exon 4 (sgTOTp53), in order to inactivate FLp53 (i.e., p53α, -β, and -γ) or FL/Δ40p53 (including Δ40p53α, -β, and -γ) isoforms, respectively (Fig. 5*A*). Moreover, both sgRNAs should not affect the expression of Δ133p53 and Δ160p53 isoforms. Then, using the CRISPR/Cas9 technique, we generated stable LHCN-M2 cell lines. Western blot analysis revealed a complete knockout of p53 isoforms in stable cells generated with sgTOTp53, whereas in cells set-up with sgFLp53 only FLp53 was deleted (Fig. 5*B*). Moreover, in these cells a band corresponding to Δ40p53α appeared, demonstrating that this CRISPR/Cas9 strategy allowed us to force LHCN-M2 cells to produce endogenous Δ40p53 instead of FLp53. Interestingly, netrin-1 and UNC5B proteins were strongly expressed in LHCN-M2 sgFLp53 cells, harboring Δ40p53 instead of FLp53, compared to control or sgTOTp53 cells (Fig. 5*B*). Moreover, quantitative RT-PCR analysis confirmed an increase in netrin-1 expression in LHCN-M2 cells expressing endogenous Δ40p53 (Fig. 5*C*). Intriguingly, gene expression of p21 and GADD45 (p53 target genes involved in cell cycle arrest) strongly decreased in both FLp53 knockout cells, compared to parental or control LHCN-M2 cells, while only complete p53 knockout drove a decrease in BAX (involved in apoptotic function of p53) gene expression (Fig. 5*C* and *SI Appendix*, Fig. S3).

Δ40p53 Expression Intensifies Netrin-1 Dependence and Sensitizes Cells to Netrin-1 Silencing-Induced Apoptosis. Since the overexpression or forced expression of Δ40p53 triggered an increase in the expression of netrin-1 and its receptor UNC5B, we hypothesized that in these cells survival dependency on netrin-1 expression may be amplified. To confirm this, we transfected stable LHCN-M2 cells with siRNA specific for netrin-1 (1). Netrin-1 silencing induced an increase in DNA fragmentation exclusively in sgFLp53 cells expressing endogenous Δ40p53 and consequently netrin-1 and UNC5B (Fig. 6*A*). Moreover, in these cells, netrin-1 silencing induced a robust caspase-3 activation, compared to parental or control LHCN-M2 cells (Fig. 6*B*). Furthermore, complete p53 knockout desensitized sgTOTp53 cells to netrin-1 silencing (Fig. 6 *A* and *B*). DNA fragmentation and caspase-3 activation observed in LHCN-M2 cells suggest that cell death triggered by netrin-1 silencing is apoptosis. Along the same line, transfection of siRNA targeting netrin-1 induced a consistent increase of the percentage of Annexin V^+ cells only in sgFLp53 cells expressing endogenous Δ40p53α (Fig. 6*C* and *SI Appendix*, Fig. S4). In line with these results, silencing netrin-1 increased DNA fragmentation upon induction of Δ40p53 in stable A549 cells (Fig. 6*D*), confirming the amplification of netrin-1 dependency in cells expressing high levels of Δ40p53. Interestingly, netrin-1 silencing had no effect on the induction of apoptosis in cells not treated with doxycycline (Fig. 6*D*).

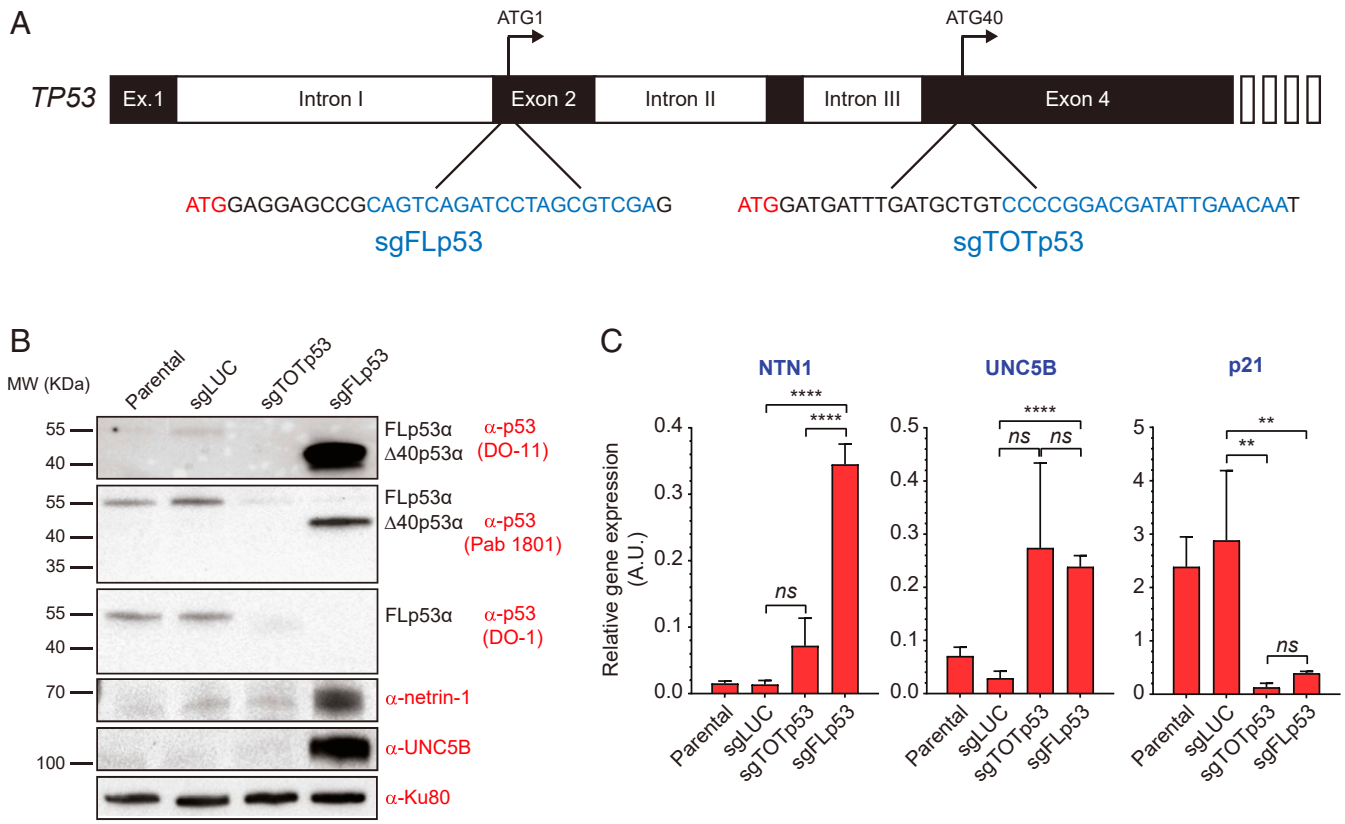

Fig. 5. Endogenous Δ40p53 regulates netrin-1 expression. (*A*) Schematic representation of the 5′ sequence of p53 gene. ATG codons for FLp53 (ATG1) and Δ40p53 (ATG40) are indicated, as well as two sgRNAs, designed near ATG1 (sgFLp53) and ATG40 (sgTOTp53). In *Insets*, sequences of sgRNAs are displayed in blue, while ATG codons are in red. (*B* and *C*) Forced endogenous Δ40p53 expression drives an increase in netrin-1 and UNC5B. Stable LHCN-M2 cells knocked out for the p53 gene were generated using firefly luciferase-control (sgLUC), ATG1- (sgFLp53) or ATG40- (sgTOTp53) targeting sgRNAs. Western blot analysis, using the pan-p53 antibodies DO-11 and Pab 1801, confirmed the complete knockout of all isoforms of p53 in LHCN-M2 cells generated with guide sgTOTp53, targeting Δ40p53 ATG40 codon, while guide sgFLp53, targeting only the ATG1 codon of FLp53 forced cells to produce a shorter p53 isoform, instead of FLp53, with a molecular weight corresponding to Δ40p53 (*B*). Western blot with the N-terminal p53-DO-1 antibody confirmed that this band corresponds to a p53 isoform lacking the very first N-terminal amino acids. Netrin-1, UNC5B and Ku80 protein levels, revealed by Western blot, are also displayed. Expression levels of netrin-1 (NTN1), UNC5B and p21 transcripts were analyzed by quantitative RT-PCR (*C*). Immunoblots in (*B*) are representative of three independent experiments. Quantitative PCR results in (*C*) are presented as mean ± SEM ($n = 3$). ns, nonsignificant; $^{**}P < 0.01$; $^{****}P < 0.0001$; Ex, exon; KDa, Kilodalton; MW, molecular weight.

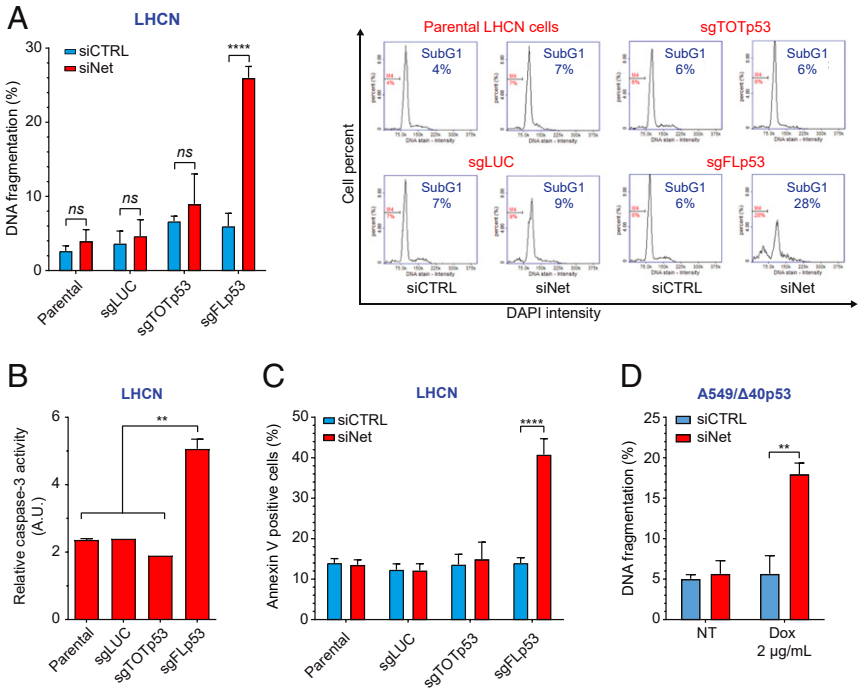

Fig. 6. Δ40p53 expression sensitizes cells to netrin-1 silencing-induced apoptosis. (*A–C*) Parental or stable LHCN-M2 cells, infected with control (sgLUC), FLp53/Δ40p53 (sgTOTp53), or FLp53 only (sgFLp53) knockout lentiviral constructs, were transfected with scramble (siCTRL) or netrin-1-specific (siNet) siRNAs. Induction of apoptosis was evaluated by DNA fragmentation assay (SubG1) (*A*), caspase-3 activity (*B*), or Annexin V and PI double staining analyzed by flow cytometry (*C*). A representative DNA fragmentation experiment is shown in *A*, *Right*. (*D*) Stable A549 cells, inducible for Δ40p53, were transfected with siCTRL or siNet siRNAs. 24 h after transfection, cells were treated with 2 μg/mL of doxycycline and induction of apoptosis was evaluated 72 h later by DNA fragmentation assay. Results are presented as mean ± SEM (n = 3). ns, nonsignificant; $^{**}P < 0.01$; $^{****}P < 0.0001$; ns, nonsignificant. Dox, doxycycline; sg, small guide.

Netrin-1 Interference Inhibits Tumor Growth in Tumor Cells Expressing Δ40p53 In Vivo. Because we observed that overexpression of Δ40p53, by inducing both netrin-1 and UNC5B, renders cancer cells more prone to die upon netrin-1 silencing, we next look at whether this can be associated with tumor growth inhibition in vivo, using a more therapeutically relevant approach. For this purpose, we evaluated the effect of NP137, a neutralizing antinetrin-1 antibody able to induce cancer cell death and tumor growth inhibition in several preclinical models (2), upon Δ40p53 induction using the chicken chorioallantoic membrane (CAM) xenograft model. This technique is well accepted to study primary tumor growth on the highly vascularized CAM of chicken embryo (40, 41). Stable A549 cells inducible for Δ40p53 were engrafted into the CAM of embryonic day (E)10 chicken embryos and primary tumors at the inoculation site were resected 7 d after (Fig. 7*A*). In the presence of doxycycline, Δ40p53α was induced (*SI Appendix*, Fig. S5*A*) and antinetrin-1 antibody (NP137) strongly reduced both the size and the weight of the tumors, compared to isotypic antibody (Iso-mAb)-treated engrafted cells (Fig. 7 *B* and *C*). Moreover, treatment with NP137 antibody did not affect CAM tumor growth in PBS-treated cells, indicating that the antitumoral effect of netrin-1 interfering is dependent on Δ40p53 expression. Interestingly, production of Δ40p53 upon doxycycline treatment slightly sustained tumor growth, even though this increase was not significant, suggesting a protumoral effect of Δ40p53/netrin-1 axis, that could be reverted by netrin-1 interference.

The antitumoral effect of antinetrin-1 antibody was also confirmed in a more conventional preclinical model of *nude* mice, engrafted with stable A549 cells inducible for Δ40p53α. As observed for the CAM model, while we noticed a substantial tumor growth in mice treated with PBS or isotypic control, antinetrin-1 antibody considerably inhibited tumor progression in mice watered with doxycycline (Fig. 7*D* and *SI Appendix*, Fig. S5*B*). This effect was associated with apoptosis induction, as indicated by the increase of cleaved caspase-3$^+$ cells in engrafted tumors treated with both doxycycline and NP137 antibody (Fig. 7*E* and *SI Appendix*, Fig. S5*C*).

Finally, we tested how endogenous Δ40p53 protein could affect sensitivity to netrin-1 inhibition. For this purpose, we used colorectal cancer HCT116 cells wild-type (HCT116$^{p53+/+}$) or deleted for FLp53 (HCT116$^{p53-/-}$) (42). Similar to the sgFLp53 LHCN-M2 cells we described above (Fig. 5*A*), HCT116$^{p53-/-}$ cells were generated by deletion of exon 2 on p53 gene, resulting in FLp53 knockout. So, in this setting the HCT116$^{p53+/+}$ parental cell line is expressing low level of wild-type FLp53 but no Δ40p53 protein, whereas HCT116$^{p53-/-}$ cells express no FLp53 but Δ40p53 protein (*SI Appendix*, Fig. S6*A*). Of interest, HCT116$^{p53-/-}$ cells showed a significant increase of netrin-1 and UNC5B transcripts (*SI Appendix*, Fig. S6*B*). Accordingly, transfection of siRNA targeting netrin-1 had no effect on cell viability in wild-type cells (i.e., supporting the view that low endogenous FLp53 does not regulate netrin-1/UNC5B activity) while it triggers DNA fragmentation exclusively in HCT116$^{p53-/-}$ cells (*SI Appendix*, Fig. S6*C*). Moreover, NP137 antibody nicely decreased the size and the weight of tumors obtained from HCT116$^{p53-/-}$ cells on chicken CAMs (Fig. 7*F* and *SI Appendix*, Fig. S6*D*).

Netrin-1 and Δ40p53 Gene Expression Are Correlated in Human Tumor Biopsies. We thus wondered whether Δ40p53 could positively regulate netrin-1 in a panel of human tumors. Indeed, conversely to FLp53, the activity of which is mainly regulated at a post-translational level in human cancers, Δ40p53 could be transcriptionally regulated and contribute to the neoplastic phenotype (29, 43–45). Moreover, netrin-1 is often dysregulated in cancers. For this purpose, we analyzed mRNA levels of netrin-1 and Δ40p53 gene expression in melanoma and colorectal cancer biopsies by

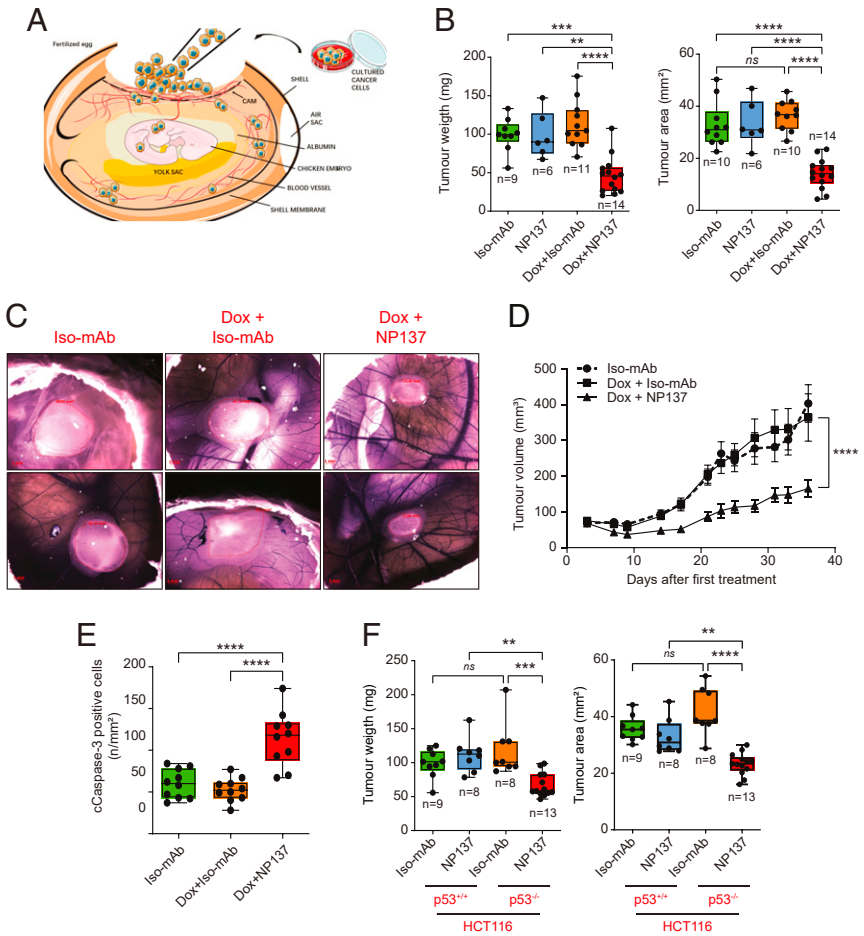

Fig. 7. Δ40p53 expression sensitizes cells to netrin-1 interference-induced tumor growth inhibition. (*A*) Schematic representation of the experimental settings of tumor growth in the chicken CAM model. A549 cells were seeded in the CAM of E10 chicken embryo, together with doxycycline and Netrin-1 neutralizing antibody. (*B*) Weight and size of CAM-engrafted tumors. Primary tumors were treated with 10 μg/mL of neutralizing antinetrin-1 antibody (NP137) or isotypic control (Iso-mAb), in presence of 2 μg/mL of doxycycline. After 7 d, primary tumors were imaged using Zeiss microscope and tumor areas were measured with AxioVision Rel software. Moreover, tumors were resected and weighted. *n* indicates the number of samples for each condition. (*C*) Representative images of A549 primary tumors, treated with PBS or doxycycline (Dox), in combination with antinetrin-1 (NP137) or isotypic antibodies (Iso-mAb). Red lines represent tumor volume automatically measured by AxioVision software. (Scale bar, 1 mm.) (*D*) Neutralizing netrin-1 antibody (NP137) inhibits tumor growth in *nude* mice engrafted with stable A549 inducible for Δ40p53 and watered with doxycycline, compared to animals treated with isotypic antibody (Iso-mAb) ($n = 10$ for each group). Tumor volume were measured at the indicated days after the first treatment with antibodies. (*E*) Antitumoral effect of NP137 antibody is associated with apoptosis induction. Engrafted tumors were resected 36 d after beginning treatments and apoptosis was evaluated by immunohistochemical staining of active caspase-3. (*F*) Δ40p53 expression in colorectal cancer cells sensitizes cells to netrin-1 inhibition in the CAM model. Wild-type or FLp53-knockout HCT116 cells were seeded in the CAM of E10 chicken embryo, together with 10 μg/mL of antinetrin-1 (NP137) or control (Iso-mAb) antibodies. Tumors were imaged after 7 d and tumor areas were measured, as well as their weight. *n* indicates the number of samples for each condition. Data are represented as mean ± SEM; ns, nonsignificant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. cCaspase-3, cleaved caspase-3; Iso, isotypic; mAb, monoclonal antibody.

quantitative RT-PCR. As shown in Fig. 8*A*, in both cohorts analyzed we found a positive and significant correlation between netrin-1 and Δ40p53. Moreover, netrin-1 appeared more expressed in human tumor materials presenting high levels of Δ40p53 (Fig. 8*B*). These results support the view that in human tumors, netrin-1 could be positively regulated by Δ40p53.

Discussion

Netrin-1 is a protein frequently up-regulated in aggressive human cancers (8–11). The protumoral function of netrin-1 mainly results from its ability to bind dependence receptors DCC and UNC5H, inhibiting their proapoptotic activity (5, 46–49). Within this context, several studies have focused on inhibiting the binding of netrin-1 to its receptors to induce cancer cell death and tumor regression (2, 8, 10–12, 50). These attempts led to the development of a netrin-1 neutralizing antibody, currently in a phase Ib/II clinical trial (2). However, netrin-1 inhibition may only be applied to the fraction of tumors expressing high levels of netrin-1. Therefore, the knowledge of which cancer cells rely on this strategy to survive and what mechanisms underlie netrin-1 up-regulation in tumor cells could be important in developing personalized therapeutic treatments. Having shown that the transcription factor p53 positively regulates netrin-1 (1), we investigated the underlying mechanisms, as such knowledge could increase the fraction of cancer patients who may be eligible for a netrin-1 interference-based treatment during early clinical evaluation.

Here we demonstrate that Δ40p53, a N-terminal truncated isoform of p53, transcriptionally regulates the gene expression of netrin-1 and its receptor UNC5B, and unexpectedly, does so independently of the presence of FLp53. Indeed, early studies reported Δ40p53 as a dominant-negative regulator of FLp53 (20), since its overexpression down-regulates FLp53-induced target genes, counteracting FLp53-dependent growth suppression

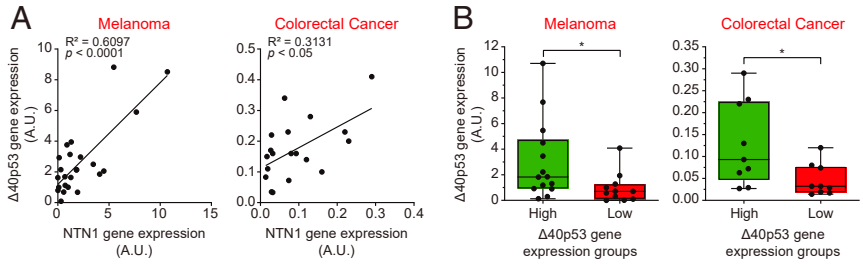

Fig. 8. Netrin-1 and Δ40p53 gene expression is correlated in human melanoma and colorectal tumor biopsies. (*A*) Correlation between Δ40p53 and netrin-1 gene expression in melanoma ($n = 24$) and colorectal cancer ($n = 19$) biopsies. mRNA levels of Δ40p53 was plotted as a function of netrin-1 (NTN1) expression, and the level of fit was obtained by calculating the coefficient of determination R^2. (*B*) Melanoma and colorectal cancer biopsies were separated according to their relative Δ40p53 gene expression, in order to form high (exceeding the median gene-expression value) or low Δ40p53 expression groups. Netrin-1 gene expression was then evaluated in Δ40p53 expression groups. $*P < 0.05$.

(23, 24). However, Δ40p53 was more recently described as a positive regulator of FLp53 functions (30), since Δ40p53 overexpression induces apoptosis in melanoma cells in cells expressing wild-type FLp53 exclusively (29). These paradoxical results have been attributed to dose-dependent effects of hetero-oligomerization of p53 isoforms, dependent on the relative expression levels (51). For example, it has been demonstrated that low Δ40p53 expression enhances FLp53 transactivation activity, whereas increasing levels suppress antiproliferative effects of FLp53 (30). As a consequence, because netrin-1 was previously described as a FLp53 target gene (1), its regulation by Δ40p53 expression could have been due to p53 stabilization and activation by hetero-oligomers Δ40p53/FLp53. However, our results, showing netrin-1 up-regulation by Δ40p53 in H1299 p53-null cells or upon FLp53 knockout in A549 p53-wild-type cells, suggest the formation of a Δ40p53-only homo-tetramer, binding to and activating the netrin-1 promoter. On the other hand, binding of Δ40p53 to p53 responsive elements located in MDM2, GADD45, and BAX genes, leading to FLp53-independent transactivation, has been already reported (32). Interestingly, our data also show that the CRISPR/Cas9 technique, using specific sgRNAs, enabled us to generate stable primary cells expressing Δ40p53 instead of FLp53. In this way, we were also able to confirm the up-regulation of netrin-1 and UNC5B by endogenously expressed Δ40p53. This information, together with gene-expression analysis on inducible stable cell lines, suggests that Δ40p53 can establish homo-tetramers, transactivating the netrin-1 promoter, in the context of both high and low Δ40p53 expression levels, at least in the absence of FLp53.

p53 isoforms are differentially expressed in several human cancers (21, 52), such as triple-negative breast and mucinous ovarian cancers and melanoma (43–45). However, the high expression of Δ40p53 and correlated cancer aggressiveness has been associated with the dominant-negative function of this isoform on FLp53 activity. Interestingly, we show here a positive correlation between Δ40p53 and netrin-1 gene expression in melanoma and colorectal cancer cohorts. Moreover, netrin-1 and its dependence receptors are involved in melanoma and colorectal cancer progression (9, 49, 53, 54). Our data thus suggest a FLp53-independent protumoral role for Δ40p53, because of its ability to transactivate an anti-apoptotic protein, such as netrin-1.

Regulation of netrin-1 and UNC5B receptor by the Δ40p53 isoform could have significant therapeutic consequences. Indeed, the fact that both the ligand and its dependence receptor are up-regulated in human cancers coexpressing Δ40p53 could enhance dependency of tumor cell survival on netrin-1 expression. Specifically, inhibition of interference of netrin-1 in these cancer cells should lead to dependence receptor-induced apoptosis and tumor regression. Our results demonstrate that silencing of netrin-1 expression by RNA interference induces cell death only in cells that are forced to produce Δ40p53 and consequently express high levels of netrin-1 and UNC5B. From a therapeutic point of view, we show here that, upon induction of Δ40p53, interference of the binding between netrin-1 and UNC5B with an antinetrin-1 neutralizing antibody is sufficient to inhibit tumor growth in two different preclinical models of chicken CAM and *nude* mice.

In conclusion, we show here that the Δ40p53 isoform could positively regulate an antiapoptotic protein, such as netrin-1, in a FLp53-independent way. In addition, this regulation of Δ40p53 could explain the increased transcript level of this survival protein, observed in several human cancers. Indeed, each mutation changing the ratio between FLp53 and Δ40p53 generation (i.e., nonsense mutations in exon 2/3) could confer a selective advantage to cancer cells. Δ40p53 could inhibit the tumor suppressor activity of FLp53, acting as a dominant-negative protein, but could also actively transactivate protumoral factors, such as shown here for netrin-1. In this context, a promising therapeutic strategy could be to inhibit the protumoral activity, as we propose for netrin-1, instead of targeting the dominant-negative function of Δ40p53.

Materials and Methods

Generation of Stable Cell Lines, Reagents, and Transfection Procedures. A549 and H1299 cell lines were obtained from the ATCC. hTERT and cdk4-immortalized LHCN-M2 cells were a kind gift from Vincent Mouly, Center for Research in Myology, Paris, France (55). HCT116$^{p53-/-}$ cells were generated previously (42) and were kindly provided by B. Vogelstein, Ludwig Center at Johns Hopkins, Baltimore, MD. N-terminal HA-tagged human FLp53, Δ40p53, and Δ133p53 coding sequences were cloned at SfiI sites in the pITR plasmid (56). Detailed cell culture conditions, reagents, transfection procedures, and protocols for generation of stable cell lines are provided in *SI Appendix, Supplementary Materials and Methods*. siRNA and small guide cloning sequences are listed in *SI Appendix*, Fig. S7.

Quantitative RT-PCR and Western Blot Analysis. Total RNAs were extracted using the NucleoSpin RNA Plus Kit (Macherey Nagel) according to the manufacturer's protocol. RT-PCR reactions were performed with the PrimeScript RT Reagent Kit (Takara Bio Europe). For immunoblotting analysis, cells were lysed by sonication in SDS buffer (10 mM Tris·HCl pH 7.4, 10% glycerol, 5% SDS, 1% TX-100, 100 mM DTT) in the presence of protease inhibitor mixture (Roche Applied Science). Antibodies and detailed protocols are supplied in *SI Appendix, Supplementary Materials and Methods*. Primer sequences are available in *SI Appendix*, Fig. S7.

Reporter Assay and ChIP Assay. Stable A549 cells inducible for FLp53 or Δ40p53 were plated in 12-well plates and transfected with the different firefly luciferase reporters containing wild-type or p53-mutated netrin-1 promoter constructs, described previously (1, 9, 39) (*SI Appendix, Supplementary Materials and Methods*). ChIP assay was performed as previously described (1), with some minor changes, described in *SI Appendix, Supplementary Materials and Methods*. Sequences of primers used for ChIP analysis are illustrated in *SI Appendix*, Fig. S7.

Cell Death Assays. For DNA fragmentation analysis (SubG1), DNA content was evaluated using the NucleoCounter NC-3000 system (ChemoMetec A/S). For AnnexinV and propidium iodide (PI) double staining, Annexin V$^+$ cells were then evaluated by FACS on FACSCanto II (BD Biosciences). The caspase-3

activity assay was performed as described previously (56). Detailed protocols are available on *SI Appendix, Supplementary Materials and Methods*.

Xenografts in Ovo and in *nude* Mice. For chick CAM assays, cells were seeded on 10-d-old CAMs, together with netrin-1–neutralizing antibody NP137 and relative control NP001 (10 μg/mL) (Netris Pharma) and doxycycline (2 μg/mL). For xenografts in *nude* mice, A549 cells were implanted in 5-wk-old female athymic *nude* Foxn1$^{nu/nu}$ mice. Procedures and treatment administration are detailed in *SI Appendix, Supplementary Materials and Methods*.

All experiments were performed in accordance with relevant guidelines and regulations of animal ethics committee (authorization APAFIS 28723; accreditation of laboratory animal care by the Comité d'Éthique en Expérimentation Animale [CECCAP], École Normale Supérieure Lyon, Plateau de Biologie Expérimentale de la Souris, France).

Patients and Tumor Samples. Twenty-four melanoma samples were collected as previously described (54), whereas 19 primary colorectal cancer tissue samples were obtained from the Biological Resource Centre of Léon Bérard Centre (protocol no.: BB-0033-00050; Lyon, France), as previously described (57) (*SI Appendix, Supplementary Materials and Methods*).

Data Availability. All study data are included in the article and *SI Appendix*.

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