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Δ160p53 is a novel N-terminal p53 isoform encoded by Δ133p53 transcript

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ABSTRACT

***p53* gene expresses several protein isoforms modulating p53-mediated responses through regulation of gene expression. Here, we identify a novel p53 isoform, Δ160p53, lacking the first 159 residues. By knockdown experiments and site-directed mutagenesis, we show that Δ160p53 is encoded by Δ133p53 transcript using ATG160 as translational initiation site. This hypothesis is supported by endogenous expression of Δ160p53 in U2OS, T47D and K562 cells, the latter ones carrying a premature stop codon that impairs p53 and Δ133p53 protein expression but not the one of Δ160p53. Overall, these results show that the Δ133p53 transcript generates two different p53 isoforms, Δ133p53 and Δ160p53.**

1. Introduction

The tumour suppressor p53 protein maintains genomic and cellular integrity [1]. Besides the classical p53 protein, the human *p53* gene expresses several isoforms [2]. Three N-termini (TA, Δ40, Δ133) can be combined to three distinct C-termini (α, β, γ), the six variations resulting from usage of alternative promoters, splicing sites or translational initiation sites [3]. TA forms contain transactivation domain (TAD), which is partially or entirely lacking in Δ40 and Δ133 forms respectively, and α forms include an oligomerisation domain, replaced by new residues in β and γ forms [2,4]. The expression of several p53 isoforms, together with their production mechanism and their structural organisation, is conserved through evolution [5]. In humans, p53 isoforms are expressed in a tissue-dependent manner and their expression is deregulated in cancers [2,6]. Altogether, these observations indicate that p53 isoforms have important functions.

The Δ133p53α isoform is generated by an internal promoter located within *p53* gene [2]. Through direct binding, p53 regulates internal promoter activity thus inducing Δ133p53 expression in response to stress, both in zebrafish and in human cells [7–9]. Furthermore, several studies suggested a role of Δ133p53 in p53-mediated response. In human fibroblasts, knockdown of Δ133p53 expression promoted replicative senescence through modulation of *mir-34* expression, a p53-target gene [10]. In response to stress, induction of Δ133p53α resulted in a p53-mediated G2 cell-cycle arrest with inhibition of p53-mediated apoptosis and G1 arrest, by modulating expression of some p53-related genes [7,8]. These observations suggest that Δ133p53α is part of an auto-regulatory feedback loop, where p53 induces Δ133p53α expression to regulate its own transcriptional activity thus triggering adequate responses to stress. In previous report, we observed that ectopic expression of Δ133p53α resulted in detection of a band doublet, which decreased in cells treated with a Δ133p53-specific siRNA [8]. Here, we identify the upper band as Δ133p53α and the lower band as a novel p53 isoform, Δ160p53α.

2. Materials and methods

2.1. Cell lines and plasmids

The human U2OS (wild-type p53), T47D (mutant p53L194F) and H1299 (p53-null) cells were maintained at 37 °C in DMEM medium supplemented by 10% foetal calf serum (FCS) and 0.5% gentamycin under 5% CO_2 atmosphere. Doxorubicin treatment was performed as previously described [8]. The U2OS-Δ133p53α cells stably express Δ133p53α isoform and selective pressure is maintained by addition of 0.5 mg/ml neomycin in culture medium [8]. The human erythroleukaemic K562 cells were maintained at 37 °C in RPMI medium supplemented by 10% FCS, 2 mM

Abbreviations: TAD, transactivation domain; DBD, DNA-binding domain; OD, oligomerisation domain

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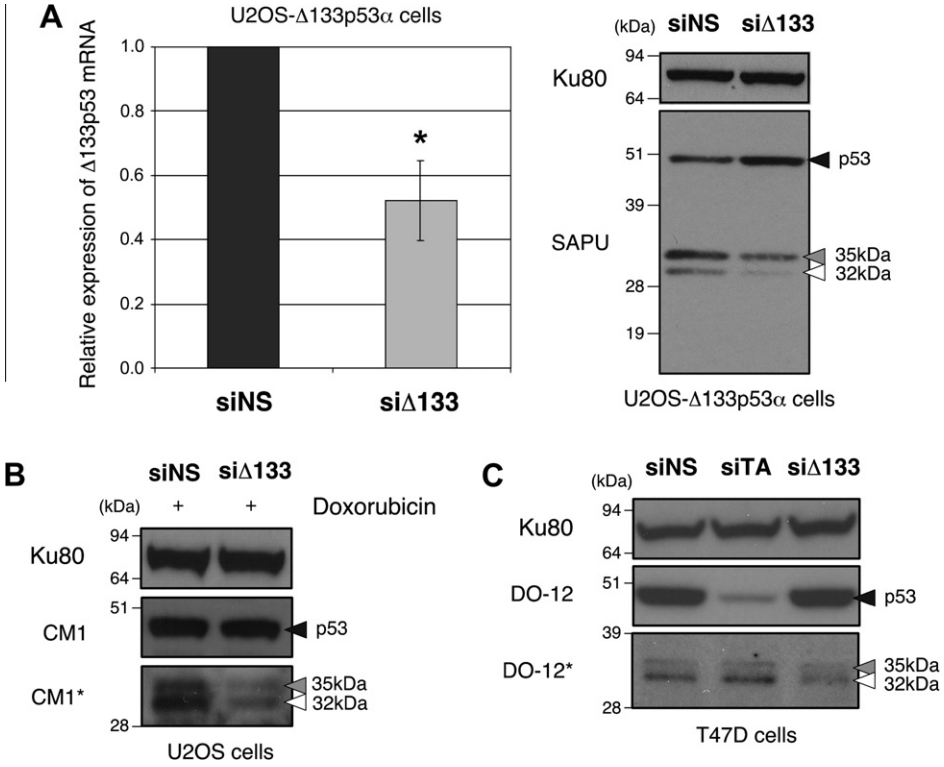

Fig. 1. Effect of Δ133p53 knockdown on the detection of a band doublet. (A) Knockdown of Δ133p53 expression in wild-type p53 U2OS-Δ133p53α cells, stably expressing Δ133p53α isoform. U2OS-Δ133p53α cells were transfected with either a non-relevant siRNA (siNS) or a siRNA targeting Δ133p53 transcripts (siΔ133). Expression of Δ133 forms was analysed at mRNA levels by real time PCR (left panel) (Supplementary Table I) and at protein levels by western blot using SAPU antibody recognising all p53 isoforms (right panel) (see [5] for description of p53 isoforms antibodies). Compared to siNS, siΔ133 treatment decreased the protein band doublet detected at 35 and 32 kDa. Ku80: loading control; *: *P*-value <0.05. (B) Knockdown of endogenous Δ133p53 expression in the parental U2OS cells in response to doxorubicin treatment. The parental U2OS cells were transfected with siRNAs as described in (A) and exposed to doxorubicin treatment to induce endogenous Δ133p53 protein, as previously described [8,9]. Analysis of protein expression using CM1 antibody specific of all p53 isoforms revealed a decrease of the band doublet in cells transfected with siΔ133 compared to the ones transfected with siNS. CM1*: long exposure compared to CM1. (C) Knockdown of endogenous Δ133p53 expression in T47D cells, as described in (A). The endogenous band doublet was detectable using DO-12 antibody specific of all p53 isoforms without any drug treatment. Compared to siNS, the band doublet signal decreased in siΔ133 treated cells but not in response to siTA, specifically targeting TAp53 mRNA forms (see Fig. 5 for localisation of p53 siRNAs). DO-12*: long exposure.

L-glutamin, 100 IU/ml penicillin and 100 μg/ml streptomycin under 5% CO_2 atmosphere. The differentiation of K562 cells was induced by addition of 50 μM Hemin for 96 h into 3.10^5 cells.

Δ133p53α transcripts issued from a mix of five normal human tissues were previously cloned in a pSV expressing vector (pSV-Δ133p53α) [2]. Δ133p53α and Δ133p53β mRNAs expressed in K562 cells were introduced into the same vector (pSV-K562α and pSV-K562β, respectively). In these plasmids, point mutations were introduced by site-directed mutagenesis to insert a premature stop codon at residue 255 (I255X) or replace methionine residue by a leucine at codon 133 (M133L) or codon 160 (M160L). pEGFP-N1 was used as a control of transfection efficiency.

Oligofectamine (Invitrogen) and Fugene (Roche) were used to introduce into cells 50 nM of siRNAs (Supplementary Table I) or 1 μg of expressing vectors, respectively.

2.2. Real-time PCR

Total RNAs were extracted using the RNeasy Mini Kit (Qiagen). Reverse transcription was performed on 1 μg of total RNAs using the SuperScriptII (Invitrogen) at 42 °C. Quantification of Δ133p53 mRNA levels was carried out by real-time PCR on MX3000P apparatus (Stratagene). Briefly, 10 ng of cDNAs were amplified using 0.8 μM primers, 0.4 μM probes (Supplementary Table I) and 1X TaqMan Universal Master Mix (Applied Biosystem). The ΔΔCt method was used to determine the fold change of Δ133p53 mRNA levels using *TBP* as the reference gene. The Δ133p53 mRNA levels were measured in triplicate on three independent experiments and compiled to perform statistical analysis (*t*-student test). A significant difference was reach for a *P*-value <0.05. Detection of p53 isoforms was assessed by nested PCR on 100 ng of cDNAs using 160 nM primers (Supplementary Table I) and 1X PCR SuperMix (Invitrogen).

2.3. Western blot and immunofluorescence

Proteins were extracted by scrapping and syringing cells in 1X NuPAGE LDS buffer (Invitrogen). Thirty micrograms of protein extracts were separated on pre-cast 10% NuPAGE gels (Invitrogen). Monoclonal mouse DO-1 antibody was used to detect the classical p53 isoform, since it epitope in TAD is lacking in Δ40p53 and Δ133p53 [5]. Polyclonal sheep SAPU and monoclonal mouse DO-12 antibodies detect all p53 isoforms, while monoclonal BP53.10 and KJC8 antibodies recognise α and β forms, respectively. Monoclonal mouse MAI antibody was specifically designed to hybridize ATG160. Globin and c-myc (Santa-Cruz) were used as controls of differentiation. Monoclonal mouse Ku80 and GFP (AbCam) were used as controls of loading and of transfection efficiency, respectively.

H1299 cells seeded on two-well glass chamber slides (Lab Tek) were transfected and 48 h latter, fixed by 1% formaldehyde. After permeabilization with 0.1% Triton X-100 in 1X PBS, cells were incubated 1 h with the monoclonal DO-12 antibody and then for 1 h with FITC-conjugated anti-IgG (Jackson Immuno-chemicals).

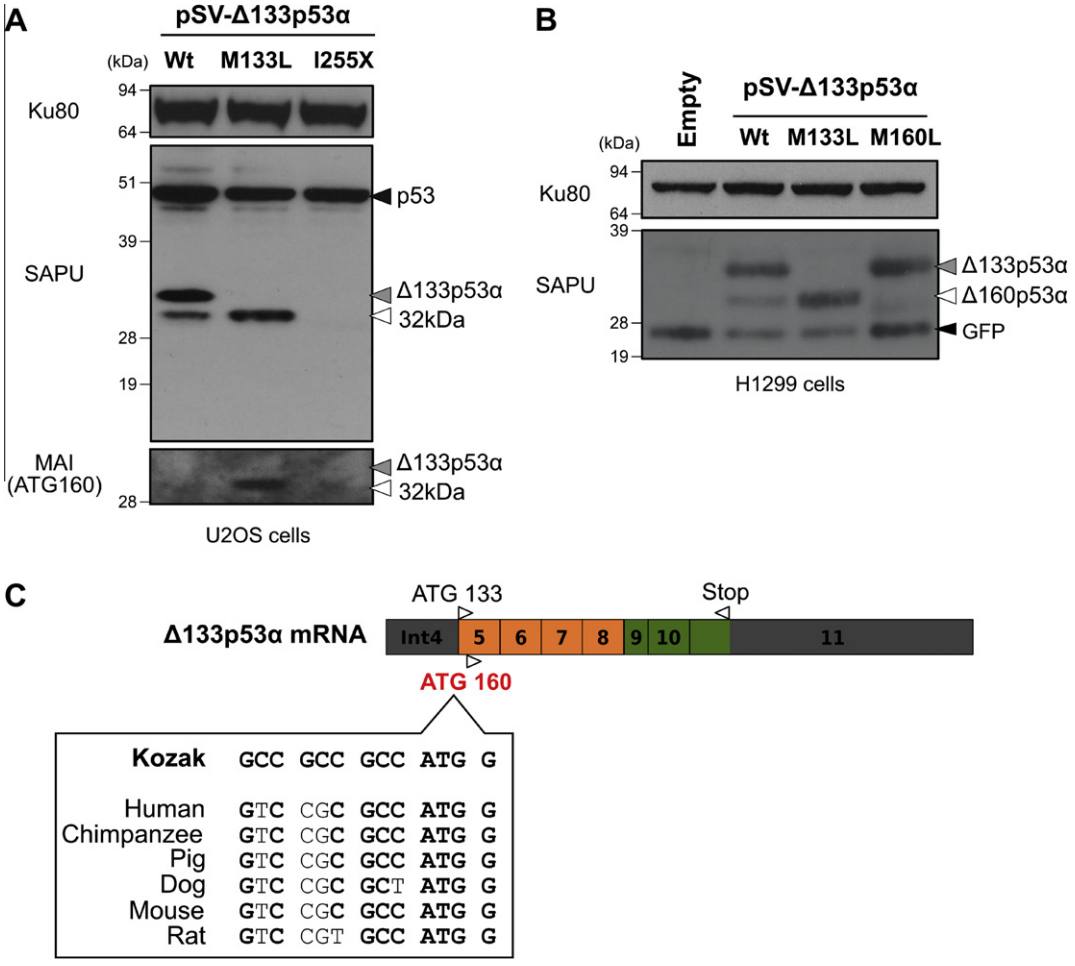

Fig. 2. Δ133p53 transcript encodes a second p53 isoform, Δ160p53. (A) Role of Δ133p53 transcript in expression of the band doublet in U2OS cells. Point mutations were introduced by site-directed mutagenesis in pSV-Δ133p53α expressing vector. Substitution of the methionine (ATG) at codon 133 by a leucin (CTG, M133L) abolished the expression of Δ133p53α protein (35 kDa-band), while introduction of a premature stop at codon 255 (I255X) abolished the expression of the band doublet. (B) Role of codon 160 in expression of the 32 kDa-protein in p53-null H1299 cells. Substitution of the methionine by a leucine at codon 160 (M160L) abolished the expression of the 32 kDa-protein but not the one of the 35 kDa-band corresponding to Δ133p53α isoform. GFP: control of transfection efficiency. (C) Schematic representation of the Δ133p53α transcript. The Δ133p53α transcript holds a downstream methionine residue at codon 160 (red) that is included in a Kozak environment conserved through evolution (bold letter).

3. Results and discussion

To determine the relation between detection of the band doublet and expression of Δ133p53α transcript, we performed siRNA experiment in wild-type p53 U2OS-Δ133p53α cells, which stably express Δ133p53α at a detectable level [8]. We observed that siΔ133 treatment targeting Δ133p53 transcripts resulted in a significant 50% decrease of Δ133p53 mRNA levels, compared to treatment with a non-relevant siRNA (siNS) (Fig. 1A, left panel). This diminution at mRNA levels was associated with a decreased intensity of a band doublet observed by western blot (35 and 32 kDa), suggesting that Δ133p53α mRNA encodes two protein products (Fig. 1A, right panel). The same decrease of the band doublet was observed in the parental U2OS expressing endogenous Δ133p53α in response to siΔ133 treatment, coupled to doxorubicin treatment (used to detect endogenous Δ133p53 levels [8]) (Fig. 1B). In T47D cells, where the endogenous band doublet is detectable without any drug treatment (Fig. 1C), its signal decreased in presence of siΔ133 but not in presence of a TAp53-specific siRNA (siTA, localisation Fig. 5). Altogether, these observations indicate that in several cells, the band doublet corresponds to p53 protein products generated by Δ133p53 transcript.

To determine whether Δ133p53α isoform corresponds to the 35 kDa-band and/or to the 32 kDa-band, we introduced point mutations in pSV-Δ133p53α expressing vector. Methionine at codon 133 was replaced by a leucine (M133L) to abolish Δ133p53α protein expression. In U2OS, introduction of wild-type pSV-Δ133p53α vector resulted in detection of the band doublet (Fig. 2A), support the notion, like for knock-down expression of Δ133p53 mRNA, that Δ133p53 transcript encodes two p53 isoforms. In addition, we observed that the 32 kDa-band was of weaker intensity than the 35 kDa one. Mutation M133L resulted in loss of the 35 kDa-band, thus corresponding to Δ133p53α. However, the 32 kDa-band was still detectable and showed an increased intensity compared to wild-type pSV-Δ133p53α transfection. To determine whether the 32 kDa-band was produced by Δ133p53 transcript, a premature stop codon was introduced at residue 255 (I255X) that led to loss of the band doublet (Fig. 2A). Taken together, siRNA and site-directed mutagenesis experiments suggest that the 35 kDa-band corresponds to Δ133p53α protein isoform. In addition, it indicates that the 32 kDa-band is a p53 protein product generated by Δ133p53α transcript that does not correspond to Δ133p53α protein. Thus, Δ133p53 transcript produces two distinct p53 protein isoforms. These observations evoke those of

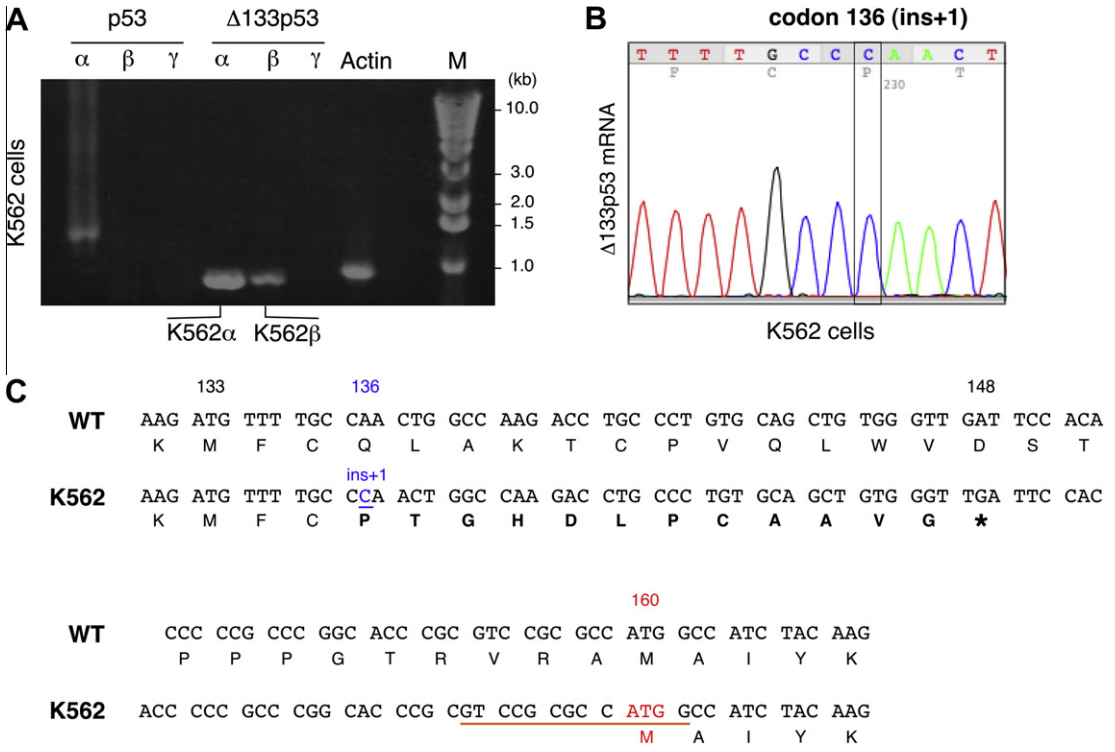

Fig. 3. p53 mutation status in K562 cell line. (A) Expression of p53 isoforms mRNAs by nested RT-PCR. K562 cells express p53α, Δ133p53α and Δ133p53β transcripts. Actin: control of reverse transcription efficiency. (B and C) p53 mutation status. Mutation in Δ133p53 transcripts expressed in K562 cells was determined by sequencing. An insertion at codon 136 (B, blue in C) introduces a frameshift leading to a premature stop at codon 148 (C, bold sequence: new residues due to the frameshift mutation) [13]. Δ133p53 transcripts expressed in K562 cells retains the open reading frame starting at codon 160 (bold red) and an intact Kozak environment (red line).

Courtois et al., who described for the first time the production of Δ40p53 isoform using ATG40 from p53 transcript [4]. In the open reading frame of the human Δ133p53α mRNA, a second methionine is present at codon 160, which is contained within a Kozak's consensus motif conserved through evolution (75% similarities) (Fig. 2B) [11]. To investigate the role of ATG160 in production of a novel p53 isoform, we raised an antibody specific of the ATG160 (MAI). We observed that MAI antibody detected the 32 kDa-band in presence of mutant M133L, which expressed the highest level of the 32 kDa-band (Fig. 2A). However, the 32 kDa-band was not detected with the MAI antibody in presence of the premature stop codon (I255X), indicating that ATG160 is accessible for antibody recognition. In addition, methionine 160 was substituted by a leucine into pSV-Δ133p53α vector. To avoid endogenous p53 isoform expression, we transfected H1299 cells with both wild-type or mutant pSV-Δ133p53α and GFP, which was detectable in all conditions indicating an effective transfection (Fig. 2C). As previously shown, pSV-Δ133p53α produced the band doublet and pSV-Δ133p53αM133L did not generate the 35 kDa-band, thus corresponding to Δ133p53α protein isoform. Moreover, while mutation M160L had no effect on Δ133p53α expression, it resulted in loss of the 32 kDa-band. This suggests that the 32 kDa-band corresponds to a p53 isoform initiated at ATG160 and we named it, Δ160p53α. We next attempted to identify cell line expressing exclusively Δ160p53 isoform. Using the IARC *TP53* Database, a list of mutant p53 cells was established that present premature stop between codons 133 and 160, either by nonsense mutation or insertion/deletion [12]. It drew our attention to the erythroleukaemic K562 described as p53-null because of the presence of a premature stop codon [13]. Using nested PCR, we first showed that K562 cells express three p53 transcripts, p53α, Δ133p53α and Δ133p53β (Fig. 3A). Sequencing of both Δ133p53α and Δ133p53β transcripts confirmed the insertion of a cytosine into codon 136 that results in a premature stop codon at residue 148 (Fig. 3B and C) [13]. As expected, this frameshift mutation avoids expression of both p53 and Δ133p53 proteins (Fig. 4A and B). Nevertheless, mutant Δ133p53 transcripts expressed in K562 cells retains the wild-type open reading frame initiated at ATG160 (Fig. 3C, red line). To determine whether K562 Δ133p53 transcripts can produce Δ160p53 protein, they were cloned into pSV expression vector (pSV-K562α or -K562β) and point mutation M160L was introduced to abolish usage of ATG160 as initiation site of translation (pSV-K562αM160L). H1299 cells transfected with pSV-K562α or pSV-K562β plasmids expressed a p53 protein product (Fig. 4B). However, p53 protein product expression was lost in cells transfected with pSV-K562αM160L, while control of transfection efficiency was still detectable (Fig. 4C). These observations indicate that mutant Δ133p53 transcripts expressed in K562 cells conserves the ability to generate p53 isoforms initiated at codon 160, Δ160p53α and Δ160p53β.

Using immunofluorescence, we compared the subcellular localisation of Δ160p53 isoforms to that of Δ133p53. To avoid any interplay with endogenous p53, analysis was performed in p53-null H1299 cells transfected either with pSV-Δ133p53α/β or with pSV-K562α/β plasmids (Fig. 4D). As already described, Δ133p53α and Δ133p53β signals were concentrated in the nucleus, the latter one presenting a foci pattern in about 10% of cells [2]. Transfection of pSV-K562α or pSV-K562β plasmids produced a strong nuclear signal, as observed for Δ133p53α and Δ133p53β isoforms. In addition, Δ160p53α presented a perinucleolar pattern, while Δ160p53β formed foci in some cells. Based on the distinct localisation of Δ133p53β and Δ160p53β, it indicates that foci formation is dependent on the β peptide in short N-terminal p53 isoforms [2].

Finally, we investigated the expression of endogenous Δ160p53 isoforms in K562 cells in response to hemin treatment. Hemin promotes erythrocyte differentiation, as shown by concomitant decrease of c-myc and increase of haemoglobin expression (Fig. 4E)

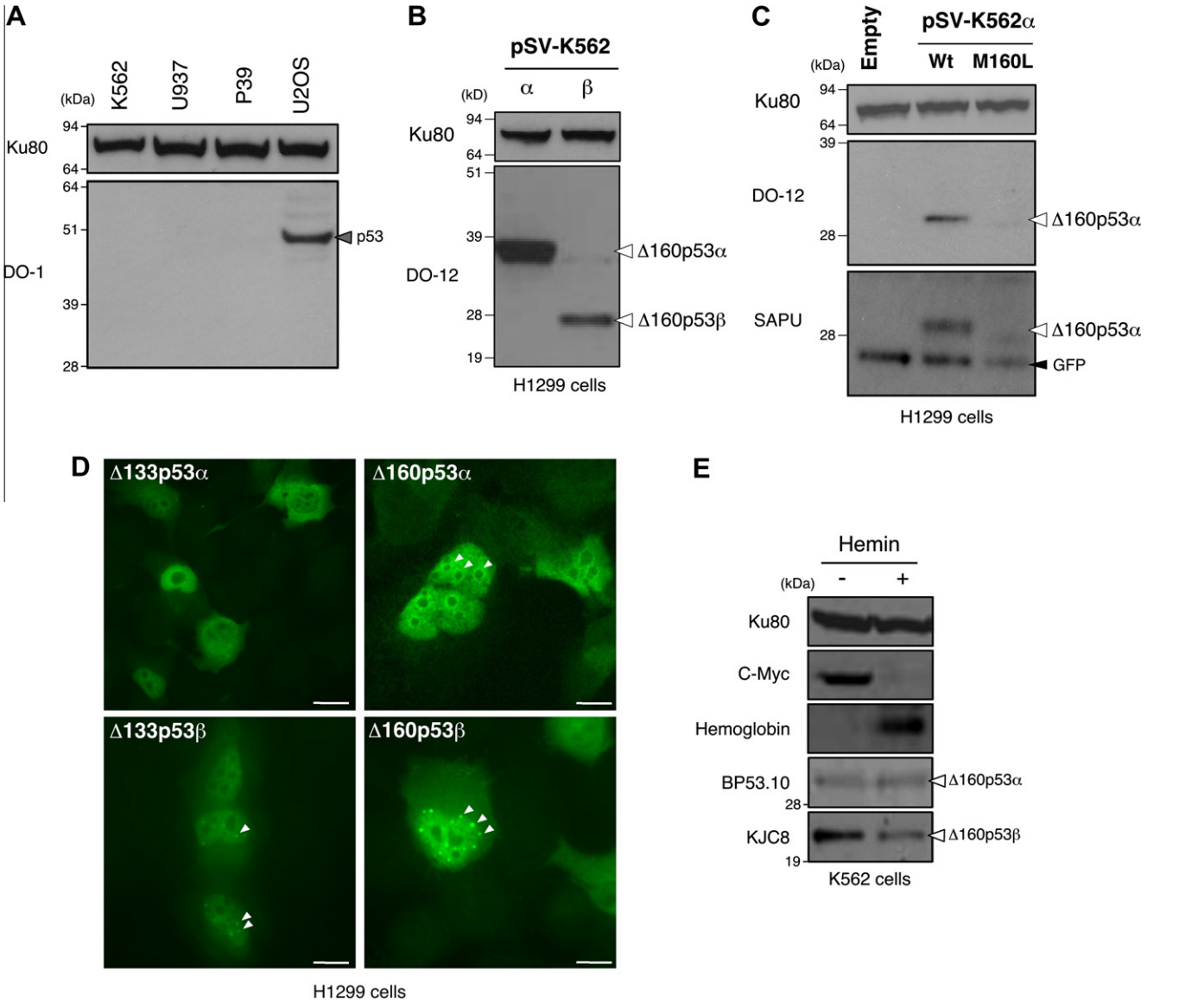

Fig. 4. Properties of Δ160p53 isoforms expressed in K562 cells. (A) Lack of the canonical p53 protein. Analysis of p53 expression profile using DO-1 antibody specific of the canonical p53 protein confirmed the lack of p53 expression in K562, like in p53-null leukaemia U937 and P39 cells. U2OS: positive p53 control. (B) Expression of p53 protein products by mutant Δ133p53 mRNAs expressed in K562 cells. Δ133p53α and Δ133p53β mRNA expressed in K562 cells were cloned into an expressing vector (pSV-K562α or pSV-K562β) and transfected in p53-null H1299 cells. pSV-K562α and pSV-K562β plasmids express p53 protein products at 32 and 26 kDa, respectively. (C) Determination of internal initiation of translation at ATG160 in K562 Δ133p53α transcript. Substitution of the methionine at codon 160 by a leucine (M160L) was introduced in pSV-K562α to abolish usage of ATG160 as internal initiation site of translation. In p53-null H1299 cells, the 32kD-band was detected in cells transfected with wild-type pSV-K562α but not with the M160L one, confirming that the 32 kDa-band corresponds to Δ160p53α. (D) Subcellular localisation of Δ160p53 isoforms. pSV-K562α and pSV-K562β expressing vectors were transfected in H1299 cells and localisation of Δ160p53 proteins analysed by immunofluorescence using DO-12 antibody. Both Δ160p53α and Δ160p53β were localised in the nucleus, like Δ133p53α and Δ133p53β. However, Δ160p53α presented a perinucleolar localisation and Δ160p53β exhibited foci pattern in some cells, as already reported for Δ133p53β [2]. White scale: 10 μM. (E) Expression of endogenous Δ160p53 isoforms. Differentiation of K562 cells was induced by hemin treatment as shown by c-myc and globin expression, as already described [14]. Δ160p53α isoform was detected using the α form-specific BP53.10 antibody and Δ160p53β isoform using the β form-specific KJC8 antibody. In response to hemin-induced differentiation in K562 cells, Δ160p53β isoform expression level was reduced compared to in K562 non-treated cells.

[14]. In K562 cells, both endogenous Δ160p53α and Δ160p53β were detected using α or β-forms specific antibodies. Moreover, Δ160p53β expression was decreased in response to hemin treatment, suggesting regulation of Δ160p53β expression during hemin-mediated differentiation (Fig. 4E).

Here, we report that Δ133p53 transcript can express two p53 protein isoforms, Δ133p53 and Δ160p53 (Fig. 5). By site-directed mutagenesis, we demonstrated that the human ATG160, present in a Kozak environment conserved through evolution, is used to produce the novel Δ160p53 isoform. Δ160p53α can be detected in several cell lines, including K562, despite the presence of a premature stop at codon 148. In addition, K562 cells express C-terminal variant Δ160p53β, which is repressed by hemin treatment. Further experiments will be required to investigate the regulation of Δ160p53β expression during hemin-mediated differentiation. In addition, it is important to note that the previous studies showing the biological implication in the regulation of p53-mediated senescence, apotosis and G1 cell cycle arrest, have been performed using knock-down approaches based on the usage of two siRNAs specific of the Δ133p53 mRNA. Thus, it will remain important to decipher whether the biological effects observed are associated to Δ133p53 and/or Δ160p53. In theory, a third Δ160p53 isoform remains to be identified exhibiting a C-terminal γ peptide (Δ160p53γ), since Δ133p53 transcript has been shown to present three different

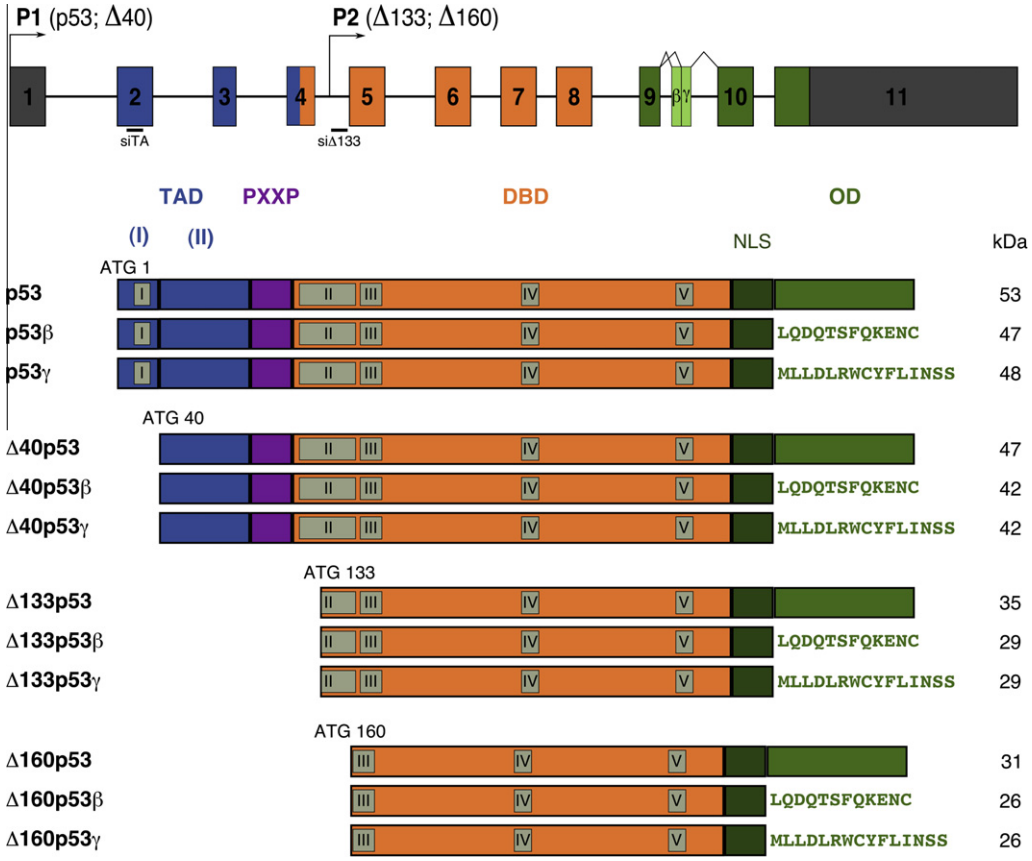

Fig. 5. The human p53 isoforms. The human *p53* gene contains a proximal (P1) and an internal (P2) promoter, regulating the expression of p53 and Δ133p53 transcripts, respectively. The p53 transcript encodes two p53 isoforms: p53 and A40p53, produced by internal initiation of translation using ATG 40 encompassing in a Kozak environment [4]. The Δ133p53 transcript encodes both Δ133p53 and Δ160p53 isoforms. Δ160p53 protein isoform lacks the entire transactivation domain and part of the DNA-binding domain, which encompasses the conserved domain II. Δ160p53 can be expressed with at least two different C-terminal domains, α and β. Δ160p53γ isoform remains to be identified. siTA and siΔ133, sequences targeted by the siRNAs used in the study; TAD, transactivation domain (I and II); PXXP, proline-rich domain; DBD, DNA-binding domain; NLS, nuclear localisation signal; OD, oligomerisation domain; grey boxes, p53 conserved domains through evolution (from I to V).

spliced 3′-end encoding α, β or γ peptides [2]. Taken together, this study shows that *p53* gene can express at least 11 isoforms, with the identification of two additional isoforms, Δ160p53α and Δ160p53β.

4. Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.10.005.

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