Nonsense mutation-dependent reinitiation of translation in mammalian cells

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ABSTRACT

In-frame stop codons mark the termination of translation. However, post-termination ribosomes can reinitiate translation at downstream AUG codons. In mammals, reinitiation is most efficient when the termination codon is positioned close to the 5′-proximal initiation site and around 78 bases upstream of the reinitiation site. The phenomenon was studied mainly in the context of open reading frames (ORFs) found within the 5′-untranslated region, or intercistronic viral mRNA. We hypothesized that reinitiation of translation following nonsense mutations within the main ORF of p53 can promote the expression of N-truncated p53 isoforms such as Δ40, Δ133 and Δ160p53. Here, we report that expression of all known N-truncated p53 isoforms by reinitiation is mechanistically feasible, including expression of the previously unidentified variant Δ66p53. Moreover, we found that significant reinitiation of translation can be promoted by nonsense mutations located even 126 codons downstream of the 5′-proximal initiation site, and observed when the reinitiation site is positioned between 6 and 243 bases downstream of the nonsense mutation. We also demonstrate that reinitiation can stabilise p53 mRNA transcripts with a premature termination codon, by allowing such transcripts to evade the nonsense mediated decay pathway. Our data suggest that the expression of N-truncated proteins from alleles carrying a premature termination codon is more prevalent than previously thought.

INTRODUCTION

As manifested in the central dogma of molecular biology, ribosome-mediated mRNA translation is the last step in the flow of genetic information (1). The fundamental and complicated cyclical mechanism of translation consists of initiation, elongation, termination and ribosome recycling phases. According to the linear scanning model, in eukaryotes, the pre-initiation complex scans the 5′-untranslated region (UTR) of the mRNA in the 5′ to 3′ direction until the first (proximal) AUG codon is recognized via base pairing with Met-tRNA\(^{\text{Met}}\) (reviewed in (2,3)). Following GTP hydrolysis-dependent structural and conformational rearrangements of the pre-initiation complex and recruitment of the 60S subunit, the resulting 80S complex enters the elongation stage (4), which terminates when the ribosome encounters an in-frame stop codon (UAA, UAG or UGA; reviewed in ref. (5)). That said, translation in eukaryotes may not always adhere to this oversimplified description (6,7). For example, following stop codon-dependent termination of translation, a fraction of 40S post-termination complexes can resume scanning and reinitiate translation at an AUG codon located downstream to the stop codon (4,8–12). Hence, reinitiation of translation defines at least two open reading frames (ORFs) in a single mRNA, namely an upstream ORF (uORF) that begins with the 5′-proximal AUG, and a downstream ORF that begins with an AUG codon located downstream to the stop codon.

The efficiency of reinitiation in eukaryotes (and consequently, translation of the downstream ORF) is dependent on the mRNA context around the downstream AUG codon, the duration of the 5′-proximal ORF translation process, and the intercistronic distance (6,9–11,13). Since the reinitiation rate is usually lower than the initiation rate, uORFs located within the 5′-UTR of human mRNA transcripts were suggested to play a regulatory role in the translation of the downstream main ORF (5,14–22). In principle, mechanisms that enable reinitiation of translation downstream of an ORF within the 5′-UTR should also enable the reinitiation of translation from an AUG codon located downstream of a nonsense mutation within the main ORF (23–29). Hence, a nonsense mutation upstream to an AUG
codon could artificially turn a monocistronic ORF into a polycistronic sequence and counterintuitively lead to the expression of an N-truncated protein (in addition to the C-truncated protein). However, reinitiation of translation is less studied in the context of nonsense mutations found within the main ORF.

The tumor suppressor protein p53 plays a pivotal role in the prevention of cancer development, with mutations in TP53 being the underlying cause of over 50% of human cancers (30–32). There are currently three known N-truncated isoforms of p53: Δ40p53, Δ133p53 and Δ160p53 (Figure 1A). These isoforms are produced through alternative translation initiation sites (Δ40p53, Δ160p53), alternative splicing (Δ40p53), and alternative promoter usage (Δ133p53, Δ160p53) (33–36). According to the IARC TP53 database (version R19, August 2018), there are hundreds of identified nonsense mutations upstream of the codon for Met160 (37). These mutated alleles are usually considered as null alleles. However, we hypothesized that the nonsense mutation-dependent expression of Δ40, Δ133 and Δ160p53 variants by reinitiation, is mechanistically feasible. Using p53 cDNA and minigene in transiently and stably transfected cell lines, together with amber suppression technology, we found significant nonsense mutation-dependent increase in the expression of N-truncated p53 variants by reinitiation of translation. We also describe, for the first time, the expression of the Δ66p53 variant by reinitiation of translation. Our data suggest that expression of N-truncated proteins by reinitiation is an underestimated phenomenon, implying that some alleles, currently regarded as null alleles due to nonsense mutations (including those introduced by CRISPR-Cas9 genome editing), may, in fact, enable the expression of N-truncated proteins.

MATERIALS AND METHODS

General

Unless stated otherwise, chemicals and DNA oligomers were ordered from Sigma Aldrich (Darmstadt, Germany) and used without further purification. Nε-acetyl lysine was purchased from Chem-Impex International (Wood Dale, IL, USA). Enzymes for molecular cloning (i.e. restriction, ligation and Phusion High-Fidelity DNA Polymerase for PCR reactions) were purchased from NEB (Ipswich, MA, USA) and used according to the manufacturer’s instructions. DNA was purified using spin columns from Macherey Nagel ( Düren, Germany). Escherichia coli DH10B cells (Life technologies, Carlsbad, CA, USA) were used for molecular cloning and plasmid propagation. Escherichia coli BL21(DE3) cells (NEB) were used for recombinant protein expression. During cloning steps, the bacteria were incubated in liquid LB media or plated on LB-agar plates supplemented with the required antibiotic (32 μg/ml zeocin or 50 μg/ml ampicillin). Anti-6×His (#G020) and anti-HA tag (#G036) antibodies were purchased from abm (Richmond, ON). Anti actin antibody (#4967) was purchased from Cell Signaling (Danvers, MA, USA). Anti-HUPF1 antibody (#ab109363) was purchased from Abcam (Cambridge, UK). Secondary horseradish peroxidase-conjugated anti-mouse IgG (#ab7068) and anti-rabbit IgG (#ab92080) were purchased from Abcam.

Vector construction

All genes for protein expression in mammalian cells were cloned into the commercially available pBudCE4.1 vector (Invitrogen-Thermo Fisher Scientific, Waltham, MA, USA), DNA encoding N′-6×His- and C′-HA-tagged WT p53 was amplified using primers 1/2 or primers 3/4 (Supplementary Table S1), and cloned between the KpnI and NotI or BamHI and EcoRI restriction sites downstream of the EF1α or CMV promoters, respectively. Point mutations were introduced by site-directed mutagenesis using primers 5–34. The DNA for N′-6×His- and C′-HA-tagged p53 Q52 TAG mini gene (IDT, Leuven, Belgium) was cloned between KpnI and NotI restriction sites downstream of the EF1α promoter. The point mutation M66L was introduced using primers 35/36. DNA for C′-HA-tagged WT Eno1 was amplified from plasmid HsCD00000030 (DNASU plasmid repository) using primers 37/38 and primers 2/37, and cloned between the KpnI and NotI restriction sites downstream of the EF1α promoter. In-frame TAG mutations at positions encoding K80 or G156 of Eno1 were introduced using primers 39/40 or 41/42, respectively. Similarly, DNA encoding C′-HA-tagged WT PFK (IDT) was amplified using primers 43/44 and 2/43, and cloned between the KpnI and NotI restriction sites downstream of the EF1α promoter. In-frame TAG mutation at position K25 was introduced using primers 45 and 46. For the expression of p53 variants in bacteria, WT and truncated TP53 were amplified using primers 47–53, and cloned between the NdeI and XhoI restriction sites of the commercially available pRSETb vector (Thermo Fisher Scientific).

Cell culture

Cells were incubated at 37°C in a humidified chamber under a 5% CO2 atmosphere. HEK293T and HCT116-p53−/− cells were maintained in DMEM (Biological Industries, Beit Haemek, Israel) and McCoy’s medium (Sigma Aldrich), respectively. Culture media were supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin G sodium, 0.1 mg/ml streptomycin sulfate and 1.25 units/ml nystatin (Biological Industries). For Western blot or RT-qPCR analyses, cells were seeded 24 h before transfection. HEK293T cells were transfected with Lipofactamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) or Lipofectamin...
Figure 1. N-truncated variants of p53. (A) Potential p53 N-truncated isoforms. Full-length p53 is expressed when translation begins at the first AUG codon. N-truncated p53 isoforms can be expressed by translation initiation or reinitiation at downstream AUG codons at positions 40, 66, 133 and 160 to produce Δ40p53, Δ66p53, Δ133p53 and Δ160p53, respectively. (B) Main TP53 mutants used in the current study. In-frame stop codon mutations (TAG) were introduced at the indicated positions of wild type TP53, as well as the codon for position K120' of M133'L p53 mutant. (C) Western blot of total cell extracts from HEK293T cells expressing WT or TAG mutants of p53. Cells were transfected with a plasmid carrying the indicated construct and expression was detected using antibodies against the C-terminal HA-tag. ‡N-truncated p53 that might be produced by initiation of translation at non-AUG codons (39), or by post-translational proteolytic cleavage (40). (D) Expression levels of N-truncated p53 variants, displayed relative to WT p53. Values were obtained by densitometric analyses of Western blot results (panel C), and normalized to actin expression (n = 5, ± SD). Statistical analyses were performed using one-way ANOVA with Dunnett’s post-test. *P < 0.05, **P < 0.01, ***P < 0.001. See Supplementary Figure S1 for a more detailed presentation of the data.
Expression of truncated p53 variants in bacteria

Different p53 variants (full length, Δ40, Δ66, Δ133, Δ160 and Δ169) were expressed in *E. coli* BL21(DE3) cells grown in 2×TY medium. At OD600 = 0.6, protein expression was induced with 1 mM isopropyl-β-D-1-thiogalactoside (IPTG) and the bacteria were incubated over night at 22°C. Cells from the over night culture were pelleted by centrifugation and lysed in 1×Laemmli sample buffer by heating to 95°C for 5 min, mixing and repeating the heating step. Cleared lysates were diluted as required before the sizes of expressed proteins were analysed by SDS-PAGE followed by Western blotting.

RESULTS

In-frame nonsense mutations promote the expression of N-truncated p53 variants

To study the effects of in-frame nonsense mutations on the expression of N-truncated p53, we introduced stop codon (TAG) mutations at different positions along *TP53* fused to C-terminal hemagglutinin (HA) and N-terminal 6×His tags (Figure 1B). To remain coherent with WT p53 amino acid numbering, we ignored the additional six histidine residues at the N-terminus of p53. The relevant numbers are marked by apostrophe, such that p53 residues 40′, 50′ and 60′ refer to positions 46, 56 and 66 of expressed p53, respectively. To identify possible effects of p53 transcriptional activity, we also cloned the transcriptionally inactive R273*H* p53 mutant (41,42). All variants were first cloned downstream of an EF1α promoter.

According to Western blot analysis using antibodies against the C-terminal HA tag, full-length p53 and Δ40′p53 were the two main variants expressed from WT and R273*H* *TP53*, in addition to Δ133*p53 and Δ160*p53 that were expressed at lower levels (Figure 1C and D, and Supplementary Figure S1) (43). These results are expected, since Δ40p53, Δ133p53 and Δ160p53 are known transcription variants of p53 (33–36,44). However, in-frame nonsense mutations changed this expression pattern. When an in-frame TAG mutation was introduced at position Q38′ (Q38′TAG), expression levels of Δ40′p53, Δ66p53 and Δ133p53 increased significantly (Figure 1D and Supplementary Figure S1). The same phenomenon was observed when an in-frame TAG mutation was introduced at position Q52′ (Q52′TAG), with increased expression levels of Δ66′p53 and Δ133′p53, relative to WT and R273’H *TP53*. When the TAG mutation was introduced at position K120′ (K120′TAG), Δ133′ and Δ160′p53 were expressed at levels similar to those measured with Q52′TAG. This can be attributed to the fact that reinitiation downstream of 57 codons long uORF (Q52′TAG) is expected to be more efficient than reinitiation downstream of 125 codons long uORF (K120′TAG) (5,10). However, when M133′ was mutated to leucine (K120 TAG M133′L), a significant increase in the expression of Δ160′p53 was observed. Such an expression pattern cannot be explained by out-of-frame AUG codons (Supplementary Figure S2), and the sizes of all truncated variants were verified using bacterially-expressed N-truncated p53 (Supplementary Figure S3). Similar to p53, an in-frame stop codon in position G156 of α-enolase promoted the expression of Δ165 enolase (Supplementary Figure S4A), and an in-frame stop codon in position K25 of human platelet-specific isoform of phosphofructokinase (PFK) promoted the expression of Δ39 PFK (Supplementary Figure S4B). These data demonstrate that in our expression system, nonsense mutation-dependent expression of N-truncated proteins is not limited to p53. Furthermore, these results clearly show that in-frame nonsense mutations can promote the expression of N-truncated proteins translated from AUG codons positioned ~160 codons downstream of the initiation site.

Nonsense mutation-dependent N-truncation is observed with different cell lines, promoters, and stop codons

To explore the generality of N-truncated protein expression, we considered the effects of the cell line and promoter on the expression of N-truncated p53. The same expression pattern was found when WT and TAG-mutants of *TP53* were cloned downstream of the cytomegalovirus (CMV) promoter in the same expression vector (Figure 2A), or when expressed in the p53-null HCT116 cell line (Supplementary Figure S5) (45). Moreover, to determine whether the observed nonsense mutation-dependent expression was unique to the TAG codon, mutations to other stop codons (TGA and TAA) were introduced at positions Q38′ and Q52′ of *TP53*. The three stop codons equally promoted the expression of N-truncated p53 variants starting at AUG codons downstream of the nonsense mutation (Figure 2B and Supplementary Figure S6). Therefore, our data show that nonsense mutation-dependent expression of N-truncated p53 variants is not limited to a specific promoter, cell line or type of in-frame stop codon.

mRNA transcripts of p53 cDNA with an in-frame nonsense mutation are not subjected to 5′-mRNA degradation

Next, we asked whether the expression of N-truncated p53 variants was the result of mRNA degradation. To answer this question, we measured p53 mRNA levels by real-time quantitative PCR (RT-qPCR) using specific primers designed to amplify a sequence close to the 3′-end of p53 mRNA (Figure 2C, p-3) and two sets of primers designed to amplify different sequences close to the 5′-end
Figure 2. Expression of N-truncated p53 by nonsense mutation-dependent reinitiation. (A) Expression of WT and N-truncated p53 from a TP53 cDNA cloned downstream of the CMV promoter (compared to genes cloned downstream of the EF1α promoter portrayed in Figure 1C). (B) Expression of N-truncated p53 as a function of premature stop codon type. Indicated stop codon mutations (TAG, TGA or TAA) were introduced at codons for positions Q38′ or Q52′, and expression of N-truncated p53 was evaluated by Western blotting using antibodies against the C-terminal HA-epitope. (C) The ratio between 5′ and 3′ p53 mRNA levels. For each indicated p53 variant, mRNA levels were quantified by RT-qPCR using primers recognizing a sequence at the 5′ (p-5′1, p-5′2) or 3′ (p-3′) end of p53 mRNA. The ratios between 5′ to 3′ mRNA levels are plotted relative to those measured for WT p53. (D) Expression of p53 variants detected using antibodies against the N-terminal 6×His-tag. Expression of p53 Q52′TAG and K120′TAG mutants of p53 yielded C-truncated p53(1–51′) and p53(1–119′), respectively. The unstable p53(1–37′) peptide was not detected. (E) Co-expression of p53 K120TAG together with the amber suppression machinery. The use of an evolved acetyl lysine synthetase and its cognate amber suppressor tRNA enabled the incorporation of the non-canonical amino acid Nε-acetyllysine in response to the in-frame TAG mutation encoding position 120. (F) The effect of frame-shift mutation on expression of N-truncated p53 variants evaluated by western blotting using antibodies against the C-terminal HA-tag.

of p53 mRNA (p-5′1 and p-5′2). We then calculated the ratio between mRNA levels (i.e. p-5′/p-3′) measured for WT p53 and p53 Q38′TAG (using p-5′1) or WT p53 and p53 K120′TAG (using p-5′2). In both cases, the normalized p-5′/p-3′ ratio calculated for the TAG mutants was similar to the ratio measured for WT p53, indicating that no mutation-dependent degradation of 5′-mRNA had occurred. Furthermore, total levels of p53 mRNA transcripts carrying an in-frame nonsense mutation were similar to each other, although lower than the levels of WT p53 mRNA transcript (Supplementary Figure S7). Thus, the expression of N-truncated p53 variants was enabled at the level of translation and was not regulated at the transcriptional level, nor was the result of mRNA degradation.

N-truncated p53 variants are expressed by nonsense mutation-dependent reinitiation of translation

Given the data presented above, we sought to describe the mechanism underlying nonsense mutation-dependent expression of N-truncated p53. Leaky scanning may contribute to the expression of N-truncated proteins, but the positive correlation between expression levels and the presence of an upstream nonsense mutation suggests that another mechanism was responsible for the observed nonsense mutation-dependent expression of N-truncated variants. One possible mechanism is initiation of translation at the 5′-proximal AUG codon, followed by termination at an in-frame stop codon and reinitiation of translation at downstream AUG codons. In such cases, the N-terminal of the protein (i.e. C-truncated p53) should be expressed. To assess the expression of C-truncated p53, we performed Western blot analysis using antibodies against the N-terminal 6×His-tag (Figure 2D). As expected, strong bands observed at ~53 kDa when WT and R273′H p53 were expressed (lanes 1 and 2), indicative of the expression of full-length p53. As the N-terminal region of p53 is natively unfolded and unstable, p53(1–37′) could not be detected (lane 3). However, weak bands of C-truncated p53 were detected, reflecting the expression of p53(1–51′) and p53(1–119′) from Q52′TAG and K120′TAG mutants of p53, respectively (lanes 4 and 5). Hence, during the expression of p53 Q52′TAG or K120′TAG mutants, translation was initiated at the 5′-proximal AUG codon and terminated at the in-frame stop codon at position 52′ or 120′, respectively. In addition, we co-expressed the p53 K120TAG mutant with amber suppression machinery for the incorporation of Nε-
acetyl-lysine at position 120 by an aminoacylated amber suppressor tRNA (Figure 2E) (46,47). Suppression of the in-frame stop codon enabled the expression of full-length K120-acetylated p53, indicating that the translation of p53 mRNA with an in-frame UAG codon at position K120 was initiated at the first AUG codon. Finally, we introduced a frame-shift between the TAG mutation at position 38’, 52’ or 120’, and the downstream ATG codon at position 40’, 66’ or 133’, respectively. In all cases, the frame-shift mutation had no effect on N-truncated p53 expression levels, to leucines in both WT and Q52

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or 120’

−

dependent expression of

pseudo-upstream initia-

tion site. Hence, nonsense mutation-

translation initiation site at position 40

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we found that expression levels of

significantly when the uORF length increases beyond 13–18

codons. Hence, we conclude that the underlying mechanism of in-frame nonsense mutation-dependent expression of N-truncated p53 is reinitiation of translation. Therefore, known N-truncated isoforms of p53 may be expressed by mechanism of reinitiation, in addition to the previously described alternative initiation from internal AUG codons.

Reinitiation from the AUG codon at position 66’ is mechanistically feasible despite the long uORF and short intercistronic distance

Previous studies found that reinitiation decreases significantly when the uORF length increases beyond 13–18 codons (11), and that reinitiation is most efficient when the intercistronic distance is 80–90 nucleotides (9). However, we found that expression levels of Δ66’p53 from Q52’TAG were ~80% of WT p53 (Figure 1D), although in this construct the length of the uORF was 58 codons and the intercistronic distance was 42 nucleotides. Therefore, we decided to check if the relatively high expression level of Δ66’p53 from Q52’TAG is indeed a unique example of reinitiation—suggesting that the previously determined constraints on reinitiation are too strict—or maybe other factors affect the reinitiation from the AUG codon at position 66’. Specifically, one may argue that the known alternative translation initiation site at position 40’ promotes the reinitiation from position 66’. According to this hypothetical model, the AUG codon at position 40’ can serve as an additional initiation site (pseudo initiation site), thereby creating an uORF between codons 40’ and 52’, with an ideal length of 12 codons, upstream to the AUG codon at position 66’. To verify that the AUG codon at position 40’ (or 44’) is not a pseudo initiation site, we mutated Met40’ and Met44’ to leucines in both WT and Q52’TAG p53. We quantified Δ66’p53 expression levels and found that these mutations had no negative effect on reinitiation of translation from the AUG codon at position 66’ of the Q52’TAG construct (Figure 3A and Supplementary Figure S8). Moreover, we found that M40’L and M44’L mutations in WT p53 had a small effect on expression levels of Δ66’p53 (Figure 3A, lanes 1–3), suggesting only a minor contribution of leaky scanning or alternative initiation to the expression of Δ66’p53. Thus, the AUG codon at position 66’ is a bona fide reinitiation site and the codon for Met40’ (or Met44’) does not serve as a pseudo-upstream initiation site. Hence, nonsense mutation-dependent expression of Δ66’p53 is mechanically feasible, and efficient reinitiation may occur when the uORF is longer than 13–18 codons and the intercistronic distance is shorter than 80–90 nucleotides.

Premature termination codon-dependent reinitiation of translation stabilises mRNA transcripts by circumventing the NMD

The data presented thus far was collected using TP53 cDNA with an in-frame nonsense mutation. However, mRNA of genomic TP53 with a premature termination codon (PTC) is expected to be recognized and degraded by the nonsense-mediated mRNA decay (NMD) pathway. According to current models, an in-frame nonsense mutation can be identified as a PTC, if positioned upstream to an exon-exon junction. However, if reinitiation is enabled by an AUG codon positioned between the PTC and the downstream exon-exon junction, the protein complex that marks the exon-exon junction should be displaced from the mRNA by the translating ribosome during the pioneering round of translation. Consequently, the in-frame nonsense mutation may not be recognized as a PTC by the NMD. Thus, reinitiation may allow mRNA transcripts with a PTC to escape degradation by the NMD pathway and thereby stabilises such transcripts (13,23,27,48–51).

To study the interplay between NMD-mediated degradation of mRNA transcripts with a PTC and nonsense mutation-dependent reinitiation of translation, we created a p53 minigene with intron 5 downstream of the nonsense mutation at position Q52’ (Q52’TAG minigene). We found that expression levels of Δ66’p53 were not affected by the addition of intron 5, when HEK293T cells were transiently transfected with Q52’TAG p53 (−intron 5) or Q52’TAG minigene (+intron 5; Figure 3B, left blot). Moreover, the addition of intron 5, which defines the UAG codon at position 52’ as a PTC, had no effect on expression levels of Δ66’p53 in HEK293T cell lines stably transfected with the same constructs (Figure 3B, right blot). Thus, p53 mRNA transcripts with a PTC upstream of the exon 4–exon 5 junction allow for the expression of Δ66’p53.

Next, we asked if TP53 mRNA transcripts carrying a PTC at position Q52’ are substrates of the NMD pathway, and if reinitiation affects NMD efficiency. To answer these questions, we first created another HEK293T cell line, stably transfected with M66’L mutant of the Q52’TAG minigene (Q52’TAG M66’L minigene). In this mutant, reinitiation from the AUG codon at position 66’ and subsequent mRNA stabilisation, is not expected. We then inhibited the NMD using siRNA directed against hUPF1, an essential factor of the NMD pathway (52). Western blot analyses showed a significant downregulation of hUPF1 in cells transfected with sequence specific siRNA, compared to random siRNA (Figure 3C). NMD inhibition following hUPF1 knockdown was also confirmed by the measured increase in mRNA levels of several known physiological NMD substrates: SC35 1.7, CARS and SMG1 (Figure 3D, left) (53,54). Importantly, NMD inhibition resulted in a significant increase in p53 mRNA levels, with a stronger effect on mRNA transcripts of the M66’L mutant. Moreover, similar effects of NMD inhibition on mRNA levels were measured in three monoclonal HCT116Δp53+/− cell
lines stably transfected with Q52'TAG minigene (Supplementary Figure S9). These data show that Δ66'p53 can be expressed via nonsense mutation-dependent reinitiation, although p53 transcripts with a PTC at position 52' are subjected to degradation by the NMD pathway.

In agreement with current models, our data show that reinitiation stabilises mRNA transcripts with a PTC, by circumventing the NMD. NMD inhibition in cells expressing Q52'TAG minigene resulted in an ∼1.5-fold increase in p53 mRNA levels, while a 1.9-fold increase in mRNA levels was measured in cells expressing Q52'TAG M66'L minigene (Figure 3D, right). Similarly, p53 mRNA levels in cells expressing Q52'TAG M66'L minigene were lower, compared to their levels in cells expressing Q52'TAG minigene. Hence, mRNA transcripts of Q52'TAG minigene that enable reinitiation from position 66' were less sensitive to NMD inhibition and accumulated to higher levels, compared to mRNA transcripts of the Q52'TAG M66'L minigene with no reinitiation site between the PTC and the downstream exon-exon junction.

DISCUSSION
Reinitiation of translation was first reported by Marilyn Kozak (8,9). Here, we provided three examples for nonsense mutation-dependent reinitiation of translation and more rigorous studies are required in order to understand how general this observation is. The phenomenon and its underlying mechanisms in mammalian cells were mainly studied in the context of a regulatory element following translation of an uORF within the 5'-UTR. The stress-dependent expression of GCN4 in Saccharomyces cerevisiae (or its mammalian homologue ATF4) via eIF2α phosphorylation-dependent reinitiation of translation is one such example (16,17,55,56). That said, in the EXAC database (57), which currently includes the exomes of 60,706 individuals, there are over 30,000 valid stop codon mutations, of which, thousands are located within the first 150 codons. Alleles with PTCs are usually considered null alleles. However, in line with our data, such alleles may enable the expression of N-truncated proteins by reinitiation of transla-
tion from a downstream AUG codon, and may even ‘rescue’ patients from potentially deleterious effects of a null allele. For example, the E12X and Y27X mutations in the aristless-related homeobox gene (ARX) promoted the expression of Δ41ARX using the Met41 codon as reinitiation site (58). Patients carrying the R37X mutation in the ATRX gene escape the rather lethal ATRX-null phenotype, due to expression of Δ40ATRX (59). Moreover, as demonstrated here, reinitiation can stabilise mRNA transcripts with a PTC by rescuing them from degradation by the NMD, thereby allowing such transcripts to accumulate in the cell. Hence, nonsense mutation-dependent reinitiation within the main ORF has the potential for either protecting against disease by enabling the expression of truncated—yet functional—proteins, or intensifying the disease phenotype by allowing expression of potentially harmful N- as well as C-truncated proteins. While nonsense mutation-dependent reinitiation was suggested to increase when the reinitiation site is close to the 5'-end of the main ORF, we found significant reinitiation of translation from AUG codons positioned over 160 codons downstream of the first initiation site. Thus, our data suggest that when referring to genes with PTCs, nonsense mutation-dependent expression of N-truncated proteins should be considered, in addition to the default assumption of a null allele.

Our results are also important for studies in cultured mammalian cells involving genetic code expansion technology, where a non-canonical amino acid is encoded by an in-frame stop codon. As also demonstrated in yeast (24), an introduced stop codon mutation can promote the expression of N-truncated proteins, which must be considered in such experiments. In addition, in recent years, we have witnessed a surge in the use of CRISPR/Cas9 technology. One of the common strategies for knocking-out specific genes using this genome editing approach is to introduce a PTC within the first exon. Given the data presented here, it may be important to confirm whether an introduced PTC promotes the expression of an N-truncated protein.

It is still not clear why nonsense mutation-dependent reinitiation of translation is mechanistically feasible. Is PTC-dependent reinitiation of translation an evolved mechanism with specific functions in the eukaryotic cell? Does it provide an evolutionary advantage? Is reinitiation of translation in mutated proteins regulated, and if so, by which mechanism(s)? We hope that future research will address these and other questions related to reinitiation of translation downstream of a PTC.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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