3'-UTR-located inverted Alu repeats facilitate mRNA translational repression and stress granule accumulation

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Alu repeats within human genes may potentially alter gene expression. Here, we show that 3'-UTR-located inverted Alu repeats significantly reduce expression of an AcGFP reporter gene. Mutational analysis demonstrates that the secondary structure, but not the primary nucleotide sequence, of the inverted Alu repeats is critical for repression. The expression levels and nucleocytoplasmic distribution of reporter mRNAs with or without 3'-UTR inverted Alu repeats are similar; suggesting that reporter gene repression is not due to changes in mRNA levels or mRNA nuclear sequestration. Instead, reporter gene mRNAs harboring 3'-UTR inverted Alu repeats accumulate in cytoplasmic stress granules. These findings may suggest a novel mechanism whereby 3'-UTR-located inverted Alu repeats regulate human gene expression through sequestration of mRNAs within stress granules.

Introduction

Alu repeats are the most abundant repetitive elements in the human genome. The roughly 1.4 million Alu repeats in human DNA account for at least 12% of the mass of the human genome.1 The vast majority of Alu repeats are found in introns; however, approximately 5% of fully spliced cDNAs harbor Alu repeats, with 82% of these Alus located in 3' untranslated regions (3'-UTRs), 14% in 5' untranslated regions (5'-UTRs) and 4% within coding regions.2 Alu repeats are proposed to form regions of double-stranded RNA (dsRNA) that are targets for adenosine deaminases acting on RNA (ADARs), RNA-editing enzymes which convert adenosine residues in dsRNA to inosine.3 Adenosine-to-inosine (A-to-I) edited CTN-RNA is then bound by p54nrb, a multifunctional nuclear RNA binding protein, and retained in nuclear speckles.4 In another study, a pair of inverted Alu repeats, the precise mechanism(s) of this repressive action remain controversial. In the present study, we utilized a reporter gene expression system to examine the molecular mechanisms underlying gene repression by 3'-UTR inverted Alu repeats, the precise mechanism(s) of this repressive action remain controversial. In the present study, we utilized a reporter gene expression system to examine the molecular mechanisms underlying gene repression by 3'-UTR inverted Alu repeats, the precise mechanism(s) of this repressive action remain controversial. In the present study, we utilized a reporter gene expression system to examine the molecular mechanisms underlying gene repression by 3'-UTR inverted Alu repeats, the precise mechanism(s) of this repressive action remain controversial. In the present study, we utilized a reporter gene expression system to examine the molecular mechanisms underlying gene repression by 3'-UTR inverted Alu repeats, the precise mechanism(s) of this repressive action remain controversial. In the present study, we utilized a reporter gene expression system to examine the molecular mechanisms underlying gene repression by 3'-UTR inverted Alu repeats, the precise mechanism(s) of this repressive action remain controversial.

Although recent findings have provided evidence for the repression of gene expression by 3'-UTR-located inverted Alu repeats, the precise mechanism(s) of this repressive action remain controversial. In the present study, we utilized a reporter gene expression system to examine the molecular mechanisms underlying gene repression by 3'-UTR inverted Alu repeats. Consistent with previous findings,5 we show that expression of an AcGFP reporter gene is significantly reduced by inverted Alu repeats in the 3'-UTR. Mutational analysis of the 3'-UTR inverted Alu repeats indicates that the secondary structure of the inverted Alu repeats, but not the primary nucleotide sequence, is critical for...
repression of reporter gene expression. The mRNAs with 3'-UTR-located inverted Alu repeats are not extensively edited and are not retained in the nucleus, but instead, accumulate in cytoplasmic stress granules. These results, together with previous studies, suggest that Alu repeats within the 3'-UTRs of human genes can modulate gene expression through multiple mechanisms.

**Results**

AcGFP reporter gene expression is strongly repressed by inverted repeat Alu elements in the 3'-UTR region of the gene. To study the effects of 3'-UTR inverted Alu repeats on gene expression, a reporter gene construct was generated by inserting inverted Alu repeats (a sense-oriented Alu repeat and an antisense AluYa5 repeat separated by 162 base pairs) into the 3'-UTR region of the AcGFP reporter gene in the expression vector pACGFP1-C1. The resulting plasmid was designated p4AluIR. To evaluate the impact of a single sense or antisense Alu on gene expression, derivative constructs containing either the single antisense AluYa5 or the sense AluAlb repeat were generated from pAluIR and designated p4AluYa5 and p4AluAlb, respectively. All constructs carried the AcGFP coding sequence driven by the human cytomegalovirus (CMV) immediate early promoter. Plasmid constructs are represented schematically in Figure 1A. The details of plasmid construction are outlined in the Materials and Methods.

The inverted Alu 3'-UTR construct (p4AluIR), its single Alu derivatives (p4AluAlb and p4AluYa5), and the parental AcGFP control vector (pACGFP1-C1) were each transfected into HEK293 cells using the calcium phosphate DNA precipitation method.16 At 48 h post-transfection, the AcGFP expression level from each plasmid was measured by western blot. AcGFP expression was normalized to β-tubulin, as a loading control. All experiments were performed at least five times. AcGFP reporter gene expression was not significantly altered by a 3'-UTR-located single sense or antisense Alu repeat (Fig. 1B and lanes 2 and 3). In contrast, AcGFP expression was significantly reduced by the 3'-UTR-located inverted Alu repeats (Fig. 1B and lane 4). These results are consistent with a previous report that 3'-UTR inverted Alu repeats reduced reporter gene expression, while single antisense or sense Alu repeats had little impact on gene expression.15

Inverted Alu repeat secondary structure contributes to AcGFP repression. A previous study9 suggested that stem-loop secondary structure contributed by 3'-UTR-located inverted Alu repeats may function to repress gene expression. Inverted Alu secondary structure formation was indirectly inferred from observations of A-to-I editing of the Alu repeats. However, the formation of inverted Alu secondary structure and its direct contribution to repression of gene expression remains unclear.

Repression of gene expression by 3'-UTR-located inverted Alu repeats could be mediated by specific nucleotide sequences within the inverted Alu repeats, such as adenine and uracil-rich elements (AREs) that have been shown to regulate mRNA stability.12,18 Alternatively, the inverted Alu sequences could provide structural motifs, such as the stem-loop required for ASH1 mRNA localization in budding yeast.20

To test whether the secondary structure or the primary nucleotide sequence of the 3'-UTR inverted Alu contributes to repression of reporter gene expression, a systematic substitution mutation approach was used. Primary sequence changes designed to disrupt a potential stem-loop arising from Alu-Alu base pairing were introduced into the 3'-UTR inverted Alu pair. In one mutant, pIRsubJb, each nucleotide within the sense AluAlb sequence of the inverted pair was altered by purine-to-purine and pyrimidine-to-pyrimidine substitutions. A second mutant, pIRsubYa5, was generated by purine-to-purine and pyrimidine-to-pyrimidine substitutions of each nucleotide within the antisense AluYa5 sequence of the inverted Alu pair. In addition, a compensatory mutant (pIRCompensatory) which alters both the AluAlb and the AluYa5 sequences of the inverted Alu pair, but restores base pairing with altered non-Alu sequences was generated. Plasmid constructs are represented schematically in Figure 2A. Details of mutant plasmid construction are described in the Materials and Methods. The complete nucleotide sequence of the wild-type Alu repeats and the synthetic substitution fragments are shown in Figure S1.

The 3'-UTR inverted Alu substitution mutants were tested by transient transfection for their ability to repress AcGFP reporter gene expression. AcGFP reporter gene expression from each plasmid was measured by western blot 48 h after transfection. Western blot analysis of cotransfected β-galactosidase was used to normalize transfection efficiency (Fig. 2B). AcGFP expression was normalized to β-tubulin, as a loading control. All experiments were performed at least five times. Substitution mutations that alter the sequence of one Alu of the inverted pair relieved the inverted Alu repeat-mediated repression of AcGFP expression. Transfection of pIRsubJb (sense Alu substitution) or pIRsubYa5 (antisense Alu substitution) into HEK293 cells resulted in similar levels of reporter gene expression compared with AcGFP control gene expression (Fig. 2B and lanes 3 and 4). In contrast, AcGFP protein levels are reduced in compensatory mutant (pIRCompensatory) transfected HEK293 cells (Fig. 2B and lane 5). The reduction of AcGFP expression in compensatory mutant (pIRCompensatory) transfected cells was comparable to that observed for inverted Alu repeat (p4AluIR) transfected cells (Fig. 2B and lanes 2 and 5). Taken together, these results demonstrate that the secondary structure, but not the primary nucleotide sequence of the 3'-UTR inverted Alu repeats sequence is important for reduced reporter gene expression.

Regulation of AcGFP expression occurs post-transcriptionally. Alu 3'-UTR sequences can influence mRNA transcript levels, stability, translational efficiency and localization.13,21-24 To determine the mechanisms that underlie gene repression by 3'-UTR inverted Alu, we examined the steady-state levels and subcellular localization of mRNAs with or without 3'-UTR inverted Alu repeats. Northern blot analysis using 32P-labeled probes for AcGFP and GAPDH mRNAs was performed on total RNA, and RNA isolated from subcellular fractions. mRNAs encoding AcGFP and GAPDH were detected in all samples (Fig. 3A and C). The
Chimeric AcGFP mRNA accumulates in cytoplasmic foci and granule-like structures. As demonstrated by northern blot analysis, chimeric AcGFP reporter mRNAs are largely distributed in the cytoplasm of transfected HEK293 cells and steady-state mRNA levels are not significantly changed. It is, therefore, unlikely that repression of AcGFP reporter gene expression is due to significant transcription inhibition or mRNA degradation. To determine the mechanism of repression, we examined the cytoplasmic fate of the reporter gene mRNA. RNA FISH using biotin-labeled AcGFP DNA as probes was used to localize AcGFP mRNA in HEK293 cells transfected by 3'-UTR inverted Alu repeats (Fig. 4A and left parts). By comparison, AcGFP mRNAs are diffusely distributed in the cytoplasm of AcGFP control vector transfected cells and in cells transfected by 3'-UTR

Figure 1. Expression of an AcGFP reporter gene is inhibited by 3'-UTR-located inverted Alu repeats. (A) Schematic representation of AcGFP expression constructs. The AcGFP-gene of all constructs is expressed from the CMV promoter. (B) Western blot analysis of total AcGFP protein shows significantly reduced AcGFP protein levels in 293 cells transfected by an AcGFP expression construct with 3'-UTR inverted Alus (lane 4) relative to cells transfected with an AcGFP control (lane 1). The expression level of AcGFP protein was determined by western blot 48 h post-transfection and normalized to that of β-galactosidase as a transfection control and to α-tubulin as a loading control. Figures are representative of five independent experiments, **p = 0.0031, paired t-test. (C) Bar graph densitometry of western blot bands expressed in arbitrary units. Data are expressed as mean ± SEM of five independent experiments. The value obtained from the control vector, pAcGF1-C1, was set to 1. Quantification of images was done by scanning densitometry with NIH Image J 1.54 software (National Institutes of Health, Bethesda, MD USA).
were more prominent in cells expressing reporter mRNAs with 3'-UTR-located \textit{Alus} \((p_{Alu})\) than in those expressing \textit{AcGFP} mRNA without 3'-UTR inverted \textit{Alus} \((p_{Alu}IR}) (Fig. 5B).

Additionally, very little of the 3'-UTR inverted \textit{Alu} mRNA was detectable outside of stress granules. These findings suggest that the 3'-UTR inverted \textit{Alu} repeats may promote the formation of stress granules, where expression of the inverted \textit{Alu}-containing mRNA is repressed.

It has been reported that RNAs with hyperedited 3'-UTR-located inverted repeats are retained within nuclear paraspeckles.\(^{13,15}\) To determine if the inverted \textit{Alu} repeat-containing \textit{AcGFP} reporter mRNA associates with nuclear paraspeckles, \textit{AcGFP} RNA FISH was combined with indirect immunofluorescence using antibodies against PSPC1 (paraspeckle protein 1), a known protein component of paraspeckles, was performed. Colocalization of \textit{AcGFP} reporter transcripts with nuclear PSPC1 was not observed, indicating that \textit{AcGFP} mRNAs with
Several studies have suggested that A-to-I hyperediting of repetitive elements in mRNA transcripts can influence gene expression.13,15,31,32 To determine if the 3'-UTR inverted Alu repeats in AcGFP reporter mRNAs are subject to A-to-I editing we compared multiple cDNA sequences of these RNAs to matching genomic sequences. Because inosine is read as guanosine by reverse transcriptase in the RT reaction, A-to-I editing events are detected as A-to-G transitions in cDNA sequences.

Promiscuous editing (50–60% deamination of adenosines) of the inverted Alus was not detected (Fig. S2). RNA editing occurred at only two positions within the Alus repeat (~1.6% of the total adenosines appeared as guanosines) and at four positions

Figure 3. AcGFP mRNAs localize to the cytoplasm. (A) Northern blot analysis of AcGFP mRNA. Equal amounts (20 μg) of total (tot), nuclear (nuc) and cytoplasmic (cyt) RNA were analyzed by northern blot using radiolabeled random-primed probes specific for AcGFP and GAPDH mRNAs. Levels and subcellular localization of AcGFP mRNA were similar for all transfected constructs. Western blot analysis against α-tubulin, a cytoplasmic protein and TATA-binding protein TFIID, a nuclear protein, was performed to check the effectiveness of fractionation. Representative northern blots are shown. n = 5 experiments. (B) AcGFP mRNA nuclear to cytoplasmic (n/c) ratios were quantified by densitometry, normalized to GAPDH, and the obtained results from five independent experiments are represented on the graph as means ± SD. The value obtained from the control vector, pAcGFP1-C1, was set to 1. (C) Northern blot analysis of AcGFP mRNA. Equal amounts (20 μg) of total (tot), nuclear (nuc) and cytoplasmic (cyt) RNA were analyzed by northern blot using radiolabeled random-primed probes specific for AcGFP and GAPDH mRNAs. Levels and subcellular localization of AcGFP mRNA were similar for all transfected constructs. Western blot analysis against α-tubulin, a cytoplasmic protein and TATA-binding protein TFIID, a nuclear protein, was performed to check the effectiveness of fractionation. Representative northern blots are shown. n = 5 experiments. (D) AcGFP mRNA nuclear to cytoplasmic (n/c) ratios were calculated as described above and the obtained results from five independent experiments are represented on the graph as means ± SD.
Figure 4. For figure legend, see page 365.
within the antisense AluYa5 repeat (9% of the total adenosines appeared as guanosines).

Discussion

The 3'-UTRs of human mRNAs frequently contain Alu sequences. Recent findings have provided evidence for the repression of gene expression by 3'-UTR-located inverted Alus. However, the precise mechanism(s) of this repression action remain unclear. To systematically examine the molecular mechanisms that underlie gene repression by 3'-UTR inverted Alus, we compared the expression, distribution, and subcellular localization of mRNAs with or without 3'-UTR inverted Alus. Consistent with previous findings, we found that 3'-UTR-located inverted Alus repeats significantly reduced reporter gene expression while, in contrast, a 3'-UTR-located single sense or antisense Alu did not alter reporter gene expression.

Steady-state abundance and subcellular distribution of AcGFp reporter mRNAs with or without 3'-UTR inverted Alus were similar; indicating that repression of AcGFp expression by 3'-UTR inverted Alus is not a direct consequence of decreased transcription or reduced mRNA stability. AcGFp reporter mRNA was predominantly localized to the cytoplasm for all transfected constructs; indicating that repression of AcGFp expression by 3'-UTR inverted Alus repeats may occur at the post-transcriptional level in the cytoplasm. Combined RNA in situ hybridization and immunofluorescence demonstrate that mRNAs with 3'-UTR inverted Alus accumulate in stress granules, as demonstrated by their colocalization with the stress granule marker, eIF3p (Fig. 5). By comparison, mRNAs with 3'-UTR single Alus and those with 3'-UTRs lacking Alus repeats do not localize to stress granules. All of the reporter mRNA, both those with 3'-UTR inverted or single Alus and those with 3'-UTRs lacking Alus, associated with the F-body marker GW182. Together these findings indicate that mRNAs with 3'-UTR inverted Alus repeats selectively associate with stress granules.

Compensatory substitution mutagenesis that restores the predicted stem-loop structure of the inverted repeats demonstrates that the secondary structure, but not the primary nucleotide sequence, of the 3'-UTR inverted Alus repeats is critical for repression of gene expression and stress granule association. RNA stem-loop structures in 3'-UTRs have been previously shown to repress mRNA expression in several ways. A-to-I hyperediting of 3'-UTR inverted Alus repeats has been linked to mRNA retention in nuclear paraspeckles. ADAR editing selectivity (the number of deamination events that occur in a substrate) is determined by the secondary structure of the substrate RNA. Perfectly base paired, thermodynamically stable dsRNAs are promiscuously deaminated at up to 50 to 60% of their adenosines. In contrast, less stable dsRNAs; those with mismatches, bulges and internal loops, are selectively deaminated at less than 10% of their adenosines.

It was shown experimentally that the size of the internal spacer or loop between the inverted repeats significantly impacts editing efficiency. Because ADAR editing efficiency is influenced by the length of the dsRNA, large internal loops decrease the number of ADAR binding sites by destabilizing the secondary structure, whereas smaller loops do not. In the study by Chen et al. two pairs of highly homologous, closely spaced 3'-UTR inverted Alus were analyzed. The sense and antisense repeats of one inverted Alu pair were separated by a 50 base pair internal spacer or loop. The oppositely-oriented Alus of the other inverted pair were separated by a 70 base pair internal loop. These inverted Alus substrates have the potential to form very stable stem-loop structures and according to the authors both pairs of inverted Alus are extensively edited. In our expression constructs, the oppositely-oriented Alus are separated by a 162 base pair internal loop generating a less stable dsRNA structure that is not extensively edited. This difference could account for the lack of nuclear paraspeckle association observed for inverted Alu-containing mRNAs in the present study.

In the present study, we show that mRNAs with 3'-UTR inverted Alus repeats co-localize with the stress granule marker eIF3p. The accumulation of transcripts with 3'-UTR inverted Alus repeats into stress granules would imply that translation of these mRNAs is repressed. Stress granules, cytoplasmic ribonucleoprotein (hnRNPs) aggregates, are implicated in the post-transcriptional regulation of gene expression. Translational repressed mRNAs associated with abortive translation initiation complexes and numerous RNA-binding proteins that affect mRNA expression are found in stress granules. Several proteins that influence mRNA stability, including HuR (Human antigen R) and TTP (tristetrapolin) accumulate in stress granules. In addition, proteins implicated in translational repression, including TIA-1, TIAR, CPEB1, RAP55 and YB-1, are also found in stress granules. Insine-containing dsRNAs have been shown to interact with components of stress granules and decrease reporter gene expression in trans. Stress granule accumulation is not entirely dependent on RNA editing, but is dependent upon stem-loop secondary structure formation. While the precise details of the 3'-UTR inverted Alus repeat-mediated repression that we observe will require further study, stress granule association may block mRNA translation as previously described in reference 42. The 3'-UTR inverted Alus repeat-mediated repression may also involve microRNAs. Alus repeats within 3'-UTRs are potential target sites for miRNAs. Additionally, effector molecules of RNAs-mediated posttranscriptional gene silencing including Argonaute proteins 1 and 2, and microRNAs, are found in stress granules.
Figure 5. For figure legend, see page 367.
In summary, our present study identifies a novel regulatory role for 3'-UTR inverted Alu repeats in the regulation of mRNA expression. Our previous studies, our work demonstrates that Alu repeats within the 3'-UTRs of human genes can modulate gene expression through multiple mechanisms; including the nuclear retention and Staufen 1 (STAU1)-mediated mRNA decay of mRNAs containing 3'-UTR Alu, and as we observe sequestration of mRNAs with 3'-UTR inverted Alu into stress granules. Given that the 3'-UTRs of human genes frequently harbor Alu sequences, it is quite likely that these sequences play important roles in regulating mRNA expression.

**Materials and Methods**

**Cell culture.** Human embryonic kidney cells (HEK293) were cultured in Dulbecco’s Modified Eagle’s Medium (Invitrogen, 11965-118) supplemented with 10% (v/v) fetal bovine serum (Gibco B-Products, cat. no. 900-108) and 5% streptomycin/penicillin (Gibco-BRL, Invitrogen). Cells were maintained at 37°C with 5% CO2.

**Plasmid construction.** To generate plasmid pΔΔIR, a genomic DNA fragment containing inverted Alu repeats (sense-oriented AluIR repeat and an antisense AluYa5 repeat separated by 162 base pairs) was PCR-amplified from intron 16 of the human Angiotensin Converting Enzyme (ACE) gene. Genomic DNA was extracted from HEK293 cells using the USB PrepEase Genomic DNA Isolation Kit (USB, 78850) according to the manufacturer’s instructions. Two oligonucleotide primers, GCC CAG CTC CCC CAT TAG AAC AAT G (forward) and GAC GTG GCC ATC ACA TTC GTC AGA TC (reverse) were used to amplify the corresponding DNA fragment by PCR. PCR reactions were performed in 50 μl volumes with Platinum Taq DNA polymerase High Fidelity (Invitrogen, cat. no. 11304-011) according to the manufacturer’s instructions. The PCR conditions were as follows: 1 min of denaturation at 94°C, followed by 35 cycles of 94°C for 30 sec, 57°C for 30 sec, 68°C for 1 min, and a final extension at 68°C for 5 min.

The 1,142-bp PCR product was gel purified from a 1% agarose gel using a QIAquick Gel Extraction kit (Qiagen Inc., cat. no. 28706) and cloned into TA cloning vector, pCR2.1 (Invitrogen, K2020-20), to generate pCR2.1ΔΔIR. Correct orientation of the subcloned ACE gene fragment, with respect to the genomic DNA, was determined by sequencing. An 1,184-bp HindIII-BglII fragment containing the inverted Alu repeat was then isolated from pCR2.1ΔΔIR and subcloned into the 3'-UTR region of the AcGFP1 gene of BamHI-HindIII-digested pAcGFP1-C1 (Clontech, 632470). Plasmid pΔΔIR was generated from pΔΔIR by XbaI digestion to remove the AluYa5 repeat and self-ligation. Plasmid pΔΔIR was generated from pΔΔIR by SfiI-Spel digestion to remove the AluYa5 repeat, blunt end-joining with T4 DNA polymerase (New England Biolabs, cat. no. M0203S) and self-ligation.

To generate the mutant plasmids pΔΔIRsubYa5 and pΔΔIRsubJb we utilized vector pΔΔIR. The wild-type full-length AluYa5 repeat or the wild-type full-length AluJb repeat of vector pΔΔIR was replaced by synthetic DNA fragments of the same length as the AluYa5 repeat or the AluJb repeat. To design the synthetic DNA fragments, we utilized the wild-type AluYa5 sequence or the wild-type AluJb sequence as templates. Each purine base (A or G) of the full-length AluYa5 and full-length AluJb repeats was substituted for another purine base. Likewise each pyrimidine base (C or T) of the synthetic sequences was synthesized and subcloned into pDTSMART plasmids by Integrated DNA Technologies (IDT).

To replace the AluYa5 repeat in plasmid pΔΔIR with a synthetic DNA fragment, pΔΔIR was first linearized with SphI and blunt-ended with T4 DNA polymerase (New England Biolabs, M0203S). Linearized pΔΔIR was then digested with SfiI to remove the AluYa5 repeat. The pΔΔIR vector backbone was then ligated to the synthetic mutant DNA fragment isolated from pΔΔIRsubYa5 digested pDTSMARTAluYa5bsb (Integrated DNA Technologies) to generate plasmid pΔΔIRsubYa5. To replace the AluYa5 repeat in plasmid pΔΔIR with a synthetic DNA fragment, pΔΔIR was ligated with SfiI to remove the AluYa5 repeat, blunt-ended with T4 DNA polymerase (New England Biolabs, M0203S) and digested with SfiI. Linearized pΔΔIR was then treated with calf intestinal alkaline phosphatase (New England Biolabs, M0203S) to prevent self-ligation and ligated to the synthetic mutant DNA fragment isolated from SfiI/SnaBl digested pDTSMARTAluYa5bsb (Integrated DNA Technologies) to generate plasmid pΔΔIRsubYa5. A third mutant plasmid (pΔΔIRcompensatory) containing substitutions of both the AluYa5 and AluJb repeats for synthetic DNA fragments was also generated. To construct pΔΔIRcompensatory, plasmid pΔΔIRsubYa5 was digested with EcoRI, blunt-ended with T4 DNA polymerase (New England Biolabs, M0203S), and digested with SfiI to remove the AluJb repeat. The isolated vector bone was then ligated to the substitution DNA fragment from pΔΔIRsubJb digested pDTSMARTAluJb-IRsub using T4 DNA ligase. Spacing between the inverted Alu repeats was recapitulated in the mutant constructs.

**Western blot.** HEK293 cells were cotransfected in 10 cm cell culture dishes by calcium phosphate precipitation with 10 μg of an AcGFP expression plasmid, together with 1 μg of pCMVβ (Clontech, PT2004-5), a β-galactosidase control plasmid. Forty-eight hours post-transfection, total proteins were isolated from transfected cells using the PARIS kit (Ambion, cat. no. AM1921) according to the manufacturer’s instructions. Cell lysates were separated by electrophoresis on 12% (w/v) SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membrane was then incubated with antibodies against β-galactosidase (1:5,000 dilution, Millipore MAB374) and AcGFP (1:10,000 dilution, Clontech). Membranes were washed with Tris-buffered saline (TBS) and incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000 dilution, BioRad). Membranes were washed and developed using ECL Western Blotting Detection Reagent (Amersham, RPN2232) according to the manufacturer’s instructions.
gels and transferred to Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore, IPVH00010). Membranes were then blocked in 5% BSA in TBS/T (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween-20) for one hour at room temperature. After blocking, the membranes were incubated overnight with primary antibodies against AcGFP (1:1,000; Santa Cruz Biotechnology Inc.), α-tubulin (1:1,000; Santa Cruz Biotechnology Inc.) and β-galactosidase (1:500; Santa Cruz Biotechnology Inc.). Blots were washed with TBS/T and then incubated with secondary antibodies conjugated to horseradish peroxidase (1:1,000; Santa Cruz Biotechnology Inc.). After a final wash, protein bands were detected by using an ECL detection kit (Thermo Fisher Scientific Inc., cat. no. 34087), and band densities were evaluated with ImageJ software (US National Institutes of Health).

Northern blot analysis. HEK293 cells were transiently transfected in triplicate using the calcium phosphate DNA precipitation method4 with AcGFP expression plasmids. At 48 h post-transfection, total RNA and protein was harvested from one dish. Cytoplasmic and nuclear RNA fractions were isolated from the remaining dishes. The PARIS kit (Ambion, AM1921) was used to isolate RNA and protein from total cell lysates or from nuclear or cytoplasmic fractions according to the manufacturer’s instructions. Isolated protein was saved for western blot analysis to confirm the purity of subcellular fractions. Total, nuclear or cytoplasmic RNA was isolated protein from total cell lysates or from nuclear or cytoplasmic fractions according to the manufacturer’s instructions. Isolated protein was saved for western blot analysis to confirm the purity of subcellular fractions. Total, nuclear or cytoplasmic RNA (20 μg) was mixed with an equal volume of Glycrol Load Dye (Ambion, AM8551) and incubated at 65°C for 15 min. After electrophoresis at 5 V/cm on a 1% agarose gel, RNA was transferred onto BrightStar-Plus positively charged nylon membranes (Ambion, AM10102) and crosslinked with UV light. Membranes were prehybridized in ULTRAhyb buffer (Ambion, AM8670) at 42°C for 30 min. Membrane bound RNA was then hybridized with 1 x 10^6 cpm/ml of 32P-ribolabeled random-primed probes specific for AcGFP and GAPDH at 42°C overnight. Following hybridization, membranes were washed with a low stringency wash buffer (Ambion, AM8673) at room temperature for 10 min, fol-

DNA and RNA extraction. Northern blot analysis. HEK293 cells were transiently transfected in triplicate using the calcium phosphate DNA precipitation method4 with AcGFP expression plasmids. At 48 h post-transfection, total RNA and protein was harvested from one dish. Cytoplasmic and nuclear RNA fractions were isolated from the remaining dishes. The PARIS kit (Ambion, AM1921) was used to isolate RNA and protein from total cell lysates or from nuclear or cytoplasmic fractions according to the manufacturer’s instructions. Isolated protein was saved for western blot analysis to confirm the purity of subcellular fractions. Total, nuclear or cytoplasmic RNA (20 μg) was mixed with an equal volume of Glycrol Load Dye (Ambion, AM8551) and incubated at 65°C for 15 min. After electrophoresis at 5 V/cm on a 1% agarose gel, RNA was transferred onto BrightStar-Plus positively charged nylon membranes (Ambion, AM10102) and crosslinked with UV light. Membranes were prehybridized in ULTRAhyb buffer (Ambion, AM8670) at 42°C for 30 min. Membrane bound RNA was then hybridized with 1 x 10^6 cpm/ml of 32P-ribolabeled random-primed probes specific for AcGFP and GAPDH at 42°C overnight. Following hybridization, membranes were washed with a low stringency wash buffer (Ambion, AM8673) at room temperature for 10 min, fol-

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RNA editing analysis. HEK293 cells were transfected in 10 cm cell culture dishes with calcium phosphate precipitation with pFlagR. After 48 h, the transfected cells were harvested and split into two equal parts, one part for genomic DNA extraction and the other for RNA extraction. Genomic DNA was prepared using the USB PrepLase Genomic DNA Isolation Kit (USB, 78850) according to the manufacturer’s instructions. RNA was isolated using the PARIS kit (Ambion, AM1921). RNAs were treated with TURBO DNA-free DNase (Ambion, AM9707) to remove genomic DNA contamination. First-strand cDNA was reverse-transcribed from RNA with the SuperScript III First-Strand Synthesis RT-PCR kit (Invitrogen, 18808-055) according to the manufacturer’s instructions using a gene-specific primer located downstream of the inverted Alu on the plasmid pFlagR. The resulting cDNA and isolated genomic DNA were PCR-amplified using Platinum Taq DNA polymerase High Fidelity (Invitrogen, 11304-011) with forward and reverse oligonucleotides corresponding to the inverted Alu repeats. The cDNA-derived and genomic DNA-derived PCR products were subcloned into TA cloning vector, pCR2.1 (Invitrogen, K2020-20), transformed into E. coli, and multiple independent clones were submitted for automated DNA sequencing.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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RNA editing analysis. HEK293 cells were transfected in 10 cm cell culture dishes with calcium phosphate precipitation with pFlagR. After 48 h, the transfected cells were harvested and split into two equal parts, one part for genomic DNA extraction and the other for RNA extraction. Genomic DNA was prepared using the USB PrepLase Genomic DNA Isolation Kit (USB, 78850) according to the manufacturer’s instructions. RNA was isolated using the PARIS kit (Ambion, AM1921). RNAs were treated with TURBO DNA-free DNase (Ambion, AM9707) to remove genomic DNA contamination. First-strand cDNA was reverse-transcribed from RNA with the SuperScript III First-Strand Synthesis RT-PCR kit (Invitrogen, 18808-055) according to the manufacturer’s instructions using a gene-specific primer located downstream of the inverted Alu on the plasmid pFlagR. The resulting cDNA and isolated genomic DNA were PCR-amplified using Platinum Taq DNA polymerase High Fidelity (Invitrogen, 11304-011) with forward and reverse oligonucleotides corresponding to the inverted Alu repeats. The cDNA-derived and genomic DNA-derived PCR products were subcloned into TA cloning vector, pCR2.1 (Invitrogen, K2020-20), transformed into E. coli, and multiple independent clones were submitted for automated DNA sequencing.
References

1. Lander ES, Linton LM, Birger B, Nusbaum C, Zody MC, Birren B, et al. International Human Genome Sequence Consortium. Initial sequence of the human genome. Nature 2001; 409:860-921; http://dx.doi.org/10.1038/35057062.

2. Eichler EE, Koren S, Sivachenko A, Smalheiser NR, Volfovsky NV, et al. Copy number variation in the human genome. Nat Genet 2010; 42:903-910; http://dx.doi.org/10.1038/ng.1019.

3. Ramadoss A, Choudhuri A, Bhattacharyya S, Bhowmick N, et al. The human microRNAome. Mol Cell 2010; 38:370-381; http://dx.doi.org/10.1016/j.molcel.2010.03.025.

4. Loeb M, Sivachenko A, Koren S, Volfovsky NV, et al. A microRNA expression atlas in human tissues and cell types. Nat Genet 2011; 43:54-61; http://dx.doi.org/10.1038/ng.1670.

5. Eichler EE, Altshuler D, Abecasis GR, et al. Copy number variation in human genome evolution and disease. Nature 2010; 461:216-222; http://dx.doi.org/10.1038/nature08278.

6. Loeb M, Sivachenko A, Koren S, Volfovsky NV, et al. A microRNA expression atlas in human tissues and cell types. Nat Genet 2011; 43:54-61; http://dx.doi.org/10.1038/ng.1670.

7. Eichler EE, Altshuler D, Abecasis GR, et al. Copy number variation in human genome evolution and disease. Nature 2010; 461:216-222; http://dx.doi.org/10.1038/nature08278.

8. Loeb M, Sivachenko A, Koren S, Volfovsky NV, et al. A microRNA expression atlas in human tissues and cell types. Nat Genet 2011; 43:54-61; http://dx.doi.org/10.1038/ng.1670.

9. Eichler EE, Altshuler D, Abecasis GR, et al. Copy number variation in human genome evolution and disease. Nature 2010; 461:216-222; http://dx.doi.org/10.1038/nature08278.

10. Loeb M, Sivachenko A, Koren S, Volfovsky NV, et al. A microRNA expression atlas in human tissues and cell types. Nat Genet 2011; 43:54-61; http://dx.doi.org/10.1038/ng.1670.

11. Eichler EE, Altshuler D, Abecasis GR, et al. Copy number variation in human genome evolution and disease. Nature 2010; 461:216-222; http://dx.doi.org/10.1038/nature08278.

12. Loeb M, Sivachenko A, Koren S, Volfovsky NV, et al. A microRNA expression atlas in human tissues and cell types. Nat Genet 2011; 43:54-61; http://dx.doi.org/10.1038/ng.1670.

13. Eichler EE, Altshuler D, Abecasis GR, et al. Copy number variation in human genome evolution and disease. Nature 2010; 461:216-222; http://dx.doi.org/10.1038/nature08278.

14. Loeb M, Sivachenko A, Koren S, Volfovsky NV, et al. A microRNA expression atlas in human tissues and cell types. Nat Genet 2011; 43:54-61; http://dx.doi.org/10.1038/ng.1670.

15. Eichler EE, Altshuler D, Abecasis GR, et al. Copy number variation in human genome evolution and disease. Nature 2010; 461:216-222; http://dx.doi.org/10.1038/nature08278.

16. Loeb M, Sivachenko A, Koren S, Volfovsky NV, et al. A microRNA expression atlas in human tissues and cell types. Nat Genet 2011; 43:54-61; http://dx.doi.org/10.1038/ng.1670.

17. Eichler EE, Altshuler D, Abecasis GR, et al. Copy number variation in human genome evolution and disease. Nature 2010; 461:216-222; http://dx.doi.org/10.1038/nature08278.

18. Loeb M, Sivachenko A, Koren S, Volfovsky NV, et al. A microRNA expression atlas in human tissues and cell types. Nat Genet 2011; 43:54-61; http://dx.doi.org/10.1038/ng.1670.

19. Eichler EE, Altshuler D, Abecasis GR, et al. Copy number variation in human genome evolution and disease. Nature 2010; 461:216-222; http://dx.doi.org/10.1038/nature08278.

20. Loeb M, Sivachenko A, Koren S, Volfovsky NV, et al. A microRNA expression atlas in human tissues and cell types. Nat Genet 2011; 43:54-61; http://dx.doi.org/10.1038/ng.1670.

21. Eichler EE, Altshuler D, Abecasis GR, et al. Copy number variation in human genome evolution and disease. Nature 2010; 461:216-222; http://dx.doi.org/10.1038/nature08278.

22. Loeb M, Sivachenko A, Koren S, Volfovsky NV, et al. A microRNA expression atlas in human tissues and cell types. Nat Genet 2011; 43:54-61; http://dx.doi.org/10.1038/ng.1670.

23. Eichler EE, Altshuler D, Abecasis GR, et al. Copy number variation in human genome evolution and disease. Nature 2010; 461:216-222; http://dx.doi.org/10.1038/nature08278.

24. Loeb M, Sivachenko A, Koren S, Volfovsky NV, et al. A microRNA expression atlas in human tissues and cell types. Nat Genet 2011; 43:54-61; http://dx.doi.org/10.1038/ng.1670.

25. Eichler EE, Altshuler D, Abecasis GR, et al. Copy number variation in human genome evolution and disease. Nature 2010; 461:216-222; http://dx.doi.org/10.1038/nature08278.

26. Loeb M, Sivachenko A, Koren S, Volfovsky NV, et al. A microRNA expression atlas in human tissues and cell types. Nat Genet 2011; 43:54-61; http://dx.doi.org/10.1038/ng.1670.

27. Eichler EE, Altshuler D, Abecasis GR, et al. Copy number variation in human genome evolution and disease. Nature 2010; 461:216-222; http://dx.doi.org/10.1038/nature08278.

28. Loeb M, Sivachenko A, Koren S, Volfovsky NV, et al. A microRNA expression atlas in human tissues and cell types. Nat Genet 2011; 43:54-61; http://dx.doi.org/10.1038/ng.1670.