Structural determinants of human ζ-globin mRNA stability

Zhenning He1, Decheng Song1, Sebastiaan van Zalen1 and J Eric Russell1,2*

Abstract

Background: The normal accumulation of adult α and β globins in definitive erythrocytes is critically dependent upon processes that ensure that the cognate mRNAs are maintained at high levels in transcriptionally silent, but translationally active progenitor cells. The impact of these post-transcriptional regulatory events on the expression of embryonic ζ globin is not known, as its encoding mRNA is not normally transcribed during adult erythropoiesis. Recently, though, ζ globin has been recognized as a potential therapeutic for α thalassemia and sickle-cell disease, raising practical questions about constitutive post-transcriptional processes that may enhance, or possibly prohibit, the expression of exogenous or derepressed endogenous ζ-globin genes in definitive erythroid progenitors.

Methods: The present study assesses mRNA half-life in intact cells using a pulse-chase approach; identifies cis-acting determinants of ζ-globin mRNA stability using a saturation mutagenesis strategy; establishes critical 3'UTR secondary structures using an in vitro enzymatic mapping method; and identifies trans-acting effector factors using an affinity chromatographical procedure.

Results: We specify a tetranucleotide 3'UTR motif that is required for the high-level accumulation of ζ-globin transcripts in cultured cells, and formally demonstrate that it prolongs their cytoplasmic half-lives. Surprisingly, the ζ-globin mRNA stability motif does not function autonomously, predicting an activity that is subject to structural constraints that we subsequently specify. Additional studies demonstrate that the ζ-globin mRNA stability motif is targeted by AUF1, a ubiquitous RNA-binding protein that enhances the half-life of adult β-globin mRNA, suggesting commonalities in post-transcriptional processes that regulate globin transcripts at all stages of mammalian development.

Conclusions: These data demonstrate a mechanism for ζ-globin mRNA stability that exists in definitive erythropoiesis and is available for therapeutic manipulation in α thalassemia and sickle-cell disease.

Keywords: RNA stability, ζ Globin, Sickle-cell disease, Thalassemia

Background

Human α-like globins are encoded by three homologous genes (5′-ζ-α2-α1-3′) arranged in order of their developmental expression [1]. Embryonic α-like ζ globin is produced during the first gestational trimester in primitive erythroblasts that originate in blood islands of the yolk sac, while fetal/adult α globin is induced at the end of this interval and continues to express in definitive erythroblasts that are initially produced in the liver and, subsequently, in the bone marrow [1,2]. Unlike α globin—which is required for normal growth and development both in the uterus and after birth—ζ globin appears to be largely dispensable to mammalian reproduction. Embryonic ζ globin is not required for viability in mice [3], and naturally occurring deletions and duplications in its encoding gene are not associated with any defined phenotype in man [2,4,5]. As a consequence of this apparent biological irrelevance, then, there has been little incentive to detail the processes that control ζ-globin expression in either primitive or definitive erythropoiesis.

Investigations of the molecular and cellular programs that regulate ζ globin, though, are recently justified by its demonstrated potential as a novel therapeutic for both α thalassemia and sickle-cell disease [6,7]. When compelled to express in definitive erythroid cells, embryonic ζ globin

* Correspondence: jeruss@mail.med.upenn.edu
1 Department of Medicine (Hematology/Oncology), Perelman School of Medicine at the University of Pennsylvania, Biomedical Research Building, Room 808, 421 Curie Boulevard, Philadelphia, PA 19104, USA
2 Department of Pediatrics (Hematology), Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, USA

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assembles with adult β globin into heterotetrameric Hb ζβ2 (Hb Portland-2), which exhibits O2-binding and allosteric properties that differ modestly from those of Hb αβ2 (Hb A, the principal adult hemoglobin), but that remain fully compatible with normal adult physiology [8,9]. The significance of this approach for treating α-globin deficiency states (α thalassemia) is illustrated by evidence that transgenic human ζ globin fully reverts the pathological phenotype of mice containing heterozygous knockout of their endogenous α-globin genes and, remarkably, restores full viability to animals with homozygous, embryonic-lethal inactivation of these same genes [6]. The developmental stage-discordant expression of ζ globin holds additional therapeutic promise for sickle-cell disease, as its low-level expression effectively mitigates the abnormal phenotype of mouse models of this disorder [7,10]. Importantly, the α → ζ exchange that converts pathological Hb αβ2S (Hb S) to non-pathological Hb ζβS does not exclude the mutant βS subunit, raising the possibility that this novel strategy can be coordinated with contemporary therapies that promote βS → γ exchange. These two developing applications for ζ globin hold additional therapeutic promise for sickle-cell disease, as its low-level expression effectively mitigates the abnormal phenotype of mouse models of this disorder [7,10]. The present work describes structures within the ζ-globin 3′UTR that are critical to its post-transcriptional regulation, illustrates their effects on the half-life value of the full-length ζ-globin mRNA in intact cells, and identifies a trans-acting factor that is likely to mediate this process. These results provide a foundation for understanding the impact of post-transcriptional activities to the developmental stage-discordant expression of ζ globin in adult erythropoiesis, as a promising therapeutic for both α thalassemia and sickle-cell disease.

**Results**

A transcriptional chase strategy identifies cis-acting regulatory determinants within the ζ-globin 3′UTR

Transgenic human ζ globin accumulates to high levels in mouse erythrocytes, consistent with the existence of cis-acting determinants that stabilize the cognate ζ-globin mRNA in transcriptionally silent progenitor cells [6,7,18]. Precedent analyses of both globin and non-globin mRNAs indicate that relevant stability determinants are commonly confined to the 3′ region of untranslated mRNA (3′ UTR), which is not subject to disruption by translating ribosomes [27-31]. We investigated the likely positioning of an mRNA stability element in the ζ-globin 3′ UTR in a systematic manner, employing a linker-scanning strategy to identify cis-acting determinants of mRNA half-life that act in vivo in intact cells. Full-length ζ-globin genes were constructed to include either the 105-nt wild-type ζ-globin 3′UTR (WT); or identically sized variant ζ-globin 3′ UTRs, each containing an 8-nt mutation at a different position between the UGA translational termination codon and the polyadenylate tail (Figure 1). Each gene was linked to a recombinant hybrid tetracycline response element (TRE) that promotes transcription in cultured cells expressing a hybrid tetracycline trans-activating protein (tTA), but is transcriptionally silent in the presence of tetracycline (tet) [32]. This approach permitted half-life values for wild-type and 3′ UTR-variant ζ-globin mRNAs to be specified in vivo in tTA-expressing cells, using a transcriptional chase strategy that assesses the temporal reduction in the level of each variant ζ-globin mRNA relative to the steady-state level of a tet-indifferent control mRNA [33,34].

To identify the positions of mRNA stability determinants within the ζ-globin 3′ UTR, we conducted a screening assay that quantified the relative decay of wild-type and 3′ UTR-variant ζ-globin mRNAs in vivo in intact cultures. Mammalian cultured-cell models for erythroid development transcribe a variety of embryonic, fetal, and adult globin mRNAs that can compete for post-transcriptional
regulatory activities [18,35,36]; consequently, we conducted our studies in tTA-expressing HeLa cells (HeLa^tTA) that do not transcribe globin mRNAs and have previously been used to characterize post-transcriptional processes affecting both native and exogenous globin genes [16,34]. We tested the relative decays of transiently expressed ζWT and 3′UTR-variant ζ-globin mRNAs, relative to that of a control β-globin mRNA transcribed from a co-transfected gene, using a two-point decay method of our design. One of the 3′UTR-variant mRNAs—containing a CCCCAGCC→agtg- caCa substitution at nts 57–64 (ζ57 mRNA)—reproducibly decayed over a 16-hour interval to a level that was approximately one-third that of ζWT mRNA (Figure 2A). The effect of the octanucleotide substitution was dependent upon its position rather than its content, as ζ-globin mRNAs containing a similar mutation elsewhere in the 3′UTR—including flanking nts 49–56 and 65–72 (ζ49 and ζ65 mRNAs, respectively)—decayed at the same rate as parental ζWT mRNA. The mutational effects were consistent over three or more replicate analyses, thus identifying and localizing a previously unknown post-transcriptional determinant of mRNA decay within the ζ-globin 3′UTR. For convenience, we now term the nt 57–64 region the ζ-globin mRNA regulatory element (ZMRE).

We posited that the kinetics of ζ-globin mRNA decay might be similarly affected by a smaller defect in the nt 57–64 target region, according with precedent studies demonstrating the deleterious effects of site-specific single- or several-nt 3′UTR substitutions on the stabilities of both α- and β-globin mRNAs [16,18,38]. This hypothesis was validated by transcriptional chase analyses of transiently expressed ζ-globin mRNAs carrying tetranucleotide substitutions at either positions 1–4 or 5–8 of the ζ57 target region (ζ57.1-4 and ζ57.5-8 mRNAs, respectively; CCCCAGCC→agtgAGGC and CCCCCGCC→CCCCCcaCa). While the relative decay of ζ57.5-8 mRNA was no different from ζWT mRNA (Figure 2B, left), the...
decay of ζ57.1-4 mRNA was significantly accelerated, and to a similar extent as that of parental ζ57 mRNA. Subsequent analyses demonstrated that smaller, dinucleotide substitutions to the nt 57–64 region did not affect mRNA decay, indicating that the ZMRE is functionally permissive for mutations of this magnitude (Figure 2B, right). Collectively, these screening data specify the importance of a site-specific tetranucleotide motif to the regulated accumulation of ζ-globin mRNA.

The ZMRE regulates the half-life of ζ-globin mRNA

Results from two-point screening analyses indicated the likelihood that the ζ57 determinant is essential for normal accumulation of ζ-globin mRNA in cell cytoplasm. We validated this hypothesis by formally assessing t1/2 values for wild-type and informative 3′ UTR-variant ζ-globin mRNAs in vivo. Analyses were conducted in triplicate in transiently transfected HeLaTAT cells using a conventional pulse-chase strategy that assesses the levels of test mRNAs at defined intervals, relative to the level of endogenous tet-indifferent β-actin mRNA [33]. Under these conditions, the ζ57 mRNA displayed a t1/2 value that was approximately one-fourth that of WT mRNA (3.7 v 15.4 hr, respectively), while ζ49 and ζ65 mRNAs—containing ZMRE-flanking mutations—displayed relatively normal t1/2 values of 11.6 and 10.2 hr (Figure 3). These data were affirmed by subsequent analyses demonstrating the deleterious effect of the ζ57.1-4 mutation (but not the ζ57.5-8 mutation) on the half-life of ζ-globin mRNA (Figure 4). In these latter studies, the t1/2 value of control ζWT mRNA (11.2 hr) was reduced to the same extent by either full (ζ57) or partial (ζ57.1-4) substitution of the nt 57–64 target region (3.6 and 3.7 hr, respectively), confirming the centrality of the CCCC tetranucleotide to ZMRE function. Additional pulse-chase studies—conducted in cells that stably express wild-type and 3′ UTR-variant ζ-globin mRNAs—revealed similar results (not shown), providing a third measure of experimental validation. Collectively, the transcriptional chase analyses agree on the positioning of a post-transcriptional regulatory element within the ζ57 region, as well on its critical importance to the constitutive half-life of ζ-globin mRNA.

The mRNA-stabilizing activity of the ZMRE is not autonomous

Previous analyses in transgenic mice demonstrated that the stability of human α-globin mRNA was significantly reduced when its 3′ UTR was exchanged for the corresponding region of ζ-globin mRNA [18]. This effect was originally attributed to a generic difference in then-unspecified determinants of α- and ζ-globin half-life [17] and, later, to sequence dissimilarities between homologous pyrimidine-rich regulatory elements within the α- and ζ-
globin 3′UTRs [18]. We posited that this effect might alternately arise from a ZMRE function that was highly sensitive to its regional mRNA structural context; i.e., that the regulatory properties of the ZMRE might be reduced when repositioned near heterologous mRNA. To test this possibility, we assessed whether the half-life of a reporter coding-region mRNA contiguous with the ζ57 3′UTR would be reduced relative to an identical mRNA flanked by a ζWT 3′UTR (Figure 5A). A two-point decay analysis indicated that the stabilities of 3′UTR-chimeric β-globin mRNAs were indifferent to the presence of stabilizing (ζWT) or destabilizing (ζ57 and ζ57.1-4) 3′UTRs, validating an activity for the ZMRE that is dependent upon its RNA context (Figure 5B). While it is difficult to generalize this effect to coding regions from all mRNAs, our observations suggest that the activity of the ZMRE is defined both by its primary structure (Figures 3 and 4) as well as by undefined effects of neighboring mRNA and/or mRNA-bound factors (Figure 5).

The ZMRE maps to a region of ζ-globin 3′UTR that displays conformational flexibility

The observation that the ζ-globin mRNA is destabilized by mutations within the ZMRE, but not by similar mutations in neighboring cytosine-rich regions, suggests a local high-order structure that favors functional interactions between the ZMRE and one or more trans-acting effector factors. An unrefined m-fold analysis of ζ-globin 3′UTR structure predicts that the ZMRE is embedded in a highly stable RNA stem, and directly participates in

Figure 4 Half-life values for ζ57-derivative globin mRNAs. Stability analyses were conducted on informative ζ-globin mRNAs as described in Figure 3. (A) Stable control ζWT mRNA. (B) ζ57 mRNA. (C) ζ57.1-4 mRNA. (D) ζ57.5-8 mRNA. (E) Half-life and R² values for curves in panels A-D, calculated from best-fit exponential decay curves.

Figure 5 Relative decay of transiently expressed βζ-chimeric mRNAs in HeLa<sup>T</sup> cells. (A) Structures of mRNAs encoded by chimeric βζ-globin genes; regions derived from β- and ζ-globin sequence are indicated in black and white, respectively. The positions of the translation initiation (I) and termination (T) sites, as well as the polyadenylate tail (An), are indicated. The ζ57, ζ57.1-4, ζ49, and ζ65 mutations are illustrated in gray. The 3′UTR of the βζARE mRNA contains a defined 59-nt adenosine/uridine-rich element, inserted 15 nts 3′ to the translation stop codon; this motif destabilizes the full-length transcript, providing an unstable mRNA that is used as a methodological control [33]. RD = relative decay values obtained using the experimental strategy described in Figure 3.
seven base-pair interactions (Figure 6A) [39,40]. We were skeptical that this arrangement would support site-specific interactions with regulatory RNA-binding proteins, and consequently employed an enzymatic mapping technique to formally characterize the high-order RNA structure that encompasses the ZMRE [41]. Our analyses revealed regions of ζ-globin 3’UTR that—by virtue of their sensitivities to single- and double-strand-specific RNases—were inconsistent with the unrefined structure (Figure 6B). A subsequent m-fold analysis, constrained by unequivocal, experimentally determined regions of structure that encompasses the ZMRE [41]. Our analyses indicated by s and d, respectively.

**Cytoplasmic mRNA-binding proteins interact with the ZMRE**

The stabilities of many mRNAs—including those encoding α globin [27], β globin [16], α(1) collagen [28], tyrosine hydroxylase [29], histone [30], and the transferrin receptor [31]—require the assembly of defined mRNP effector complexes on specific determinants within their 3’UTRs. To identify candidate trans-acting factors that might functionally interact with the ZMRE, we conducted affinity chromatography analyses on cell extracts from both non-erythroid HeLa and erythroid K562 cells, using three 32-nt single-stranded (ss) DNAs corresponding to comparable regions of ζ57, control (WT), and control ζ65 3’UTRs. Both the control (WT) and (65 ssDNAs retained fewer than 10 distinct factors—two of which were not retained by the ζ57 ssDNA—suggesting site-specific trans-acting interactions and potential regulatory functions (Figure 7A). Excised bands were subjected to nanoLC/MS/MS and provisionally identified as isoforms of AUF1 (hnRNP D), a protein that is ubiquitously expressed [44], displays known mRNA-binding characteristics [45,46], participates in post-transcriptional regulatory events that effect both mRNA stabilization [47] and destabilization [48], and has been implicated as a determinant of β-

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**Figure 6 High-order structure of the ζ-globin 3’UTR.** (A) A thermodynamically favored structure resulting from unrefined m-fold analysis of the full-length ζ-globin 3’UTR (detail). The ZMRE is indicated in boldface. Double and triple hydrogen bond interactions are indicated by thin and thick connectors, respectively. (B) Enzymatic secondary-structure mapping of the ζ-globin 3’UTR (detail). 5’-end [32P]-labeled RNAs corresponding to the ζ-globin 3’UTR (and contiguous 18-nt polyadenylate tail) were digested with RNases A, T1, and V1 at four different concentrations (wedges), then resolved on an acrylamide-urea gel. Nucleotide assignments (right) were deduced by aligning the known 3’UTR sequence to specific bands corresponding to guanine bases (lane G), which were generated by RNase T1 digestion of denatured 3’UTRs. An alkaline hydrolysis ladder (lanes A) provides additional sequence information at single-nucleotide resolution. Regions of 3’UTR that exhibit stem- and loop-like characteristics (i.e., sensitivities to double- and single-strand-specific nucleases, respectively) are indicated to the left. (C) Thermodynamically favored structure resulting from m-fold analysis of the full-length ζ-globin 3’UTR, where base-pairing is enforced for nts 20–21, 24–26, and 38; and prohibited for nts 29–34 (detail). Nucleotides that displayed less-exacting single- or double-strand characteristics were not used for predictive purposes but are indicated by s and d, respectively.
globin mRNA half-life [16]. The identity of AUF1—and its relative affinities for ζWT, ζ57, and specificity-control ζ65 3′UTRs—was subsequently validated by immunoblot analyses of the three retenates (Figure 7B). Additional analyses demonstrated that AUF1 immunoprecipitate of extract prepared from WT-expressing HeLaTTA cells is enriched for both WT and positive control c-myc mRNAs (Figure 7C), demonstrating the assembly of ζ-globin/AUF1 mRNPs in vivo. These results document the interaction of AUF1 with the ZMRE and provide an attractive mechanistic link between the structure of the ζ-globin 3′UTR and its mRNA-stabilizing functions. The importance of AUF1 to this process also suggests that the half-life of ζ-globin mRNA may be regulated in definitive erythropoiesis through post-transcriptional programs that act on other mRNAs, including β-globin mRNA, that are central to normal red cell development.

Discussion

Recent reports that ζ-globin acts as a physiological surrogate for deficient α-globin [8,9] and efficiently inhibits the pathological polymerization of deoxyHb S [7,10] have engendered substantial interest in developing this embryonic globin as a unique and highly effective therapeutic for α-thalassemia and sickle-cell disease, respectively. The premise that developmentally silenced globin genes can be derepressed in definitive erythroid cells is consistent with observations that ζ-globin is expressed at high levels in several congenital and acquired conditions in humans [24-26] and can be reactivated by mutations that target transcriptional regulatory elements in animals [22-24]. Developmental stage-discordant embryonic globins can also be expressed from transgenes that have been modified to contain adult-stage promoters and enhancers [6,17,22]. While the specific mechanisms that underlie transcriptional repression of embryonic globin genes in definitive erythropoiesis remain a matter of active investigation, it is clear that this process can be reversed in adult erythroid progenitors.

An equally important determinant of ζ-globin expression—its post-transcriptional regulation—has been studied less extensively in both primitive and definitive erythropoiesis. Processes that impart high stability to globin mRNAs are particularly important in adult erythroblasts, permitting these transcripts to accumulate to high levels and to translate globin protein for 3–5 days following nuclear condensation and extrusion from orthochromatophilic erythroblasts [1]. As might be predicted, mutations that impair the high stabilities of globin mRNAs in transcriptionally silent cells will disproportionately impact the levels of their encoded proteins. For example, a naturally occurring antitermination mutation that shortens the t1/2 of α-globin mRNA to ~25% of its normal value (αConstant Spring) coordinately reduces expression of the cognate globin monomer to ~2% of the wild-type value [49,50]. Without some knowledge of the half-life of ζ-globin mRNA, on the mechanism through which it is stabilized in definitive erythroid cells, it is difficult to predict whether transcriptional derepression of the ζ-globin gene transcription will necessarily achieve the desired therapeutic effect.

We previously observed a modest difference in the stabilities of human α- and ζ-globin mRNAs that were compelled to express in definitive mouse erythroid cells [18]. This effect, which mapped to single-nt differences in homologous pyrimidine-rich elements (PREs) positioned within the two 3′UTRs, was attributed to a 6-fold reduction in the affinity of the ζ-PRE for αCP (hnRNP E), a cytoplasmic mRNA-binding protein that effects the high
stability of α-globin mRNA [12,18]. Importantly, ζ-globin mRNA was not fully stabilized by exchange of the ζ-PRE for the corresponding α-globin determinant, suggesting the activities of other, structurally dissimilar mRNA-stabilizing elements [18]. The present study validates this hypothesis, as it identifies a unique site-specific region of 3′ UTR that is essential for the normal cytoplasmic accumulation of ζ-globin (Figures 1 and 2). Subsequent half-life analyses, conducted in vivo in intact cultured cells, directly demonstrate the importance of the ZMRE (and, specifically, its 4-nt cytosine-rich core) to the cytoplasmic stability of ζ-globin mRNA (Figures 3 and 4).

Our several measures suggest a t1/2 value of 11–15 hr for ζ-globin mRNA in HeLa cells (Figures 3 and 4), which is surprisingly close to a t1/2 value of ~11 hr for α-globin mRNA obtained in MEL cells using a similar strategy [50]. The similar stabilities of α- and ζ-globin mRNAs in these cultured cells—which do not express other globin mRNAs in significant quantities—contrasts sharply with their discordant stabilities in primary mouse cells that co-express high levels of endogenous α-globin mRNA [18]. The cell context-dependent difference in the relative stabilities of the α- and ζ-globin mRNAs suggests that they may be co-regulated through a shared post-transcriptional mechanism. We previously described a similar relationship among developmentally related β-like globin mRNAs, where the stabilities of transgenic embryonic ε- and fetal γ-globin mRNAs are reduced in the presence of adult β-globin mRNA [35,36]. A similar relationship between the ζ- and α-globin mRNAs would account for the significant reduction in the half-life of ζ-globin mRNA when co-expressed with α-globin mRNA of either human or mouse origin. The implications of this mechanism vis-à-vis α thalassemia are substantial, as expression of ζ-globin mRNA from a therapeutic form that is targeted by trans-acting factors might bind and subsequently alter the regional structure to reveal (or conceal) co-determinants of mRNA stability.

Conclusion

Collectively, the present studies address the relevance of post-transcriptional regulatory events to ζ-globin expression in definitive erythroid cells, by defining structures and implicating mechanisms of ζ-globin mRNA stability. Our functional analyses identify a previously unrecognized determinant of mRNA stability within the ζ-globin 3′ UTR whose position can be narrowed to a 4-nt site-specific cytosine sequence. Coordinate structural studies recognize that this region maps to a functionally germane mRNA form that is targeted by trans-acting regulatory factors that have been previously been implicated in stabilizing both globin and non-globin mRNAs. This information provides a foundation for subsequent mechanistic studies that will be critical for understanding the full utility of developmental stage-discordant ζ-globin mRNA as a therapeutic for important disorders of globin gene expression.

Methods

Gene construction

Parental pTRE2Aζ was derived from pTRE2 (Clontech) by inserting the full-length human ζ-globin gene (including ~100 nt of contiguous 5′- and 3′-flanking regions)
into the polylinker SacII-BamHI site. A ~1.0 kb NcoI-SapI vector fragment was subsequently deleted to elimination a default polyadenylation signal that competes with the native ζ-globin poly(A) signal. 3′ UTR-derivative ζ-globin genes were constructed from pTRE2Aζ by exchanging the exon III BstEII-polylinker BamHI fragment with 330-bp syntheticDNAs (Genscript), each encompassing a mutated ζ-globin 3′ UTR. The construction of a corresponding full-length β-globin gene (pTRE2Aβ) is described elsewhere [15]. Genes encoding chimeric β-globin mRNAs were generated from pTRE2Aβ by exchanging the parental EcoRI-EcoNI DNA fragment (comprising β-globin exon III and contiguous 3′-flanking region) for a corresponding synthetic DNA containing the desired wild-type or derivative ζ-globin 3′ UTR. All recombiantDNAs were validated by automated sequencing.

Cell culture
HeLaTATα cells expressing the tetracycline transactivator fusion protein (tTA; Clontech) were maintained in DMEM supplemented with 10% FBS and antibiotics, at 37°C in a humidified 5% CO2 environment. Wild-type and 3′ UTR-derivative pTRE2Aζ vectors used for stable transfections were modified by inserting a 1.5-kb DNA fragment encoding hygromycin (hyg) resistance into a unique vector XhoI site. Approximately 6×10⁵ cells were transfected with 50 μg of DNA using Superfect reagent under conditions recommended by the manufacturer (Qiagen), and selected with 400 μg/mL hyg. Hyg-resistant clones were tested by RT-qPCR for levels of ζ-globin mRNA and control endogenous β-actin mRNA. Tetracycline (tet) response was assessed by quantifying the level of ζ-globin mRNA, relative to the level of tet-indifferent β-actin mRNA, following a 48-hr incubation in tet-supplemented media (2 μg/mL).

mRNA decay analyses
Two-point decay analyses were conducted on 5×10⁵ pre-plated HeLaTATα cells maintained in tet-supplemented medium (0.5 μg/mL). Cells were transfected with 50 μg DNA comprising equal quantities of wild-type (or 3′ UTR-derivative) TRE2Aζ and control pTRE2Aβ using Superfect reagent, and replated as two aliquots for overnight growth in tet-supplemented medium (0.03 μg/mL). PBS-washed aliquots were then incubated for five hr in tet-free medium; one aliquot was sacrificed immediately (t = 0) and a second aliquot after an additional 16-hr incubation in tet-supplemented medium (2.0 μg/mL). Conventional half-life analyses were conducted on 2×10⁶ HeLaTATα cells maintained in tet-supplemented medium (0.5 μg/mL), transfected with 10 μg DNA using Superfect reagent, and then replated in aliquots in tet-supplemented medium (0.03 μg/mL) for overnight growth. PBS-washed aliquots were then incubated in tet-free medium for five hr, supplemented with tet (2.0 μg/mL) and sacrificed at defined intervals. For both transient and stable analyses, cells were sacrificed by immersion in Trizol, and RNA prepared as recommended by the manufacturer (Invitrogen). Purified RNA was resuspended at 10 μg/mL in H2O, and stored at −80°C.

RT-qPCR
Purified RNA (50 ng) was assayed using Taqman One-Step reagents on a model 7500 real-time PCR system, using protocols recommended by the manufacturer (Applied Biosystems; AB). Analyses were conducted using assays for human ζ-globin (AB catalogue HS00923579_m1), β-globin (HS00747223_g1), and β-actin (HS9999990_3_m1), and quantified by ΔΔCt methodology that we describe elsewhere [34]. Relative decay values were calculated as the quantity of each ζ-globin mRNA remaining after a 16-hr transcriptional chase interval, relative to transiently co-expressed β-globin mRNA; the relative decay of (WT mRNA was arbitrarily assigned unit value.

Affinity enrichment
Custom 5′-terminal biotinylated single-strand (ss) DNAs corresponding to 3′ UTRs from ζWT (5′GGAGGTTCCTC CACGCCTACTTACCAGGAATT3′), ζ57 (5′GGAG GAGGTGA TATGTCACACCA CTACCAGCAAGAAT3′), and ζ65 (5′TG GAGGTTCCTCAGCAGGTCA CGGTAAT3′) mRNAs were commercially sourced (IDT). Approximately 1 μg of each ssDNA was incubated overnight at 4°C in 500 μL cytoplasmic extract supplemented with 50 μL ImmunoPure Avidin Agarose beads (Pierce). The pelleted beads were washed 2× with PBS + Triton-x100 (0.05%) and 2× with PBS + Triton-x100 (1.0%), resuspended in 10 μL loading buffer, and resolved on a precast 4-12% gradient SDS-polyacrylamide gel (Invitrogen). Cytoplasmic extracts were prepared from ~1×10⁷ cells lysed in buffer (1 mM Hepes pH 7.9, 0.15 mM MgCl2, 1 mM KCI) and clarified by centrifugation at 13000xg for five minutes at 4°C; extract was stored in aliquots at −80°C.

Proteomics
Analyses were conducted by the University of Pennsylvania Proteomics Core Facility. Tryptic digests were studied by nanoLC/MS/MS using Thermo LTQ and Eksigebt nano LC-2 Da instruments, and data analyzed from the Uniprot_Sprot database using Sequest and Scaffold software packages. Statistical p-value cutoffs of 95.0% and 99.0% were applied for peptides and proteins, respectively.

Western transfer
Antibodies were used at the following dilutions: AUF1 (kind gift of G Dreyfuss; 1:2000), replication protein A (Santa Cruz SC-81373; 1:300), and hnRNP-H (Santa
Cruz SC-10042: 1:500). Cytoplasmic extracts were resolved on precast 4-12% SDS-polyacrylamide gels, then transferred to nitrocellulose using an XCell II Module per manufacturer recommendations (Invitrogen). Membranes were blocked for 30 minutes at room temperature (RT) with Superblock T20 (Thermo Scientific), then supplemented with primary antibody for 60 min and with secondary HRP-conjugated antibody for an additional 30 minutes. Immunoblots were washed thrice for five minutes at RT with PBS + Tween-20 (0.1%), and analyzed by ECL chemiluminescence (GE Healthcare).

**mRNA secondary structure**

A DNA template for in vitro transcription of the \( c^{WT} \) 3′ UTR, containing an 18-nt polyadenylate tail, was directionally inserted into the Xhol-BgIII polynucleotide site of pSP72. The BgIII-linearized plasmid was transcribed in vitro as previously described [41], and the purified RNA then 5′-end labeled with \([γ-32P]ATP\) using a Kinase Max kit (Ambion). The \([γ-32P]\)-labeled RNA was digested with RNase A (100, 10, 1.0, and 0.1 ng/mL), RNase T1 (100, 10, 1.0, and 0.1 μg/mL), or RNase V1 (10, 1.0, 0.1, and 0.01 μg/mL) as directed by the supplier (Ambion). RNases A and T1 exhibit cleavage specificities for pyrimidine and guanosine bases in single-stranded regions of RNA, respectively, while RNase V1 cleaves 3′ to nucleotides within double-stranded regions of RNA. Reaction products were resolved on a 33 cm \( \times \) 40 cm 6%:8 M acrylamide:urea gel, in parallel with a migration-control ladder generated by alkaline hydrolysis of \([32P]-labeled transcripts [41].

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

Participated in research design: ZH, DS, SvZ. Performed data analysis: ZH, JER. Wrote or contributed to the writing of the manuscript: JER. All authors read and approved the final manuscript.

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