SMG6 Cleavage Generates Metastable Decay Intermediates from Nonsense-Containing β-Globin mRNA

Roshan Mascarenhas1,2,*, Julie A. Dougherty1,2,3,*, Daniel R. Schoenberg1,2,3,*

1 Center for RNA Biology, The Ohio State University, Columbus, Ohio, United States of America, 2 Department of Molecular and Cellular Biochemistry, The Ohio State University, Columbus, Ohio, United States of America, 3 Biomedical Sciences Graduate Program, The Ohio State University, Columbus, Ohio, United States of America

Abstract

mRNAs targeted by endonuclease decay generally disappear without detectable decay intermediates. The exception to this is nonsense-containing human β-globin mRNA, where the destabilization of full-length mRNA is accompanied by the cytoplasmic accumulation of 5′-truncated transcripts in erythroid cells of transgenic mice and in transfected erythroid cell lines. The relationship of the shortened RNAs to the decay process was characterized using an inducible erythroid cell system and an assay for quantifying full-length mRNA and a truncated RNA missing 169 nucleotides from the 5′ end. In cells knocked down for Upf1, a reciprocal increase in full-length and decrease in shortened RNA confirmed the role of NMD in this process. Kinetic analysis demonstrated that the 5′-truncated RNAs are metastable intermediates generated during the decay process. SMG6 previously was identified as an endonuclease involved in NMD. Consistent with involvement of SMG6 in the decay process full-length nonsense-containing β-globin mRNA was increased and the Δ169 decay intermediate was decreased in cells knocked down for SMG6. This was reversed by complementation with siRNA-resistant SMG6, but not by SMG6 with inactivating PIN domain mutations. Importantly, none of these altered the phosphorylation state of Upf1. These data provide the first proof for accumulation of stable NMD products by SMG6 endonuclease cleavage.

Introduction

Endonuclease decay was thought to play a minor role in mRNA turnover before results from deep sequencing showed widespread evidence for endonuclease cleavage throughout the mammalian mRNA transcriptome [1,2]. Despite this relatively little is known about the enzymes that generate these cleavages, and only a few bona fide mRNA endoribonucleases have been identified and characterized [3]. A major complication to the study of endonuclease-mediated mRNA decay is the rapidity with which cleavage products are cleared by 5′-3′ and 3′-5′ exoribonucleases [3]. For the most part decay intermediates are only detected by knocking down Xrn1 to stabilize the downstream fragment [4] or by PCR amplification after ligating a primer to the newly formed 3′ ends of cleavage products [5]. A possible exception to this is the decay of nonsense-containing β-globin mRNA in erythroid cells.

In 1989 Lim and Maquat [6] showed that 5′-truncated forms of human β-globin mRNA accumulate in erythroid cells of mice that are transgenic for several nonsense containing alleles. The same 5′-truncated RNAs accumulate in murine erythroleukemia cells that are stably transfected with wild type and nonsense-containing human β-globin genes [7,8]. We showed previously that these shortened RNAs were generated by endonuclease cleavage [7], but because they were only seen in erythroid cells it was unclear if these are intermediates in the decay process or the products of a cell type-specific processing that is unique to β-globin mRNA in its native cell environment. Complicating matters further the same 5′-truncated RNAs were also seen in cells expressing wild type β-globin mRNA, albeit at a much lower level [7], and their quantity is increased by coexpressing Xenopus PMR1 in these cells [8]. This was originally interpreted as evidence that erythroid cells employ a PMR1-like endonuclease to degrade β-globin mRNA, but that finding preceded the identification of SMG6 as an endonuclease that catalyzes the degradation of nonsense-containing mRNA [9,10].

Progress in studying endonuclease decay has been limited by the challenges inherent in quantifying short-lived decay intermediates. Thus, if the shortened forms of β-globin mRNA are indeed decay intermediates we could take advantage of their appearance to address several questions about the decay process. To address this we developed an inducible line of erythroid cells which were used to monitor the cytoplasmic appearance of full-length normal (WT-βG) and PTC-containing (PTC-βG) human β-globin mRNA after treating cells with doxycycline to induce transcription of their

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* E-mail: schoenberg.3@osu.edu
¤ Current address: Department of Pharmacology, The Ohio State University, Columbus, Ohio, United States of America
¶ These authors contributed equally to this work.
respective genes. Changes in full-length mRNA and one of the 5'-truncated RNAs were determined using a modification of Molecular Beacon Rapid Amplification of cDNA Ends (MBRACE) [11], a qRT-PCR-based assay for quantifying products after ligation of a common primer to uncapped 5' ends.

**Materials and Methods**

**Plasmid Constructs**

A wild type (WT) human β-globin gene and a gene with a nonsense codon at position 60/61 (PTC60/61, [7]) were cloned into a modified form of pcDNA3 (pcDNA3/TO) with a tetracycline operator element upstream of the multiple cloning site. Destabilized forms of each of these were generated by site-directed mutagenesis of the nucleolin/σ-CP binding site (H124 mutation) [12]. Plasmids expressing wild type and PTC-containing TCRβ mRNA (pAc/IF-TCRβ) [13] were provided by Miles Wilkinson. pcDNA3-HA-SMG6 and SMG6-m4 provided by Oliver Muhlemann were used for complementation experiments. All of the primers described here and below are listed in Table S1.

**Cell Culture**

Murine erythroleukemia (MEL) cells that were stably transfected with wild type (Norm2) or nonsense-containing (Thal10) β-globin genes were described previously [7]. Thal10 cells express a form of β-globin mRNA with a single base deletion that results in a nonsense codon at position 60/61 in the mature transcript. Norm2 and Thal10 cells were cultured in Minimal Essential Medium alpha (alpha MEM) supplemented with 10% FBS, and β-globin gene transcription was induced by treating for 48 hr with 1.5% DMSO. K562 human erythroleukemia cells were obtained from the American Type Culture Collection and cultured in the same medium as Norm2 and Thal10 cells. A line of tetracycline inducible K562 cells was generated by transduction with a tetracycline repressor-expressing lentivirus (pLenti6/TR, Invitrogen). Transduction was performed by adding viral supernatant to cells in a 24-well culture plate and centrifuging at 300 × g for 4 min, the cell pellet was washed with cold PBS. Norm2 and Thal10 cells were resuspended in lysis buffer containing 0.1 M NaCl, 10 mM Tris-HCl pH 8.0, 2 mM EDTA, 1% NP-40, 1% 2-mercaptoethanol, 80 units/ml RNaseOUT. K562 cells were resuspended in modified lysis buffer containing 0.15 M NaCl, 0.9% NaCl, 1 mM dithiothreitol, and 80 units/ml RNaseOUT. These were placed on ice for 5 min, the tubes were gently flicked and incubated on ice for an additional 5 min. The lysates were centrifuged at 4000 × g at 4°C for 10 min to pellet the nuclei, supernatant (cytoplasmic) fractions were transferred to chilled microcentrifuge tubes and RNA was extracted using Trizol Reagent (Life Technologies) as recommended by the manufacturer’s protocol. Cell lysate was used directly for protein quantification and visualization by western blot analysis.

**Isolation of Cytoplasmic RNA and Protein**

Cell were harvested by centrifugation and washed twice with ice cold PBS. Norm2 and Thal10 cells were resuspended in lysis buffer containing 0.1 M NaCl, 10 mM Tris-HCl pH 8.0, 2 mM EDTA, 1% NP-40, 1% 2-mercaptoethanol, 1 mM dithiothreitol, and 80 units/ml RNaseOUT. K562 cells were resuspended in modified lysis buffer containing 0.15 M NaCl, 90 mM Tris-HCl pH 7.5, 10 mM KCl, 10 mM MgCl2, 0.2% NP-40, 2 mM dithiothreitol and 80 units/ml RNaseOUT. These were placed on ice for 5 min, the tubes were gently flicked and incubated on ice for an additional 5 min. The lysates were centrifuged at 5000 × g at 4°C for 10 min to pellet the nuclei, supernatant (cytoplasmic) fractions were transferred to chilled microcentrifuge tubes and RNA was extracted using Trizol Reagent (Life Technologies) as recommended by the manufacturer’s protocol. Cell lysate was used directly for protein quantification and visualization by western blot analysis.

**Antibodies**

Affinity-purified rabbit polyclonal antibody to the tetracycline repressor protein was obtained from MoBiTec. Rabbit polyclonal antibodies to hUpf1 and hSMG6 were provided by Jens Lykke-Andersen, and rabbit polyclonal anti-phosphoUpf1 was obtained from Millipore, Inc. Antibody to ribosomal protein S6, horseradish peroxidase (HRP)-coupled goat anti-rabbit IgG and goat anti-mouse IgG were obtained from Santa Cruz Biotechnology.

**Western Blot Analysis**

Samples were denatured in 2× Laemmli sample buffer (Bio-Rad Laboratories) with β-mercaptoethanol. 10 μg of cytoplasmic protein was separated on 10% Mini-PROTEAN® TGX™ precast gels (Bio-Rad Laboratories) and transferred onto Immobilon™-P PVDF membrane (EMD Millipore). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween-20 (TBS-T), incubated with primary antibody (1:1000 dilution) in the same solution, washed with TBS-T, then incubated with horseradish peroxidase-coupled secondary antibody (1:10000 dilution). For experiments with anti-phospho-Upf1 antibody blots were blocked with 1× TBS +3% BSA for 2 hr at 25°C. They were incubated overnight at 4°C in the same solution with a 1:250 dilution of anti-phospho-Upf1 antibody and secondary antibody incubation was performed in 1× TBS +3% BSA. Membranes were developed with ECL-plus or for phospho-
Up1 Western blotting a 1:1 mixture of ECL-prime solutions A and B, and visualized on X-ray film (GeneMate).

S1 Nuclease Protection Assay

S1 nuclease protection assay was performed as described in [14]. RNA was dissolved in S1 hybridization buffer (80% formamide, 40 mM PIPES, pH 6.4, 0.4 M NaCl, 1 mM EDTA) and incubated overnight at 52°C with 3–5 × 10^{-5} pmol of [32P]-end-labeled antisense DNA probe. The antisense probe was obtained by asymmetric PCR using the human β-globin cDNA plasmid pSPKβG or the mouse β-actin cDNA plasmid pBSβactin as templates. T4 polynucleotide kinase was used to label the 5’ end of the antisense β-globin primer HBB-AS: beginning at position 250, primer 1249: beginning at position 346 and the antisense β-actin primer YO-41 with γ-[32P] ATP (3,000 Ci/mmol). The unlabeled β-globin sense primer (HBB-S) and β-actin sense primer (ACT-S) correspond to sequences just upstream of the β-globin or β-actin cDNA in the expression plasmid. The [32P] labeled probe generated by this reaction was purified by electrophoresis on a 6% polyacrylamide/urea gel. After hybridization, S1 nuclease solution (0.28 M NaCl, 0.05 M sodium acetate, pH 5.2, 4.5 mM ZnSO4, 20 μg/ml sheared salmon sperm DNA, 100 units S1 nuclease, Invitrogen) was added to each reaction and the mixture was incubated at 28°C for 2 hr. Samples were precipitated with ethanol and electrophoresed on denaturing 6% polyacrylamide/urea gels. Protected fragments were visualized and quantified by PhosphorImager.

MBRACE, Modified MBRACE and qRT-PCR Assays

1 μg of DNase I-treated cytoplasmic RNA was incubated with 3 units of tobacco acid pyrophosphatase (TAP, Epicentre) at 37°C for 2 hr in 1 × TAP buffer. An RNA adapter (RNA ADP) was ligated to this using T4 RNA ligase I (New England Biolabs) in T4 RNA ligase reaction buffer supplemented with 1 mM ATP at 37°C for 2 hr. cDNA was prepared using SuperScript® III first-strand synthesis system (Life Technologies) and the product was purified using QiAquick® PCR purification columns (Qiagen). Approximately 20 ng of cDNA was used per qPCR reaction containing 500 nM forward (MBRACE-F) and reverse primers (HBB-FL-R1, HBB-A169-R1), 250 nM molecular beacon (HBB-FL-MB, HBB-A169-MB) in 1 × PerfectTaq qPCR FASTmix (Quanta Biosciences). PCR was performed using an Eco Real-Time PCR system (Illumina® and the following thermal profile: 95°C for 3 min, 95°C 10 sec, 61°C 30 sec with signal detection, 72°C 15 sec) for 40 cycles. Ct baseline and threshold were automatically determined by Eco software. Molecular beacons were designed to the junction sequence between the RNA adapter and full-length or Δ169 β-globin mRNA. HEX-BHQ1 and 6FAM-Dabcyl fluorophore-quencher pairs were used to label the full-length and decay product beacons respectively. HPLC purified beacons were resuspended in 1 × TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) buffer and stored in aliquots at −80°C. Modified MBRACE assay used primers designed to the junction between the ligated adapter RNA and full-length (HBB-JS-F) and Δ169 (HBB-A169 -JS-F) RNA. β-globin specific primers (HBB-FL-R1, HBB-A169-R1 in MEL, HBB-FL-R2, HBB-A169-R2 in K562) were designed to generate similar length products that could be verified by gel electrophoresis. The locations of these primers on hG mRNA are shown in Figure S1. Reaction conditions to detect full-length β-globin and its decay product were as follows: 20 ng cDNA, 375 nM junction-specific and reverse primers in 1 × SYBR Green PCR Master Mix (Applied Biosystems/Life Technologies) in 10 μl reaction volume. The control mRNA TCRβ (TCRβ-F, TCRβ-R), GFP (emGFP-F, emGFP-R), and β-actin (MA-F, MA-R) levels were also quantified by similar method using indicated primers. qPCR was performed on the Eco Real Time PCR system with thermal profiles for all SYBR Green reactions as follows: 95°C for 10 min, [95°C for 10 sec, 60°C 30 sec with signal detection] for 40 cycles followed by a melt curve. ROX present in master mix acted as well-to-well normalization control. The Eco software automatically determined Ct baseline and threshold. To determine the fold change in transfection experiments, β-globin mRNA levels were normalized to the levels of co-transfection control GFP mRNA. In MEL cells β-globin mRNA levels were normalized to β-actin mRNA.

Data Analysis

Experiments were conducted in biological triplicate and qPCR reactions were performed in sample triplicate. Ct values for each replicate were averaged, ΔCt was calculated by subtracting the control (β-actin or GFP) Ct from the sample Ct. ΔCt values were averaged over the replicates and the average ΔCt for WT β-globin acted as the reference value. The ΔΔCt value was calculated by subtracting the ΔCt of WT from the sample ΔCt value. Fold expression was calculated using the 2−ΔΔCt method [15]. Statistical analysis was performed using JMP9 (SAS, North Carolina) software. Data distribution and variance determined the appropriate method of analysis, all of which were performed 2-tailed with an α=0.05. Graphs were generated using GraphPad Prism 5 (GraphPad Software, Inc.) and bars represent standard deviation.

Results

Actinomycin D Chase Shows Prolonged Presence of 5’ Truncated RNAs in the Erythroid Cell Cytoplasm

Our previous work used murine erythroleukemia cells that were stably transfected with WT-hβG (Norm2) or PTC-hβG (Thal10) transgenes to study β-globin mRNA decay [7,8]. Using Actinomycin D and Northern blotting we showed that full-length wild type mRNA has a half-life of 12 hr in Norm2 cells and PTC-containing mRNA decays with biphasic kinetics and a half-life of 100 min [8]. Neither of the earlier studies looked at the fate of the 5’-truncated RNAs, and because these are more abundant in Thal10 cells we repeated Actinomycin D chase but this time used S1 nuclease protection to monitor changes in full-length and 5’-truncated RNAs (Figure 1). Again, full-length PTC-hβG mRNA showed a biphasic curve similar our previous results and to PTC-hβG mRNA in non-erythroid cells [16]. Although they appeared to be more stable than full-length mRNA there were differences in the patterns seen for each of the 5’-truncated RNAs. The transcripts whose 5’ ends were closest to that of full-length mRNA disappeared at a faster rate than those whose 5’ ends were further away. Although it does not constitute proof, this is consistent with multiple endonuclease cleavage events happening over time until a limit digest is reached.

A Quantitative Assay for Full-length and 5’-truncated β-globin RNAs

Given the challenge presented by the prolonged presence of 5’-truncated RNAs we sought an alternative approach for studying hβG mRNA decay in erythroid cells. The first step involved the development of a method for quantifying full-length mRNA and one of the truncated transcripts, and for this we selected Molecular Beacon Rapid Amplification of cDNA Ends (MBRACE) [11] (outlined in Figure 2A). In this assay, cytoplasmic RNA is first treated with a phosphatase to prevent adapter ligation to uncapped ends. The cap is removed with tobacco acid pyrophosphatase
The discovery of cytoplasmic capping [17] was the result of following the 5'-ends of PTC-hbG mRNA decay intermediates in untreated THAL10 cells as a function of time and measured by MBRACE (Figure 2D). Our results confirmed that the junction with the ligated adapter of the PTC-hbG transcript with 5'-capped ends was protected from digestion by S1 nuclease protection (Figure 2C). The transcript with 5'-capped ends was sequenced to confirm that the junction with the ligated adapter was protected from digestion by S1 nuclease protection (Figure 2C). The transcript with 5'-capped ends was sequenced to confirm that the junction with the ligated adapter was protected from digestion by S1 nuclease protection (Figure 2C).

NMD is Responsible for the Increase in 5' Truncated RNAs from PTC-hbG mRNA

Although K562 cells are an erythroid cell line they do not express hbG mRNA. We developed a line of tetracycline inducible K562 cells to circumvent complications resulting from the prolonged cytoplasmic lifetime of 5'-truncated RNAs. In each of the following experiments these cells were electroporated with plasmids expressing tetracycline inducible full-length WT- and PTC-hbG genes, and changes in full-length and 5'-truncated RNAs were quantified by MBRACE assay after inducing their transcription with doxycycline. In the experiment in Figure 3 electroporated K562 cells were treated with Accell® control or Upf1 siRNAs before inducing WT- or PTC-hbG mRNA. This reduced Upf1 to 10% of control (Figure 3A), and the stabilization of nonsense-containing TCRβ mRNA (Figure 3B) demonstrated its effectiveness in inactivating NMD. Knocking down Upf1 had little impact on WT-hbG mRNA or the amount of 5'-truncated RNAs in cells expressing WT-hbG mRNA; however, the stabilization of full-length PTC-hbG mRNA and reduction in the level of 5'-truncated RNA to that observed with WT-hbG mRNA (Figure 3C and D) confirm that NMD is responsible for the increased appearance of 5'-truncated transcripts from PTC-hbG mRNA.

The 5' Truncated RNAs are Metastable Decay Intermediates

The prolonged cytoplasmic residence of 5'-truncated RNAs after Actinomycin D (Figure 1) ruled out the use of transcription inhibitors in determining whether these are intermediates of PTC-hbG decay. Instead we monitored the appearance of both forms of hbG mRNA over time after inducing transcription of both WT- and PTC-hbG genes (Figure 4). If the truncated RNAs are decay intermediates the expectation is their appearance in the cytoplasm should follow that of full-length mRNA. Changes in each transcript over 12 hr after induction are shown in Figure 4A, and the first 3 hr after induction, where initial differences are more evident, are shown enlarged in (Figure 4B). The appearance of 5'-truncated RNAs in cells expressing WT-hbG mRNA (Figure 4C and D) confirm that NMD is responsible for the increased appearance of 5'-truncated transcripts from PTC-hbG mRNA.

Whereas TAP treatment of RNA from PTC-hbG-expressing cells resulted in an almost 800-fold increase in signal for 5'-truncated RNAs, the applicability of MBRACE assay for quantifying changes in full-length and 5'-truncated RNAs was confirmed by the comparable results obtained by MBRACE assay (Figure 2D) and S1 nuclease protection (Figure 2C). As anticipated, this reduced the degree to which of the 5'-ends of PTC-hbG mRNA decay intermediates were protected from digestion by S1 nuclease protection (Figure 2C). The presence of a cap on 5'-truncated RNAs is evident in the right panel of Figure 2B, where TAP treatment of RNA from PTC-hbG-expressing cells resulted in an almost 800-fold increase in signal for 5'-truncated RNAs.
these data confirm that the 5’-truncated RNAs are endonuclease-generated decay intermediates.

SMG6 is the Endonuclease that Generates 5’-truncated forms of PTC-hβG mRNA

We next sought to determine the identity of the endonuclease responsible for generating the 5’-truncated decay intermediates. Our previous work suggested this was PMR1 or a PMR1-like endonuclease [7,8]. However this was ruled out by the lack of any impact on WT- or PTC-hβG mRNA of inhibiting PMR1 activation or overexpressing human PMR1 (Figure S2). SMG6 is a PIN-domain containing endonuclease that participates in the degradation of nonsense-containing mRNAs [9,10]. It lacks sequence selectivity, and specificity for nonsense-containing mRNAs results from its binding to the exon junction at the same site as Upf3b [20]. In the experiment in Figure 5 K562 cells electroporated with plasmids expressing WT- and PTC-hβG mRNA were knocked down for SMG6 before adding doxycycline to induce WT- and PTC-hβG mRNA. Knockdown depleted ∼90% of SMG6 (Figure 5A) and this had the anticipated effect of stabilizing PTC-containing TCRβ mRNA (Figure 5B). SMG6 knockdown had no impact on WT full-length or Δ169 RNA, increased full-length PTC-hβG mRNA and returned the amount Δ169 RNA to the level generated from WT-hβG mRNA.

Although these data are consistent with SMG6 generating Δ169 RNA it was formally possible that the observed effects were secondary to changes in Upf1 phosphorylation [21]. This was first addressed by a complementation experiment similar to that performed in [10]. In the experiment in Figure 6 tetracycline-inducible K562 cells were electroporated with control or SMG6 siRNAs, then a second time with the same siRNAs together with empty vector or plasmids expressing siRNA-resistant SMG6 or an inactive form of SMG6 with the...
3 active site aspartic acid residues changed to asparagine (SMG6-m4). The second round of transfection included tetracycline-inducible plasmids expressing WT- and PTC- \( \beta \)-G mRNA, and cells recovered 6 hr after induction were analyzed by Western blotting for changes in SMG6 and by modified MBRACE assay.

Figure 3. Evidence that NMD is responsible for the accumulation of \( \Delta 169 \) RNA from PTC-\( \beta \)G mRNA. Tet-inducible K562 cells in antibiotic-free medium were electroporated with plasmids expressing constitutive wild type or nonsense-containing TCR\( \beta \) genes, or each of the inducible \( \beta \)-globin genes, and a GFP control. Sixteen hr later they were transfected with Accell SmartPool→ Upf1 or control siRNAs, cultured for an additional 48 hr, then induced with doxycycline for 6 hr. A. Cytoplasmic extracts from WT- and PTC-\( \beta \)G expressing cells were assayed by Western blotting for efficiency of Upf1 knockdown. B. The effectiveness of Upf1 knockdown in inhibiting NMD was determined by qRT-PCR analysis of changes in WT- versus PTC-TCR\( \beta \) mRNA. C. The modified MBRACE assay was used to quantify the impact of Upf1 knockdown on full-length WT- and PTC-\( \beta \)G mRNA. D. Modified MBRACE assay was used to monitor the impact of Upf1 knockdown on the production of \( \Delta 169 \) RNA. The results represent the mean ± standard deviation of triplicate cultures, *indicates p<0.05 by two-tailed Student’s T-test. doi:10.1371/journal.pone.0074791.g003

Figure 4. Evidence that 5'9-truncated RNAs are decay intermediates. A. Doxycycline was added at time 0 to tet-inducible K562 cells that were electroporated 16 hr earlier with plasmids bearing inducible WT- or PTC-\( \beta \)G genes and a GFP control. Cytoplasmic RNA isolated at intervals over 12 hr of induction was analyzed by modified MBRACE assay for changes in full-length and \( \Delta 169 \) forms of h\( \beta \)G mRNA. B. The first 3 hr of induction is shown enlarged. C. The experiment was repeated except that the plasmids that were electroporated into tet-inducible K562 cells expressed a destabilized form of \( \beta \)-globin mRNA (H124 mutation) as a result of disruption of the 3'-UTR nucleolin binding site [12]. Each point represents the mean ± standard deviation for triplicate determinations. doi:10.1371/journal.pone.0074791.g004
SMG6 knockdown reduced levels of the endogenous protein to ~30% of control (Figure 6A, compare lane 3 with lanes 4 and 5), and in each transfectant siRNA-resistant SMG6 was overexpressed compared to endogenous protein (Figure 6A, compare lanes 1,2,6–9 with lane 3). The similar expression of each form of recombinant SMG6 confirmed that each of these is resistant to SMG6 siRNA and therefore capable of complementing the impact of SMG6 knockdown on PTC-h\beta G NMD. Results in Figure 6B show that neither SMG6 knockdown nor its complementation with exogenous SMG6 had any impact on wild-type full-length or \( \Delta 169 \) RNA (grey bars). As in Figure 5, SMG6 knockdown increased the amount of full-length PTC-h\beta G mRNA and decreased the amount of \( \Delta 169 \) RNA (SMG6 siRNA+ vector). This was reversed by co-expression of siRNA-resistant SMG6, but not by co-expression of catalytically-inactive SMG6-m4, thus confirming the identity of SMG6 as the enzyme that is responsible for generating stable decay products from PTC-h\beta G mRNA.

Discussion

To the best of our knowledge the 5’-truncated forms of PTC-h\beta G mRNA examined here are the only example of metastable products of nonsense-mediated mRNA decay. They were first identified in erythroid cells of mice expressing a nonsense-containing transgene [6], and to date have only been detected in erythroid cells. Like the parent mRNA the 5’ ends of these RNAs are capped [17,18] and they have an intact 3’-poly(A) tail [7]. S1 nuclease protection assays performed on cytoplasmic RNA recovered over 8 hr of actinomycin D treatment showed biphasic kinetics (Figure 1)
that resemble the decay of PTC-hβG mRNA in non-erythroid cells [16]. The decay curves in Figure 1B indicated that the 5′-truncated RNAs disappear more slowly than full-length PTC-hβG mRNA. However, the transcripts with 5′ ends closer to the cap appear to decay more quickly than transcripts whose 5′ ends are further from the cap, the result one might expect for RNA undergoing multiple cleavage events until reaching some limit digest. The complexity of this precluded the use transcriptional inhibition to study the relationship of the 5′-truncated transcripts to PTC-hβG mRNA decay. This was instead addressed through the use of an inducible erythroid cell line and a modification of the MBRACE assay for quantifying changes in full-length mRNA and one of the truncated RNAs (Δ169). In the course of qualifying this assay we reaffirmed results in [17] showing the 5′-truncated RNAs are capped (Figure 2B).

Nonsense-containing mRNAs have been reported to undergo deadenylation, decapping, 5′-exonucleolytic decay, 3′-exonucleolytic decay and endonucleolytic decay [2,22]. The rules by which any given mRNA selected for a particular degradation pathway have not been determined. Results presented here confirm that NMD is responsible for the decay of full-length PTC-hβG mRNA and identify the 5′-truncated RNAs as decay intermediates. Three different experiments demonstrated that SMG6 is the endonuclease that generates these intermediates. The first (Figure 5) looked at the impact of SMG6 knockdown alone, and the most straightforward interpretation of these data identifies SMG6 as the enzyme that cleaves PTC-hβG mRNA to generate the stable Δ169 intermediate. However, SMG6 has been reported to function in the dephosphorylation of Upf1, and its knock down in HeLa cells was reported to inhibit NMD secondary to the accumulation of phospho-Upf1 [21]. To address this we performed a complementation experiment in which we knocked down SMG6 and expressed siRNA-resistant forms of SMG6 or SMG6 with the 3 aspartic acid residues in the PIN domain catalytic core changed to asparagine (SMG6-m4). Results in Figure 6 show that expression of siRNA resistant SMG6 reversed the effect of SMG6 knockdown on the generation of Δ169 RNA from PTC-hβG mRNA, but expression of SMG6-m4 did not, thus supporting the identification of SMG6 as the responsible endonuclease.

Results in Figure 6 also brought to light a difference between our results and those in [21] regarding the impact of overexpressing inactive forms of SMG6. In that study NMD was inhibited by overexpression of SMG6 with a single aspartate-to-alanine PIN
domain mutation (D1251A). We saw no evidence for inhibition of NMD with overexpression of SMG6-m4 in K562 cells. In Figure 6 each form of SMG6 was clearly overexpressed (Figure 6A); however, as evident in the first 3 datasets in Figure 6B neither of these had any impact on full-length or Δ169 RNA. We do not know why these results differ from those in [21], but this may be due to differences in the form of inactive SMG6 used in our study, or to the fact that our experiments examined hβG mRNA in its native cell context.

It remained formally possible that SMG6 knockdown still resulted in the accumulation of phospho-Upf1 and expression of SMG6-m4 just helped to keep Upf1 in the phosphorylated state. This was ruled out by the results of Figure 7, where we saw no evidence for changes in the phosphorylation state of Upf1 regardless of whether SMG6 was knocked down or an inactive form of SMG6 was expressed in these cells. Together with the preceding experiments these data provide proof that metastable decay intermediates are generated by SMG6 cleavage of PTC-hβG mRNA.

Finally, K562 cells do not natively express β-globin mRNA but they do express detectable levels of δ-globin mRNA [23]. In the course of this work we identified a form of δ-globin mRNA whose 5' end matched that of Δ169 RNA (Figure S1). This raised a question that was not answered by this study; to wit, why are the same 5'-truncated RNAs also detectable in cells expressing WT-hβG mRNA? These were seen in [7] and [8], and again here by both S1 nuclease protection and by the modified MBRACE assay (Figure 2). Although the endonuclease responsible for generating these fragments has yet to be identified, the fact that both WT- and PTC-hβG mRNA are cleaved at the same sites suggests features of the mRNP play a major role in determining the location of endonucleolytic cleavage sites.

Supporting Information

Figure S1 Sequence alignment of human beta- and delta-globin mRNA and locations of MBRACE primers. Human beta-globin (HBB) and delta-globin (HBD) mRNAs are shown aligned. The yellow highlights identify the locations of primers used to quantify full-length mRNA and the green highlights identify the locations of primers used to quantify Δ169 RNA. Note that the sequence at the 5' Δ169 primer binding site is identical for beta- and delta-globin mRNA. (TIF)

Figure S2 Impact of changes in PMR1 on full-length and Δ169 hβG mRNA. A and B. Tet-inducible K562 cells electroporated with inducible WT- and PTC-hβG genes were treated with DMSO (vehicle), PP3 (an inactive analog of PP2), or PP2 c-Src inhibitor to inactivate PMR1 targeting to polysomes [24,25]. Cytoplasmic RNA recovered 6 hr after induction was assayed by modified MBRACE for changes in full-length (A) and Δ169 RNA (B). C and D. Tet-inducible K562 cells were electroporated with WT- and PTC-hβG expressing plasmids together with plasmids expressing GFP, active hPMR1 or inactive hPMR1. Cytoplasmic RNA recovered 6 hr after induction was analyzed by modified MBRACE for changes in full-length (C) and Δ169 RNA (D). The results represent the mean ± standard deviation of triplicate cultures, *indicates p<0.05 by two-tailed Student’s t-test. (TIF)

Table S1 Oligonucleotides and primers. (XLSX)

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Author Contributions

Conceived and designed the experiments: RM JAD DRS. Performed the experiments: RM JAD DRS. Analyzed the data: RM JAD DRS. Contributed reagents/materials/analysis tools: RM JAD DRS. Wrote the paper: RM JAD DRS. Prepared the figures: JAD DRS.

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Table S1 Oligonucleotides and primers. (XLSX)

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