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A chemiluminescence-based reporter system to monitor nonsense-mediated mRNA decay

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Abstract

Nonsense-mediated mRNA decay (NMD) is a surveillance pathway that mediates rapid degradation of transcripts bearing premature translation termination codons (PTCs) and thereby limits the expression of unproductively processed mRNAs and the synthesis of C-terminally truncated peptides. Both its importance as a means to control gene expression and in the context of genetic and acquired human diseases call for an exploration of the mammalian NMD pathway using chemical biology approaches. Here, we describe a novel cell-based chemiluminescence reporter system that recapitulates the hallmark features of mammalian NMD. The assay is characterized by its high sensitivity, robustness, and its potential for automated handling. Limiting NMD efficiency by RNAi-mediated depletion of the essential NMD factor UPF1 markedly and specifically increased the NMD reporter mRNA level and resulted in a proportional increase in protein expression reflected by *Renilla* luminescence. The PI 3-kinase inhibitor wortmannin has previously been found to up-modulate PTC-containing transcripts by inhibiting the UPF1 kinase SMG1. Wortmannin treatment enhanced NMD reporter expression in our system in a dose-dependent way, illustrating its utility for small molecule screening.

Keywords: Nonsense-mediated mRNA decay; Reporter assay; UPF1 phosphorylation; Chemical biology; Luciferase; Wortmannin

In eukaryotes, many processing steps during gene expression are monitored by surveillance mechanisms that ensure their accurate execution or remove faulty products. One of these processes is the nonsense-mediated mRNA decay (NMD) pathway, which mediates rapid degradation of transcripts bearing premature translation termination codons (PTCs) [1,2].

Initially, NMD was solely considered to be a quality control mechanism that eliminates PTC-containing messages coding for non-functional or potentially harmful polypeptides. However, recently it has been increasingly appreciated that NMD is an important post-transcriptional mechanism to regulate physiological gene expression. NMD has thus been implicated in regulating and balancing the expression of alternatively spliced mRNA isoforms [3,4]. Furthermore, the abundance of NMD target transcripts varies considerably [5–7]. NMD efficiency has been reported to be tissue-specific [8], and to differ between individuals [9].

Both, the function of NMD as a quality control mechanism and as a mechanism for post-transcriptional control of gene expression is of medical relevance. The quality control function is clearly beneficial in many hereditary disorders by preventing heterozygotes from the detrimental effects of dominant negative polypeptides [10]. In contrast, NMD has also been shown to eliminate potentially useful mRNAs as has been reported for cystic fibrosis or Duchenne muscular dystrophy (for reviews see [11,12]). For this reason, NMD has become a focus for the development of

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new therapeutic strategies for inherited genetic diseases that are caused by PTCs [13–15]. Therapeutic strategies have previously aimed to circumvent NMD and to (partially) repair gene function by nonsense suppression [13,14] or by altering splicing to bypass the PTC-mutated exon of the transcript [16,17]. Recently, it has become apparent that a modulation of NMD itself may carry significant therapeutic potential [15].

A second important area in the field of NMD research focuses on the regulation of NMD and on signaling pathways that may modulate NMD by external stimuli. Specifically, one of the key proteins of the NMD pathway, UPF1, is known to be a phosphoprotein that is phosphorylated by the kinase SMG1 [18,19] and dephosphorylated by a complex consisting of PP2A, SMG5, SMG6 and SMG7 [19]. SMG1 contains a conserved kinase domain that belongs to the family of phosphatidyl-inositol-3-kinase (PIK)-related kinases and its activity is wortmannin-sensitive [20]. Recent reports identified roles for the phosphorylation of Upf2p [21] and post-translational modifications of Y14 [22] for NMD in yeast and in humans, respectively. The identification of functionally important post-translational modifications of UPF1 and other NMD proteins underscores the potential importance of signaling pathways and, consequently, of potential intra- and extracellular stimuli in NMD.

Chemical biology approaches involving screening for small molecules that can modulate NMD will prove powerful to examine the role of signaling in NMD. It may also contribute to the identification of lead compounds that could be developed into NMD therapeutics. To this end, we developed a reliable, chemiluminescence-based NMD reporter assay in mammalian cells that can readily be adapted to a high throughput format. We show that this cell-based assay system reflects key characteristics of NMD in HeLa cells, such as its down-modulation by UPF1 depletion via RNAi or by wortmannin treatment. A GFP based reporter assay has been described recently by Paillusson et al. [23]. Yet, the relatively low sensitivity of GFP restricts its applicability for high throughput screening. This difficulty is overcome here by employing *Renilla* and firefly luciferase reporters thus significantly increasing the system's sensitivity.

Materials and methods

*Plasmids.*The *Renilla* sequence coding for amino acid (aa) 1–311 of *Renilla* luciferase and the firefly sequence coding for aa 1–551 of firefly luciferase were PCR amplified from pCREL [24] and inserted between the *Nhe*I and *Xho*I sites of pCI-neo (Promega) to yield pCI-*Renilla* and pCI-firefly, respectively. pCI-*Renilla*/β-globin was created by inserting the open reading frame of human β-globin into the *Xho*I/*Not*I sites of pCI-*Renilla* to yield a fusion of human β-globin with *Renilla* luciferase. The sequences of all oligonucleotides that were used for cloning are available upon request. Expression plasmids for β-globin wt and NS39 were described previously [25]. An elongated human β-globin gene (wt + 300 + e3) served as a control for transfection efficiency where indicated [26].

Cell culture, plasmid and siRNA transfection. HeLa cells were grown in DMEM supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were transiently transfected by calciumphosphate precipitation as described previously [25]. Unless stated otherwise, cells were transfected in six-well dishes. 2 μg β-globin reporter (pCI-*Renilla*/β-globin for luminometry and pCI-β-globin for Northern blotting) was co-transfected with 0.7 μg control plasmid (pCI-firefly for luminometry and β-globin wt + 300 + e3 for Northern blotting) and 0.2 μg GFP-expression vector for visual estimation of transfection efficiency. Twenty hours post-transfection, cells were washed twice with Tris-buffered saline (pH 7.4) and 2 ml DMEM was added to each well. 24 h later, cells were harvested for analysis of RNA- and protein-expression. For dose dependency studies of wortmannin with pCI-*Renilla*/β-globin and pCI-firefly constructs, cells were co-transfected in 10 cm dishes with 10 μg pCI-*Renilla*/β-globin (wt or NS39, respectively), 3.3 μg of the control (pCI-firefly) and 1 μg of a GFP-expression vector. Sixteen hours post-transfection, cells were trypsinized, seeded in 96-well plates at a density of 2.3×10^4 cells/well, and incubated for 8 h to allow the cells to attach. Inhibitor treatment was then performed for 16 h. In 96-well plates, the vehicle DMSO concentration was 1% (v/v) and in 6-well plate experiments 0.5% (v/v).

Transient transfection of siRNAs was carried out using Oligofectamine reagent (Invitrogen) in Opti-MEM I reduced medium (Invitrogen) without serum and antibiotics. HeLa cells were seeded at a density of 1.4×10^5 cells per well in six-well plates. After overnight incubation, the cell culture medium was replaced with 800 μl Opti-MEM, and siRNA transfection was performed according to the manufacturer's recommendations using 10 μl siRNAs (20 μM stock) and 3 μl Oligofectamine reagent. After 4 h, the medium was supplemented with 1 ml of DMEM containing 20% FCS. 20 h later, the HeLa cells were transfected with plasmid DNA using calciumphosphate precipitation as described earlier.

Luminometry. Cells were lysed with Passive Lysis Buffer (Promega) and luminescence was measured in the Centro LB 960 luminometer (Berthold Technologies, Germany) with the Dual-Luciferase Reporter Assay System (Promega). Thirty microliters Luciferase Assay Substrate and Stop& Glo Buffer (Promega) were automatically injected at medium speed. The integration time was 1 s after a 4 s delay time. *Renilla* signals were normalized to the firefly control.

RNA analysis. Total cytoplasmic RNA was isolated from the supernatant of homogenized cells as previously described [27]. For the siRNA experiments, a slightly modified lysis buffer (10 mM Tris–Cl (pH 7.5), 8 mM $MgCl_2$, 10 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.1% sodium-deoxycholate, and 5 mM vanadyl–ribosyl-complex) was used. When subsequent protein analysis was required, the protease-inhibitors *complete* (Roche Applied Science) and PMSF were included in the lysis buffer. Northern blot analysis and ribonuclease protection assays (RPA) were performed with 2 μg of total cytoplasmic RNA according to standard protocols.

For the RPA, cytoplasmic RNA was analyzed by using excess of complementary RNA β-globin exon 3 and firefly probes. Hybridization was carried out at 50 °C overnight, ribonuclease treatment was done for 45 min at 30 °C, and the protected fragments were analyzed on a 6% denaturing polyacrylamide gel.

Signal quantification. Radioactive signals were quantified by phosphoimaging in a FLA-3000 fluorescent image analyzer (Raytest, Fujifilm). The indicated expression levels were calculated after correction for transfection efficiency. Mean values and standard deviations (SD) of all experiments shown were calculated from at least three independent experiments.

Quantitative real-time PCR (LightCycler). Real-time PCR (LightCycler, FastStart DNA Master SYBR Green Kit; Roche Diagnostics) was used to quantify mRNA levels. Two micrograms of total cytoplasmic RNA was reverse transcribed in the presence of 0.5 μg oligo$(dT)_{20}$ primer with 200 U RevertAid™ H Minus M-MuLV Reverse Transcriptase according to the manufacturer's protocol (Fermentas). SC35 mRNA was measured using 1 μM forward primer gtgtccaagagggaatccaa and 1 μM reverse primer ctacacaactgcgccttttc. RPL3 mRNA was measured using the primers described earlier [4]. GAPDH mRNA was used for normalization.

Protein analysis. Immunoblot analysis was performed using 10–30 μg of cytoplasmic extracts for 10% SDS–polyacrylamide gel electrophoresis. Subsequently, proteins were transferred to PVDF-membranes using a semi-dry electroblotting system. Membranes were blocked with 5%

non-fat skimmed milk in TBS–Tween (0.1%). The anti-UPF1 rabbit and anti-tubulin mouse antibodies were used at a dilution of 1:2000 and 1:10,000, respectively. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies (Sigma) were used as secondary antibodies at a dilution of 1:5000 and 1:10,000, respectively. Blots were developed with ECL Western Blotting Detection Reagent (Amersham–Pharmacia Biotech).

Results and discussion

The NMD reporter system described here is based on nonsense-mutated human β-globin (NS39) transcripts, which have been extensively studied and shown to cause a robust and highly reproducible reduction of mRNA abundance via NMD [25,28,29]. *Renilla* luminescence was used as a read-out for the steady-state mRNA levels of the NMD reporter. Specifically, the human β-globin gene with or without a nonsense mutation at position 39 was fused to the 3′ end of the *Renilla* luciferase open reading frame. Co-transfected firefly luciferase served as a normalization control (Fig. 1 A). This dual luciferase assay system thus enables monitoring of the ratio between NS39 and wildtype (wt) β-globin chemiluminescence. This approach

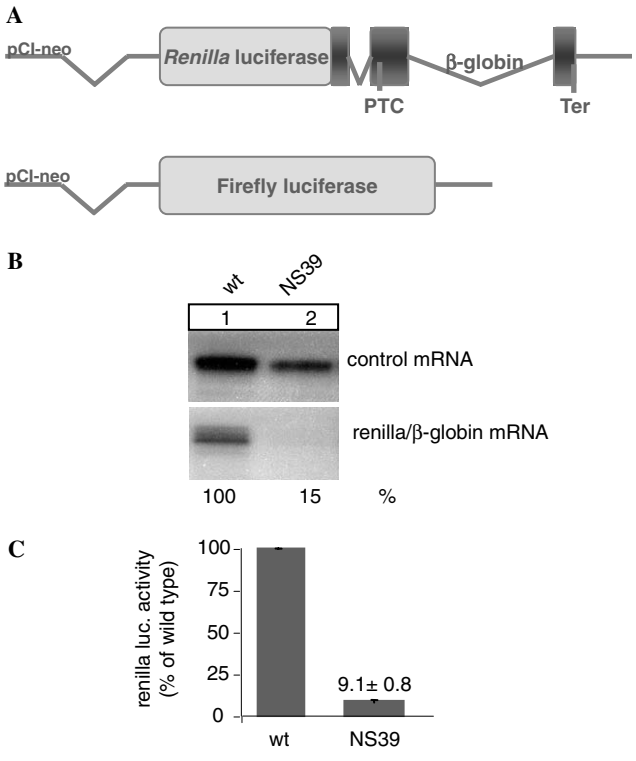

Fig. 1. The chemiluminescence-based NMD assay reflects nonsense-mutated mRNA abundance. (A) Schematic representation of the mammalian NMD reporter. The NMD reporter gene consisted of an in-frame *Renilla* luciferase/β-globin fusion construct with or without a nonsense mutation at codon 39 of the β-globin open reading frame (PTC for premature termination codon or Ter for wild type stop signal, respectively). The *Renilla* sequence is shown in light grey and β-globin sequences in dark grey (upper panel). Co-expressed firefly luciferase activity was used to normalize the level of *Renilla* luciferase activity (lower panel). The mRNA and protein expression levels of the reporter were measured by ribonuclease protection assay (B) and by luminometry (C), respectively.

conceptually overcomes the disadvantages of traditional or recently described co-reporters (e.g., CAT, β-Gal or GFP [23]) by integrating the assays of two luciferase reporters having compatible chemistries, handling conditions, sensitivities, linear ranges, and instrumentation requirements.

A reliable luminescence-based NMD reporter system requires that the reporter gene read-out is proportional to its mRNA levels. The expression of the NMD reporter in transiently transfected HeLa cells was thus measured at the level of protein expression by luminometry and compared with *Renilla* luciferase mRNA levels by ribonuclease protection assay. The ratio between NS39 and wildtype expression was similarly low (0.09 and 0.15, respectively) at the protein and mRNA level (Fig. 1B and C). These data demonstrate that (a) the hybrid *Renilla*/β-globin reporter mRNA displays expression differences that are specifically caused by the presence of the NS39 PTC and (b) that *Renilla* luminescence readings suitably reflect these changes.

Next, the reporter system was validated by testing its ability to recapitulate well established molecular characteristics of NMD. The depletion of UPF1 by RNA interference significantly down-modulates NMD efficiency [26,30]. Thus, we assessed the effect of siRNA-mediated UPF1 depletion on both the chemiluminescence β-globin reporter system and on β-globin mRNA levels. UPF1 was efficiently depleted to <10% of the level in cells that were transfected with β-gal control siRNA (Fig. 2C), which resulted in the down-modulation of NMD efficiency by approximately 4.3-fold (Fig. 2A). Reflecting these mRNA changes, the luminescence generated by the NS39 reporter increased 5.6-fold from 7% of wildtype in β-gal control siRNA-treated cells to 39% in UPF1-depleted cells (Fig. 2B). The luminescence based reporter thus reliably reflects the up-modulation of NS39-mutated mRNA levels in UPF1-depleted cells.

Wortmannin treatment down-modulates NMD efficiency by interfering with UPF1 phosphorylation at S1078 and S1096 [31] by the PIK-related kinase SMG1 [18,19]. We used this pharmacological effect of wortmannin as a positive control to assess the suitability of the NMD reporter system for small molecule screening.

HeLa cells transfected with the NMD reporters were treated for 16 h with 5 μM wortmannin and subjected to NMD analysis by northern blotting and luminometry. The treatment of transfected cells with the drug vehicle DMSO served as a negative control.

Wortmannin treatment resulted in an increase of the NS39 transcript from 8% to 26% of wildtype levels, corresponding to a ~3.3-fold down-modulation of NMD efficiency (Fig. 3A). In accordance with these mRNA measurements, luminescence increased from 10% to 46% of wildtype levels (Fig. 3B).

Next, we adapted the system to a 96-well format that is suitable for automated handling in large scale screens using plate readers. Cells transfected with the NS39 and wildtype reporter constructs were seeded in 96-well plates and wort-

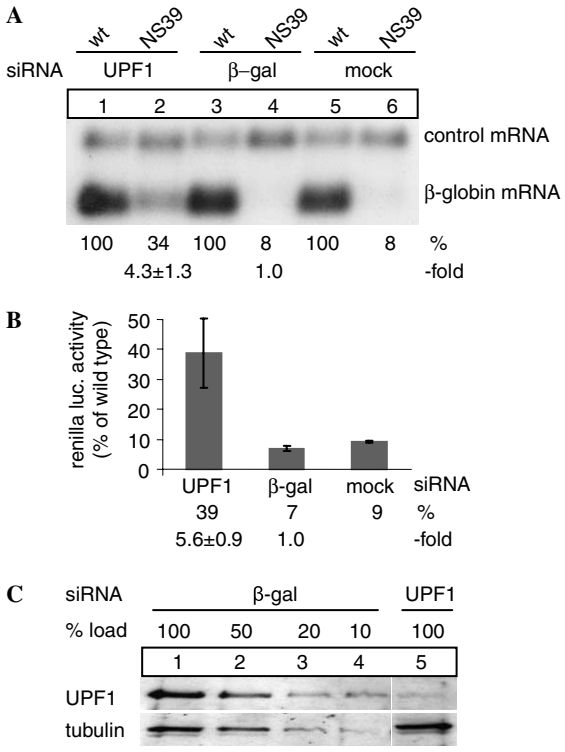

Fig. 2. The reporter assay recapitulates the effect of UPF1 depletion on NMD. NMD was limited by RNAi-mediated depletion of the essential NMD factor UPF1. HeLa cells were transfected with siRNA directed against UPF1 or β-galactosidase as a negative control, and subsequently with the indicated reporter plasmids and analyzed by Northern blotting (A) or luminometry (B). RNAi-mediated depletion of UPF1 increased the NMD reporter ~4–5-fold both on the mRNA (A) and protein level (B). Representative results are shown from at least three independent experiments. Fold up-modulation signifies the change in the ratio of NS39 to wt of UPF1 depleted cells with reference to the ratio of NS39 to wt of cells treated with siRNA directed against β-galactosidase. (C) Immunoblot analysis of protein lysates from HeLa cells transfected with siRNAs against β-galactosidase (lanes 1–4) or UPF1. Dilutions corresponding to 50%, 20%, or 10% (lanes 2–4) of the initial protein amount (lane 1) from β-gal-siRNA-transfected cells were loaded to assess the efficiency of the UPF1 depletion. Reprobing with a tubulin-specific antibody was performed to control for loading. UPF1 was reduced >10-fold as determined by comparison with the dilution series of negative control siRNA.

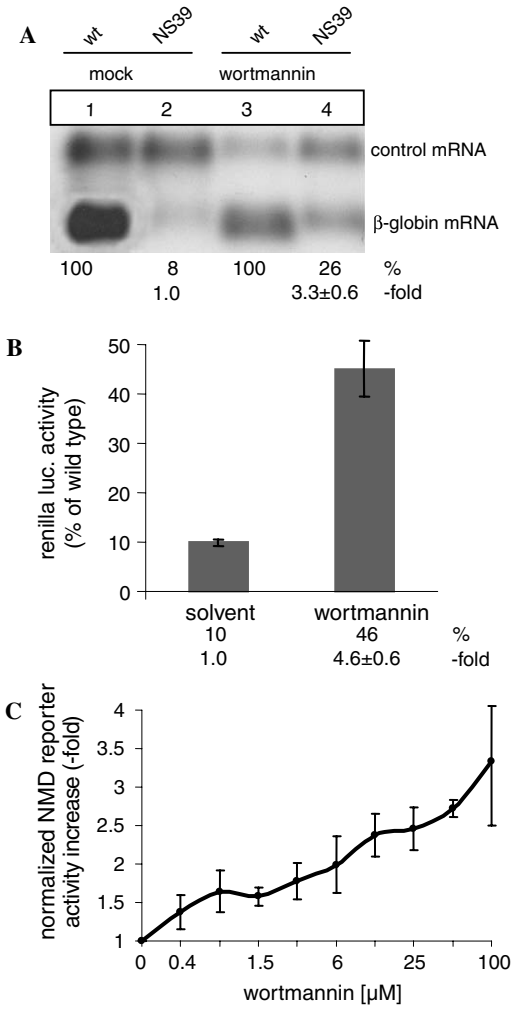

Fig. 3. The reporter assay recapitulates the effect of wortmannin on NMD. (A) HeLa cells were co-transfected with plasmids for the control and the NMD reporter. Cells were treated with the inhibitor wortmannin or DMSO (mock). Cytoplasmic mRNA was analyzed by northern blotting. (B) *Renilla* luminescence intensity was normalized to the firefly luciferase control and the ratio of NS39 to wild type is depicted. At an inhibitor concentration of 5 μM, the nonsense-mutated mRNA increased on average 3.3-fold (A) and the protein activity 4.6-fold (B). The percentage values refer to the mean of four independent experiments after normalization for transfection efficiency. Fold up-modulation signifies the change in the ratio of NS39 to wt of wortmannin-treated cells with reference to the ratio of NS39 to wt of cells treated with the drug vehicle DMSO. (C) The assay was adapted to a 96-well format and a wortmannin dose response curve (0.4–100 μM final concentration) was generated. Transiently transfected HeLa cells were treated with wortmannin, lysed with Passive Lysis buffer and the signal was recorded with a luminometer. Wortmannin treatment of transfected cells increased the luminescent NMD reporter in a dose-dependent manner up to ~4-fold in the 96-well format. The average of five independent experiments with standard deviations is depicted as fold up-modulation relative to DMSO-treated samples.

mannin dose response curves were generated from 0.4 to 100 μM final inhibitor concentration, normalized for non-specific or toxic effects of the inhibitor using the β-globin wildtype reporter. These analyses reproduced the dose dependence of wortmannin inhibition of NMD (Fig. 3C), re-affirming the reliability of the chemiluminescence-based assay system.

Finally, we extended the findings regarding the small molecule wortmannin on NMD by analyzing the up-modulation of the endogenous NMD substrates SC35 (SFRS2) and RPL3 as a secondary specificity assay. The splicing factor SC35 controls its own expression by promoting splicing events that destabilize its mRNA [3] (Fig. 4A). Two of seven alternative splicing forms are preferentially produced in response to SC35 overexpression. These splice forms contain an intron in their 3′ UTR at an NMD-competent position and are validated NMD targets. Inhibition of UPF1 phosphorylation by 5 μM wortmannin resulted in a ~4-fold increase of these SC35 transcripts as analyzed by real-time PCR (Fig. 4B). A second endogenous NMD substrate, RPL3 mRNA, was analyzed after wortmannin

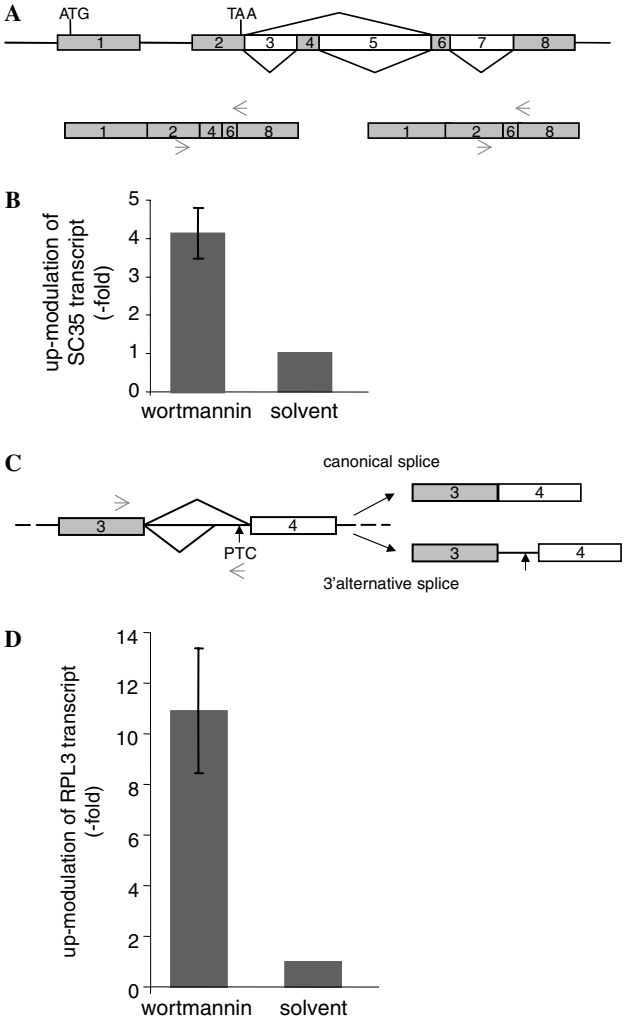

Fig. 4. Up-modulation of cellular NMD targets by wortmannin treatment can serve as a secondary specificity control. (A) Schematic representation of the SC35 gene. The intron between exon 1 and 2 is constitutively spliced. Exons 3–8 display alternative splicing patterns. The two splice variants that are NMD targets and the hybridization position of the primers used in PCR experiments are depicted. (C) Schematic representation of the alternative splicing of the human RPL3 gene. The intron 3 and flanking exons 3 and 4 are depicted. HeLa cells were treated with 5 μM wortmannin or DMSO (mock). The abundance of the cellular NMD-regulated transcripts SC35 (B) and RPL3 (D) was determined by quantitative RT-PCR. Relative expression levels were normalized to GAPDH mRNA levels. Fold increase relates to DMSO-treated cells. The average of at least three independent experiments with standard deviations is depicted.

treatment of HeLa cells. For this transcript, inclusion of the 3′-most 180 nt of intron 3 by alternative splicing leads to the generation of an in-frame translation termination codon subjecting the aberrant mRNA to the wortmannin-sensitive NMD pathway [4] (Figs. 4C and D). These data suggest that both SC35 and RPL3 are suitable secondary specificity controls for potential NMD-interfering compounds identified in small molecule screens.

A recent report describes monitoring of NMD with a GFP-based reporter system [23] that has been proposed to be adaptable for large scale screenings. However, as mentioned by the authors, its application to reliably monitor NMD is limited by high background fluorescence of whole cell lysates. Here, we present an NMD reporter system that overcomes the problem of low GFP signal intensity that is emitted by the nonsense reporter by substituting the fluorescent reporter by more sensitive luminescence based reporters. In our system, untransfected cells generate virtually no luminescence background signal. Therefore, both the down- and the up-modulation of the luminescence NMD reporter by small chemical compounds that potentially interfere with the NMD pathway can be reliably measured. Furthermore, the design of our reporter system enables to specifically control for non-specific effects of small chemical compounds, because NMD independent effects on the NS39 reporter expression is filtered by normalizing to its wild type counterpart.

Conclusion

We devised a sensitive and easy-to-monitor cell-based chemiluminescence reporter assay for mammalian NMD with superior characteristics compared to state-of-the art NMD reporter assays. We show that the intensity of the *Renilla* luciferase signal proportionally reflects the reporter mRNA levels. We further validated this system by UPF1 depletion and wortmannin treatment, which both lead to the expected specific increase in reporter activity. The cell-based assay was adapted to an automatable 96-well format suitable for large scale screens of small molecule libraries as well as large scale RNAi screens. Secondary assays to validate potential hits are available making this system very useful for identifying and studying signaling pathways affecting NMD.

Acknowledgments

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