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# A luciferase reporter assay to investigate the differential selenium-dependent stability of selenoprotein mRNAs

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**Abstract**

The mechanisms regulating the differential selenium (Se)-dependent stability of selenoprotein mRNAs are partially characterized. To further study the Se-dependent regulation of selenoproteins, we developed a novel chemiluminescent reporter to monitor the steady-state mRNA level of an artificial selenoprotein. Our reporter is a fusion of the *Renilla* luciferase gene and of the β-globin gene, but contains features required for incorporation of selenocysteine (SEC), namely, a UGA-SEC codon and a 3′ untranslated region RNA stem loop called a SEC incorporation sequence (SECIS). At various levels of Se, the activity of reporters containing *GPX1* or *GPX4* SECIS elements is proportional to the steady-state mRNA level of the reporter construct and reflects the level of the corresponding endogenous mRNA. In a reporter containing a UGA codon and a functional *GPX1* SECIS, Se-dependent nonsense-mediated decay (NMD) occurred in the cytoplasm, as opposed to the more typical nuclear location. To validate the reporter system, we used genetic and pharmacologic approaches to inhibit or promote NMD. Modulation of UPF1 by siRNA, overexpression, or by inhibition of SMG1 altered NMD in this system. Our reporter is derived from a Renilla luciferase reporter gene fused to an intron containing B-globin gene and is subject to degradation by NMD when a stop codon is inserted before the second intron.


*Keywords:* Selenium; Selenoprotein; Glutathione peroxidase; Nonsense-mediated decay; Reporter; Luciferase

## 1. Introduction

Selenoproteins are a small group of proteins that incorporate selenocysteine (SEC), a selenium (Se)-containing amino acid, into their primary structure [1]. The 25 known human selenoproteins include several with important antioxidant properties, including five glutathione peroxidases (GPXs), three thioredoxin reductases and selenoprotein P (a major plasma Se-delivery molecule with GPX-like activity). While the standard 20 amino acids all have codons of their own, SEC appears to be a late addition to the genetic code [2,3]. In this regard, SEC does not have a codon of its own, but rather must recode an in-frame UGA "stop" codon to allow incorporation of SEC. The recoding of the UGA "stop" codon is mediated by a unique stem-loop structure in the 3′ untranslated region (UTR) of the selenoprotein mRNA called a SEC insertion sequence or SECIS [4–6]. SECIS elements are necessary for the insertion of SEC at a UGA codon, but they are not sufficient [4]. A number of protein factors assemble on the SECIS to form an insertion complex [7–9]. Essential factors in the complex include a SECIS binding protein (SBP2), a specific elongation factor (EFSEC) and the $tRNA^{SEC}$ (encoded by *TRSP*). When this complex assembles on the SECIS, SEC is delivered to the ribosome and incorporated into the growing peptide chain when directed to do so by the presence of a UGA codon [7].

An interesting phenomenon first noted in rats and later confirmed in tissue culture experiments is that, under conditions of Se deficiency, the expression of *GPX1* mRNA is reduced [10,11]. In contrast to what is observed with the level of *GPX1* mRNA, the level of *GPX4* mRNA remains relatively stable under conditions of Se deficiency [12]. Like the *GPX1* mRNA, some other selenoprotein mRNAs including *SelH* and *SelW* are also regulated by Se deficiency, and a few mRNAs are resistant to Se-dependent degradation [13]. Based on their structure, some, but not all, selenoprotein mRNAs are predicted to be degraded by a process known as nonsense-mediated decay (NMD) [12,14]. NMD is a surveillance pathway used by the cell to detect and degrade aberrant mRNA transcripts containing premature termination codons (PTCs). In general, termination codons are usually situated within the last exon and typically lack a downstream exon–junction complex, a feature required for the initiation of NMD, and therefore do not generally trigger the degradation of the mRNA [15]. According to the classic rules, in order for an mRNA to be flagged for destruction by NMD, it must have a PTC followed by an appropriately spaced intron, features present in the pre-mRNAs of both *GPX1* and *GPX4* [16]. One hypothesis is that the differential susceptibility of selenoprotein mRNAs to Se-dependent NMD is a regulatory mechanism by which a limited supply of Se may

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be preferentially used to support the production of more essential selenoproteins [13,17].

The mechanisms that allow the *GPX4* mRNA to resist being degraded by NMD are partially understood. A distinguishing feature between the *GPX1* and *GPX4* selenoprotein mRNAs is that they have distinct SECIS forms. SECIS elements come in two forms [5]. Form 1 (*GPX1*) SECIS elements have a 10–14-nucleotide adenosine loop. Form 2 (*GPX4*) SECIS elements have an adenosine bulge and an upper stem loop. Most selenoproteins have just one SEC codon and one SECIS element. It is possible that the efficiency of NMD is mediated in part by different proteins that assemble on the various SECIS forms, by the affinity of specific SECIS binding proteins for the SECIS or by the efficiency of SECIS-mediated SEC incorporation. All SECISs differ in sequence and structure; however, only some selenoprotein mRNAs are subjected to degradation under Se deficiency; in addition, some selenoprotein mRNAs are regulated by Se but do not follow the classical rules for NMD [13]. Other cell studies have shown that the GPX4 3′ UTR is as effective as the GPX1 3′ UTR in mediating the incorporation of SEC at UGA codons and that some of the difference in mRNA stability resides in the coding region [18]. Others hypothesize that $tRNA^{SEC}$ isoforms play a role in selenoprotein mRNA stability [19] or that additional stem-loop structures in the coding region might account for differences in stability [20]. Moreover, data from Müller et al. indicate that the stabilizing efficiency of an individual selenoprotein 3′ UTR does not necessarily correlate with the efficiency of read-through at the UGA codon, suggesting that Se-responsive elements may be located in both the translated and untranslated region of the gene [21]. Improved reporter systems will facilitate studies aimed at further characterizing mechanisms regulating the stability of selenoprotein mRNAs and, in the future, could be used to characterize mechanisms regulating the stability of additional members of the selenoprotein family.

Here we describe the development and validation of a chemiluminescence-based selenoprotein reporter system that is susceptible to Se-dependent NMD. To validate our reporter, we confirm that a *GPX1* SECIS dictates in the absence of optimal supplemental Se that a transcript containing both an intron and a UGA-SEC codon is susceptible to NMD. In the presence of optimal supplemental Se, a functional *GPX1* SECIS element inhibits the NMD of the artificial selenoprotein reporter. Our validated reporters are powerful tools that may be useful for rapidly characterizing mechanisms regulating selenoprotein mRNA stability and cytoplasmic NMD.

## 2. Methods and materials

### 2.1. Plasmids

A reporter for monitoring the NMD of an artificial selenoprotein mRNA was created by modifying a pCI-*Renilla*/β-globin NMD reporter plasmid developed by Dr. Andreas E. Kulozik [22]. The original reporter is a fusion of the *Renilla* luciferase open reading frame and the human β-globin gene, with or without a nonsense mutation at amino acid position 39 (NS39). The presence of an intron downstream of the PTC renders the NS39 mRNA susceptible to NMD. To allow monitoring of selenoprotein mRNA decay, the β-globin gene was made into an artificial selenoprotein by mutating the premature UAA termination codon located at position 39 to a UGA codon and by inserting a SECIS element into the 3′ UTR of the construct (Fig. 1A). To facilitate the insertion of a SECIS, site-directed mutagenesis was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). We sequentially introduced *Xba*I and *Bgl*II restriction sites into the proximal 3′ UTR region of the NS39 plasmid and then introduced a UGA codon at position 39. As the original plasmid contained an additional *Bgl*II restriction site upstream of the reporter, this site was removed by site-directed mutagenesis. The human *GPX1* SECIS element was amplified from human genomic DNA (Coriell sample ID# NA10861; Coriell Institute for Medical Research, Camden, NJ, USA) using polymerase chain reaction (PCR) primers containing *Xba*I and *Bgl*II restriction sites in their tails. Restriction-digested PCR products were ligated into the *Xba*I/*Bgl*II-digested and gel-purified reporter constructs. Control plasmids containing an ACC codon or true stop codon (UAA) were constructed from the parental plasmid using site-directed mutagenesis. Plasmids with mutant SECIS elements were generated by mutating the four-base SECIS core (UGAA) to CCCC or by deleting these four bases. The primers used are reported in Supplemental Table 1.

### 2.2. Cell culture and plasmid transfection

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Media Core, Lerner Research Institute) supplemented with 10% fetal calf serum (USA Scientific, Orlando, FL, USA), 100 U/ml penicillin and streptomycin. In six-well plates, cells were transiently transfected using Lipofectamine 200 (Invitrogen, CA, USA) as described in the manufacturer's protocol. Where appropriate, tissue culture medium was supplemented with 1 μM of selenomethionine (SeMet), a concentration of Se empirically determined to be optimal for the incorporation of SEC in a read-through reporter assay (Fig. S1A) and by Western blot (Fig. S1 B). We elected to use SeMet rather than an inorganic form of Se, as SeMet may be less toxic to cells [23] and has been used in human clinical trials [24].

### 2.3. Luminometry

Cells were lysed with Passive Lysis Buffer (Promega, Madison, WI, USA), and luminescence was measured in a luminometer (Victor $R^{3v}$, Perkin Elmer) using the Dual-Luciferase Reporter Assay System (Promega). *Renilla* luciferase signals were normalized to a cotransfected firefly luciferase control.

### 2.4. Protein analysis

Immunoblot analysis was performed using 60 μg of total cell extract electrophoresed on 12% sodium dodecyl sulfate–polyacrylamide gels. Subsequently, proteins were transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA) using a semidry electroblotting system. Membranes were blocked with 5% nonfat dry milk (Bio-Rad). Antibodies were used that recognized UPF1, GAPDH, β-actin (dilution, 1:1000; Santa Cruz Biotech Inc., Santa Cruz, CA, USA), GPX1 (dilution, 1:1000; Cell Signalling Technology Inc., Danvers, MA, USA) and GPX4 (dilution, 1:1000; Santa Cruz Biotech Inc.). Reactivity to each antibody was detected using 1:2000 dilution of horseradish-peroxidase-conjugated donkey anti-rabbit (Cell Signaling Technology Inc.) or 1:5000 dilution of anti-mouse antibodies and anti-goat (Santa Cruz Biotech). Reactivity of the secondary antibody was visualized by SuperSignal West Pico solution (Pierce, Thermo Scientific, Rockford, IL, USA).

### 2.5. Quantitative real-time PCR

Total RNA was purified from cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). One microgram of total RNA from transiently transfected cells was subjected to reverse transcription using an i-script cDNA synthesis kit (Bio-Rad). PCR amplification was carried out using iQ-SYBR Green Supermix (Bio-Rad) and an i-Cycler (Bio-Rad). Primers were designed to amplify a 100- to 200-base-pair (bp) product. Before being used in real-time PCR, each primer pair was authenticated by standard reverse transcription-PCR (RT-PCR). The primers used are reported in Supplemental Table 2. For real-time PCR assays, all values were normalized to the ACTB housekeeping gene. The ΔCt method was used for relative quantification of transcripts. Endogenous *GPX1* (Hs 00829989_gh; Applied Biosystems, Carlsbad, CA, USA) and *GPX4* (Hs00157812_ml; Applied Biosystems) levels were determined using Taqman real-time PCR probes and master mix (Applied Biosystems). For relative quantification of the transcripts, we compared the ΔCt value normalized to the transcript level of GAPDH (Hs99999905_ml; Applied Biosystems).

### 2.6. Nuclear and cytoplasmic separation

Nuclear and cytoplasmic total RNA was isolated from cells grown in 10-cm plates. Cells were transiently transfected with the appropriate plasmid and incubated in the presence or absence of 1 μM SeMet. After 72 h of SeMet supplementation, the total RNA was extracted from both cytoplasmic and nuclear fractions using the Sure Prep Nuclear or Cytoplasmic RNA Purification Kit (Fisher BioReagents, USA). The total RNA was subjected to cDNA synthesis as described above.

### 2.7. LY294002 treatments

HeLa cells transfected with the NMD reporters were treated for 24 h, and then the medium was replaced by serum-free medium for another 24 h. The cells were preincubated with 20 μM of LY294002 for 2 h in serum-free medium, and then they were incubated for 24 h in serum-containing medium; thereafter, reporter activity was analyzed by luminometry. The treatment of transfected cells with the drug vehicle dimethyl sulfoxide served as a negative control.

### 2.8. UPF1 knockdown and overexpression

To knock down the expression of *UPF1*, HeLa cells were transiently transfected with a pool of four UPF1 shRNAs (Origene; TR308482) or a GFP-specific control shRNA plasmid (Origene). Twenty-four hours later, cells were subjected to another round of transfection with two plasmids, RenβG-ACC *GPX1* (wild-type control) or RenβG-TGA *GPX1* (UGA at NS 39 position), and a firefly luciferase control plasmid. Western blotting indicated that, relative to GFP-specific shRNA, UPF1 shRNA reduced the level of cellular UPF1 by 95%. *Renilla* luciferase activity was normalized to the firefly luciferase

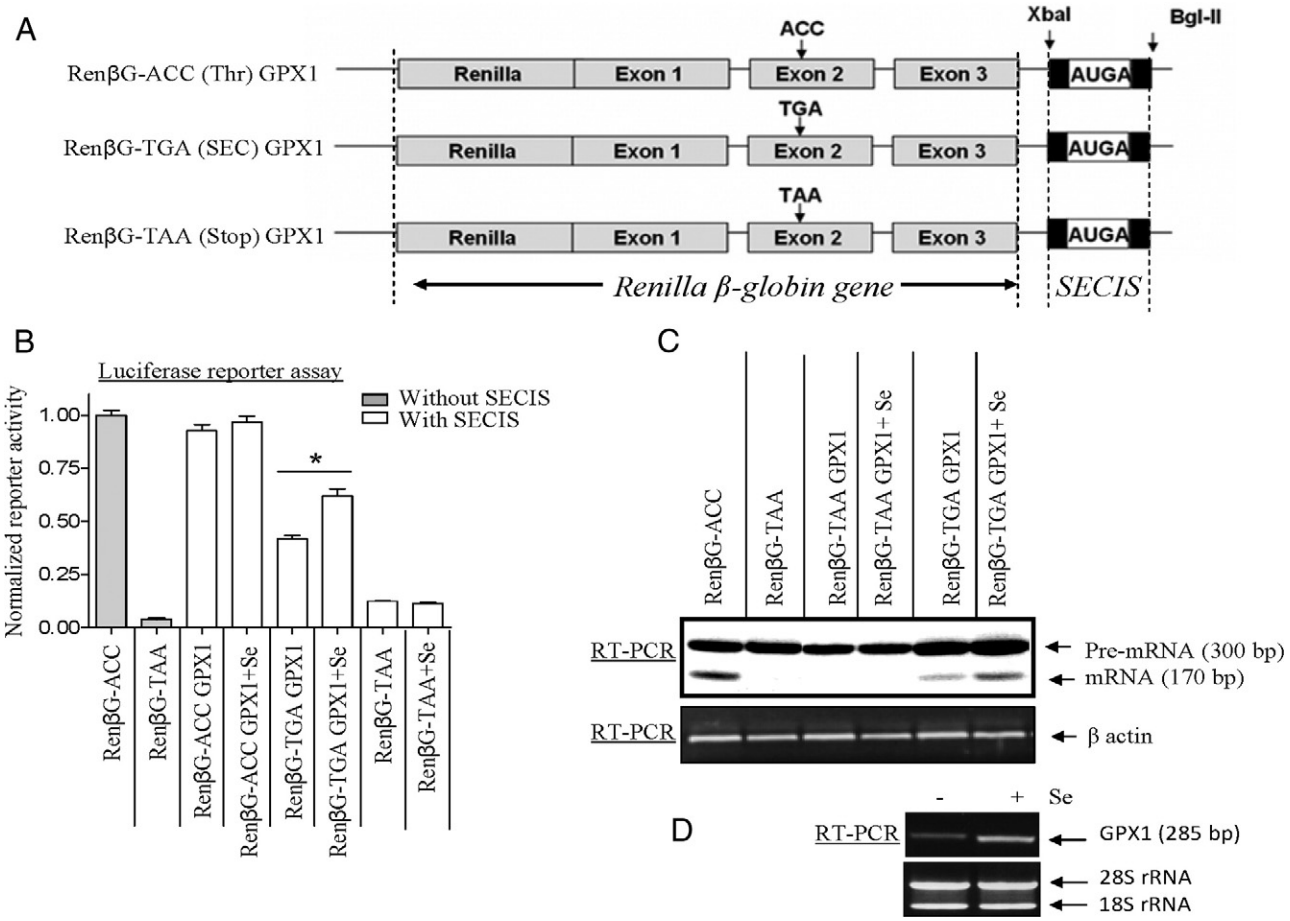


Fig. 1. RenβG reporter for monitoring the steady-state mRNA level of an artificial selenoprotein. (A) Key features of the reporter include a *Renilla* luciferase gene fused to a β-globin gene, the presence or absence of a nonsense codon at amino acid position 39 (NS39) of the β-globin gene and a *GPX1* SECIS element ligated into the construct's 3′ UTR. Exon sequences are indicated by boxes, and intron sequences are indicated by solid lines. Reporter constructs were created that contained a true stop codon (TAA), a SEC codon (TGA) or a threonine codon (ACC) at amino acid position 39. The *Xba*I and *Bgl*II restriction endonuclease sites used to incorporate the SECIS are shown. The solid black box indicates the location of the *GPX1* SECIS. The highly conserved SECIS core sequence is depicted as "AUGA." (B) In the absence of added SeMet (1 μM), a *GPX1* SECIS fails to protect the RenβG-TGA construct from NMD. A TAA nonsense mutation located at NS39 promotes the NMD of the mother vector. Se-dependent protection from NMD is achieved by mutating the TAA codon to TGA and by adding a 3′ UTR *GPX1* SECIS. Each data point is the mean of six wells. Error bars indicate 1 standard deviation. (*$P$<.05). (C) To validate the RenβG luciferase reporter, we used a single set of RT-PCR primers to simultaneously detect the processed reporter mRNA (170 bp) and the unspliced reporter pre-mRNA (300 bp). The 300-bp pre-mRNA band confirms robust and equal reporter transcript expression. The RT-PCR assay confirms that the RenβG-TGA *GPX1* reporter is degraded by NMD in the absence of supplemental SeMet (1 μM). (D) Endogenous *GPX1* mRNA levels were determined by RT-PCR. The upper panel shows the *GPX1* mRNA level, and lower panel shows the level of rRNA, the loading control. The figure is from one of three representative experiments.

control, and then the relative normalized luciferase activity of the TGA plasmid to the ACC plasmid was determined. To overexpress UPF1, HeLa cells were transiently transfected with UPF1 cDNA (Origene; SC118343) or with an empty vector control plasmid. Twenty-four hours later, cells were transfected with two plasmids, a RenβG-ACC *GPX1* or RenβG-TGA *GPX1*, and the firefly luciferase control reporter plasmid. The luciferase activity was measured after 48 h of retransfection and normalized as described above.

## 3. Results

### *3.1. A reporter for monitoring the steady-state mRNA level of an artificial selenoprotein*

To better understand the cellular mechanisms regulating the Se-dependent NMD of *GPX1* mRNA transcripts, we developed a luciferase-based reporter to monitor the steady-state mRNA levels of an artificial selenoprotein (Fig. 1A). Our reporter is derived from an NMD reporter developed in Dr. Andreas E. Kulozik's laboratory (Fig. S2 A) [22]. The parental reporter is a fusion of a *Renilla* luciferase gene and of an intron containing β-globin gene (Fig. S2 A); a TAA mutation at amino acid 39 (NS39) renders the construct susceptible to NMD. The steady-state level of the *Renilla* β-globin mRNA is directly proportional to the relative *Renilla* luciferase activity and the reporter mRNA level as shown (Fig. S2 B, C and D). The efficiency of NMD differed among cell lines; NMD was more efficient in HeLa cells than in either Hek293 or HepG2 cells (Fig. S2 E). Since the efficiency of NMD was greatest in HeLa cells, we used this cell line for our further studies.

To study the NMD of an artificial selenoprotein, a *GPX1* SECIS element was inserted into the 3′ UTR of the wild-type and the mutant NMD plasmid (Fig. 1A). In the wild-type reporter construct, the *GPX1* SECIS had no effect on either the relative luciferase activity or the steady-state mRNA level of the reporter (Fig. 1B and C) when compared to the original wild-type reporter (Fig. S2 A). Similarly, in the mutant NMD plasmid, which contains a TAA stop codon, the *GPX1* SECIS element did not influence the luciferase activity or mRNA level of the reporter (RenβG-TAA GPX1 in Fig. 1) compared to the original PTC-containing reporter (Fig. S2). Although the presence of a functional *GPX1* SECIS did not confer Se responsiveness to either the wild-type reporter or the reporter with a TAA stop codon (Fig. 1B and C), when the ACC at position 39 was mutated to a TGA codon, the presence of a functional *GPX1* SECIS reduced luciferase reporter

activity by 55%, but supplemental 1 μM Se resulted in a 48% increase in activity relative to this reporter in unsupplemented media (Fig. 1B), clearly showing that Se regulated expression of this reporter. In this regard, the Se responsiveness of our reporter recapitulates fairly accurately the Se responsiveness of the endogenous *GPX1* mRNA, although the change in the level of the endogenous mRNA appears to be more pronounced (Fig. 1D). When the *GPX1* SECIS was replaced by a *GPX4* SECIS in the reporter assay, less Se responsiveness was observed (Fig. 2A; 48% for GPX1, $P<.005$; 8% for GPX4, $P<.07$). The relative Se responsiveness of the *GPX1* (85%, $P<.005$) and *GPX4* (41%, $P<.05$) SECIS-containing reporters, as determined by quantitative (Q)-RT-PCR, closely mimicked the Se responsiveness of the endogenous *GPX1* (89%, $P<.001$) and *GPX4* (47%, $P<.05$) mRNA (Fig. 2C). Interestingly, our reporter data (Fig. 2A) suggest that the *GPX4* SECIS ($P<.005$), compared to the *GPX1* SECIS ($P<.05$), may have a greater ability to stabilize a transcript containing a UAA true stop codon.

We next explored the effect of decreasing the percentage of fetal bovine serum, which contains Se, on the activity of the *GPX1* and *GPX4* containing reporters (Fig. S3). In the absence of supplemental SeMet, but in the presence of 5% or 10% added fetal bovine serum, the activity of our *GPX4* reporter was comparable to that of our wild-type construct. In the absence of fetal bovine serum, the activity of our *GPX4* SECIS-containing reporter decreased by approximately 30% ($P<.01$), suggesting that although the *GPX4* SECIS-containing construct is stable under conditions of moderate Se insufficiency, more profound Se insufficiency promotes the degradation of the reporter.

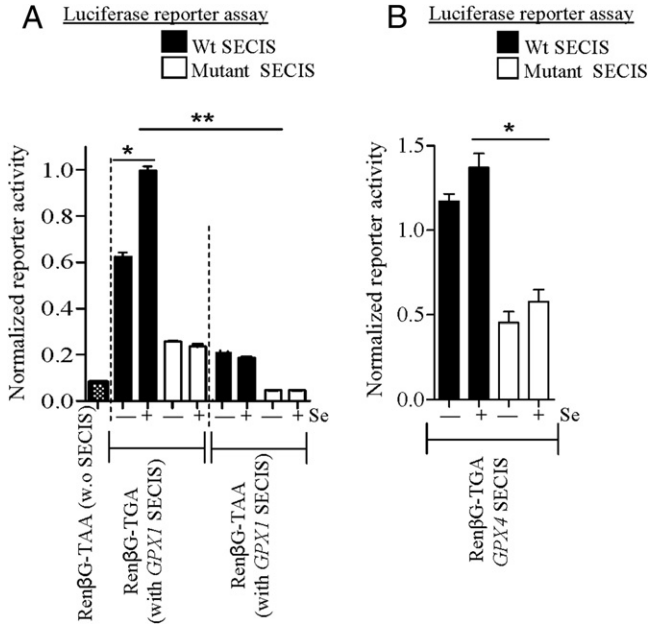


Fig. 3. Effect of SECIS core mutations on the efficiency of selenoprotein reporter NMD, as determined by luciferase reporter assay. Se=SeMet (1 μM). (A) HeLa cells were transiently transfected with RenβG-TGA *GPX1* or RenβG-TAA *GPX1* reporter constructs with or without a *GPX1* SECIS core mutation. Each data point is the mean of six wells. Error bars indicate 1 standard deviation (*$P<.005$, **$P<.001$). (B) Effect of mutation of *GPX4* SECIS core sequence on RenβG-TGA *GPX4* reporter NMD efficiency (*$P<.005$). Each data point is the mean of six wells. Error bars indicate 1 standard deviation.

### 3.2. Validation of the reporter assay by mutating the SECIS sequence

Mutating the highly conserved SECIS core from AUGA to AAAA (RenβG-TGA GPX1mut1) abrogated the Se-dependent protection against NMD (Fig. 3A). In the presence of Se, reporter plasmids containing a UGA codon and a mutated *GPX1* SECIS were efficiently degraded by NMD. In the absence of a functional SECIS, luciferase activities were decreased by 80% ($P<.001$). Therefore, the stability of the artificial reporter selenoprotein mRNA is dependent upon not only supplemental Se, but also the presence of a UGA codon and an intact SECIS. Mutating the SECIS core sequence (AUGA) of the *GPX4* SECIS-containing plasmids reduced the relative *Renilla* luciferase activity of the reporter plasmids by 62% ($P<.005$), suggesting that a functional SECIS element is also important for maintaining steady-state mRNA level of the *GPX4* reporter (Fig. 3B).

### 3.3. Validation of reporter assay by modulating SBP2 level

SBP2 is an essential transacting factor required for selenoprotein translation and is known to influence the stability of selenoprotein mRNAs [25]. Using a commercially available SBP2 cDNA (Origene;

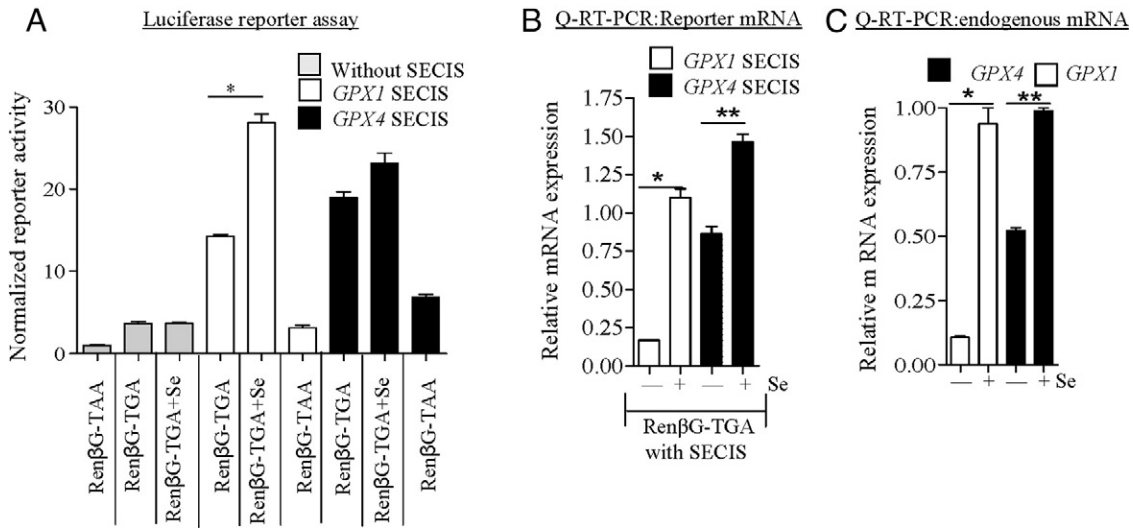


Fig. 2. The effect of GPX4 SECIS on reporter RenβG-TGA or RenβG-TAA NMD efficiency. (A) HeLa cells were transiently transfected with the RenβG reporter constructs with either a *GPX1* or *GPX4* SECIS in the presence of Se (SeMet 1 μM). Each data point is the average of three independent experiments (*$P<.001$). (B) Level of RenβG-TGA *GPX1* and *GPX4* SECIS-containing reporters, as determined by Q-RT-PCR. Each data point is the average of three independent experiments (*$P<.005$, **$P<.05$). (C) Level of endogenous *GPX1* and *GPX4* mRNA in the presence and absence of supplemental SeMet (1 μM), as determined by Q-RT-PCR. Each data point is the average of three independent experiments. (*$P<.001$, **$P<.05$). All error bars indicate 1 standard deviation.

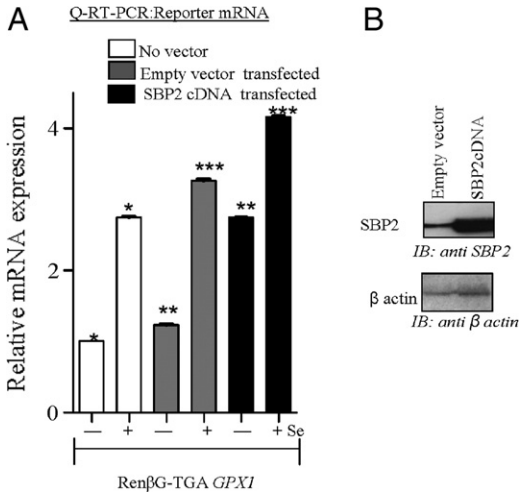


Fig. 4. SBP2 protects the RenβG-TGA *GPX1* SECIS-containing reporter from NMD. SBP2 is an essential transacting factor required for SEC incorporation. To determine whether overexpressing SBP2 would stabilize an NMD reporter with a *GPX1* SECIS, HeLa cells were transiently transfected with either empty vector or a cDNA for SBP2. Se=SeMet (1 μM). (A) After transfection with the RenβG-TGA *GPX1* reporter, the cells were incubated for 72 h either with or without SeMet (1 μM). SBP2 and RenβG-TGA *GPX1* mRNA levels were quantified by Q-RT-PCR analysis. The data were normalized with β-actin. Each data point is the average of three independent experiments (*$P$<.05, **$P$<.05, ***$P$<.05). (B) SBP2 protein levels were measured by Western blot. The figure is from one of three independent experiments.

SC112316), we explored whether overexpression of SBP2 in HeLa cells would influence the activity of our reporter construct (RenβG-TGA *GPX1*). At 72 h, total mRNA was isolated and subjected to RT-PCR. The level of *SBP2* and the *GPX1* reporter mRNA was then measured by Q-RT-PCR assay. Overexpression of SBP2 enhanced the relative mRNA level of the reporter (Fig. 4A) both in the presence (22%, $P$<.05) and in the absence (54%, $P$<.05) of supplemental Se. These data confirm that SBP2 may play an important role in protecting selenoprotein mRNAs from NMD. However, in contrast to what was observed with the RenβG-TGA *GPX1* mRNA, the activity and mRNA level of the wild-type RenβG-ACC *GPX1* SECIS did not show any difference after over-expression of SBP2 (data not shown).

### 3.4. UPF1 modulates reporter NMD

To further validate the reporter system, we monitored luciferase activity in cells over- or underexpressing the NMD factor UPF1. shRNA mediated down-regulation of UPF1-enhanced RenβG-TGA *GPX1* reporter activity by 45% (Fig. 5A), consistent with reports that UPF1 is required for NMD [26,27]. We confirmed UPF1 down-regulation by Q-RT-PCR (94%, $P$<.001) and Western blot (Fig. 5B and D). UPF1 down-regulation also up-regulates (94%, $P$<.01) the endogenous *GPX1* mRNA level (Fig. 5C), supporting previous observations that *GPX1* mRNA is subjected to NMD in the absence of supplemental Se. Next, we overexpressed UPF1 in HeLa cells using a UPF1 cDNA. We confirmed UPF1 overexpression by Q-RT-PCR (97%, $P$<.001) and Western blot (Fig. 5F and H). In cells overexpressing UPF1, normalized RenβG-TGA

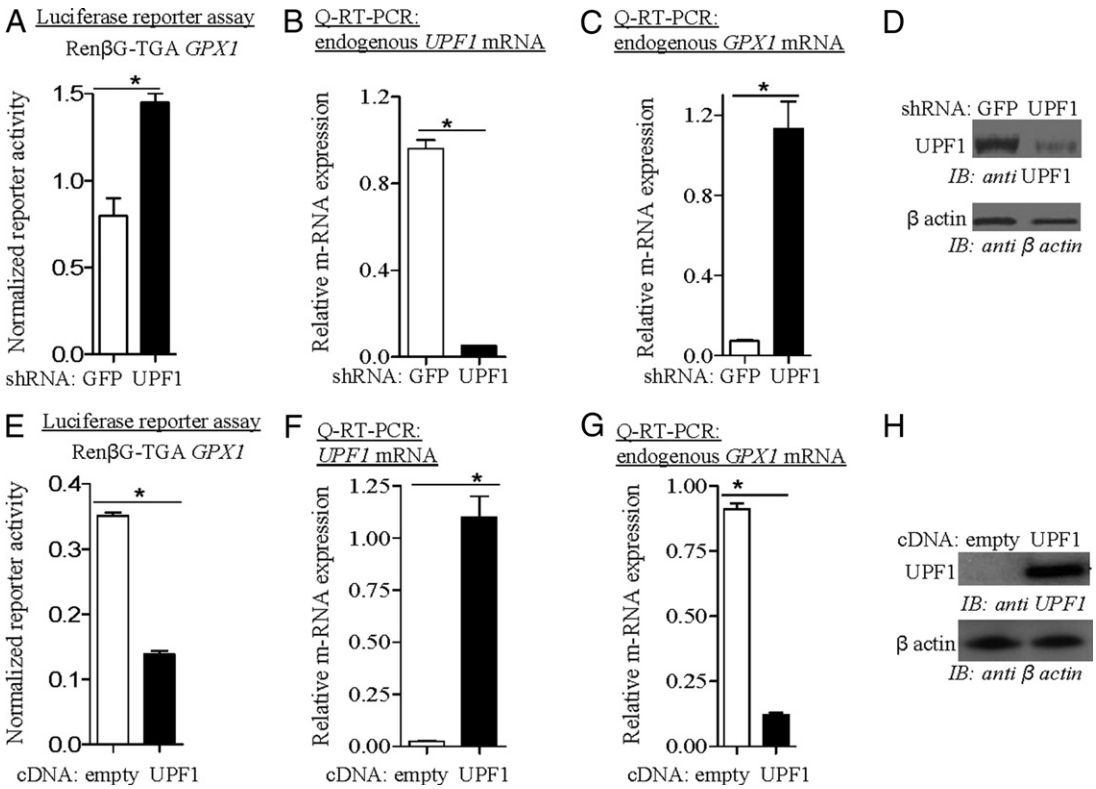


Fig. 5. *UPF1* modulates the Se-dependent NMD of the RenβG-TGA *GPX1* reporter mRNA. (A) Reporter activity following shRNA-mediated knockdown of *UPF1*. Each data point is the average of six biological replicates (*$P$<.05). (B) Confirmation of shRNA-mediated knockdown of UPF1 by Q-RT-PCR measurement of UPF1 mRNA (*$P$<.001). (C) Knockdown of UPF1 enhances endogenous *GPX1* mRNA expression, as determined by Q-RT-PCR. Each data point is the average of three independent experiments (*$P$<.001). (D) Western blot confirmation of UPF1 knockdown. (E) Reporter activity following transient transfection with empty vector or a plasmid for *UPF1* cDNA. Each data point is the average of three independent experiments (*$P$<.005). (F) Confirmation of cDNA-mediated overexpression of *UPF1* by Q-RT-PCR. Each data point is the average of three independent experiments (*$P$<.001). (G) Overexpression of UPF1 decreases the expression of endogenous *GPX1* mRNA, as determined by Q-RT-PCR. Each data point is the average of three independent experiments (*$P$<.001). (H) Western blot confirmation of UPF1 overexpression.

*GPX1* reporter activity decreased approximately 50% (Fig. 5E, $P<.005$). Up-regulating UPF1 in Se-insufficient cells also decreased endogenous *GPX1* mRNA levels by approximately 86% (Fig. 5G, $P<.001$), indicating that *GPX1* mRNA is subjected to NMD.

### 3.5. Inhibiting SMG1 blocks reporter NMD

SMG1 is a phosphatidylinositol 3-kinase (PI3K)-related kinase capable of regulating the phosphorylation of UPF1 [22,23]. We therefore tested whether LY294002, a PI3K and SMG1 inhibitor, would inhibit the NMD of endogenous *GPX1* mRNA (Fig. 6A). In the absence of supplemental Se, the levels of both *GPX1* (88%, $P<.0001$) and *GPX4* (47%, $P<.05$) mRNA declined, although the decline in *GPX1* mRNA was much more pronounced than the decline of *GPX4*. In a manner similar to SeMet, LY294002 enhanced both *GPX1* (80%, $P<.001$) and *GPX4* (38%, $P<.05$) mRNA levels. The combination of SeMet and LY294002 was especially effective at enhancing the mRNA levels of the two selenoproteins (*GPX1* 93%, $P<.0001$; *GPX4* 63%, $P<.05$). Since the PI3K and SMG1 inhibitor LY294002 enhances endogenous selenoprotein mRNA levels, we tested the ability of LY294002 to inhibit reporter NMD. In the absence of sufficient Se, LY294002 inhibited by 50% the NMD of the RenβG-TGA *GPX1* construct (Fig. 6B, $P<.005$). LY294002 also inhibited by 90% the NMD of the RenβG-TAA *GPX1* construct (Fig. 6C, $P<.001$). These data support the utility of our reporter in characterizing the role of signaling pathways in the regulation of selenoprotein mRNA stability and suggest that an effective way to optimize selenoprotein mRNA levels might be to use a combination of approaches that involves both the correction of nutritional Se deficiency and the inhibition of cellular signaling pathways that promote NMD.

### 3.6. Localization of reporter NMD

Most mRNAs that undergo NMD do so in the nucleus. However, under conditions of Se insufficiency, endogenous *GPX1* mRNAs are degraded in association with the cytoplasmic fraction of the cell [28,29]. To determine whether the behavior of the reporter with regard to the location of NMD recapitulated the behavior of endogenous GPX1, we monitored reporter mRNA levels in the nuclear and cytoplasmic fractions of HeLa cells. We found that the original PTC-containing reporter (RenβG-TAA) mRNA is subjected to nucleus-associated NMD. In a Se-dependent manner, the reporter containing

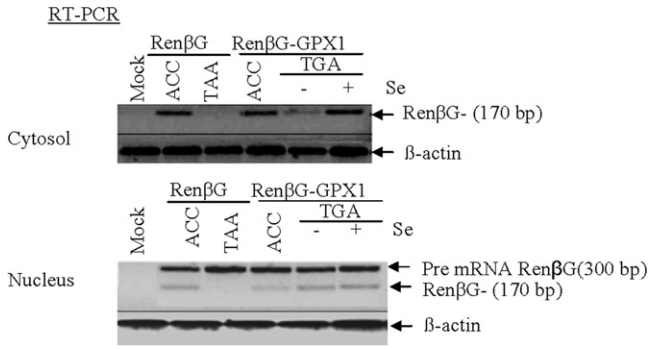


Fig. 7. A *GPX1* SECIS influences the nuclear versus cytoplasmic location of a reporter for monitoring NMD. Endogenous *GPX1* mRNA is known to undergo NMD in the cytoplasm [28]. To determine whether a *GPX1* SECIS would alter the location of reporter NMD, HeLa cells were transiently transfected with RenβG-ACC, RenβG-TAA, RenβG-ACC-*GPX1* or RenβG-TGA *GPX1* reporter constructs. After 72 h of incubation in the presence or absence of Se (SeMet 1 μM), total RNA was isolated from the cytoplasmic and nuclear fractions. The level of reporter mRNA in each fraction was determined by RT-PCR. The figure is from one of three independent experiments. An RT-PCR for β-actin was used to confirm equal loading.

a UGA codon and a *GPX1* SECIS underwent NMD in the cytoplasm (Fig. 7). In this regard, the reporter might be a valuable tool for characterizing cytoplasmic NMD. As expected, unprocessed pre-mRNA was only detected in the nuclear fraction.

## 4. Discussion

To better understand the mechanisms regulating the stability of selenoprotein mRNAs, we have developed a sensitive and easy-to-monitor cell-based chemiluminescence reporter assay that can be used to measure the steady-state level of an artificial selenoprotein mRNA. Our reporter is distinct from luciferase-UGA-SECIS and other read-through reporters commonly used by researchers in the selenoprotein field to study the efficiency of SEC incorporation and read-through at UGA codons [20,30–32] in that reporter activity is present regardless of whether read-through occurs at the UGA codon and therefore reflects the steady-state level of the selenoprotein mRNA. Our reporter is derived from a *Renilla* luciferase reporter gene fused to an intron containing human β-globin gene and, when a stop codon is inserted before the second intron, is subject to degradation by NMD [22].

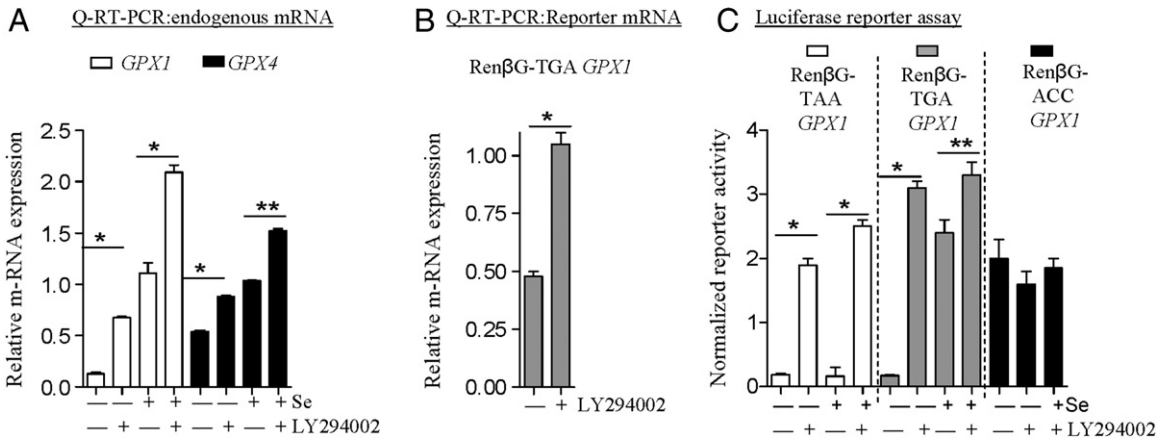


Fig. 6. The SMG1 and PI3K inhibitor LY294002 prevents Se-dependent NMD of a selenoprotein reporter. (A) The expression of endogenous *GPX1* or *GPX4* mRNA following treatment with Se (SeMet 1 μM) or LY294002, as determined by Q-RT-PCR. The data points are the average of three independent experiments. Error bars indicate 1 standard deviation (*$P<.005$, **$P<.05$). (B) Reporter mRNA levels following treatment with LY294002, as determined by Q-RT-PCR. Each data point is the average of three independent experiments. Error bars indicate 1 standard deviation (*$P<.005$). (C) Effect of Se or LY294002 on normalized reporter luciferase activity for selenoprotein NMD reporters with TAA, TGA or ACC codons at NS39. Each data point is the average of six biological replicates. Error bars indicate 1 standard deviation (*$P<.001$, **$P<.05$).

In contrast to classic NMD reporters [22], our reporter construct contains two selenoprotein specific features; these include a SECIS element in the 3′ UTR and a UGA codon, which may be interpreted as either a SEC codon or as a true stop codon depending upon whether translation read-through is supported. The β-globin gene contains two introns. The insertion of an appropriately spaced premature termination codon within the second exon of the β-globin gene renders the construct susceptible to NMD. Since the PTC is located after the coding sequence for the *Renilla* luciferase reporter gene, reporter protein is produced from any mRNA transcript not degraded by NMD. In this regard, we demonstrate that the *Renilla* luciferase reporter activity of our artificial selenoprotein gene is proportional to the steady-state mRNA level of the reporter. To confirm that reporter mRNA degradation is by NMD, genetic and pharmacologic approaches were used to inhibit NMD, using UPF1 depletion and LY294002 treatment; both treatments lead to the expected specific increase in reporter activity.

Previously, an intron containing *GPX1* construct was used by Weiss and Sunde to investigate the role of SECIS elements on mRNA stability. Our reporter construct differs from the construct used by Weiss and Sunde in that ours has a β-globin backbone and theirs is a *GPX1*-based construct and therefore contains additional exonic and intronic selenoprotein sequences that might influence Se responsiveness. Using their reporter, Weiss and Sunde found that exchanging only the *GPX1* and *GPX4* 3′ UTR imparted nearly identical regulation by Se, with decreases to 44% and 45%. In contrast, using our β-globin-based construct, we found that the reporter with a *GPX1* SECIS was more Se responsive than the reporter with a *GPX4* SECIS (Fig. 2A and B). Under more profound Se-deficient conditions induced by culturing the cells in the absence of fetal bovine serum, the activity of our *GPX4* SECIS-containing reporter did decrease by approximately 30%, suggesting that although the *GPX4* SECIS-containing construct is stable under conditions of moderate Se insufficiency, more profound Se insufficiency promotes the degradation of the reporter. This effect is unlikely to be the consequence of serum starvation because the effect was dramatically observed in the TGA-containing construct and not in the TGT-containing construct, indicating preservation of protein synthesis. Our data do not address the extent to which the apparent increased Se responsiveness of Weiss and Sunde's *GPX4* SECIS-containing construct might be due to Se-responsive instability elements present within exonic or intronic regions of their GPX1-based construct's backbone but absent in our β-globin-based construct [21].

To determine whether a specific SECIS was able to protect our artificial selenoprotein from NMD, we cloned the SECIS elements from either the human *GPX1* or the human *GPX4* gene into the 3′ UTR of the reporter plasmid. For the transcript with a UGA codon, in the presence of a *GPX1* SECIS, the steady-state selenoprotein mRNA levels declined in the absence of optimal supplemental Se. In the presence of a *GPX4* SECIS, however, reporter activity was resistant to the effects of Se insufficiency, decreasing only under conditions of more profound Se insufficiency. Interestingly, our data also suggest that the *GPX4* SECIS might partially protect a reporter transcript containing a UAA true stop codon (Fig. 2A). While several laboratories have shown that the *GPX4* SECIS is more efficient than the *GPX1* SECIS at promoting translation read-through at UGA codons [21,32], since in the absence of a UGA codon there is no SEC incorporation, the stabilizing effect of the *GPX4* SECIS in the UAA-containing construct is likely due to the appended RNA sequence and not to enhanced translation read-through or SEC incorporation.

The observation that a *GPX4* SECIS is able to partially protect an mRNA from NMD even when the PTC is not a UGA codon may shed light on the mechanisms by which selenoproteins avoid NMD. Since translation is likely to stall at a PTC when the UGA codon is replaced by a true stop codon or when there is a UGA codon but insufficient Se, the *GPX4* SECIS might help prevent NMD by mechanisms that are not dependent upon promoting read-through at the PTC. In the presence and absence of optimal Se, perhaps the *GPX4* SECIS recruits protein factors that allow the transcript to assume a conformation that facilitates the avoidance of NMD regardless of whether translation is efficient. In contrast, the *GPX1* SECIS appears to protect the reporter from NMD only in the presence of both a UGA codon and sufficient supplemental Se (Fig. 2A).

According to the classic model for NMD, an mRNA is flagged for degradation during a pioneer round of translation, which generally is believed to occur as an mRNA bound to the cap binding protein (CBP) heterodimer exits the nucleus [33]. If the mRNA is recognized during this pioneer round of translation as having a nonsense codon, it is flagged for degradation. Generally, degradation by NMD takes place in association with the nuclear fraction of the cell. If the mRNA avoids NMD, it exits the nucleus, the CBP is replaced by eIF4E, and the mRNA subsequently becomes immune to NMD. While most mRNAs that are degraded by NMD do so in association with the nuclear fraction of the cell, when the *GPX1* mRNA undergoes NMD, it does so in association with the cytoplasmic fraction of the cell [12,28,33,34]. A role for the *GPX1* SECIS in the cytoplasmic localization of *GPX1* NMD is suggested by our finding that, under conditions of Se insufficiency, the *GPX1* containing selenoprotein reporter, but not the parental *Renilla* luciferase reporter gene, undergoes cytoplasmic NMD.

In the future, our reporter might be used to help decipher the molecular mechanisms underlying the differential Se-dependent stability of the mRNAs corresponding to the various selenoproteins. With regard to the differential susceptibility of the *GPX1* and *GPX4* SECIS-containing transcripts to NMD, it will be of particular interest to explore the possibility that conformational changes associated with the methylation status of charged or uncharged $tRNA^{SEC}$ influence the displacement of specific SECIS binding proteins such as eIF4a3 or SBP2 from the *GPX1* SECIS [8,35,36]. Since SBP2 has a stronger affinity for the *GPX4* SECIS than for the *GPX1* SECIS [16], it might be more difficult to displace and consequently might remain bound to the SECIS both when Se is abundant and when Se is limited. In contrast, since the affinity of SBP2 for the *GPX1* SECIS is lower, SBP2 may be more readily displaced from the *GPX1* SECIS when the uncharged tRNA assumes a specific conformation.

Since selenoproteins such as *GPX1* play important roles in antioxidant defense, therapeutic approaches to augment selenoprotein production might be used to treat disorders associated with oxidative stress, to modify the course of severe sepsis, to chemoprevent cancer or to decrease the toxic effects of tobacco smoke exposure. One mechanism by which selenoprotein production might be enhanced is by modulating the NMD of selenoprotein mRNAs. In this regard, we find that the PI3K and SMG1 inhibitor LY294002 prevents the Se-dependent NMD of our selenoprotein reporter construct. This observation demonstrates the utility of our reporter for rapidly characterizing signaling pathways regulating selenoprotein mRNA stability and raises the possibility that conditions associated with aberrant activation of the corresponding signaling pathways might influence selenoprotein levels. In the future, it will be of interest to use our reporter in a high-throughput small molecule screen to identify less toxic inhibitors of selenoprotein NMD. Since some cancer cells have an impaired ability to express specific selenoproteins [37], the reporter might also be used in combination with a reporter for monitoring SEC incorporation efficiency at a UGA codon to rapidly characterize the general integrity of selenoprotein production pathways in specific cancer cell lines or to define molecular signaling pathways that regulate selenoprotein levels.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jnutbio.2011.07.010.

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