

**MECHANISTIC REGULATION OF SELENOPROTEIN MRNAS  
DURING SELENIUM DEFICIENCY**

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*To my uncle Omid*

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## ABSTRACT

The essential trace element selenium is present in selenoproteins via the unique amino acid selenocysteine (Sec). Most often, selenoproteins have a single Sec residue that utilizes the electrochemical properties of selenium to catalyze crucial biochemical reactions. Characterized selenoproteins possess antioxidant functions that play integral roles in various aspects of human health. The molecular biology of Sec incorporation is also unique because UGA serves as its triplet. In order to recode this UGA codon, all selenoprotein mRNAs have a specialized secondary structure in their 3'un-translated region (UTR) which facilitates the placement of Sec into the ribosome. In addition, Sec is synthesized directly on its tRNA and selenoprotein synthesis is thus sensitive to selenium availability. Since UGA is a common stop codon, selenoprotein mRNAs are potential targets of the nonsense-mediated decay pathway (NMD). This pathway targets aberrant transcripts with premature termination codons (PTCs) for degradation in order to prevent the production of potentially toxic truncated proteins. Several groups have observed a hierarchy of selenoprotein mRNA abundance during selenium deficiency whereby the levels of certain transcripts decline while others do not. Since the cellular machinery cannot distinguish Sec codons from UGA stop codons, it is generally postulated that NMD is involved in this response to selenium deficiency. While this assumption is logical, there has been little evidence to support it. This primary aim of this dissertation is to evaluate the role of the NMD pathway in the regulation of selenoprotein mRNAs during selenium deficiency. The overarching hypothesis of this thesis is that selenoprotein mRNA that are predicted sensitive to NMD will decrease in abundance during selenium deficiency. The established rules that govern the mammalian model for NMD were utilized to assess the susceptibility of the human selenoprotein transcriptome to the NMD pathway. About half the mRNAs were predicted sensitive to NMD while the other half were predicted resistant. Those that were predicted sensitive decreased significantly in abundance during selenium

deficiency while those that were predicted resistant did not. In addition, the mRNAs that were sensitive to NMD and likewise responded to selenium status, were also more abundantly bound to central NMD regulator UPF1 during selenium deficiency. Furthermore, siRNA depletion of SMG1, the kinase responsible for UPF1 phosphorylation and NMD activation, abrogated the selenium response of the NMD-sensitive transcripts. These results strongly suggest that NMD is involved in the decrease of selenoprotein transcript abundance observed during selenium deficiency. The stability of GPx4 mRNA presents an exception to the expected responses to selenium status and NMD predictions. GPx4 mRNA is predicted to be sensitive to NMD but does not respond to selenium deficiency and likewise remains stable with knockdown of SMG1. A consensus sequence in the 5'UTR of GPx4 was previously shown to facilitate the translation of its mRNA. The 5'UTR of GPx4 was thus analyzed in order to investigate its potential role in GPx4s enigmatic response to cellular selenium status. Our analysis of this consensus sequence suggests that it is not involved in the observed stability of GPx4 mRNA during selenium deficiency although it may be increasing the translatability of the transcript.

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## LIST OF ABBREVIATIONS

Aly	T7 epitope-tagged mREF
BTZ	Barentz
CBC	Cap binding complex
CBP	Cap binding protein
cDNA	Complementary deoxyribonucleic acid
DIO	Deiodinase
DMEM	Dulbecco's modified eagle medium
EFSec	Elongation factor selenocysteine
EIF4A3	Eukaryotic initiation factor
EIF4E	Eukaryotic initiation factor 4E
EJC	Exon junction complex
FBS	Fetal Bovine Serum
FLAG	Asparagine Tyrosine Asparagine(4x) Lysine
GDP	Guanosine diphosphate
GPx	Glutathione peroxidase
GRSF1	Guanine-rich sequence binding factor 1
GTP	Guanosine triphosphate
HEK	Human embryonic kidney
HIS4	Histidine 4
HIV	Human immunodeficiency virus
kDA	Kilo dalton
mM	Milli molar
mRNA	Messenger ribonucleic acid
mRNP	Messenger ribonucleoprotein
nM	Nano molar
NMD	Nonsense-mediated decay

PSTK	Phosphoseryl tRNA kinase
PTC	Premature termination codon
qPCR	Quantitative polymerase chain reaction
REF	RNA and export factor binding protein
RIP	RNA immunoprecipitation
RNA	Ribonucleic acid
Rnps1	RNA-binding protein with serine-rich domain
SBP2	SECIS binding protein
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Se	Selenium
Sec	Selenocysteine
SECIS	Selenocysteine insertion sequence
SecS	Selenocysteine synthase
SelH	Selenoprotein H
SelI	Selenoprotein I
SelK	Selenoprotein K
SelM	Selenoprotein M
SelN	Selenoprotein N
SelO	Selenoprotein O
SelP	Selenoprotein P
SelW	Selenoprotein W
SerRS	Seryl tRNA synthetase
siRNA	small-interfering RNA
SKAR	S6 kinase 1 Aly/Ref-like protein
SMD	Staufen-mediated decay
SMG1	Suppressor of morphogenic effect on genitalia 1
SPS2	Selenophosphate synthetase 2
SRm160	SR related nuclear matrix protein of 160 kDA
SUF1	Frameshift suppressor

T4	Thyroxine 4
tRNA	transfer RNA
tRNA <sup>sec</sup>	transfer RNA selenocysteine
Txnrd	Thioredoxin reductase
UTR	Un-translated region
UPF	Up-frameshift
UV	Ultraviolet
WT	Wild Type

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## CHAPTER 1

### INTRODUCTION

#### 1.1 A brief history of selenium

Elemental selenium (Se) was discovered in 1817 by the prominent Swedish chemist Jons Jacob Berzelius. He was studying a byproduct from a sulfuric acid preparation produced by a local factory that he thought might be causing illness to the factory workers. He named it after Selene, the Greek moon goddess, and since then much has been uncovered regarding its chemical function and biological role (Hatfield *et al.* 2012). It wasn't until a century and a half later that Se was identified as a component of an essential antioxidant protein, glutathione peroxidase (GPx)(Rotruck *et al.* 1973, Flohe *et al.* 1973). Our understanding of the importance of Se for proper health has progressed dramatically since its initial discovery by Berzelius. In 1937, A.L. Moxon published the first paper linking Se with toxicity when he identified it as the component responsible for livestock poisoning in cattle ranges in the west. The livestock were consuming a variety of the *Astragalus* plant which accumulates Se in toxic amounts from the soil and they suffered significant hair loss and cracking of hoofs. This finding by Moxon put Se in the category of dangerous and harmful substances for two decades, until Schwarz and Foltz uncovered a beneficial role for it in 1957 (Schwarz & Foltz 1999). Liver necrosis was a common problem in their laboratory rats and they found that after switching their feed the problem was alleviated. They ultimately learned that Se was deficient in the original feed and that the deficiency was causing the necrotic livers. This finding spearheaded subsequent studies investigating the health benefits of Se. Shortly thereafter, Se was reported to play a role in protecting newborn lambs from developing white muscle disease and in 1969, McCoy and Westwig identified Se as having essential nutritional benefits (McCoy & Westwig 1969, Oldfield *et al.* 1958). Since then, Se has been considered an essential micronutrient and reports describing protective health benefits have abounded (Roman *et al.* 2013, Schmidt & Simonovic 2012).

## 1.2 The biochemistry of selenium

GPx became the first characterized Se-containing protein and its efficiency in protecting red blood cells from oxidative damage (Mills 1957) ultimately led to the identification of selenocysteine (Sec) as the Se-containing amino acid responsible for the catalytic efficiency of the enzyme (Cone *et al.* 1976). Almost identical to cysteine, Sec differs by having a Se atom in place of cysteine's sulfur. Although there isn't a definitive conclusion explaining the catalytic superiority of Se to sulfur at the active sites of enzymes, several theories have developed. Se is in the same group in the periodic table as sulfur and therefore has similar properties. They each have two unpaired electrons in respective p orbitals making them reactive species. A telling difference between the two however, is the size of the nucleus and thus the electronic properties of the atom as a whole. The bigger size of the Se nucleus makes it more electrophilic than cysteine and this is believed to underlie its catalytic superiority.

Well-characterized Sec-containing enzymes (selenoproteins) are oxidoreductases that make use of Se's efficiency in accepting electrons to facilitate the reduction of harmful reactive oxygen species within the cellular environment. The Sec residues in selenoproteins are subject to various oxidation states, most commonly the seleneninic (Enzyme-SeOH), seleninic (Enzyme-SeO<sub>2</sub>) and selenonic (Enzyme-SeO<sub>3</sub>) acid forms. The reduction potential of these oxidation states provides some evidence for the catalytic efficiency of Sec over cysteine (Reich *et al.* 2002, Steinmann *et al.* 2010). Experiments involving enzymatic inactivation reactions have shown that inactivated oxidized selenoproteins are readily reduced back to their active form by the addition of exogenous thiols while inactivated cysteine-containing enzymes are not. In addition, further oxidation of the Sec in a selenoprotein proceeds slowly while that of sulfur in a cysteine counterpart is rapid. Importantly, the pKa of selenol is lower than that of thiol (5.2 vs 8.3) making ionization energetically efficient at physiological pH for Sec in comparison to cysteine (Johansson *et al.* 2005). These properties of Se together create a marked difference between the structurally similar amino acids Sec and cysteine. They

also underlie the significant differences between the nutritional requirements and effects on human health in relation to sulfur and Se consumption. Se is considered an essential trace element because of the relative amounts present on earth in comparison to other essential elements. It enters an organism through the diet as a result of consuming plants or animals that have assimilated it from the soil. While sulfur is of extremely low toxicity, Se is very toxic if consumed in excess (Koller & Exon 1986, Mosen 2000).

### **1.3 Selenoprotein biosynthesis**

The essentiality of Se and its unique properties are further exemplified by the molecular biology of selenoprotein synthesis. Although there are various Se containing molecules that contribute to the overall Se pool (Burk *et al.* 2001, Suzuki & Ogra 2002), the bulk of health benefits are attributed to the function of Sec in selenoproteins. Selenoprotein biosynthesis is anomalous because the triplet that codes for Sec is UGA, which is more commonly known to function as a stop codon. Because of this, all selenoprotein genes have specialized 3' untranslated regions (UTRs) that facilitate the recoding of UGA to incorporate Sec into the nascent peptide chain. This region in the 3'UTR, known as the SECIS element (Sec Insertion Sequence element), is a secondary structure in the mRNA that forms a hairpin which interacts with several proteins to coordinate the insertion of a Sec-specific tRNA into the ribosome (Berry *et al.* 1991). There are two types of SECIS elements depending on the specific selenoprotein. Characterized as type 1 and type 2, the two forms differ in structure where type 1 has a single internal loop and an apical loop, and type 2 has two internal loops as well as an apical loop (Figure 1). With the exception of selenoprotein P (SelP), all selenoproteins have one SECIS element. SelP is unique from all the other selenoproteins due to the presence of both SECIS elements in order to facilitate the incorporation of 10 Sec residues. After this unique aspect of selenoprotein gene architecture was discovered, conserved sequences in the SECIS element were used to identify other selenoprotein genes.



In addition to the SECIS element, there are several other factors that play a critical role in the generation of a selenoprotein. These include a sec-specific elongation factor (EFSec), a SECIS binding protein (SBP2), Sec synthase (SecS), selenophosphate synthetase (SPS2), phosphoseryl tRNA kinase (PSTK) and seryl tRNA synthetase (SerRS). These factors work in concert to generate the mature Sec-specific tRNA (tRNA<sup>Sec</sup>) (Figure 1.1), and to place it into the A site of the ribosome during translation (Tujebajeva *et al.* 2000, Small-Howard *et al.* 2006). The synthesis of a selenoprotein begins with tRNA<sup>Sec</sup> being charged with serine by SerRS followed by the phosphorylation of this serine by PSTK. If cellular Se status is sufficient, then SPS2 will generate selenophosphate from selenide. If Se is low, it has been shown that tRNA<sup>Sec</sup> can be charged with cysteine (Lu *et al.* 2009, Xu *et al.* 2010). SecS has been shown to accept selenophosphate and it plays a critical role in the generation of selenocysteine from phosphoserine by a reaction mechanism that was recently modeled (Ganichkin *et al.* 2008). Once tRNA<sup>Sec</sup> has been generated, the SECIS element works together with SBP2 and EFSec to place it into the ribosome. It is likely that there are other proteins involved in this, however, these are the only two that have been deemed necessary so far. SBP2 is a 120 kDa protein that was discovered by UV crosslinking to bind SECIS elements (Lesoon *et al.* 1997). It is a member of the L7Ae RNA binding protein family and it contains three distinct domains, two of which, the Sec incorporation domain and the RNA binding domain, are in the C-terminal region (Caban *et al.* 2007). The N-terminal domain has no known function and several organisms lack it completely (Donovan & Copeland 2009). EFsec is an elongation factor with GTPase activity that, together with SBP2, carries tRNA<sup>Sec</sup> to the ribosome (Fagegaltier *et al.* 2000). A guanine nucleotide exchange factor is yet to be discovered for EFSec but its unusually high affinity for GTP over GDP makes it likely that it can function without one. The exact order of the progression of events, spatially and temporally, is yet to be understood, and since UGA is a stop codon, this process is in competition with termination. Such a complex system having persisted through years of evolution is evidence itself of the value selenoproteins hold in the proteome. When Se is adequately available, selenoprotein synthesis would

likely be rapid and efficient to generate antioxidant proteins that constantly protect the organism from harmful damage and death. However, when Se becomes limiting, the sophisticated machinery required to recode UGA would likely yield to termination. The efficiency of Sec incorporation has been studied and it has been shown that low Se influences this efficiency (Howard *et al.* 2013). In addition, groups assaying levels of selenoprotein mRNAs in relation to cellular Se status have observed a hierarchy of abundance where the levels of certain transcripts drop with decreasing Se while others do not (Bermano *et al.* 1996b, Sunde *et al.* 2009). The reason for this hierarchical distribution is not understood and it is quite possible that there are multiple factors that contribute to it. An obvious candidate that has been implicated in this regulation is the nonsense-mediated mRNA decay pathway (NMD) because it functions to degrade mRNAs that harbor a premature termination codon (PTC). The primary focus of this thesis is to evaluate the influence of the NMD pathway on selenoprotein mRNAs when Se is limiting.

#### **1.4 tRNA<sup>sec</sup>**

Not surprisingly, tRNA<sup>sec</sup> is unique from all the other tRNAs; it is the longest and exhibits distinct structural features (Carlson *et al.* 2004). While the standard tRNA is around 75 nucleotides in length, tRNA<sup>sec</sup> is 90 nucleotides long, the longest known mammalian tRNA. tRNA<sup>sec</sup> is unique not only because of its length but also because it exists as two separate isoforms. The two isoforms differ in the methylation state of the wobble base, U34, named methylcarboxymethyl-5'-uridine and methylcarboxymethyl-5'-uridine-2'-O-methylribose (Figure 1.2)(Small-Howard *et al.* 2006). The methylation state of the tRNA has been shown to correlate with cellular Se status where under conditions of limiting Se, the non-methylated form is more abundant than the methylated form. It has been shown that certain selenoproteins depend on the methylated isoform for their synthesis while others can utilize either form (Carlson *et al.* 2009). Since tRNA<sup>sec</sup> functions as the site of Sec synthesis, as well as the molecule responsible for donation of the amino acid to protein, it plays a key role in our

understanding of selenoprotein mRNA regulation in response to cellular Se status. This tRNA isoform specificity, as well as NMD, is likely involved in the hierarchical response of selenoprotein mRNAs to Se deficiency.

### **1.5 The nonsense-mediated mRNA decay pathway (NMD)**

NMD functions to degrade mRNAs that harbor PTCs. A PTC can arise in a transcript *de novo* due to a mutation that is a product of errors in processes such as transcription and splicing. They can also occur naturally due to physiological processes such as gene rearrangements, upstream open reading frames, introns in UTR's, alternative splicing, and Sec codons. Occasionally, PTC-generating mutations can also be genetic and potentially disease-inducing depending on the location within a gene. A rare form of  $\beta$ -thalassemia exemplifies this when a mutation in the last exon of the  $\beta$ -globin gene evades degradation by NMD (the reason for this evasion will be explained shortly) and produces truncated globin, which aggregates in toxic amounts. Initially, it was believed that NMD's predominant purpose was to degrade transcripts that had generated a PTC due to mutation, and thus to protect the cell from the mass production of potentially toxic truncated proteins. As research progressed, however, evidence emerged identifying roles for NMD in the regulation of PTC-containing transcripts that are naturally occurring (Mendell *et al.* 2004, Yepiskoposyan *et al.* 2011). It is currently a matter of debate whether NMDs evolutionary fixation is due to its role in protecting the cell from potentially toxic truncated proteins versus the maintenance of cellular homeostasis by regulating transcript abundance. Proponents of the latter model argue that the frequency of *de novo* mutations that generate PTCs are inadequate to provide enough selective pressure for full evolutionary preservation of NMD. While this is still a relatively new debate, it seems likely that both purposes have contributed to the maintenance of the NMD pathway over time.

The cascade of events that leads to the decay of a transcript has been the topic of extensive study over the past decade and while much remains to be elucidated, several necessary factors have been identified. The observation that mRNAs with PTCs

lose stability was initially made in yeast by Losson and Lacroute (Losson & Lacroute 1979). They noticed that nonsense mutations reduced the levels of a specific mRNA without lowering its rate of synthesis. This study provided the first clue suggesting that PTCs could destabilize mRNAs. Another study in yeast around the same time found that strains with frameshift mutations in the HIS4 gene were deficient in histidine biosynthesis due to decreased levels of HIS4 mRNA. The same HIS4 mutants that also possessed SUF1, a glycine tRNA frameshift suppressor, were able to synthesize histidine at 30°C but not at 37°C due to low-level readthrough. Additional research identified mutations at specific loci in these strains that increased transcript stability enough to allow for histidine biosynthesis at 37°C as well. These mutations were labeled “upf” for “up-frameshift”, due to their presumed ability to enhance frameshift suppression (Culbertson *et al.* 1980). Ten years later, another group found that the upf mutations cause a more pronounced increase in HIS4 mRNA than SUF1 and thus began investigating the roles of the upfs in transcript stability (Leeds *et al.* 1991). They noticed that constructs with nonsense mutations consistently accumulated in yeast strains devoid of the protein UPF1 while only some with frameshift mutations behaved similarly. They concluded that UPF1 played an important role in an unknown pathway that leads to the degradation of transcripts carrying nonsense codons. UPF1 is currently regarded as the central regulator of NMD. It is a 140 kDa member of the RNA helicase superfamily 1 and since its initial discovery much has been learned regarding its role in NMD as well as several other cellular processes (reviewed in Imamachi *et al.* 2012).

NMD is evolutionarily conserved across eukaryotes and exists in all species examined to date (Maquat 1995). Although the key players that participate in the decay of a transcript are similar between species, there are some important differences that distinguish mammalian NMD from that in lower eukaryotes. This thesis will focus on the mammalian model of NMD. The current model holds that the path to degradation begins in the nucleus during splicing. The spliceosome deposits a multimeric complex of proteins 20-24 nucleotides upstream of exon-exon junctions (Le Hir *et al.* 2000). This complex has been termed the exon junction complex (EJC), and while all of its

constituent proteins are yet to be defined, many of the core proteins have been identified. The EJC core consists of the ATP-dependent RNA helicase eIF4A3, the heterodimer MAGOH-Y14 and the RNA transport protein Barentz (BTZ) (Buchwald *et al.* 2010, Gehring *et al.* 2009a). In addition to these core factors, several others have been proposed to be a part of the EJC as well. These include Aly/Ref, UPF2, UPF3b, splicing factor rnp1, SKAR, and SRm160 (Chang *et al.* 2007). Initially it was assumed that the spliceosome deposits EJCs upstream of every exon junction but recent reports have questioned that notion (Muhlemann 2012, Sauliere *et al.* 2012, Singh *et al.* 2012). After EJC deposition, the mRNA is transported through the nuclear pore complex and the cap-binding complex (CBC) is replaced by eIF4E. eIF4E is understood to facilitate bulk translation in the cytoplasm and it is just before this exchange that NMD is believed to take place. Evidence in support of this comes from coimmunoprecipitation experiments that have found EJCs to accompany the cap-binding protein CBP80, but not eIF4E (Lejeune *et al.* 2002). Before the exchange of mRNA cap binding proteins, the first ribosome will begin a pioneer round of translation. If it arrives at a PTC, it will stall and a release factor will bind (Ishigaki *et al.* 2001). CBP80 is then thought to facilitate the association of UPF1 and its kinase, SMG1, with the release factor to form the SURF (SMG1-UPF1-Release Factor) complex (Hwang *et al.* 2010). SMG1 is a phosphatidylinositol-kinase-related kinase responsible for phosphorylating UPF1 to initiate the decay cascade (Yamashita *et al.* 2001). Once UPF1 associates with the EJC, SMG1-mediated phosphorylation of UPF1 initiates mRNP remodeling which allows for the degradation of the transcript by endonucleases and exonucleases (Kashima *et al.* 2006, Lejeune *et al.* 2003, Isken *et al.* 2008) (Figure 1.3). It has been shown that EJCs are deposited 20-24 nucleotides upstream of exon junctions and that they are displaced by a protein associated with the ribosome from a distance of approximately 30 nucleotides (Gehring *et al.* 2009b, Le Hir *et al.* 2000). For this reason, it has been established that a PTC located in the last exon, or within approximately 50 nucleotides of the last exon, evades NMD. It is unclear whether any EJC downstream of a PTC is capable of eliciting NMD, or whether it is restricted to the next proximate EJC. It is also unclear whether

EJCs are deposited on every exon junction as was initially believed. Additionally, EJC independent degradation of PTCs has been reported *in vitro* (Buhler *et al.* 2006), as well as a few other exceptions to the 50 nucleotide rule (Zhang *et al.* 1998, Rajavel & Neufeld 2001). While it is unclear whether EJCs are absolutely necessary to facilitate NMD, they appear to significantly enhance the process.

### **1.6 The hierarchy of selenoprotein synthesis**

The human selenoproteome currently consists of 25 selenoproteins (Reeves & Hoffmann 2009). Few have been functionally characterized but it is presumed that Sec provides them with an oxido-reductase function. The focus of initial studies involving Se began with investigations attempting to understand Se utilization as a means towards elucidating the molecular interactions that underlie clinical conditions associated with Se deficiency and toxicity. While this understanding is still in the process of being developed, there are certain observations and conclusions that have contributed significantly towards shaping the field. Several groups have noted a hierarchy of selenoprotein mRNA abundance whereby the levels of certain transcripts decrease with decreasing Se while others remain steady (Bermano *et al.* 1996a, Sunde *et al.* 2009, Weiss & Sunde 1998, Sun *et al.* 2001). Due to the ease of primer design in comparison to the production of antibodies, protein data to accompany the mRNA data is lacking. The few proteins whose expression has been analyzed respond to Se in most tissues examined with the exception of the brain (Allan *et al.* 1999). The observation that some selenoprotein mRNAs remained stable with changes in Se supply suggests unique modes of regulation for various transcripts.

GPx1 mRNA is a good example of a selenoprotein transcript that is significantly regulated by Se status. Weiss and Sunde performed mutational analysis of a GPx1 construct in an attempt to elucidate portions of the mRNA that are integrally involved in the transcripts sensitivity to Se (Weiss & Sunde 1998). They found that the position of the Sec codon and the presence of an intron played a crucial role. GPx1 genomic DNA has two exons separated by a single intron. Constructs in which the intron was deleted

were no longer sensitive to Se status as well as constructs which contained the intron, but had the Sec codon relocated to the second exon instead of the first. In addition, they showed that the SECIS element is necessary for Se sensitivity of the mRNA and that the GPx4 3'UTR (which contains a different SECIS) is not able to confer a unique response to Se. Several groups have shown independently that GPx4 mRNA does not change with Se, although the structure of the transcript is similar to that of GPx1, which does respond to Se (Bermano et al. 1996a, Sun et al. 2001, Weiss & Sunde 1998, Moriarty *et al.* 1998). Since GPx1 and GPx4 differ in their SECIS elements, the initial hypothesis was that the 3'UTR of each is involved in this discrepancy. This report by Weiss and Sunde provided evidence against this, thereby leaving the unusual difference of the transcripts' response to Se enigmatic.

A more recent report which also investigated the role of the differing SECIS elements provided evidence in opposition to that of Weiss and Sunde (Budiman *et al.* 2009). Budiman *et al.* used UV crosslinking to identify SECIS-binding proteins which bind to the GPx1 and GPx4 SECIS element. They found that eIF4A3, the RNA binding helicase which lies at the core of the exon junction complex, was bound to the SECIS of GPx1 in nuclear extracts of a rat hepatoma cell line (McArdle 777 cells). They show with subsequent experiments that eIF4A3 is upregulated during Se deficiency and that it competes with SBP2 for SECIS binding. They conclude that this may be involved in the regulation of GPx1 mRNA during Se deficiency. Although their model is plausible, it is in direct conflict with Weiss and Sunde, as well as another report by Sun *et al.* who provide additional support for the NMD of GPx1 mRNA (Sun *et al.* 2000). In addition, there are a few key points in their methods, as well as the theory as a whole, that may be criticized. First, their definition of Se-deficiency and Se-sufficiency is somewhat misleading because standard cell culture media with 10% FBS was used as representative of Se-deficiency. FBS at 10% is a widely accepted concentration for cell culture and since cells can grow comfortably in this media it is generally regarded as Se-sufficient. Further, when UV cross-linking is performed on nuclear cell extracts it is possible that proteins and molecules would associate with each which would not normally do so in vivo. Since

eIF4A3 lies at the core of the EJC, and locked onto the RNA within a large complex, it would theoretically have to bind the SECIS element directly after EJC removal by the ribosome. While current evidence is in support of NMD as the mechanism by which GPx1 mRNA declines during Se deficiency, it is possible that there may be other factors involved which contribute to its regulation.

Since the NMD pathway functions to degrade mRNAs with PTCs, researchers attributed the Se-dependent regulation of selenoprotein mRNAs to NMD. While this assumption was logical, experimental evidence was lacking, and certain results presented an exception. Several groups showed independently that GPx4 mRNA does not change with Se, although the structure of the transcript is similar to that of GPx1, which does respond to Se (Bermano *et al.* 1996b, Sun *et al.* 2001, Weiss & Sunde 1998). As the model for mammalian NMD has progressed, it has provided a framework to investigate its potential role in the regulation of selenoprotein mRNAs.



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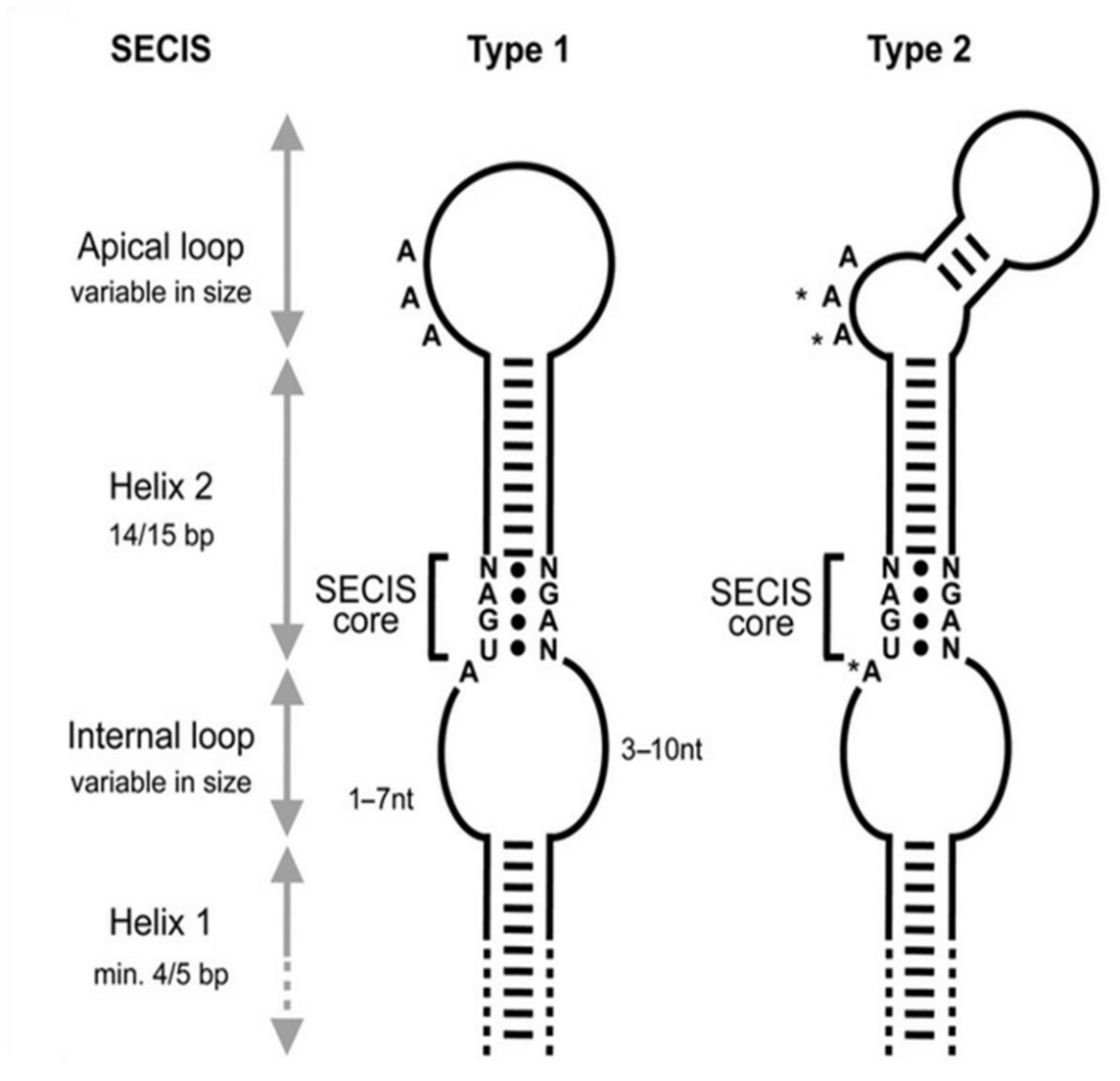
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1.8 FIGURES



**Figure 1. The two forms of SECIS elements.** The conserved sequence AUGA lies at the core of all SECIS elements. (Latreche *et al.* 2009)

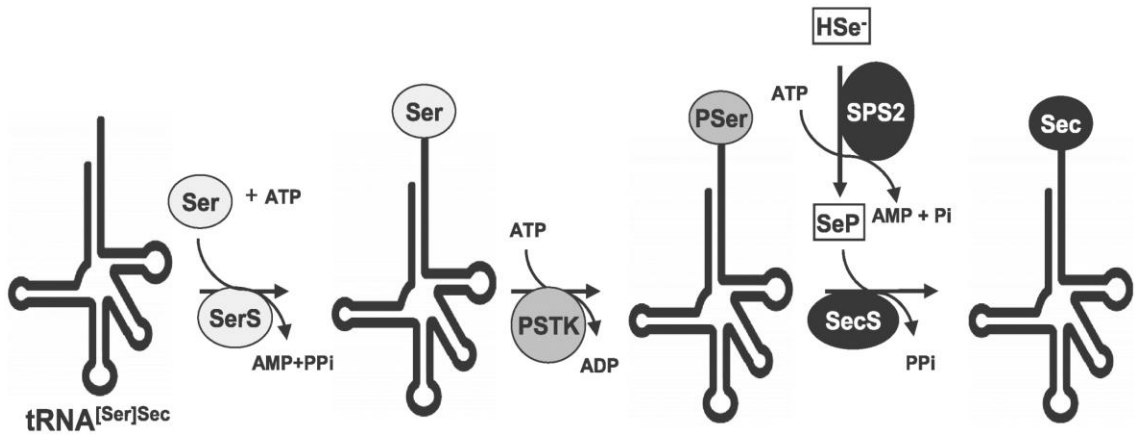
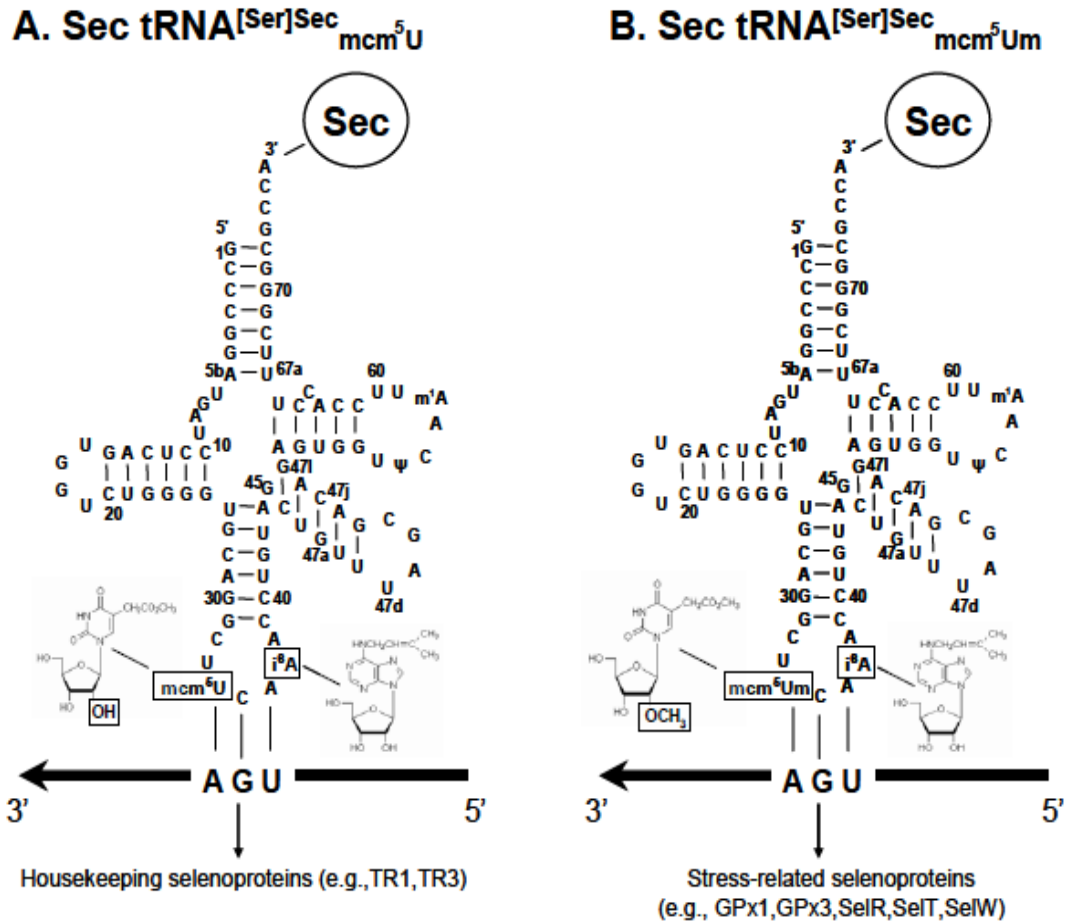
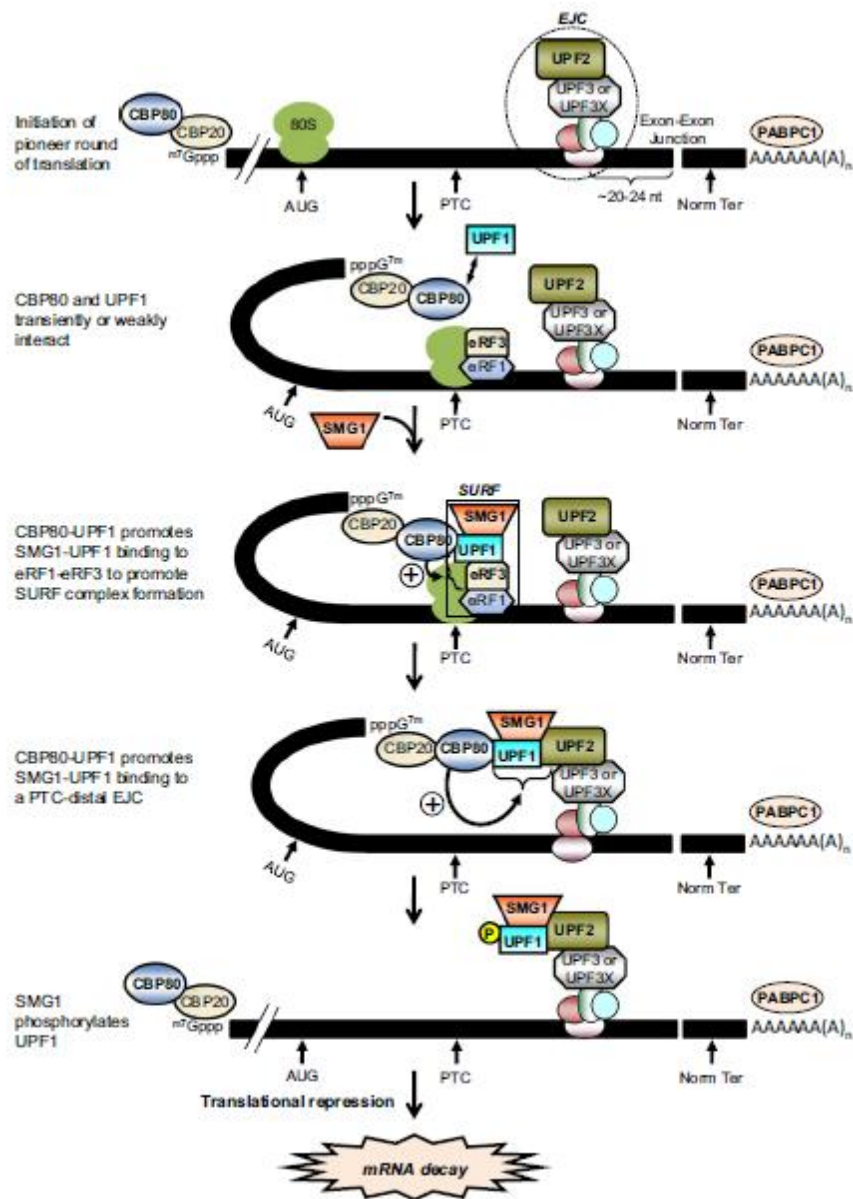


Figure 1.1 Biosynthesis of tRNA<sup>Sec</sup> (Hatfield *et al.* 2007)



**Figure 1.2 Isoforms of tRNA<sup>Sec</sup>.** A) The non-methylated isoform, designated mcmU, facilitates the synthesis of housekeeping selenoproteins such as TR1 and TR3. B) The methylated isoform, designated mcmUm, facilitates the synthesis of stress related selenoproteins such as GPx1 and SelW. (Carlson *et al.* 2009)



**Figure 1.3 Mammalian NMD.** EJCs are deposited on nascent mRNAs during splicing. If a PTC is present sufficiently upstream of an EJC, the ribosome will stall and CBP80 will facilitate the interaction of UPF1/SMG1 with the EJC to initiate the decay cascade. (Maquat *et al.* 2010)



## CHAPTER 2

### SE DEFICIENCY REDUCES THE ABUNDANCE OF SELENOPROTEIN mRNAs PREDICTED SENSITIVE TO NMD BUT NOT THOSE PREDICTED RESISTANT AND PRE-mRNAs

#### 2.1 ABSTRACT

Selenoprotein mRNAs were categorized as being predicted sensitive or resistant to the NMD pathway using the current mammalian model. Our categorization is based on the distance of the Sec UGA to the next proximate exon junction. HEK293T cells were cultured under conditions of high Se and low Se. Since Sec incorporation is in constant competition with termination, conditions of low Se would alter the availability of tRNA<sup>sec</sup> and this in turn would alter any existing equilibrium between Sec incorporation and termination towards the latter. Our categorization of NMD susceptibility roughly divides the selenoprotein transcriptome in half, one half being predicted sensitive and the other predicted resistant. Under conditions of limiting Se, mRNA abundance of selenoprotein transcripts predicted sensitive to NMD shows significant changes while those predicted resistant do not. In addition, pre-mRNAs were also analyzed to confirm that the observed changes were post-transcriptional. None of the pre-mRNAs showed a response to cellular Se status. These results suggest that NMD may be involved in the regulation of selenoprotein mRNAs during Se deficiency.

#### 2.2 INTRODUCTION

Se deficiency has been linked to several human health conditions. Among these are Keshan disease, a cardiomyopathy first noted in northeast China, Kashin-Beck disease, an osteochondropathy observed in China, Siberia, and North Korea, as well as the more prevalent hypothyroidism (Rayman 2000, Schomburg & Kohrle 2008). Although little research exists towards understanding the molecular biology underlying these conditions, it is likely that Se deficiency affects the essential properties of vital antioxidant selenoproteins and thus cellular equilibrium and the organism as a whole. The response of the entire selenoproteome and selenoprotein transcriptome to changes

in cellular Se status has not yet been investigated. Although complete protein analysis is lacking, there are however, a few studies that report responses of several mRNAs to cellular Se supply. These studies report a hierarchy of mRNA abundance whereby the levels of certain transcripts decrease with Se deficiency while others do not (Bermano et al. 1996b, Sunde et al. 2009, Yan *et al.* 2013). This hierarchy is not understood but could possibly represent a mechanism that maintains the expression of selenoproteins most vital to the organism when Se is limiting in the environment. The mechanism by which selenoprotein mRNAs are degraded is also not understood and since Sec is encoded by UGA, the NMD pathway is naturally assumed to be involved. The current model for mammalian NMD holds that a transcript will be targeted for decay if a PTC is located 50-55 nucleotides upstream of an exon junction (Nagy & Maquat 1998, Maquat 2004, Chang *et al.* 2007). This is because EJs are deposited 20-24 nucleotides upstream of exon junctions and they are removed by the first translocating ribosome during a pioneer round of translation from a distance of approximately 30 nucleotides (Fig. 2.1)(Le Hir et al. 2000, Gehring et al. 2009b, Bono & Gehring 2011). Because of this, any PTC located beyond the last exon junction, or within 50-55 nucleotides of it, will not render the transcript sensitive to NMD.

When we categorize the selenoprotein transcriptome based on this rule, we find that about half of the mRNAs are predicted to be resistant to NMD while the other half are predicted to be sensitive (Figure 2.2). In order to preliminarily assess this categorization, Se deficiency was modeled and qPCR primers were designed to 18 selenoprotein genes expressed in HEK293T cells (Figure 2.3). mRNA abundance was compared in cells cultured under high Se conditions versus low Se conditions. In addition, pre-mRNA abundance was also measured to help clarify the nature of the changes observed with the mRNAs. We see that the majority of mRNAs predicted sensitive to Se status respond significantly to changes in Se while those predicted resistant do not. In support of those changes being post-transcriptional, all of the pre-mRNAs remained stable regardless of Se concentration.

## 2.3 MATERIALS AND METHODS

### *Selenium treatment, RNA extraction, cDNA synthesis and RT-PCR*

HEK293T cells were plated in flat bottom 35 mm 6-well tissue culture plates (Corning Inc.) and were grown to confluence in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) with 10% Fetal Bovine Serum (FBS). FBS was measured for Se content and at 10% the final Se concentration due to FBS is approximately 30 nM. Media was then switched to 1% FBS with or without the addition of sodium selenite (final concentration of 3 nM Se or 60 nM Se) for 48 h. Cells were washed with phosphate buffered saline (PBS) and RNA was extracted with Trizol (Invitrogen) according to manufacturer's instructions. RNA quantity and quality were measured using a ND1000 spectrophotometer (NanoDrop Technologies). For cDNA synthesis, 1 µg of RNA was used in a total reaction volume of 20 µL using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). cDNA was diluted 5x and for qPCR a volume of 0.5 µL was used per final 5µL reaction volume. One µM specific primer pair and PerfeCTa® SYBR® Green FastMix were used (Quanta Biosciences) according to manufacturer's instructions on a Light Cycler 480® II thermal Cycler (Roche). The sequences of all primers used in this study are shown in Table 1.

## 2.4 RESULTS

*Selenoprotein mRNAs predicted to be sensitive to NMD exhibit significant changes in response to Se status while those predicted to be resistant remain stable.*

We characterized all selenoprotein mRNAs and categorized them as being predicted sensitive or resistant to NMD based on the location of the Sec UGA relative to exon-exon junctions. In accordance with the current model for mammalian NMD, Sec codons in the last exon are immune to NMD due to the displacement of all upstream EJs by the first translocating ribosome. Interestingly, about half of selenoprotein mRNAs have their Sec codon in the last exon while the other half are in either the second or third exon. Selenoprotein K (SelK) and Thioredoxin reductase 2 (Txnrd2) are

exceptions, as they have their Sec codons in the penultimate exon. However, they are both categorized as being predicted resistant to NMD due to the proximity of the Sec codon to the last exon junction. Cellular Se status directly influences tRNA<sup>Sec</sup> biosynthesis and thus its availability (Carlson *et al.* 2004, Jameson & Diamond 2004). We hypothesized that conditions of limiting Se would alter the availability of the Sec-tRNA and shift any existing equilibrium between termination and Sec insertion towards the former. Since NMD initiates with the binding of a release factor to a stop codon, it would presumably ensue if a release factor were to bind a Sec UGA codon.

To test this hypothesis, we modeled conditions of low or high Se, assessed mRNA levels, and grouped based on our categorization of predicted NMD susceptibility. HEK293T cells were cultured under standard conditions until confluent and then switched to Se-deficient (3 nM Se) or Se-supplemented (60 nM Se) media. After 48 hours cells were collected and qPCR was performed on 18 selenoprotein mRNAs that are detectable in this cell line (Fig. 2.3). Of the transcripts predicted to be vulnerable to NMD, selenoprotein W (SelW) mRNA was the most sensitive to Se status, showing a reduction by a factor of 4 in 3 nM versus 60 nM Se. Selenoprotein P (SelP), glutathione peroxidase 1 (GPx1), glutathione peroxidase 3 (GPx3), selenoprotein M (SelM) and selenoprotein H (SelH) mRNAs also showed a statistically significant response to low Se albeit to a lesser degree than SelW. Glutathione Peroxidase 4 (GPx4) and selenoprotein N (SelN) mRNAs did not show a statistically significant response to Se status. The observation that GPx4 mRNA remains stable under conditions of varying Se has previously been reported (Bermano *et al.* 1996b, Sunde *et al.* 2009, Sun *et al.* 2001) and will be discussed further below. Of all the transcripts predicted resistant to NMD, none were decreased in 3 nM versus 60 nM Se (Fig. 2.4). Only SPS2 mRNA abundance changed and this change was inversely related to Se concentration.

*Selenoprotein pre-mRNAs are not affected by Se status, indicating changes are post-transcriptional*

To help clarify the nature of the observed changes in mRNA levels, we sought to address the role transcription could be playing within the context of our model. It has previously been shown that changes in GPx1 mRNA levels in response to Se are not due to transcription but similar studies have not been reported for other selenoprotein mRNAs (Christensen & Burgener 1992, Moriarty *et al.* 1998). To examine this we designed primers to introns of a representative sample of transcripts from each category and performed qPCR. Regardless of their predicted sensitivity to NMD, none of the pre-mRNA transcripts responded to changes in Se (Fig. 2.5), suggesting the observed effects in Figure 2.4 to be post-transcriptional.

## **2.5 DISCUSSION**

Using mouse models for Se deficiency is relatively straight forward. The mice are fed a diet containing varying amounts of Se and the organs are then harvested and processed for RNA analysis. In cell culture this process is slightly hindered due to the naturally occurring presence of Se in FBS. A recent study investigating the influence of Se on selenoprotein 3'UTR-binding proteins uses Se-supplementation to standard media containing 10% FBS as representative of "High Se" and non-supplemented as "Low Se" (Budiman *et al.* 2009). Since 10% FBS has been widely accepted as the standard concentration of growth factors and hormones necessary for a healthy cell line, the Se content in such media is in turn considered adequate. While Se supplementation provides a change in the Se content, the absence of supplementation doesn't accurately represent Se deficiency. In order to create such a model, we reduced the concentration of FBS to 1% in our media and waited until the cells reached confluence before applying the reduced serum media to them. Since comparing 1% FBS with any higher concentration of FBS would present too many variables due to the presence of several factors in addition to Se, we supplemented 1% FBS with sodium selenite. Growing the cells to confluence before switching media minimizes the potential detrimental effect of reduced serum and this was confirmed by maintaining the cells in media plus 1% FBS for

five additional days, over which they appeared healthy and did not detach from the dish (Data not shown).

Our analysis of RNA abundance after 48 hours of being incubated in reduced serum with or without additional Se showed significant changes in most of the mRNAs predicted sensitive to NMD. None of the mRNAs that were predicted to be resistant to NMD changed in response to Se, which supports a role for NMDs involvement under conditions of limiting Se. Of those that were predicted sensitive, only GPx4 and SelN mRNAs did not respond to Se. GPx4 mRNA's stability has been reported in other studies but a mechanism underlying the effect is yet to be elucidated. In terms of GPx4's function, remaining stable under low Se would prove beneficial as it is essential towards maintaining cellular integrity by relieving oxidative stress in lipids that make up crucial biomembranes. A possible explanation for this observed stability will be discussed further in Chapter 3. In addition to the correlated response of the mRNAs predicted sensitive and resistant to NMD, the response of the pre-mRNAs provides additional support for a role that NMD could be playing when Se is deficient. Since the pre-mRNA transcripts remain steady in abundance with respect to Se status, the mRNA changes are likely due to a post transcriptional effect. While these data provide support for a role of the NMD pathway in regulating selenoprotein mRNAs under Se deficiency, mechanistic evidence is still lacking. A few key factors have been identified as necessary for NMD activation and these will be utilized to further assess the role of NMD in regulating Se-responsive selenoprotein mRNAs.

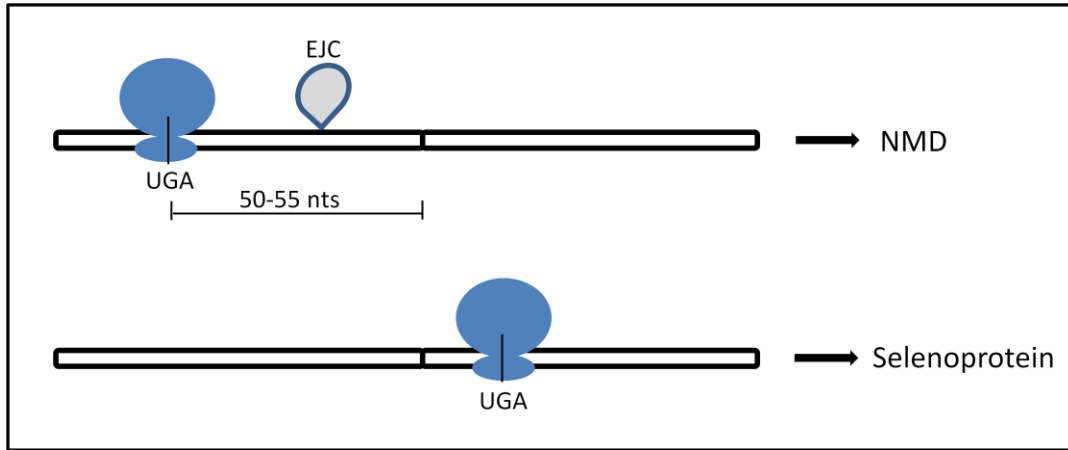
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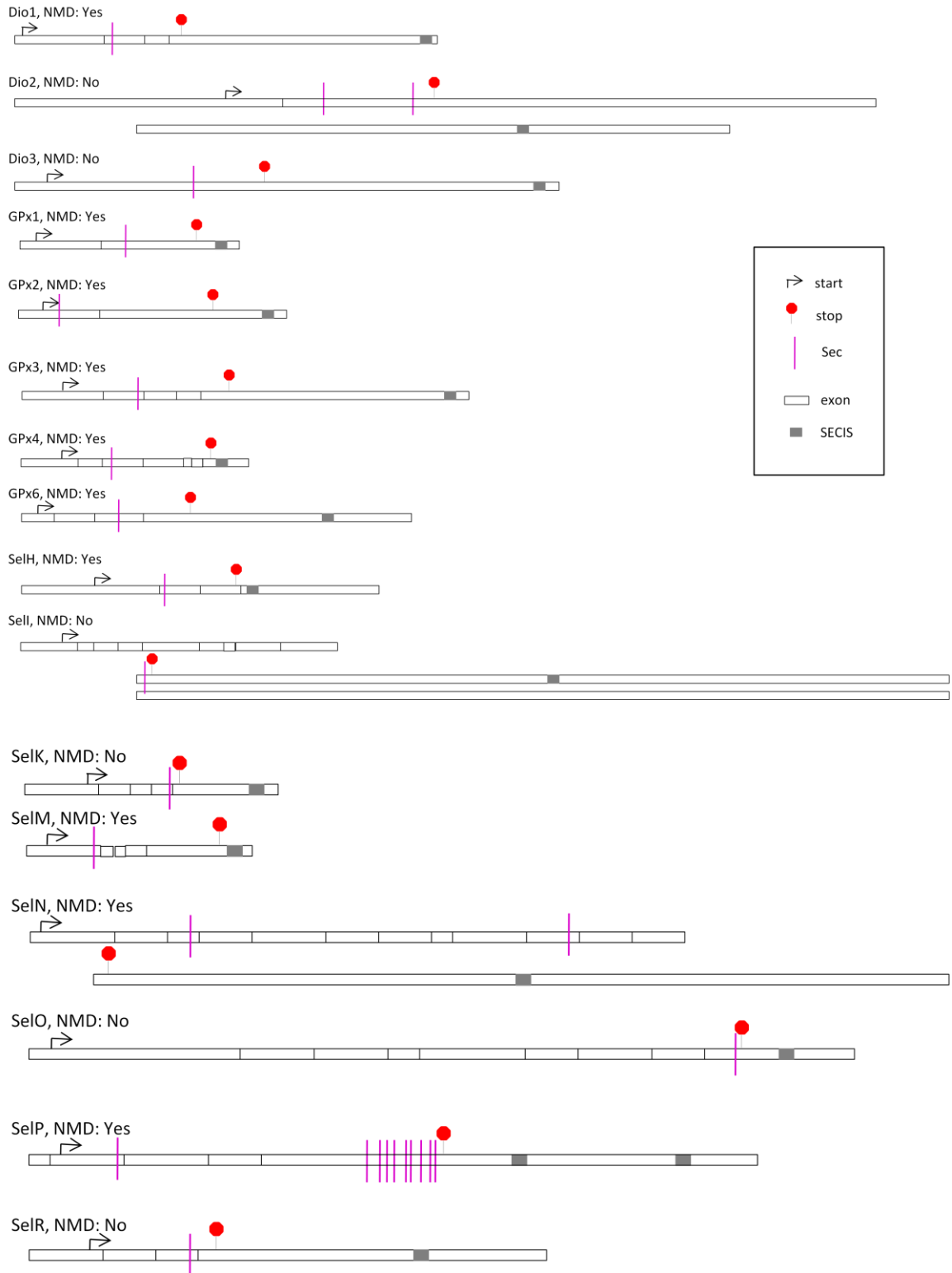
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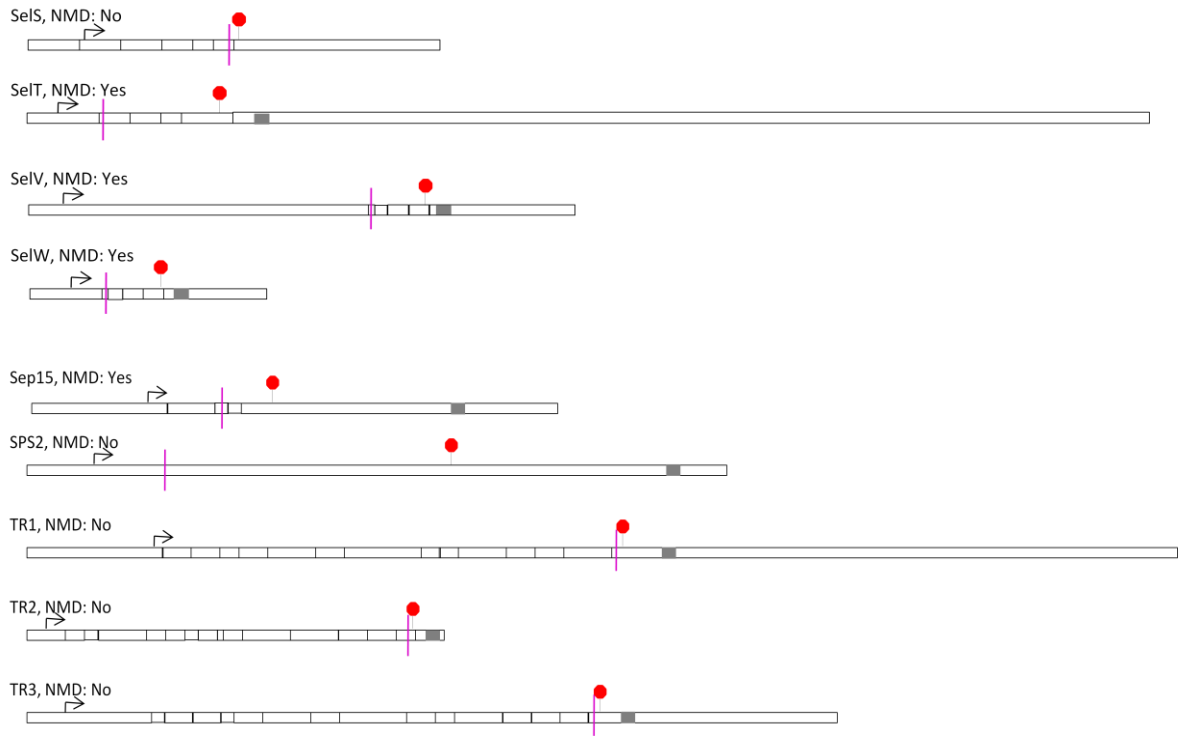


## 2.7 FIGURES

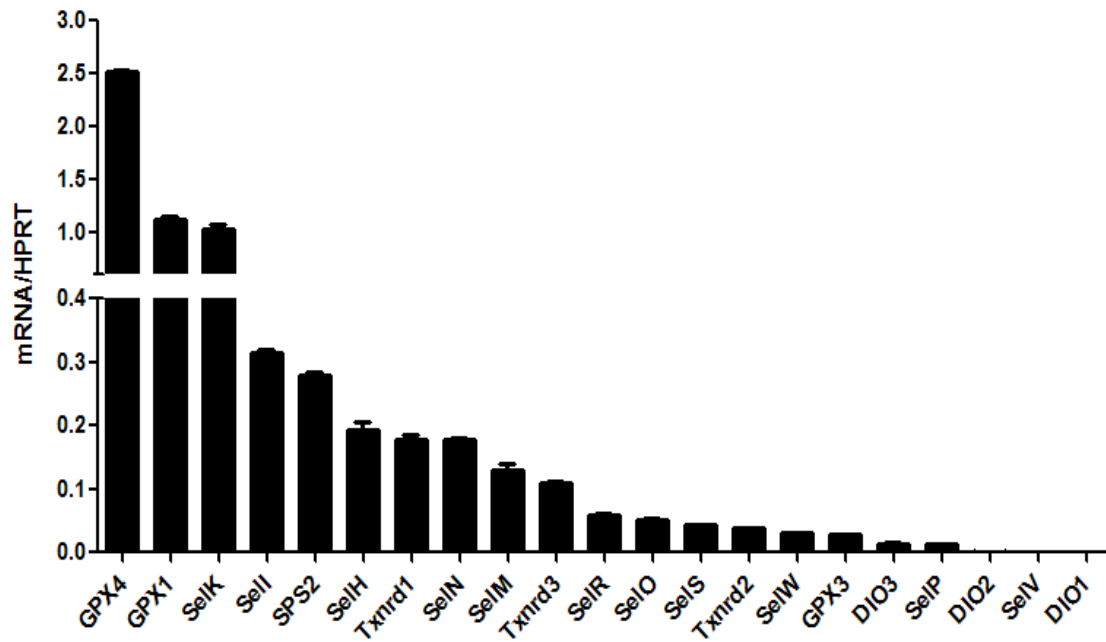


**Figure 2.1 Location of Sec UGA codon determines fate of transcript.** Sec codons located within 50-55 nucleotides of the last exon are predicted to be sensitive to NMD.

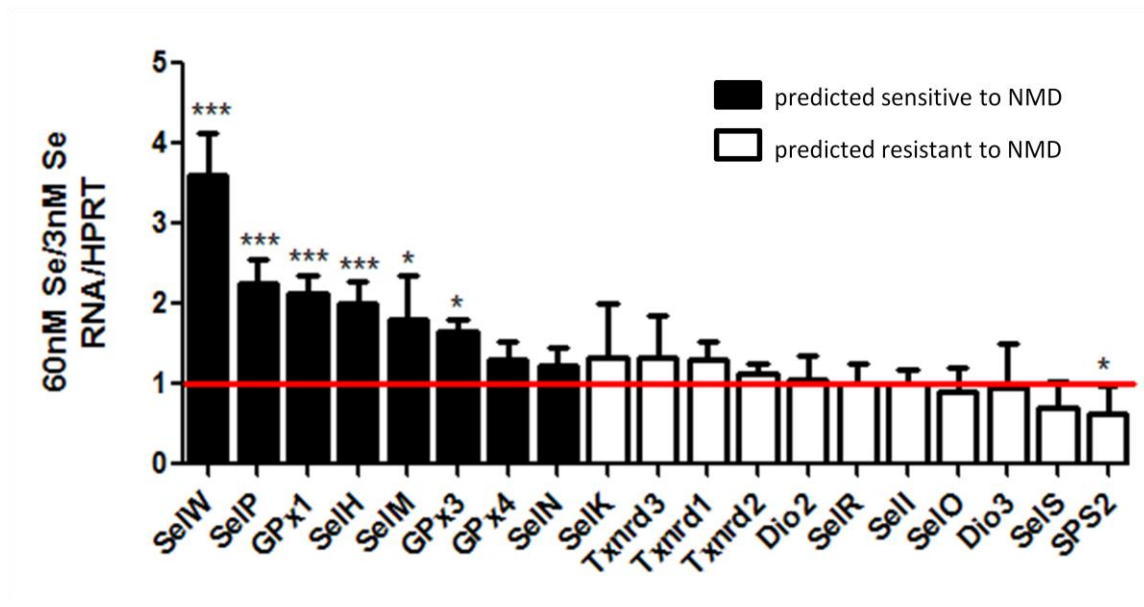




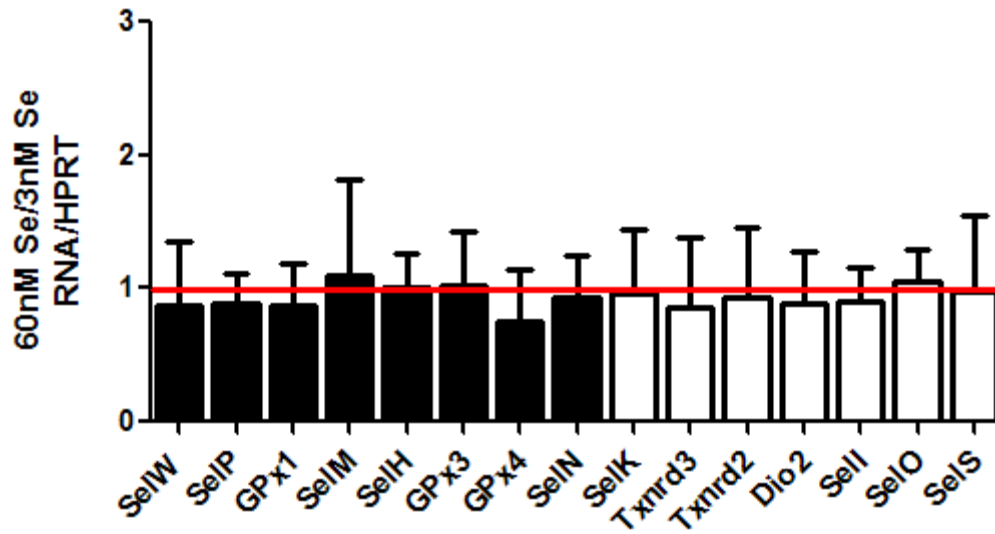
**Figure 2.2. mRNA maps of the human selenoprotein transcriptome.**



**Figure 2.3 Selenoprotein mRNA abundance in HEK293T cells.** HEK293T cells were grown to confluence in Dulbecco's modified eagle medium with 10% Fetal Bovine Serum and harvested for total RNA.



**Figure 2.4. Fold change of a subset of selenoprotein mRNAs in high versus low Se.** Levels of selenoprotein mRNAs in 60nM Se versus 3nM Se in HEK293T cells. n=6, transcript levels are normalized to HPRT. \*\*\* p<.001, \*p<.05, error bars represent standard deviation of the mean.



**Figure 2.5. Fold change of a subset of selenoprotein mRNAs in high versus low Se.**

Levels of selenoprotein pre-mRNAs in 60nM Se versus 3nM Se in HEK293T cells. n=6, transcript levels are normalized to HPRT. Error bars represent standard deviation of the mean.

**TABLE 1.** PCR primer sequences, written 5' to 3'

Gene	Primer Sequence	
	Forward	Reverse
Dio1	CACTGCCTGAGAGGCTCTACATA	TGTAGTTCCAAGGGCCAGAT
Dio2	CCTCCTCGATGCCTACAAAC	TCCTTCTGTAAGGAGACATGC
Dio3	AACTCCGAGGTGGTTCTGC	TTGCGCGTAGTCGAGGAT
GPx1	GCAACCAGTTTGGGCATCAG	CGTTCACCTCGCACTTCTCG
GPx3	CCAGTACATTGAACTGAATGCAC	CTGGTCGGACATACTTGAGG
GPx4	GTAACCAGTTCGGGAAGCA	CTTCATCCACTTCCACAGCG
HPRT	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
SelH	ACCCAAATCTCCCTACGACAGG	GCTTCCAGTAAAGGTGAACCCG
SelI	GAGACCTATTCACTGCAATG	AAACTCATTGGAAGAGTCACAC
SelK	ATCTGATTCCAGATATGATGATGG	TGATTGATTCTACCCATTCTTCG
SelM	TCCCGATGAGCCTCCTGTTG	ATAGAATGTCCTGCGTGACG
SelN	AAGGGCAAGGAGGTCATCATCC	AGGGGAGAACCAAAAGGGGAA
SelO	CAGAAGATGCGCAGGAAGCTG	GCTCAGCAAGTAGAAGGTGTTTGTG
SelP	AATGTGGAAACTGCTCTCTCACG	GCTCCTGGTTGCTGATTCTCTG
SelR	GAGGTTTTGCAGAATCACTTTGA	GGCCATGGAGACGAGTGT
SelS	GGAGACAGAAGATTGAAATGTGGG	CAGGACAGATGAAGTGGGAAGG
SelW	GGCTACAAGTCCAAGTATCTTCAG	CACTTCAAAGAACCCGGTGG
SelV	GACCTACTGTGGCCTCTGAA	CCCGTTCACAAACACCTCAAAC
SPS2	TGGAAAAGGGAAACCGAACGG	ACGAGCTAGGCTCAGAGGAG
Txnrd1	CCTTATCATCATTGGAGGTGGCTC	AAGAGGGGTGGGAGTGACAAAGTC
Txnrd2	CACATCTACGCCATTGGTGACG	AGACGGTCGTGGGAACATTGTC
Txnrd3	AAGGAAATTTGGCTGGGAAT	GGTTCTGAATCGCTTTTGTCA

## CHAPTER 3

### NONSENSE-MEDIATED DECAY FACTORS ARE INVOLVED IN THE REGULATION OF SELENOPROTEIN mRNAs DURING SELENIUM DEFICIENCY

#### 3.1 ABSTRACT

Selenoprotein mRNA susceptibility to the nonsense-mediated mRNA decay pathway during Se deficiency was further assessed using key NMD factors. RNA immunoprecipitation using central NMD factor UPF1 was performed during Se deficiency as well as siRNA-mediated knockdown of the UPF1 kinase, SMG1. We found that selenoprotein mRNAs that were predicted sensitive to NMD, and responded significantly to cellular Se status, were more abundantly bound to UPF1 during Se deficiency than those that showed no Se response and were predicted resistant to NMD. In addition, siRNA-mediated knockdown of SMG1, the kinase responsible for UPF1 phosphorylation and NMD activation, abrogated the Se response of the NMD-sensitive mRNAs.

#### 3.2 INTRODUCTION

Se deficiency has been linked to several human health conditions including Keshan disease, Kashin-Beck disease, hypothyroidism, male infertility, Graves orbitopathy, and an increased potential to miscarriage (Rayman 2000, Schomburg 2012, Mistry *et al.* 2012, Nicoll *et al.* 1999, Marcocci *et al.* 2011, Chen 2012, Foresta *et al.* 2002). While the molecular mechanisms underlying most of these conditions are unknown, they are undoubtedly related to the essential properties of key selenoproteins. Although most selenoproteins have uncharacterized functions, the best studied are enzymes that possess an oxido-reductase function that utilizes the catalytic efficiency of Sec at the active site. For example, the GPxs play an important role in maintaining cellular homeostasis by reducing harmful reactive oxygen species to water using glutathione as the reducing agent (Brigelius-Flohe & Maiorino 2013). In addition to catalase and superoxide dismutase, the GPx system comprises the main antioxidant



system that a cell possesses (Fig 3.0). A somewhat different example of how Sec is utilized in essential enzymatic reactions is demonstrated by the iodothyronine deiodinases. There are currently three known iodothyronine deiodinases (DIO1, DIO2 and DIO3) which are all selenoproteins that play a crucial role in thyroid hormone activation and deactivation. The major thyroid hormones, triiodothyronine and thyroxine (T3 and T4 respectively), which are produced by the thyroid gland, play integral roles in metabolism. The prohormone T4, is converted to the active and more potent T3 by DIO1 and DIO2 while DIO3 functions in deactivation (Fig 3.1)(Darras & Van Herck 2012). These two systems exemplify the importance of selenium in a healthy functioning organism.

While the macromolecular level of selenoproteins demonstrates how a lack of Se would influence vital physiological processes, the molecular mechanisms underlying these effects are complicated due to the unusual nature of Sec's incorporation into a polypeptide. Since Sec cannot be synthesized *de novo* like most of the other essential amino acids, its unique and stringent biosynthetic pathway, which takes place on tRNA<sup>sec</sup> (See Ch.1, section 1.4), makes cellular Se status crucial. Although pathways for Se recycling and metabolism are yet to be elucidated, it is presumed that Se must go through selenophosphate, tRNA<sup>sec</sup>, and a full selenoprotein, before it can be liberated for re-use after protein degradation. The lack of a free floating Sec pool makes Se utilization a sensitive process that is subject to the limits of environmental Se status. Experiments investigating the effects of Se deficiency at the molecular level have found a non-uniform response whereby certain selenoprotein mRNAs decline in abundance while others do not (Sunde *et al.* 2009, Yan *et al.* 2013). The NMD is generally implicated in this response because the canonical stop codon UGA serves redundantly as the triplet for Sec. When we categorized selenoprotein mRNAs as being predicted sensitive or resistant to NMD based on the current mammalian model, we found that about half were predicted sensitive while the other half were predicted resistant (Ch.2, Fig 2.1). After modeling Se deficiency in HEK293T cells, we observed a significant decline in the majority of the mRNAs that were predicted sensitive but not in those predicted resistant

(Ch.2, Fig 2.3). While this is indicative of NMD's involvement in the decline of the transcripts during Se deficiency, further examination is necessary to provide mechanistic evidence.

The NMD pathway has been extensively studied in the past decade and it is still currently under scrutiny. While many details remain unclear, much has been elucidated regarding the requirements that predispose an mRNA for targeted degradation. In addition, the pathway differs slightly between lower organisms such as yeast, *Drosophila* and *C. elegans* and that of mammals (reviewed in Schweingruber *et al.* 2013). This dissertation will focus on the mammalian model of NMD. It is currently understood that mammalian NMD begins in the nucleus during splicing. The spliceosome deposits a multimeric complex of proteins about 20-24 nucleotides upstream of exon junctions that has been termed the exon junction complex (EJC) (Le Hir *et al.* 2000). The exact constituents of this complex are not clear but the core include the D-E-A-D box RNA helicase eIF4a3, the heterodimer MAGOH/Y14, RNA transport protein Barentz (BTZ), as well as UPF2 and UPF3 (Maquat 2004). eIF4a3 is an ATP-dependent RNA helicase that remains clamped onto the RNA as long as the ATP is unhydrolyzed (Shibuya *et al.* 2006). EJC crystal structure shows MAGOH/Y14 bound to eIF4a3 in a manner that stabilizes the ATP (Buchwald *et al.* 2010, Bono *et al.* 2006). If this association is disrupted, the ATP will be hydrolyzed and eIF4A3's helicase activity is thought to be initiated as a result. The exact spatial and temporal order of EJC assembly is not known, however, it is believed that eIF4A3, MAGOH/Y14, UPF2, and UPF3 are deposited during splicing in the nucleus while BTZ joins after export (Gehring *et al.* 2009a).

Once the EJC-containing mRNAs are exported for translation, the first ribosome will dock to begin a pioneer round of translation (Ishigaki *et al.* 2001). It is during this initial round of translation that the mRNA may be recognized as aberrant. The ribosome associated protein PYM functions to disassemble EJCs from a distance of approximately 30 nucleotides (Gehring *et al.* 2009b). All EJCs will be removed from normal mRNAs and

bulk translation will then ensue. If the ribosome arrives at a premature termination codon (PTC) it will stall and a release factor will bind along with another D-E-A-D box RNA helicase, UPF1, and UPF1's kinase, SMG1. It is currently believed that the UPF1/ribosome/EJC complex initiates UPF1 phosphorylation by SMG1 and because of this, UPF1 is widely regarded as the central regulator of NMD. This phosphorylation event in turn begins a cascade toward mRNA degradation that signifies NMD.

In order to further assess NMDs involvement in regulating selenoprotein mRNA levels during Se deficiency, we sought to investigate the role of the key factors UPF1 and SMG1. We performed RNA immunoprecipitation with UPF1 in order to assess potential differences in UPF1-bound RNAs during Se deficiency. We followed this by siRNA knockdown of SMG1 also under conditions of Se deficiency. We found that when Se is low, selenoprotein mRNAs predicted to be sensitive to NMD are more abundantly bound to UPF1 compared to those predicted resistant. In addition, siRNA-mediated knockdown of SMG1 abrogated the Se response of the selenoprotein mRNAs that were predicted sensitive to NMD.

### **3.3 MATERIALS AND METHODS**

#### *RNA Immunoprecipitation (RIP)*

UPF1 antibody (anti-RENT1, A301-902A, Bethyl labs) was conjugated to magnetic Dynabeads® (Invitrogen) using 5 mM BS3 (Proteochem), reaction was quenched with Tris-HCl pH 7.5, washed twice with cold PBS and resuspended in cytosolic lysis buffer (50 mM HEPES, 5 M NaCl, 75 mM NaF, 10 mM iodoacetamide, 0.05% Triton X-100, 1x Protease Inhibitor Cocktail set II [EMD Biosciences], 240 Units RNase Inhibitor per sample [Applied Biosystems]).

HEK293T cells were plated in 10 cm tissue culture plates, and cultured with sodium selenite as described above. After the 48 h incubation with 1% FBS in the presence or absence of Se (60 nM Se or 3nM Se), cells were washed twice with cold PBS. Cold cytosolic lysis buffer was then added directly to each dish. The dishes containing

lysis buffer were gently rocked for 2.5 min, and then incubated for another 2.5 min. The supernatant was aspirated and cellular debris was removed after 10 seconds of snap centrifugation on a table-top microcentrifuge. Remaining lysate was collected and split in half. Trizol was added directly to one half for total RNA analysis (Supplemental Fig. S1) and the other half was used for the RIP. Protein was quantified using an ND1000 spectrophotometer. Lysate was added to the antibody-magnetic bead conjugate described above followed by incubation on ice with rotation for 1 h. Samples were then split in half into individual tubes and placed on the DynaMag™-2 magnet (Invitrogen) for collection. RNA/bead conjugates were washed twice with cold PBS and one half was used for SDS-PAGE and western blot (Supplemental Fig. S2). Trizol was added directly to the other half for RNA extraction. A random mouse DNA plasmid was used for in vitro RNA synthesis and this RNA was added to the Trizol to serve as an internal control for the RNA extraction (Labeled mG1 in Table 1). RNA extraction was then carried out according to manufacturer's instruction and cDNA was synthesized followed by qPCR as previously described.

#### *SMG1 knockdown*

HEK293T cells were cultured in DMEM and 10% FBS until approximately 40% confluence and then transfected with SMG1 siRNA (Qiagen) at a final concentration of 20 nM using RNAimax reagent (Invitrogen) according to manufacturer's instructions for a forward transfection. After 24 h, cells were washed with PBS and transfected with SMG1 siRNA again under the same conditions as the previous day. Another 24 h later media was changed to fresh DMEM with 10% FBS. Twenty-four h later media was changed to DMEM plus 1% FBS with or without the addition of sodium selenite and cells were cultured for 48 h. Cells were then washed with PBS and harvested for RNA and protein analysis. Protein was extracted using Cell Lytic MT Cell Lysis Reagent (Sigma-Aldrich) according to manufacturer's instructions and RNA was extracted with Trizol according to manufacturer's instructions. RNA quantity and quality were measured

using a ND1000 spectrophotometer. cDNA synthesis and qPCR were carried out as previously described.

#### *SDS-PAGE and Western Blot*

Protein extracted using the above method was added to reduced Laemmli buffer (Bio-Rad), boiled for 5 min and loaded into 4-20% gradient polyacrylamide gels (Bio-Rad). Following electrophoresis, gel contents were transferred to a PVDF membrane (Millipore) and blocked with Odyssey Blocking Buffer (Li-Cor Biosciences) for 30 min. Membranes were then probed for proteins with the following primary antibodies: SMG1 (dilution: 1:500; A300-394A, Bethyl labs) UPF1 (dilution: 1:1000; anti-RENT1, A301-902A, Bethyl labs), Grb2 (dilution: 1:1000; Upstate Cell Signaling), alpha-Tubulin (1:10,000; Novus Biologicals); and with the following secondary antibodies: goat anti-rabbit IRDye® 680 (dilution: 1:10,000, Li-Cor Biosciences), goat anti-mouse IRDye® 680 (dilution: 1:10,000, Li-Cor Biosciences), goat anti-mouse IRDye® 800 (dilution: 1:10,000, Li-Cor Biosciences). All protein quantification was carried out using Odyssey's Image Studio version 3.0 (Li-Cor Biosciences).

### **3.4 RESULTS**

#### *Selenoprotein mRNA enrichment on UPF1 correlates with mRNA responses to selenium status*

Although the data from Chapter 1 suggests a relationship between NMD and selenoprotein mRNAs, they lack a mechanistic link. In order to investigate a potential mechanism, we directed our focus to key NMD mediator protein UPF1. UPF1 belongs to superfamily I of RNA helicases and studies have identified it as the central regulator of NMD (Chakrabarti *et al.* 2011, Hwang *et al.* 2010). We reasoned that if the transcripts that decreased in low Se were undergoing NMD, then they should similarly be enriched on UPF1. The same experiment modeling conditions of low Se as in Chapter 2 was repeated and RNA immunoprecipitation was performed with UPF1. The cytoplasmic nucleoprotein complexes were immunoprecipitated with an anti-UPF1 antibody,

followed by quantification of coimmunoprecipitated RNAs by qPCR. A representative set of RNAs from each category of NMD susceptibility was analyzed including those most responsive to Se status (SelW, SelP, GPx1) as well as GPx4, which has been consistently unresponsive to Se status in past research. Interestingly, all of the selenoprotein mRNAs we analyzed coimmunoprecipitated with UPF1 to some degree in agreement with UPF1's general role in translation (Ghosh *et al.* 2010). A strikingly similar pattern to the profile in Figure 1A was observed in response to Se status. In this case, the RNAs predicted resistant to NMD showed no difference in their enrichment on UPF1 with respect to Se status. The RNAs that were the most responsive to Se status however, were also the most enriched on UPF1 under conditions of low Se (Fig. 2.1A). Importantly, UPF1 levels did not change with Se, ruling out the possibility that changes in immunoprecipitated RNAs could have been due to changes in total UPF1 protein (Figs. 2.1B, C). Overall, the degree of responsiveness to Se status directly correlated with the degree of enrichment on UPF1, suggesting the involvement of NMD in the regulation of selenoprotein mRNAs during Se deficiency.

#### *SMG1 knockdown abrogates Se responsiveness of mRNAs sensitive to NMD*

To further investigate the mechanistic link between NMD and selenoprotein mRNAs, we knocked down expression of SMG1 under the same Se conditions. SMG1 is a phosphatidylinositol-kinase-related kinase that phosphorylates UPF1 to initiate the decay cascade and has been shown to be necessary for NMD (Usuki *et al.* 2011, Yamashita *et al.* 2001). If NMD is involved in Se regulation of responsive transcripts such as SelW and SelP, then knocking down SMG1 should alter their response. HEK293T cells were again cultured under standard conditions to approximately 40% confluence and transfected with SMG1 siRNA using a two hit protocol (see Materials and Methods). Two days after the second transfection, the media was changed to Se-deficient or Se-supplemented as in the previous two experiments. qPCR was performed on the same representative transcripts from each category of predicted NMD susceptibility. SMG1 did not change with Se status and protein quantification after the final 48 h of

incubation showed almost 90% knockdown regardless of Se conditions (Fig. 2.2). Of those transcripts that were predicted sensitive to NMD and were responsive to Se, SMG1 knockdown restored SelW, SelP and GPx1 mRNAs to levels that were no longer statistically different from Se-supplemented levels. GPx1 and SelP mRNAs in 3 nM Se were increased with SMG1 knockdown to levels nearly identical to the levels in 60 nM Se. SelW mRNA was also increased with SMG1 knockdown in 3 nM Se albeit not to levels equivalent to that of 60 nM Se. The levels of these NMD sensitive mRNAs in 60 nM Se were unchanged with SMG1 knockdown suggesting that under conditions of Se-supplementation they are not undergoing NMD (Fig. 2.3A). SMG1 knockdown did not have an effect on the Se response of the transcripts predicted to be NMD resistant (Fig. 2.3B). Surprisingly, it significantly upregulated SelO mRNA independent of Se concentration. This result is unexpected and will require further research to elucidate the mechanism underlying this effect.

### **3.5 DISCUSSION**

Se-dependent regulation of selenoprotein mRNAs has long been observed and NMD is generally regarded as a factor contributing to this (Sun *et al.* 2001, Banerjee *et al.* 2012, Howard *et al.* 2013, Sunde *et al.* 2009, Sun & Maquat 2002). Since all selenoprotein mRNAs essentially have at least one PTC, it is logical to assume that NMD would target these transcripts for decay. In accordance with the mammalian model of NMD, our categorization of selenoprotein mRNAs as being predicted sensitive or resistant depends on the location of the Sec codon relative to exon junctions. Since almost half of selenoprotein mRNAs have their Sec codon in the last exon they are automatically considered immune to being degraded by NMD. This distribution of NMD susceptibility may be an evolutionary development that could represent the relative importance of each selenoprotein when environmental constraints limit the availability of Se. When we modeled conditions of low Se, we see that those transcripts predicted resistant to NMD remain stable while those predicted sensitive respond to varying degrees (Ch.2, Fig. 2.1). SelW responds to the greatest extent, followed by SelP, GPx1

and SelH. SelW has similarly been reported by others to be the most sensitive to changes in Se status and the biological significance of this extreme sensitivity is worth investigation (Howard et al. 2013, Sunde et al. 2009, Yan et al. 2013). Conversely, GPx4 is predicted to be sensitive to NMD but it remains steady with changes in Se status. Of the selenoprotein mRNAs predicted to be resistant to NMD, none responded to changes in Se except SPS2. SPS2 mRNA is the only one that increases under conditions of low Se, which suggests autoregulation, possibly linked to the fact that it is an essential factor in Sec-tRNA biosynthesis (Ehrenreich *et al.* 1992, Itoh *et al.* 2009, Veres *et al.* 1994).

When selenoprotein mRNAs were coimmunoprecipitated with NMD factor UPF1, all transcripts analyzed were observed to coprecipitate to some degree. UPF1 is a member of the DEAD box family of RNA helicases and in addition to its role in NMD, it has also been implicated in several other cellular functions such as translation stabilization, E3 ubiquitin ligation, cell cycle progression, HIV RNA metabolism, Staufen-mediated decay, telomere stability and splicing (Takahashi *et al.* 2008, Ajamian *et al.* 2008, Azzalin & Lingner 2006, Chawla *et al.* 2011, de Turrís *et al.* 2011, Ghosh et al. 2010, Kim *et al.* 2005, Reviewed in Imamachi et al. 2012). UPF1 has been shown to associate with unspliced pre-mRNAs in the nucleus in addition to mature mRNAs in the cytoplasm (de Turrís *et al.* 2011) and it likely binds a large variety of cellular mRNAs. When conditions of low Se were modeled, the selenoproteins that responded the most to Se status were also the ones that were the most enriched on UPF1 (Fig. 2.1A). Although the magnitude of response to Se status in Figure 1 is not identical to the degree of enrichment on UPF1 in low Se, the overall profile of the two results taken together provides a strong correlation between NMD and selenoprotein mRNA levels when Se is limiting.

Knocking down SMG1 under conditions of low Se restored the mRNAs of the Se sensitive transcripts to levels comparable to those at 60 nM Se (Fig. 2.3C), strongly suggesting a role for NMD in decreasing Se sensitive transcript levels during Se deficiency. The knockdown had no effect on the mRNAs when Se was supplemented,



suggesting that when Se is readily available the mRNAs bypass NMD. This agrees with the current understanding of the NMD pathway taken together with selenoprotein biosynthesis. These two are constantly in competition with each other since Sec is encoded by UGA. GPx1 mRNA has been shown to be subject to NMD in other reports (Moriarty *et al.* 1998, Sun *et al.* 2001, Sun *et al.* 2000, Usuki *et al.* 2011) and it would not be surprising that other selenoprotein mRNAs would similarly be targeted. What remains puzzling is how GPx4, whose transcript is a predicted NMD target, remains stable regardless of Se status or SMG1 knockdown (Figs. 1.1A, 2.1A, 2.3A). One possible explanation would be that it is under the influence of some other mode of regulation that takes precedence over NMD. A recent study posits that under Se deficiency, eIF4A3, a core component of the EJC, is upregulated and binds the SECIS element of GPx1 but not that of GPx4, competing for binding of the Sec-incorporation factor SBP2, and ultimately inhibiting Sec incorporation (Budiman *et al.* 2009). Although this is a plausible theory, it conflicts with a previous study (Weiss & Sunde 1998) showing that the GPx4 3'UTR is not able to stabilize the Se responsiveness of GPx1 mRNA. Another explanation could relate to the relative abundance of each of two isoforms of tRNA<sup>Sec</sup>. tRNA<sup>Sec</sup> exists as two isoforms that differ in the methylation state of the wobble base, U34. The abundance of each isoform has been shown to correlate with Se status and this could be an intermediate factor in susceptibility to NMD. The methylated isoform has been shown to be abundant when Se is sufficient and diminished when Se is limiting (Carlson *et al.* 2009, Jameson & Diamond 2004, Howard *et al.* 2013). Further, the synthesis of stress related selenoproteins such as SelW and GPx1 requires the methylated isoform while the synthesis of housekeeping selenoproteins such as GPx4 and Txnrd3 do not (Carlson *et al.* 2009). SelW and GPx1 are predicted to be sensitive to NMD while Txnrd3 is predicted to be resistant. Degree of sensitivity to Se status is thus potentially a result of both mRNA architecture in predisposing transcripts to NMD as well as tRNA isoform specificity. This means that when Se is limiting, the abundance of the methylated isoform is decreased and those mRNAs that require this isoform and are also sensitive to NMD will be rapidly degraded (e.g. SelW, GPx1). In the case of GPx4,

although it is predicted susceptible to NMD, it does not require the methylated tRNA for its synthesis. Since the non-methylated tRNA is abundant in low Se, it could potentially facilitate GPx4 mRNA translation and prevent degradation of the mRNA via NMD. This combination of tRNA isoform abundance and NMD susceptibility likely contributes significantly to the observed hierarchy of selenoprotein mRNA abundance when Se is limiting. In summary, this study is the first to present evidence supporting the involvement of the NMD pathway in the Se-dependent regulation of selenoprotein mRNAs.

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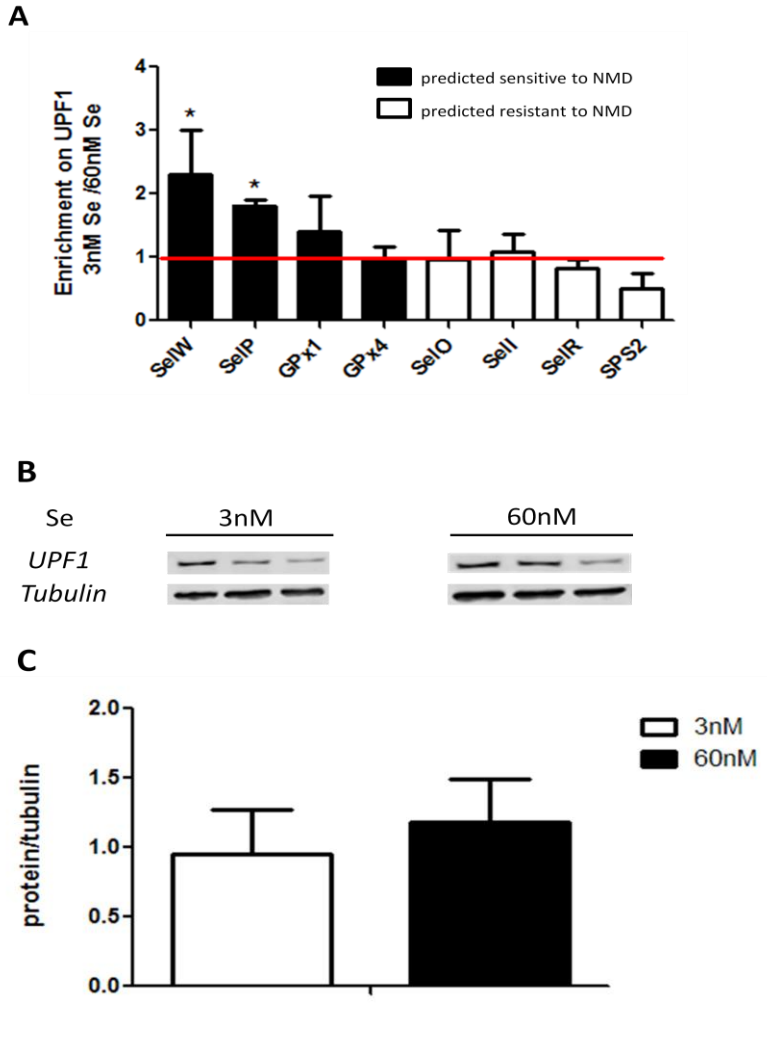
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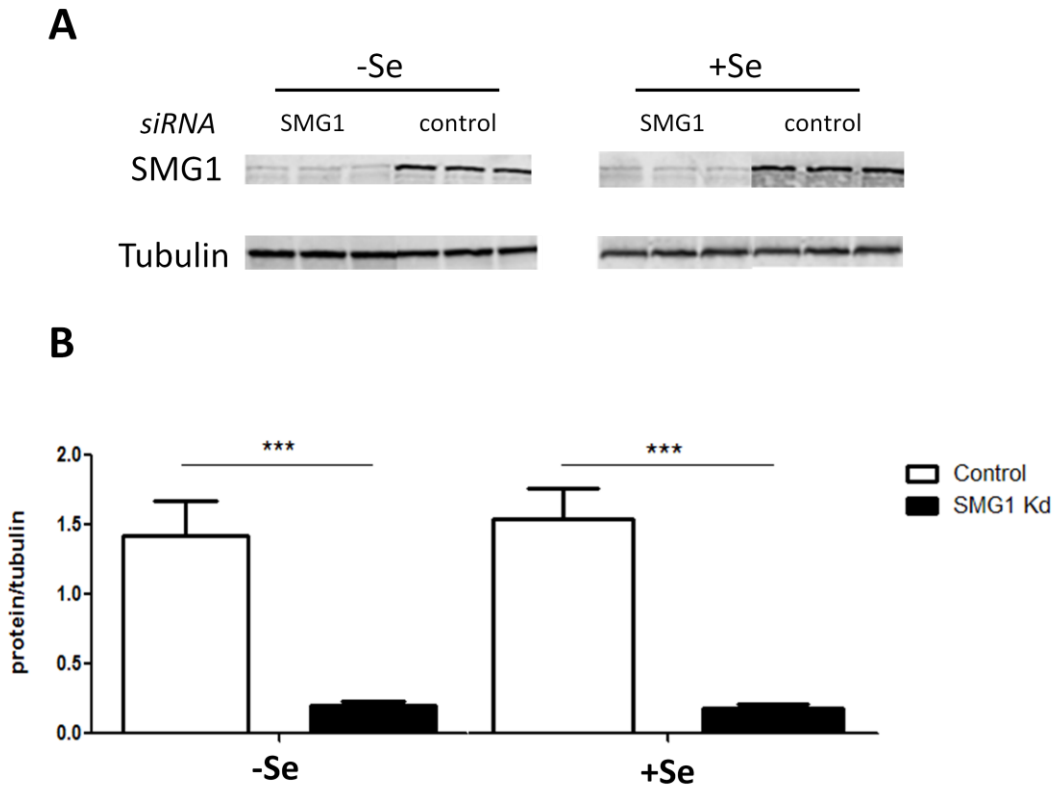
### 3.7 FIGURES



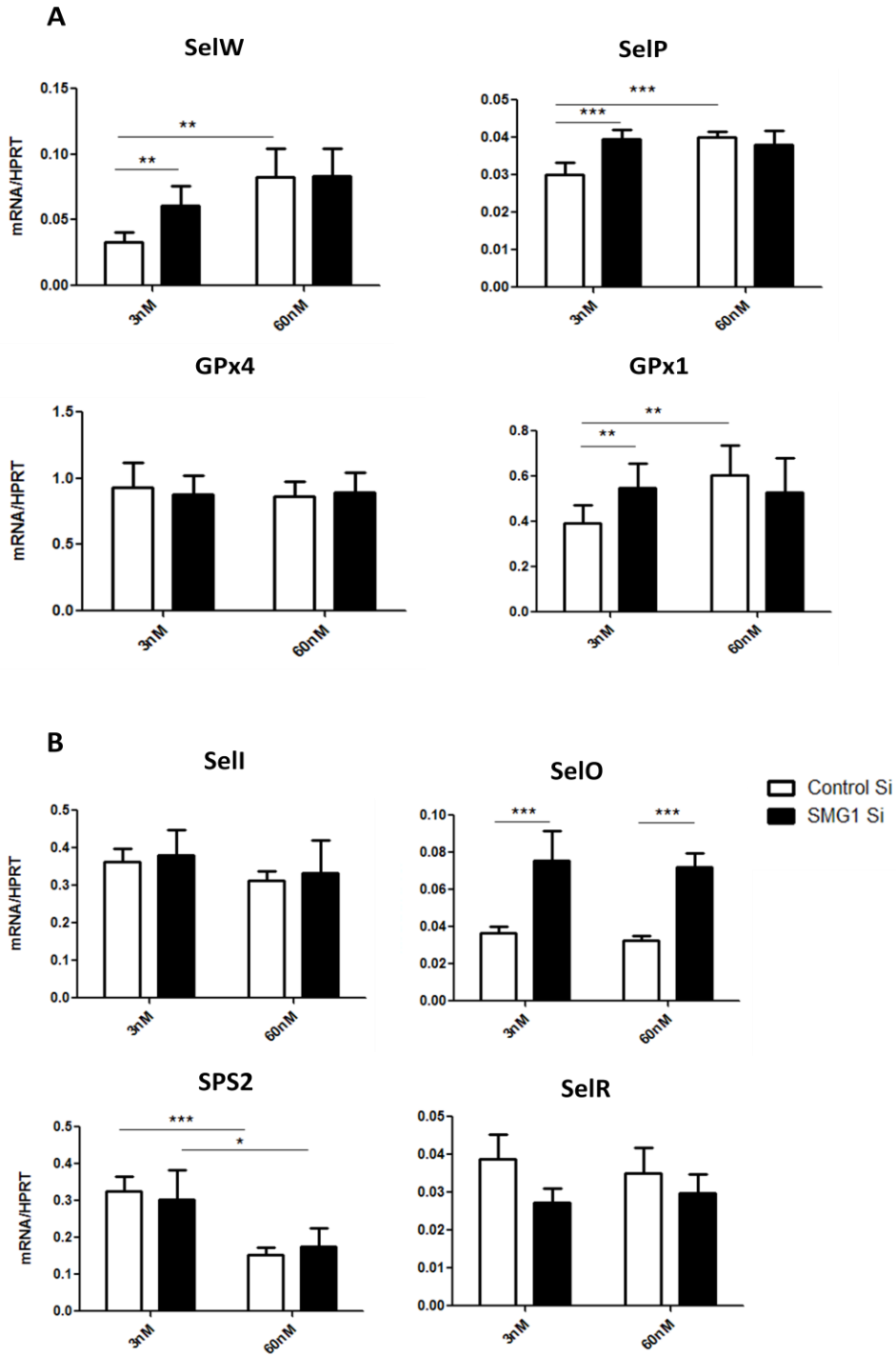
**Figure 3.1 Fold enrichment of selenoprotein mRNAs on UPF1 in low versus high Se.** (A) Levels of selenoprotein mRNAs that coprecipitated with UPF1 following culturing HEK293T cells in 3nM Se or 60nM Se. Transcript levels are normalized to a synthetic in vitro transcribed RNA and then to the value of the corresponding sample from the total RNA extraction (see methods and supplementary figure 3). (B) UPF1 protein levels in

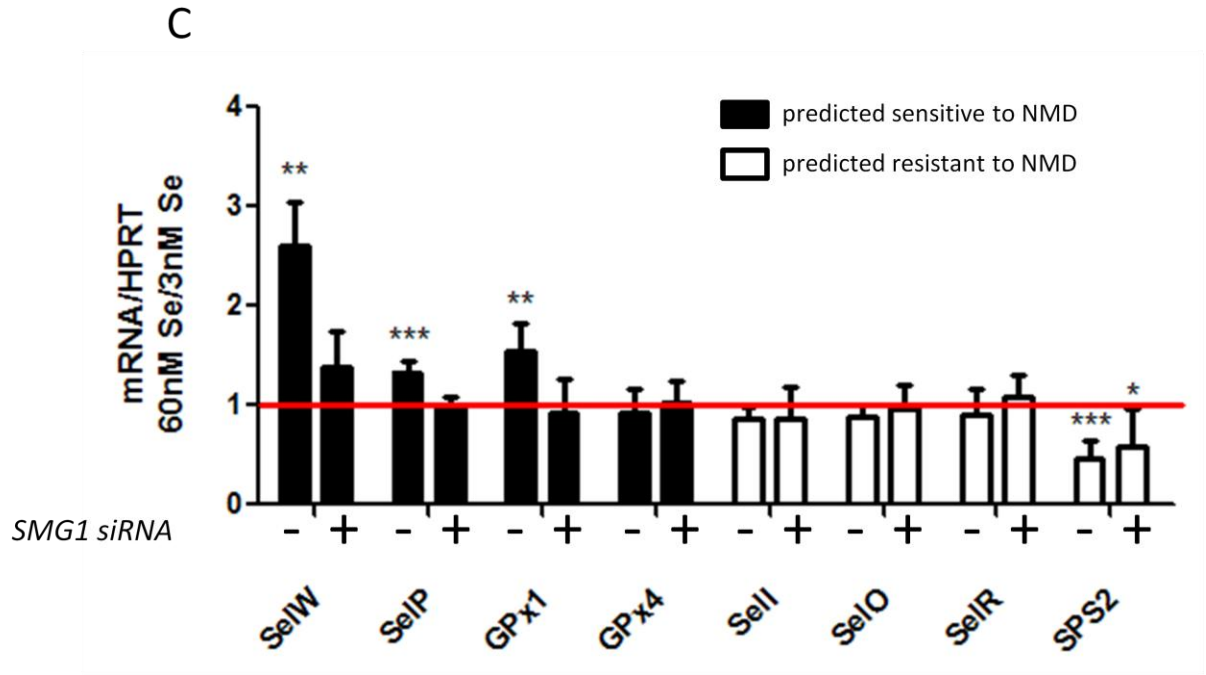


3nM Se or 60nM Se. (C) Quantitation of (B). Error bars represent standard deviation of the mean; n=4 for (A), and 6 for (B) and (C); \*p<0.05

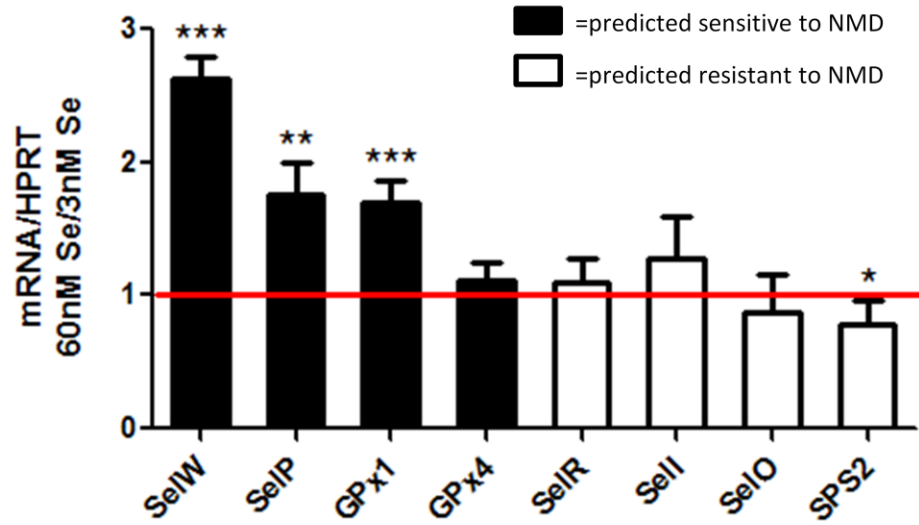


**Figure 3.2 SMG1 knockdown in HEK293T cells.** (A) Western blot of SMG1 protein after knockdown with SMG1 siRNA or non-specific control siRNA. Alpha tubulin was used as a loading control. (B) Quantitation of (A). Error bars represent standard deviation of the mean; n=5; \*\*\*p<0.001

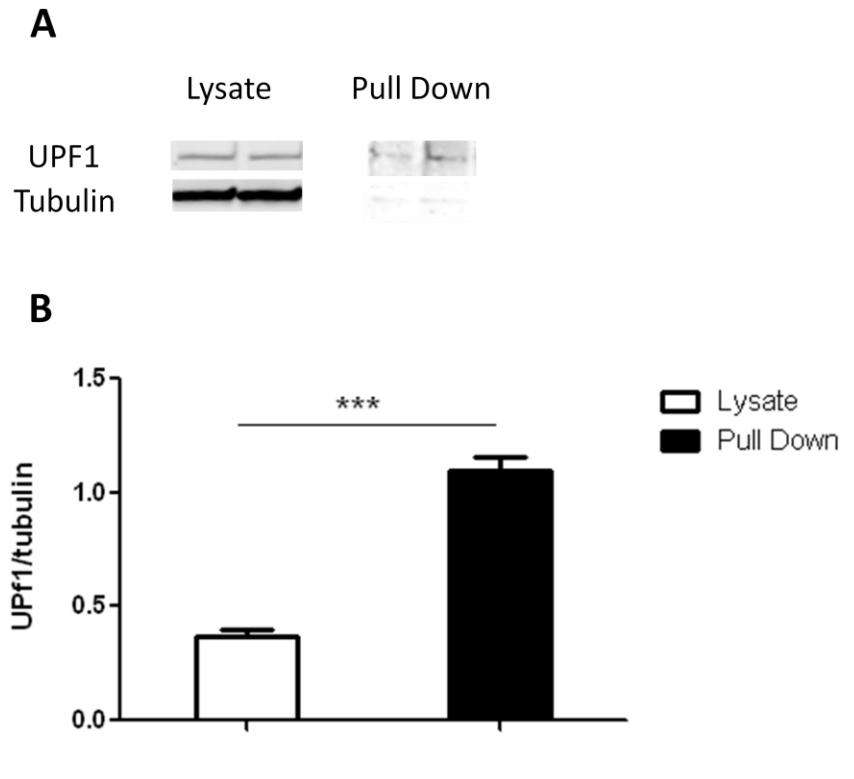




**Fig. 3.3 Effect of SMG1 knockdown on selenoprotein mRNAs.** (A) mRNAs predicted sensitive to NMD (B) mRNAs predicted resistant to NMD. (C) Fold change representation of data from (A) and (B). Transcript levels are normalized to HPRT. Error bars represent a combined standard deviation of the compared means; n=5; \*\*\* p<0.001, \*\*p<0.01, \*p<0.05



**FIGURE S1. Total RNA from RIP.** Total RNA levels from cytosolic lysate preparation. Transcript levels are normalized to HPRT. Error bars represent standard deviation of the mean; n=4; \*\*\* p<.001, \*\*p<.01, \*p<.05



**FIGURE S2. UPF1 in immunoprecipitated fraction vs lysate.** (A) UPF1 is significantly more abundant relative to tubulin in immunoprecipitated fraction compared to whole cell lysate. (B) is quantitation of western blot shown in (A) n=4, \*\*\* p<.001, error bars represent standard deviation of the mean.

## CHAPTER 4

### ANALYSIS OF THE 5'UTR OF GPX4 IN THE STABILITY OF GPX4 MRNA

#### 4.1 ABSTRACT

GPx4 mRNA is similar in structure to that of GPx1 and is predicted to be sensitive to NMD. Significant decreases in abundance have been observed in response to Se deficiency for most of the selenoprotein mRNAs predicted sensitive to NMD, with GPx4 being an exception. Ufer *et al.* in 2008 identified a consensus sequence in the 5'UTR of GPx4 which associates with the protein GRSF1. GRSF1 was reported to recruit GPx4 mRNA to active polysomes and depletion of GRSF1 had similar effects to those observed after depleting GPx4. In addition, of three GPx4 transcript variants, one does not contain the GRSF1 consensus sequence. We sought to investigate the role of the GRSF1 binding site in GPx4's stability in response to Se deficiency. We cloned the GRSF1 consensus sequence onto GPx1 cDNA and saw that there was no difference between the resulting mRNA from this mutant construct and that from the wild type construct in terms of Se responsiveness. In addition, all three GPx4 transcript variants remained stable with changes in Se. Taken together, these data suggest that the GRSF1 consensus sequence is not involved in GPx4 mRNA stability under Se deficiency.

#### 4.2 INTRODUCTION

Selenoproteins are characterized by the presence of the amino acid Sec. Sec is considered the 21<sup>st</sup> amino acid and the stop codon UGA serves as its triplet. Because of this, all selenoprotein mRNAs have a specialized structure in the 3'UTR that facilitates the incorporation of Sec into the peptide chain at designated UGA codons (Tujebajeva *et al.* 2000, Small-Howard *et al.* 2006). The Sec UGA codon which lies within the reading frame may also be considered a nonsense codon and because of this, selenoprotein mRNAs are potential targets of the NMD. Categorization of selenoprotein mRNAs as being predicted sensitive or resistant to NMD based on the rules for the mammalian model identify half of the transcripts as targets for degradation (Ch.2, Fig. 2.1). Modeling

Se deficiency as a preliminary bases to assess NMD predictions showed that the transcripts which were predicted sensitive to NMD change significantly in response to Se while those predicted resistant do not (Ch.2, Fig. 2.3). In addition, immunoprecipitation of the key NMD factor UPF1 showed that transcripts which decreased in abundance with Se deficiency were also more abundantly bound to UPF1 under Se deficiency (Ch.3, Fig. 3.1A). Furthermore, siRNA-mediated knockdown of SMG1, the kinase responsible for UPF1 phosphorylation and NMD activation, abrogated the Se-dependent changes of the NMD-sensitive transcripts (Ch.3, Fig. 3.3C). These results suggest that selenoprotein mRNAs are being degraded by NMD when Se is deficient.

An exception to the expected results is the stability of GPx4 mRNA during Se deficiency (Sunde *et al.* 2009, Sun *et al.* 2001, Yan *et al.* 2013). Although GPx4 mRNA is predicted to be sensitive to NMD, it does not decrease in abundance with Se deficiency (Ch.2, Fig 2.1). Furthermore, GPx4 mRNA coprecipitated with UPF1 equally under Se-replete and Se-deplete conditions and knockdown of SMG1 had no effect on its abundance (Ch. 3, Figs. 3.1A, 3.3C). A report by Ufer *et al.* in 2008 identified a g-rich sequence in the 5'UTR of GPx4 that may potentially be involved in this response (Ufer *et al.* 2008). They found that the protein GRSF1 binds this sequence and recruits GPx4 mRNA to active polysomes for translation. In addition, they found that depletion of GRSF1 in developing mouse brains had similar biochemical effects to depletion of GPx4 and furthermore, these effects were prevented by overexpression of GPx4.

In order to investigate the possibility of GRSF1 being involved in GPx4 mRNA stability, GPx4 transcript variants were analyzed for the presence of the GRSF1 consensus sequence. One of three GPx4 transcript variants differs in the structure of the 5'UTR and lacks the GRSF1 consensus sequence. Primers were designed to the three individual variants and their abundance under Se deficiency was measured. Additionally, mutational analysis of the GRSF consensus sequence in GPx4's 5'UTR was utilized. The g-rich region which GRSF1 binds was cloned onto the 5'UTR of a mouse GPx1 construct. If this region is involved in conferring stability to GPx4 mRNA during Se deficiency, then

we hypothesize that it will abolish the Se-sensitivity of this GPx1 construct. Also, since this consensus sequence has been shown to recruit GPx4 mRNA to active polysomes, we might also predict that it would increase the amount of protein expression of the mutant construct.

Our data show that the GRSF1 consensus sequence has no effect on GPx1 mRNA, however, it appears to be increasing the translatability of the mRNA as shown by protein data. qPCR of the three GPx4 transcripts show none of them to change in abundance in response to Se. Taken together, these results suggest that while this consensus sequence may not be involved in the Se response of GPx4 mRNA, it is likely involved in increasing the translatability of the transcript.

### **4.3 MATERIALS AND METHODS**

#### *Construct design*

Two complimentary oligonucleotides were ordered from IDT that span 72 bases of the GPx4 5'UTR and contain the GRSF1 consensus sequence. The oligonucleotides were rehydrated and 50 mM of each was added to a 1.5 mL eppendorf tube with 1x annealing buffer (5x: 50 mM tris pH 7.5, 100 mM NaCl, 5mM EDTA) and dH2O to a final volume of 75  $\mu$ L. Tubes were placed in a beaker of water at 95°C for 15 minutes followed by cooling to room temperature. A construct containing a FLAG-tagged mouse GPx1 gene was then digested with the restriction endonuclease AfeI according to manufacturer's instructions. The double stranded DNA was then ligated into the linearized mGPx1 vector using T4 DNA ligase (New England Biolabs) according to manufacturer's instructions. The mutant construct was transformed into Library Efficiency DH5a Competent Cells (Invitrogen) according to manufacturer's instructions. Transformants were selected and grown in 5 mL Luria Broth media overnight and minipreped the following day using the Zyppy Plasmid Miniprep Kit (Zymo Research) according to manufacturer's instructions. Isolated plasmid DNA was sequenced (Snyder Hall Sequencing Facility, University of Hawaii at Manoa) to confirm proper ligation of the GPx4 5'UTR insert.



### *Se treatment, Transfection and qRT-PCR*

HEK293T cells were plated in 6-well tissue culture plates in DMEM and 10% FBS until confluence. Two  $\mu\text{g}$  of the mutant or wild type mGPx1 construct were transfected using lipofectamine 2000 reagent (Invitrogen) according to manufacturer's instructions. In parallel with the last step of the transfection, media was then switched to DMEM and 1% FBS with or without the addition of sodium selenite as previously described (Ch.2, Materials and Methods). After 48 hours cells were washed with phosphate buffered saline (PBS) and RNA was extracted with Trizol (Invitrogen) according to manufacturer's instructions. RNA quantity and quality were measured using a ND1000 spectrophotometer (NanoDrop Technologies). For cDNA synthesis, 1  $\mu\text{g}$  of RNA was used in a total reaction volume of 20  $\mu\text{L}$  using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). cDNA was diluted 5x and for qPCR a volume of 0.5  $\mu\text{L}$  was used per final 5 $\mu\text{L}$  reaction volume. One  $\mu\text{M}$  specific primer pair and PerfeCTa<sup>®</sup> SYBR<sup>®</sup> Green FastMix were used (Quanta Biosciences) according to manufacturer's instructions on a Light Cycler 480<sup>®</sup> II thermal Cycler (Roche). The sequences of all primers used in this study are shown in Table 1.

### *SDS-PAGE and Western Blot*

Total protein was extracted using Cell Lytic MT reagent (Invitrogen). Twenty  $\mu\text{g}$  of total protein lysate was added to reduced Laemmli buffer (Bio-Rad), boiled for 5 min and loaded into 4-20% gradient polyacrylamide gels (Bio-Rad). Following electrophoresis, gel contents were transferred to PVDF membranes (Millipore) and blocked with Odyssey Blocking Buffer (Li-Cor Biosciences) for 30 min. Membranes were then probed for proteins with the following primary antibodies: anti-FLAG antibody (Dilution: 1:1,000), anti-Tubulin antibody (Dilution: 1:10,000).

## **4.4 RESULTS**

*All three Gpx4 transcript variants remain stable under conditions of Se deficiency*

GPx4 has three known transcript variants that are generated through alternative splicing and alternative promoter use. In rat, these splice variants are confirmed to localize to the mitochondria, nucleus and cytoplasm (Bai & Cederbaum 2000, Kelner & Montoya 1998, Esworthy *et al.* 1994). In humans however, these subcellular localizations still need to be experimentally verified. Close examination of the 5'UTR of these transcript variants shows that two of them possess the g-rich GRSF1 binding site while the third does not. We questioned whether GPx4 mRNAs observed stability under Se deficiency, as well as its cryptic resistance to NMD, may have been an artifact of a lack of specificity in primer design. We thus designed primers that were specific to each of the three variants individually and repeated the initial experiment modeling Se deficiency. Since Selenoprotein W (SelW) was consistently the most responsive to Se, it was included as a reference. Figure 4.1 shows that none of the variants change in response to Se. SelW responds significantly in agreement with the data from Chapter 1. Since the variant without the GRSF1 binding site remain stable, this suggests that GRSF1 may not be involved in GPx4 mRNA stability.

*Cloning the GPx4 5'UTR onto a GPx1 construct does not confer stability to GPx1 mRNA*

To further assess the role of the g-rich sequence in GPx4's 5'UTR, a 72 nucleotide sequence of GPx4's 5'UTR which spans this region was cloned onto a FLAG-tagged mouse GPx1 construct (mG1) (Fig. 4.2). The mutant construct (mG1G4) was then transfected into HEK293T cells under Se deficiency, along with the WT construct, to assess any differences in mRNA and protein abundance. qRT-PCR analysis shows that the abundance of the mutant construct is not significantly different than the abundance of the WT construct regardless of Se (Fig. 4.3A). Interestingly, SDS-Page and western blot using a FLAG antibody show that the levels of protein from both constructs change dramatically with Se (Fig. 4.3B). Furthermore, the fold change of the protein levels associated with the mutant construct is slightly higher than that of the WT construct. This suggests that while the GPx4 5'UTR may not play a role in the stability of the mRNA, it may function in increasing GPx4 translatability.

## 4.5 DISCUSSION

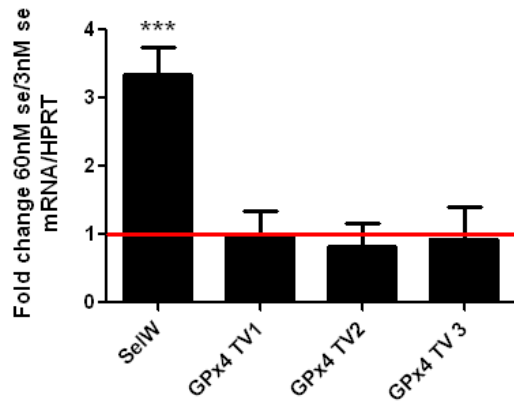
GPx4 mRNA stability under Se deficiency has been reported in the past and remains a mystery due to its expected susceptibility to the NMD pathway. The report by Ufer *et al.* provides a novel finding that implicates a g-rich sequence in the GPx4 5'UTR to be involved in GPx4 mRNAs translation. While this finding is a rare identification of a factor that is intimately associated with GPx4 during mouse embryonic brain development, further investigation is required to assess its role in Se-dependent responses. Our data show that mutant mG1 mRNA abundance is not significantly different than that of its WT counterpart in response to conditions of varying Se. One thing that is important to note is the abundance of the WT construct with respect to Se. Human GPx1 mRNA, as well as mouse GPx1 mRNA, has been shown to be sensitive to Se status. In this case, the mRNA of the WT construct does not respond to Se even though the protein changes significantly (Fig 4.3). This mRNA stability would be expected for the mutant construct, however, since the mRNAs of both constructs remain stable with Se, a definitive conclusion cannot be drawn at the mRNA level. Both constructs have introns and are presumed to go through RNA processing events, which would load EJC and predispose the transcripts to NMD. This observation makes it clear that the mRNAs are behaving in a manner that differs from the endogenous transcripts. siRNA-mediated knockdown of GRSF1 or removal of the consensus sequence from a GPx4 construct would provide further insight into the potential regulation of GPx4 mRNA by GRSF1.

Conversely, the protein data shows that the constructs are indeed being translated and that Se significantly influences the amount of protein being produced. Although subtle, the difference in the amount of protein produced by the mutant construct compared to the WT construct is statistically significant. This may confirm a previously made finding by Ufer *et al.* that GRSF1 increases the translatability of the mRNA. Whether this effect on mRNA translation protects the mRNA from NMD will require further examination.

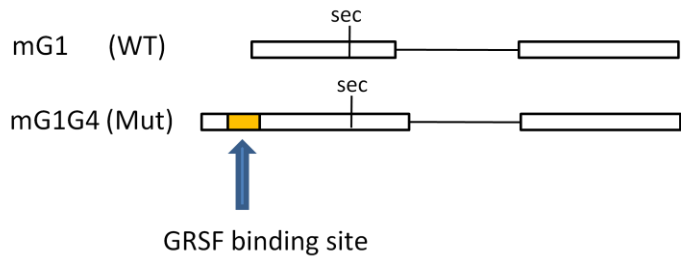
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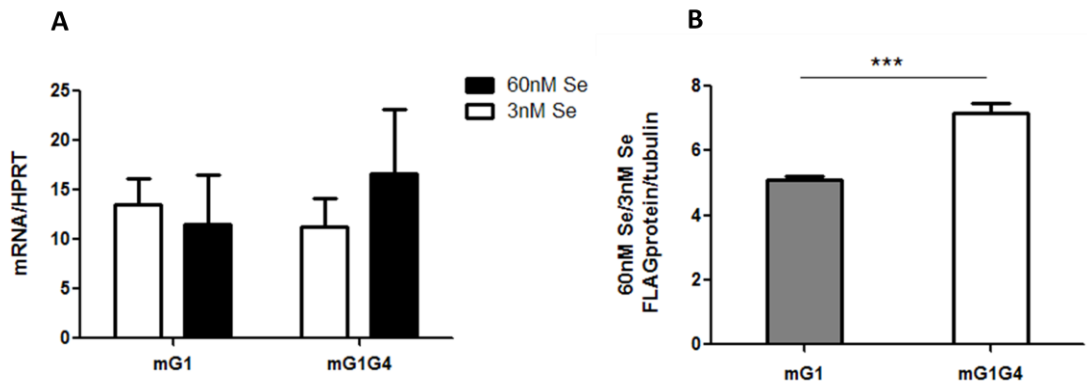
#### 4.7 FIGURES



**Figure 4.1 GPx4 transcript variants remain stable during Se deficiency.** HEK293T cells were grown to confluence in standard media and then switched to Se-deficient or Se-supplemented media for 48 hours. mRNA abundance of GPx4 transcript variants remain stable during Se deficiency. SelW mRNA changed significantly with Se and was used as a positive control for the Se treatment. N= 6, error bars represent standard deviation of the mean, \*\*\*p<.001



**Figure 4.2 Schematic representation of mG1 WT and mutant constructs.** The 5'UTR of GPx4 which contains the GRSF1 binding site was cloned onto a mouse GPx1 (mG1) construct.



**Figure 4.3 mRNA and Protein levels of WT and mutant mGPx1 constructs** (A) mRNA levels of transfected mG1 construct with or without the 5'UTR of human GPx4. (B) Fold change representation of the protein levels resulting from mGPx1 construct transfection. N=3, error bars represent standard deviation of the mean, \*\*\*p<.001.

**TABLE 1.** PCR primer sequences, written 5'to 3'

Gene	Primer Sequence	
	Forward	Reverse
SelW	GGCTACAAGTCCAAGTATCTTCAG	CACTTCAAAGAACCCGGTGG
<b>GPx4</b>	GTGATCTCAGCACCATCATTGGTCGGCTG	ATCATCGCGGCGGGGCTGGGAGCC
<b>5'UTR</b>	GACGAGGGGAGGAGCCGCTGGCTCCCAG	AGCGGCTCCTCCCCTCGTCCAGCCG
<b>oligo</b>	CCCCGCCGCGATGAG	ACCAATGATGGTGCTGAGATCAC
GPx4 TV1	TCACCAAGTTCCTCATCGACAAGA	GAAATAGTGGGGCAGGTCCTTC
GPx4 TV2	TTTGGACACCGTCTCTCCACAG	GAAATAGTGGGGCAGGTCCTTC
GPx4 TV3	CGGAAGGCCCCAGCGTGCA	CACGGGTCCTGGGACTTGGGAC



## CHAPTER 5

### SUMMARY

#### 5.1 CONCLUDING REMARKS

The aim of this work was to gain a better understanding of the molecular mechanisms that are involved in the regulation of selenoprotein mRNAs during selenium (Se) deficiency. Selenoproteins are essential to eukaryotic life and although many have functions that are yet to be characterized, the well studied proteins are oxido-reductases that utilize the electrochemical properties of Se at their active site to catalyze crucial biochemical reactions (Gromer *et al.* 2005, Johansson *et al.* 2005, Papp *et al.* 2010, Roman *et al.* 2013). The reactive Se atom in selenoproteins is present in the unique amino acid selenocysteine (Sec), which is structurally similar to cysteine, and is incorporated into the growing peptide chain via a redefined UGA codon and a specialized 3'UTR (Fletcher *et al.* 2001, Tujebajeva *et al.* 2000, Small-Howard *et al.* 2006, Berry *et al.* 1991). Several studies have reported a hierarchy of selenoprotein mRNA abundance whereby the levels of certain transcripts decline with Se deficiency while others do not (Sunde *et al.* 2009, Bermano *et al.* 1996, Yan *et al.* 2013). Since UGA is a stop codon, the decline in transcript abundance has generally been attributed to the nonsense-mediated mRNA decay (NMD) pathway since it functions to degrade mRNAs harboring premature termination codons (PTC). While this assumption is logical, experimental evidence in support of it has been lacking.

In order to address this question, we first characterized the human selenoprotein transcriptome in terms of the rules that have been elucidated to predispose a transcript to degradation via NMD. According to the current model for mammalian NMD, PTC's that lie in the last exon, or within 50-55 nucleotides of the last exon, are incapable of subjecting a transcript to NMD (Schweiggruber *et al.* 2013). About half of the mRNAs in the selenoprotein transcriptome have their Sec codon in this

position and are therefore defined as being resistant to NMD (Ch.2, Fig. 2.2). After categorizing the transcripts as being predicted sensitive or resistant to NMD, Se deficiency was modeled and transcript abundance was measured. Figure 2.4 shows that the selenoprotein mRNAs which were predicted to be resistant to NMD did not change in abundance with respect to Se. Conversely, the majority of mRNAs that were predicted sensitive to NMD changed significantly in response to Se. GPx4 mRNA and SelN mRNA remained stable although they were predicted sensitive and expected to change with Se. A possible theoretical explanation in regards to GPx4's observed stability is presented in the discussion in chapter 3. SelN's enigmatic response to Se status will require further investigation to be elucidated. To help clarify the nature of the changes in mRNA abundance, pre-mRNAs were additionally analyzed, none of which responded to changes in Se (Ch.2, Fig. 2.5). These data suggest that the mRNA changes associated with Se deficiency are post-transcriptional and NMD related.

To continue investigating the role of NMD in the observed changes that selenoprotein mRNAs exhibit during Se deficiency, two key NMD factors were utilized. RNA helicase UPf1, which in addition to NMD, is also implicated in several other cellular processes (Reviewed in Imamachi *et al.* 2012), was used for RNA immunoprecipitation. Figure 3.1 shows that the Se-sensitive mRNAs are more abundantly bound to UPF1 when Se is limiting. Interestingly, the degree of sensitivity to Se correlated with the degree to which the respective transcripts coprecipitated with UPF1. For example, SelW was the most sensitive to Se and it was also the most abundantly coprecipitated mRNA. Finally, knocking down SMG1 during Se deficiency abrogated the decline in transcript abundance of the Se sensitive mRNAs. The increase in abundance of these mRNAs during Se deficiency after depletion of SMG1 is strong evidence in support of NMDs regulation of selenoprotein mRNAs.

While the discussion in chapter 3 regarding the abundance of tRNA<sup>sec</sup> in relation to Se status provides a theoretical explanation for GPx4s stability during Se deficiency, we sought to investigate another potential mechanism for this effect. The protein GRSF1

was found to bind GPx4's 5'UTR to recruit it to active polysomes. In addition, developmental retardations associated with GRSF1 deficiency were rescued with overexpression of GPx4 (Ufer *et al.* 2008). We wondered whether this sequence in GPx4's 5'UTR was involved in the mRNAs stability that was observed in response to Se status. To address this, the GRSF1 consensus sequence was cloned into the 5'UTR of a mouse GPx1 plasmid. Since GPx1 mRNA is sensitive to Se status, we thought that the GPx4 5'UTR could abrogate this sensitivity. Transfecting the mutant GPx1 construct into HEK293T cells during Se deficiency showed no difference in mRNA levels between WT and mutant constructs. Importantly, Se did not have any effect on mRNA abundance which is an indication that our construct is not an accurate representation of endogenous GPx1 mRNA since both mouse and human mRNAs have been shown to be significantly Se responsive (Weiss & Sunde 1998, Sun *et al.* 2001, Sunde *et al.* 2009). At the level of translation, the mutant construct produced significantly more protein than the WT construct when Se was supplemented. Although the data is inconclusive in determining the role of GPx4's 5'UTR in the stability of its mRNA, it suggests that it may be increasing its translatability.

Among the transcripts that do not respond to Se and have their Sec codons out of NMD's reach are the deiodinases and the thioredoxin reductases (Txnrd). These two types of selenoproteins have vital functions in metabolism and Txnrd1 and Txnrd2 knockout mice die during embryonic development. GPx4 knockout mice also die during embryonic development and these embryonic deaths exemplify the importance of preserving the expression of vital selenoproteins (Jakupoglu *et al.* 2005, Conrad 2009). In contrast, Selenoprotein P (Selp) and GPx1 are both predicted sensitive to NMD and likewise decline in abundance under Se deficiency. Although Selp knockout mice exhibit a neurological phenotype, neither them nor the GPx1 knockout mice are embryonic lethal (Raman *et al.* 2012, Cheng *et al.* 1997). In fact, GPx1 knockout mice do not show a deleterious phenotype unless challenged with oxidative stress (Fu *et al.* 1999). This split between selenoprotein mRNAs that have their Sec codon in an NMD-resistant location and those which do not may reflect an evolutionary development that could have

occurred in response to Se-related selective pressure. NMDs function was initially thought to be that of protecting cells from mass producing potentially toxic truncated proteins that would result from the synthesis of a mutant mRNA. As research has progressed however, the control of gene expression is being considered as NMD's predominant purpose. Microarray analysis and transcriptome-wide profiling of mRNAs have identified numerous physiological substrates that are regulated by NMD (Mendell *et al.* 2004, Guan *et al.* 2006, He *et al.* 2003, Rehwinkel *et al.* 2005, Wittmann *et al.* 2006, Tani *et al.* 2012). These transcripts encompass a wide variety which, in addition to selenoprotein genes, includes genes with upstream open reading frames, products of alternative splicing, transposons or retroviruses, genes with introns in UTRs as well as several others. It's likely that over evolutionary time, these genes and NMD have been coevolving. It is also unlikely that the frequency of de novo nonsense mutations would be sufficient to provide enough selective pressure for full evolutionary maintenance of NMD.

While the NMD pathway has regularly been implicated in the hierarchy of selenoprotein mRNA abundance during Se deficiency, experimental evidence has been lacking. The data here present novel evidence supporting a role for NMD in this hierarchy.

## **5.2 FUTURE DIRECTIONS**

Although UPF1 has been extensively studied in regards to the NMD pathway, it was also found to be involved in another RNA degradation pathway referred to as Staufen-mediated decay (SMD) (Kim *et al.* 2005). Staufen is an RNA binding protein that was initially discovered as being responsible for posterior pole localization of OSKAR mRNA in *Drosophila* oocytes (St Johnston *et al.* 1991). Subsequent investigations elucidated a role for Staufen in transporting neuronal mRNAs to sites of localized translation away from the nucleus, usually at dendrites (Kim & Kim 2006, Kiebler *et al.* 1999, Miki *et al.* 2005). The overarching theme of initial reports investigating Staufen is one of mRNA transport. Most recently however, it was found to associate with UPF1 to

facilitate the degradation of certain mRNAs. While a common feature identifying these mRNAs has not been identified, alu elements and short inverted repeats have been implicated. Interestingly, a recent microarray analysis of substrates up-regulated after siRNA-mediated knockdown of Staufen showed a significant increase in abundance of the Selenocysteine lyase enzyme (Seclyase) mRNA (Kim *et al.* 2007). Seclyase was shown to cleave selenocysteine into alanine and selenide and it is currently under investigation as the putative selenium recycling enzyme (Esaki *et al.* 1982).

In response to this finding, we questioned whether there was any relationship between UPF1, Staufen, NMD, SMD and the hierarchy of selenoprotein mRNA abundance during Se deficiency. Analysis of Seclyase mRNA abundance during Se deficiency shows Seclyase mRNA to be inversely correlated to Se status (Fig. 5.1). This response is similar to that of SPS2 (Ch.2, Fig. 2.4) which also increases in abundance during Se deficiency. Since both of these are factors involved in selenoprotein metabolism, and SPS2 is itself a selenoprotein, these data suggest an autoregulatory pathway. RNA immunoprecipitation of Seclyase mRNA with UPF1 shows that it is significantly enriched on UPF1 during Se sufficiency in comparison to Se deficiency (Fig. 5.2). Further analysis of Seclyase mRNA abundance in response to UPF1 knockdown and SMG1 knockdown shows a significant increase with respect to each (Fig 5.3A, B). The former validates the initial microarray data while the latter is novel albeit not surprising since SMG1 is downstream of UPF1. The increase in abundance in response to SMG1 knockdown has never been reported and suggests that the two RNA degradation pathways converge at the stage of UPF1 phosphorylation.

In terms of the hierarchy of selenoprotein mRNA abundance, we have developed a hypothesis based on the assumption that Sec lyase functions to recycle Se for selenoprotein biosynthesis (Fig. 5.4). Putting the data together, when Se is deficient, UPF1 predominantly functions to degrade NMD-sensitive selenoprotein mRNAs (SelW, SelP, GPx1) and SMD of Sec lyase mRNA is downregulated resulting in the observed increase in Sec lyase mRNA abundance. Conversely, when Se is sufficient, NMD-sensitive

selenoprotein mRNAs will not undergo NMD thereby allowing for the upregulation of UPF1 dependent SMD of Sec lyase mRNA. A recent report which claims the efficiency of SMD to be influenced by the efficiency of NMD and that the two are constantly in competition supports this model (Maquat & Gong 2009). This type of regulation is logical because during Se deficiency it would be beneficial to upregulate Se recycling as a compensatory mechanism to preserve the function of vital selenoproteins. Conversely, upregulating recycling would no longer be necessary if the environment provides adequate amounts of Se.

### 5.3 REFERENCES

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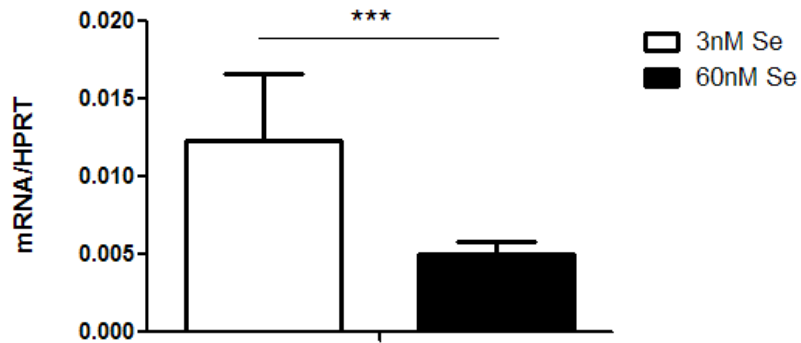
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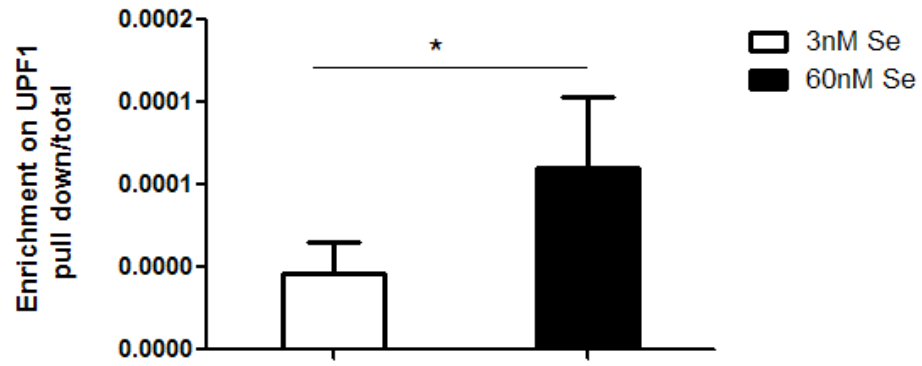
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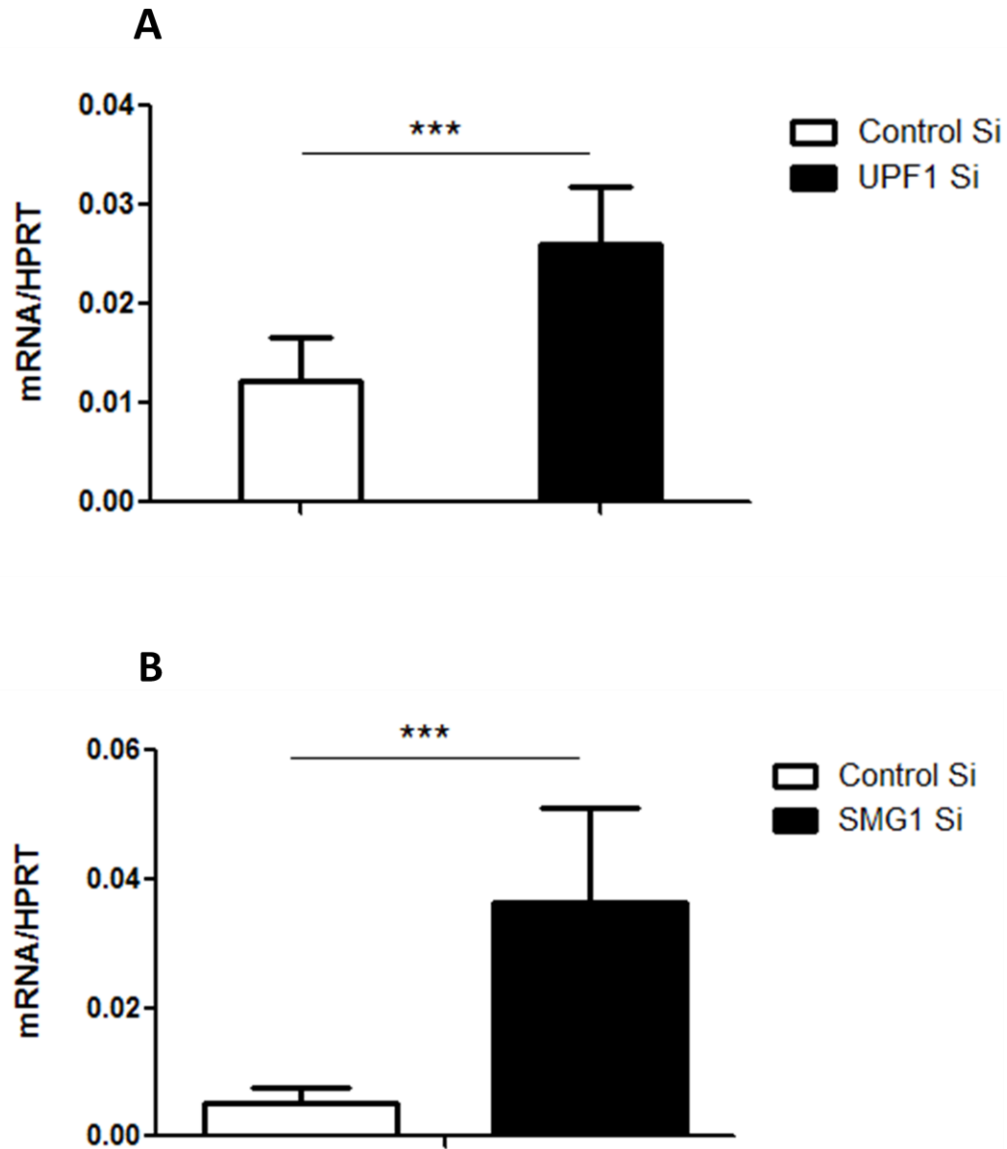
## 5.4 FIGURES



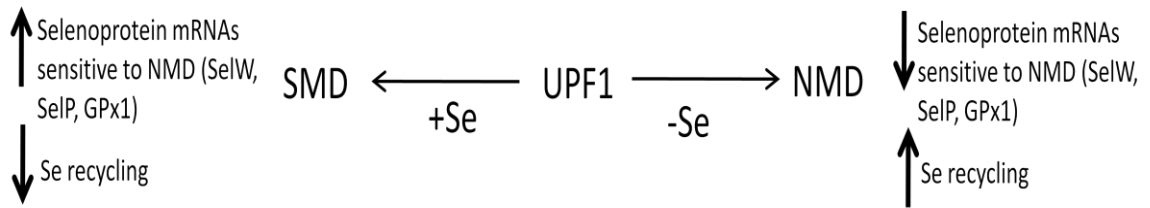
**Figure 5.1 Sec lyase mRNA abundance in response to Se status.** Sec lyase mRNA is significantly higher in abundance when Se is deficient. Error bars represent standard deviation of the mean, \*\*\* $p < .001$



**Figure 5.2 Sec lyase mRNA enrichment on UPF1 in low versus high Se.** Sec lyase mRNA is significantly enriched on UPF1 when Se is sufficient. Error bars represent standard deviation of the mean, \* $p < .05$



**Figure 5.3 Sec lyase mRNA abundance after depletion of UPF1 and SMG1.** Sec lyase mRNA abundance is significantly increased with knockdown of either (A) UPF1 or (B) SMG1. Error bars represent standard deviation of the mean, \*\*\*p<.05



**Figure 5.4 Model representing potential influence of Se on utilization of UPF1 for NMD and SMD.** During Se deficiency, UPF1 functions in NMD to degrade selenoprotein mRNAs and SMD of Sec lyase mRNA is decreased thereby increasing Se recycling. When Se is sufficient, selenoprotein mRNAs do not undergo NMD and UPF1 can function in SMD to degrade Sec lyase mRNA resulting in decreased Se recycling.