MULTIPLE LEVELS OF REGULATION OF HUMAN SECIS BINDING PROTEIN 2, SBP2

BY

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ABSTRACT

Selenium is an essential trace mineral of fundamental importance to human health. Its beneficial functions are largely attributed to its presence within a group of proteins named selenoproteins in the form of the amino acid selenocysteine (Sec). Recently, it was revealed that the human selenoproteome consists of 25 selenoproteins, and for many of them their function remains unknown. The most prominent known roles of selenoproteins are to maintain the intracellular redox homeostasis, redox regulation of intracellular signalling and thyroid hormone metabolism. Sec incorporation into selenoproteins employs a unique mechanism that involves decoding of the UGA stop codon. The process requires interplay between distinct, intrinsic features such as the Sec Insertion Sequence (SECIS) element, the tRNA\textsuperscript{Sec} and multiple protein factors. The work presented in this thesis has focused on characterising the regulation of human SECIS binding protein 2, SBP2, a factor central to this process.

Experimental approaches combined with bioinformatics analysis revealed that SBP2 is subjected to alternative splicing. A total of nine alternatively spliced transcripts appear to be expressed in cells, potentially encoding five different protein isoforms. The alternative splicing events are restricted to the 5′-region, which is proposed to be dispensable for Sec incorporation. One of the variants identified, contains a mitochondrial targeting sequence that was capable of targeting SBP2 into the mitochondrial compartment. This isoform also appears to be expressed endogenously within the mitochondria in cells.

Previous reports have depicted SBP2 as a ribosomal protein, despite the presence of a putative Nuclear Localisation Signal (NLS). In this study it was found that SBP2 subcellular localisation is not restricted to ribosomes. Intrinsic functional NLS and Nuclear Export Signals (NESs), enable SBP2 to shuttle between the nucleus and the cytoplasm via the CRM1 pathway. In addition, the subcellular localisation of SBP2 appears to play an important role in regulating Sec incorporation into selenoproteins. The subcellular localisation of SBP2 is altered by conditions imposing oxidative stress. Several oxidising agents induce the nuclear accumulation of SBP2, which occurs via...
oxidation of cysteine residues within a novel redox-sensitive cysteine rich domain (CRD). Cysteine residues were to form disulfide bonds and glutathione-mixed disulfides during oxidising conditions, which are efficiently reversed \textit{in vitro} by the thioredoxin and glutaredoxin systems, respectively. These modifications negatively regulate selenoprotein synthesis.

Cells depleted of SBP2 are more sensitive to oxidative stress than control cells, which correlated with a substantial decrease in selenoprotein synthesis after treatment with oxidising agents. These results provide direct evidence that SBP2 is required for Sec incorporation \textit{in vivo} and suggest that nuclear sequestration of SBP2 under such conditions may represent a mechanism to regulate the expression of selenoproteins.

Collectively, these results suggest that SBP2 is regulated at multiple levels: by alternative splicing, changes in subcellar localisation and redox control.
DECLARATION

This is to certify that:

(i) the thesis comprises only my original work towards the PhD except where indicated in the Preface,

(ii) due acknowledgement has been made in the text to all other material used

(iii) the thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Laura V. Papp
PREFACE

All work presented in this thesis is my original research except for the following experiments which were performed by Dr Jun Lu at the Department of Biochemistry, Karolinska Institute, Stockholm, Sweden:

- Preparation of the glutathionylated NES-His(6) protein for Mass Spectrometry analysis described in Chapter 5, Section 5.3.5

- Enzyme kinetics with Glutaredoxin1 described in Chapter 5, Section 5.3.5

- Thioredoxin fluorescent spectra measurements in Chapter 5, Section 5.3.4
PUBLICATIONS ARISING FROM THIS THESIS

ACKNOWLEDGEMENTS

The work presented in this thesis would not have been possible without the invaluable contribution, support and encouragement of a number of people that I would herein like to acknowledge.

I would like to firstly express my sincere gratitude to my supervisors Dr. Derek Kennedy and Prof. Kum Kum Khanna. Derek, thank you for making sure I stayed on and started this PhD project, for your belief in me as a scientist, for trusting my ideas, for your encouragement along the way in particular when things weren’t looking their brightest, for intellectual discussions and input, they all contributed to the success of this project. Kum Kum, thank you for making this project available, for providing me with a scholarship while writing this thesis, for all your ideas about this project, even the ones I have not even started, for the intellectual input and for sharing your scientific knowledge, and for also allowing me to follow my ideas and believing in me as a scientist.

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Thank you to Dr. Beric Henderson for providing the pRev1.4-GFP plasmid.

Other members in the labs where I conducted my work contributed in many ways to this project, that I would like to acknowledge. In particular, many thank you to Dr. Jun Lu for your invaluable help with experiments, for teaching me biochemistry, for staying with me in the lab till late night on numerous occasions to help finish experiments and for sharing your passion for science and reminding me that I do the same when I was ready to give up. I am truly grateful for meeting you and for sharing your friendship.

Many thanks to current and past members of the signal transduction group at QIMR: Dr. Aaron Urquhart, Dr. Derek Richard, Dr. Emma Bolderson, Christian Jekimovs, Jesse Kelly, Karen Hobson, Jyoti Jonnalagadda, Dr. David Young, Masroor Shariff, Julia Pagan, Mathew Jones, Dr. Sergei Tsvetanov, Kienan Savage, Dr. Megan Fabbro, Dr. Darren Krause, Dr. Katie Sloper and Kim Hanchard for helping out when
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Many thanks to all my friends outside the scientific world, for being understanding and supportive and for making sure I kept my sanity during the course of this PhD! In particular, Dean, for always being there for me and for the entertainment while writing this thesis! Thanks Diana for help with editing the thesis. Chris, thanks for cooking those delicious meals and for bringing them to the lab during the last hectic week of preparation of this thesis. You are wonderful, I have a good feeling about this…

Last but not least, I would like to thank my family, for being with me, encouraging and supporting me over the great physical distance, but next to me in my heart.

After a four year journey, it finally comes to an end and I realise it’s all just about to begin…
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<tr>
<td>Ab</td>
<td>Antibody</td>
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<tr>
<td>Amp</td>
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<td>ARM</td>
<td>Armadillo-like</td>
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<td>BRCA1</td>
<td>Breast Cancer Susceptibility 1</td>
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<td>bp</td>
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<td>BARD1</td>
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<td>Degrees Celsius</td>
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<tr>
<td>CO₂</td>
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<td>Cysteine rich domain</td>
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<td>Chromosome region maintenance 1</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>Deionised Water</td>
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<td>Deoxyribonucleic Acid</td>
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<tr>
<td>DIOs</td>
<td>Iodothyronine deiodinase</td>
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<tr>
<td>EDTA</td>
<td>Ethylene Diaminetetra–acetic Acid, Disodium Salt</td>
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<td>EtBr</td>
<td>Ethidium Bromide</td>
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<td>eIF</td>
<td>Eukaryotic initiation factor</td>
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<td>eEFSec</td>
<td>Eukaryotic elongation factor selenocysteine</td>
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<td>ER</td>
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<td>FCS</td>
<td>Foetal Calf Serum</td>
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<td>g</td>
<td>Gram</td>
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<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<td>GSH</td>
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<td>GSSG</td>
<td>Oxidised glutathione</td>
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<td>Glutathione reductase</td>
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Grx Glutaredoxin
GPx Glutathione peroxidase
Hr Hour
H$_2$O$_2$ Hydrogen Peroxide
HCl Hydrochloric Acid
HRP Horseradish Peroxidase
Ig Immunoglobulin
Imp-α Importin-α
Imp-β Importin-β
IPTG Isopropyl-β-D-galactopyranoside
IRE Iron response element
IRES Internal ribosomal entry site
IRP Iron regulatory protein
kb Kilobase
kDa Kilo Dalton
L Liter
LB Luria Broth
L30 Ribosomal protein L30
M Molar
Min Minute
µg Microgram
µl Microlitre
ml Milliliter
mM Millimolar
MTS Mitochondrial Targetting Sequence
NADPH Nicotinamide-adenine dinucleotide phosphate
NADP Oxidized nicotinamide-adenine dinucleotide phosphate
NADH Nicotinamide-adenine dinucleotide
NAD Oxidized nicotinamide-adenine dinucleotide
NLS Nuclear localisation signal
NMD Nonsense Mediated Decay
<table>
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<td>ng</td>
<td>Nanogram</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
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<td>O$_2^-$</td>
<td>Superoxide anions</td>
</tr>
<tr>
<td>OH</td>
<td>Hydroxyl radicals</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenyl Methyl Sulphonyl Fluoride</td>
</tr>
<tr>
<td>PS</td>
<td>Penicillin-Streptomycin</td>
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<tr>
<td>PRF</td>
<td>Programmed ribosomal frame-shift</td>
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<td>PVDF</td>
<td>Polyvinyladine Fluoride</td>
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<td>Peroxiredoxin</td>
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<tr>
<td>Rpm</td>
<td>Revolutions per Minute</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>RNS</td>
<td>Reactive nitrogen species</td>
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<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulphate-PolyacrylAmide Gel Electrophoresis</td>
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<td>S-O$_3$H</td>
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<tr>
<td>SECIS</td>
<td>Selenocysteine insertion sequence</td>
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<td>Soluble liver antigen</td>
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<td>SBP2</td>
<td>SECIS binding protein 2</td>
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<tr>
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<td>Description</td>
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<tr>
<td>TBE</td>
<td>Tris-borate</td>
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<td>N’N’N’N’-Tetra-Methylenediamine</td>
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<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) Aminomethane</td>
</tr>
<tr>
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CHAPTER 1.

LITERATURE REVIEW
1.1 INTRODUCTION

Organisms growing in the presence of oxygen are continuously exposed to oxidative challenge caused by reactive oxygen species (ROS) generated either endogenously through oxygen metabolism or exogenously from environmental stress. These events lead to changes in the intracellular redox balance which in turn affect protein function, interactions, activity and subcellular localisation. The intracellular redox homeostasis is maintained by the concerted action of antioxidant systems including thioredoxin (Trx), thioredoxin reductase (TR), glutathione peroxidase (GPx), glutaredoxin (Grx) and glutathione (GSH). TR and GPx are both enzymes that contain selenium in the form of the amino acid selenocysteine (Sec) within their catalytic site. These selenoenzymes use the redox potential of Sec to maintain a reducing intracellular environment which is crucial for optimal cellular function. Sec- the 21st amino acid is unique in that it is encoded by the UGA codon which usually serves as a termination codon. Its incorporation into protein therefore imposes a challenge to the translation machinery in determining which function to perform at each UGA codon. Translation of Sec is dictated by the presence of a Sec insertion element (SECIS) located within the 3’ UTR of selenoprotein mRNAs. Several protein factors are involved in this process and they include the Sec specific elongation factor (EFSec), the SECIS binding protein 2 (SBP2) and the ribosomal protein L30.

Despite recent progress in the selenoprotein field, understanding the regulation of selenoprotein synthesis is still in the early phases of discovery. It was recently suggested that Sec incorporation is inherently inefficient and a rate limiting factor has not yet been determined in vivo. Compelling in vitro evidence indicates SBP2 as a central factor in the process, making SBP2 an obvious candidate for further investigation. The subcellular localisation of SBP2 within cells represents an important aspect of SBP2 regulation and has so far remained somewhat controversial. A putative nuclear localisation signal (NLS) within SBP2 was reported earlier, suggesting that SBP2 may localise within the nuclei of cells. In later studies, endogenous SBP2 was detected solely within the ribosomal fraction of cells and over-expressed SBP2 was primarily found within the cytoplasm, generating confusion about the localisation of SBP2 within cells. Elucidating the regulation of SBP2 during
normal cellular conditions as well as during conditions of cellular stress is essential to understanding the mechanism of selenoprotein synthesis.

This review will describe the mechanisms of protein transport between the nucleus and the cytoplasm with an emphasis on their regulation. Oxidative stress and redox regulation will also be addressed, along with a brief description of selenoproteins and the proposed models for selenoprotein synthesis.

1.2 NUCLEAR-CYTOPLASMIC TRANSPORT AT A GLANCE

The separation of the nucleus and the cytoplasm by the nuclear envelope is a defining feature of the eukaryotic cell. This provides a high degree of complexity through a sophisticated regulation of gene expression by spatial separation of transcription in the nucleus and protein synthesis in the cytoplasm (Gama-Carvalho and Carmo-Fonseca, 2001). Relay of information between the two compartments thus depends on a constant, bi-directional flow of molecules into and out of the nucleus, which has set the need for high fidelity transport mechanisms across the nuclear envelope. Largely, this is achieved by so called “shuttling proteins”, the presence of intrinsic signal sequences such as the nuclear localisation signal (NLS) and the nuclear export signal (NES) and their specific recognition by transport receptors and adaptors. Transport of all molecules between the nucleus and the cytoplasm takes place through nuclear pore complexes (NPC), large macromolecular structures that perforate the nuclear envelope (Cronshaw et al., 2002; Suntharalingam and Wente, 2003). This is mediated by an evolutionary conserved superfamily of transport factors: the karyopherin-β family (Mosammaparast and Pemberton, 2004). Karyopherins are divided into two sub-families: importins and exportins which mediate nuclear import and export, respectively (Pemberton and Paschal, 2005). The mechanistics of nuclear transport occurs in several sequential steps as schematically presented in Figure 1.1 and include: 1) recognition of the cargo’s NLS/NES by importins/exportins, 2) docking of the cargo-receptor complex at the NPC, 3) translocation across the NPC, 4) release of the cargo, and 5) recycling of the receptor for the next round of transport (Ferrando-May, 2005). The direction of transport across the nuclear envelope is regulated by the Ras-like GTPase molecule, Ran. The nuclear form, Ran-GTP, promotes the release of
cargo in the nuclear compartment, whereas the cytoplasmic form Ran-GDP, promotes the cargo release into the cytoplasm (Melchior et al., 1993; Moore et al., 2004).

Nuclear transport allows the nucleus and the cytoplasm to respond in concert to changes affecting the cell. Therefore, by determining the distribution of proteins between the nucleus and the cytoplasm, nucleocytoplasmic transport enables the spatial regulation of protein activity and may serve as an integration point for signalling systems (Ferrando-May, 2005).
Figure 1.1 Overview of some major nuclear transport pathways in eukaryotic cells. (A) Some proteins such as the nuclear transcription factor 2, NTF2, can translocate into the nucleus via intrinsic signals, a process driven by the association with the cytoplasmic RanGDP. Inside the nucleus, exchange of GDP for GTP mediated by the GTP exchange factor, RanGEF, leads to dissociation of Ran and release of the protein. (B) Most cellular proteins however, are transported into the nucleus via intrinsic nuclear localisation signals (NLS) that are recognised by transporter molecules such as Importin-α (Imp-α) and Importin-β (Imp-β). Export to the cytoplasm is mediated by nuclear export signals (NES) that are bound by export protein such as (C) CAS or (D) CRM1 on the nuclear side and released in the cytoplasm by RanGTP hydrolysis by the GTPase activator protein RanGAP. The RanGDP/RanGTP gradient across the nuclear envelope determines the direction of the loading, transport and release of cargo (Pemberton and Paschal, 2005).
This may prove of particular importance in response to biological changes, when the cell needs to respond swiftly and precisely to external stimuli or when the cell is subject to stress-induced alterations in the intracellular homeostasis.

The most studied shuttling mechanism is the trafficking between the nucleus and the cytoplasm. Shuttling of proteins between these two compartments can either be a continuous process such as for transport receptors and adaptors, which can relocate from one compartment to another within minutes (Gama-Carvalho and Carmo-Fonseca, 2001), or a slow process that only occurs upon induction by certain stimuli, such as described for the cell cycle protein cyclin B1 which only localises to the nucleus during mitosis (Ookata et al., 1992; Pines and Hunter, 1991). The availability of the NLS and NES sequence within the shuttling protein itself or, within a binding partner, and its interaction with the transporter molecule provides the first level of control. Several molecular mechanisms have been described that regulate the availability of these signals and include post translational modifications such as phosphorylation, ubiquitination and sumolation (Poon and Jans, 2005), or reduction or oxidation of cysteine residues, a process known as redox regulation.

A group of proteins that have been extensively studied and shown to be regulated by nuclear transport are transcription factors, which under basal conditions are found in the cytoplasm in an inactive form and in response to stimuli, they translocate into the nucleus where they activate target gene expression (Johnson et al., 2004; Kabe et al., 2005; Xu and Massague, 2004). Similarly, translation factors that function in the cytoplasm have been shown to be negatively regulated by transport into the nucleus during conditions unsuitable for protein synthesis (Cumming et al., 2004). The list of shuttling proteins whose functions are regulated by nuclear transport between subcellular compartments, is constantly growing and now includes members of family of proteins with diverse cellular functions such as: cell cycle regulators (Pines, 1999; Yang and Kornbluth, 1999), transcription factors (Cartwright and Helin, 2000), tumour suppressors (Fabbro and Henderson, 2003), RNA binding proteins (Nakielnny and Dreyfuss, 1999), signal transducers (Xu and Massague, 2004) and apoptotic factors (Ferrando-May, 2005). Defects in regulation of nucleocytoplasmic transport leading to mis-localisation and altered function of proteins may therefore have a direct link to disease. One such link was described for tumour suppressor proteins including
the breast cancer susceptibility gene BRCA1, p53, the adenomatous polyposis coli protein APC and their link to cancers (reviewed in Fabbro and Henderson, 2003).

1.2.1 MECHANISMS OF NUCLEOCYTOPLASMIC PROTEIN TRANSPORT

1.2.1.1 Nuclear Import

Although molecules smaller than ~40 kDa can passively diffuse between the nucleus and the cytoplasm via the NPC, larger molecules and proteins require an active transport to gain nuclear access (Johnson et al., 2004). This process is predominantly achieved in an NLS- and energy-dependent manner; however, NLS-independent pathways have also been described (Pollard et al., 1996). These pathways are addressed below.

1.2.1.1.1 NLS-dependent nuclear import

Classical NLS motifs consist of short sequences (5–20 residues) containing several lysine and arginine residues (Dingwall and Laskey, 1991). When fused to a heterologous protein, these small NLS motifs are sufficient to direct the chimeric polypeptide to the nucleus (Kalderon et al., 1984). The simplest NLSs contain a single cluster of positively charged residues and are generally referred to as monopartite signals similar to the first NLS identified within the SV40 large T antigen (Kalderon et al., 1984). A second class consists of bipartite NLSs where two stretches of basic residues are close together in the amino acid sequence but not actually adjacent, as described for nucleoplasmin (Robbins et al., 1991). Both variants of the classical NLS are recognised by members of the importin family of carrier proteins, which are divided into importins-α (Imp-α) with 8 members in humans, and importins-β (Imp-β) with >20 identified members. Importins-α contain a central domain composed of eight, approximately 40 amino acid long, tandemly arranged armadillo-like (ARM) repeats and importins-β are characterised by 14-15 tandemly arranged HEAT sequence motifs (Andrade and Bork, 1995; Malik et al., 1997). Importin-mediated nuclear import is predominantly carried out by the IMPα/IMPβ-1 heterodimer (Enenkel et al., 1995; Gorlich et al., 1995), with the IMPα moiety binding to the NLS.
within the cargo protein (Conti et al., 1998; Kobe, 1999) and the IMPβ-1 subunit facilitating the translocation of transporter-cargo complex through the NPC via interactions with nucleoporins (Stewart et al., 2001). Inside the nucleus, the cargo is released by the interaction between IMPβ-1 and Ran-GTP, and the transporters are recycled to the cytoplasm for another round of transport (Chook and Blobel, 2001; Lee et al., 2005; Mosammaparast and Pemberton, 2004; Weis, 2003). Nuclear import does not always require the IMPα/IMPβ-1 heterodimer, in some instances members of the IMPβ family have been shown to interact directly with NLSs within cargo proteins and this interaction is mediated via the HEAT repeat motifs (Pemberton and Paschal, 2005). NLSs recognised by the IMPβ members show greater variety than the classical NLS and have been identified within core histones (Muhlhauesser et al., 2001), ribosomal proteins (Jakel and Gorlich, 1998), and arginine-glycine-rich NLSs observed in some RNA-binding proteins (Leslie et al., 2004; Senger et al., 1998).

1.2.1.1.2 NLS-independent nuclear import

Alternative means that enable proteins that lack intrinsic NLSs to gain nuclear entry have also been described. These involve a so called “piggy-back” mechanism where nuclear translocation of cargo is facilitated by complex formation with an interactor protein with intrinsic NLS. One such mechanism was described for the breast cancer-associated tumour suppressor protein BRCA1 that was shown to enter the nucleus by an alternative route, independent of its two NLSs (Fabbro et al., 2002). This alternative nuclear targeting element was identified as the N-terminal RING-finger domain, previously shown to serve as interaction domain for the BRCA1 binding partner BARD1 (Brzovic et al., 2001; Wu et al., 1996). BARD1 was shown to actively carry BRCA1 into the nucleus and this action was found to be of functional importance in recruiting BRCA1 to DNA-repair associated nuclear foci (Fabbro et al., 2004). β-catenin, SMAD2, SMAD3, SMAD4 and ERK2 are additional proteins for which NLS-independent nuclear import has been reported (Xu and Massague, 2004). All of these proteins contain ARM or HEAT-repeat like motifs which through direct interactions with nucleoporins were shown to be necessary and sufficient for their nuclear import (Xu and Massague, 2004).
1.2.1.2 Nuclear export

1.2.1.2.1 CRM1-dependent nuclear export

The mechanism of nuclear export is a more recent subject of investigation than nuclear import, nevertheless, a number of exportin molecules have been identified, namely CAS, CRM1, exportin-t, Exp4, Exp5, and Imp13 (Kutay and Guttinger, 2005). CRM1/exportin1 is so far the best characterised exporter protein in mammalian cells and is believed to be the predominant export receptor of proteins, having the broadest substrate specificity range. CRM1 recognises and binds to classical NESs, which are resembled by short peptide sequence with closely spaced large hydrophobic amino acids of the consensus \((L_{x(1-3)}L_{x(2-4)}LxL)\) (L can be replaced by V, I, F or M) (Fornerod et al., 1997; Fukuda et al., 1997; Kutay and Guttinger, 2005; Ossareh-Nazari et al., 1997; Stade et al., 1997). One of the first CRM1-dependent NESs characterised was the NES of the HIV Rev1 protein (Fischer et al., 1995) and since then, at least 75 HIV Rev-like NESs have been identified within cellular proteins with diverse functions (la Cour et al., 2003; la Cour et al., 2004), such as signal transduction proteins, cell cycle regulators and RNA-binding proteins (Jakel and Gorlich, 1998; Nakielny and Dreyfuss, 1999; Senger et al., 1998). Translation factors such as eIF4A1, eIF2, eIF2B and eIF5 have also been shown to shuttle between the nucleus and the cytoplasm via the CRM1 pathway however, other parallel export pathways are also involved (Bohsnack et al., 2002). Similar to nuclear import, the driving force for nuclear export is provided by the Ran-GTP/Ran-GDP gradient across the NPC, with the nuclear Ran-GTP form promoting interaction between CRM1 and the NES-containing cargo thus facilitating export. GTP hydrolysis on the cytoplasmic side promotes CRM1-cargo complex disassembly thus terminating the export reaction (Rout et al., 2003; Weis, 2003). One valuable tool for identifying CRM1-dependent NESs within proteins has been provided by the findings that the fungicide Leptomycin B (Wolff et al., 1997) acts as a direct inhibitor of CRM1 (Kudo et al., 1998). This compound is now used extensively in the process of isolating novel CRM1-dependent NES containing proteins.
1.2.1.2 CRM1-independent nuclear export

CRM1-independent protein export pathways are less well characterised, however several proteins with export receptor activities have been identified. Calreticulin is one such protein that was shown to act as a Ran-GTP associated receptor for some NES sequences in the glucocorticoid hormone receptor (GR) (Holaska et al., 2001). Another exporter shown to transport cargo-free IMPα to the cytoplasm is the IMPβ family member CAS (Cse1p in yeast) (Kutay et al., 1997). Structural studies of CAS in complex with Ran-GTP and its cargo IMPα has provided further insight into the mechanistics and the role of Ran-GTP in nuclear import and export (Matsuura and Stewart, 2004). Some members of the IMPβ family can function bidirectionally and thus operate as both importins and exportins. Such examples include Msn5, an export receptor for phosphoproteins (Strom and Weis, 2001) and importin 13 that mediates export of the translation initiation factor eIF1A, independent of Ran-GTP (Mingot et al., 2001).

1.2.2 REGULATION OF NUCLEOCYTOPLASMIC TRANSPORT

The regulation of protein function by nucleocytoplasmic shuttling not only provides an efficient way for the cell to rapidly respond to sudden intracellular changes, but is also a fundamental requirement of the compartmentalised eukaryotic cell. This regulation can broadly be accomplished by three, sometimes overlapping, mechanisms: 1) masking of transport signals, 2) retention within subcellular compartments or 3) by post-translational modification affecting signal availability. Although sometimes overlapping, these mechanisms will be discussed individually below.

1.2.2.1 Masking of transport signals

One of the most examined transcription factors whose biological activity is regulated through nucleocytoplasmic shuttling is NFkB. Crystal structure analysis of the NFkB/IκB complex showed that the interaction between the two proteins induces a conformational change in the NFkB subunit that masks the NLS-containing region of
The NFκB heterodimer is usually detected predominantly in the cytoplasm, where it is kept inactive by complex formation with inhibitor proteins such as IkB (Beg et al., 1992). In response to appropriate stimuli, the IkB kinase phosphorylates IkB to promote its ubiquitin-dependent degradation. The liberated NFκB then translocates into the nucleus where it binds to target genes and activates their transcription (Karin and Ben-Neriah, 2000).

An interesting and so far unique model of reciprocal regulation through masking of transport signals was recently proposed for the BRCA1/BARD1 heterodimer (Henderson, 2005). Both BRCA1 and BARD1 contain NESs, which enable them to independently shuttle between the nucleus and the cytoplasm, and as a heterodimer, both proteins mask each other’s NESs (Rodriguez and Henderson, 2000; Rodriguez et al., 2004). Direct evidence for reciprocal masking of the NESs within both proteins was also provided by NMR studies of the BRCA1-BARD1 heterodimer (Brzovic et al., 2001), implying that heterodimerisation regulates the nuclear retention of both BRCA1 and BARD1 which may in turn be linked to their function (Henderson, 2005). A thorough understanding of the functional implications of this level of regulation for the BRCA1-BARD1 heterodimer remains unclear at present.

### 1.2.2.2 Retention

Two modes by which a cargo is retained or “trapped” in either the nucleoplasmic or the cytoplasmic compartments have been proposed (Rout et al., 2003). Trapping can occur if active transport in one direction is faster than passive diffusion in the other, which was shown using an NLS-tagged GFP protein that efficiently accumulated in the nucleus even though the GFP does not bind there and is small enough to passively diffuse out (Shulga and Goldfarb, 2003; Shulga et al., 2000). The second way of trapping involves sequestration of a macromolecule to binding sites on one side of the nuclear envelope that prevents it from diffusing back. One such example is β-catenin which is a multifunctional protein implicated in transcriptional activation by the Wnt-signalling pathway and cell-cell adhesion (Peifer and Polakis, 2000). β-catenin is capable of nucleocytoplasmic shuttling independent of transporter molecules, through its affinity for nucleoporins in the nuclear pores (Eleftheriou et al., 2001; Fagotto et al., 1998; Yokoya et al., 1999). The cytoplasmic pool of β-catenin is inhibited from
nuclear entry and activation of gene expression through anchorage to the plasma membrane via interactions with E-cadherin and α-catenin (Morin, 1999; Peifer and Polakis, 2000). This interaction leads to masking of the NPC contact surface of β-catenin, inhibiting its nuclear import (Suh and Gumbiner, 2003). Other proteins subject to this mode of regulation are the tumour suppressor protein APC which is anchored in the cytoplasm through association with cytoskeletal microtubules (Munemitsu et al., 1994; Smith et al., 1994). Transcription factors such as the STAT family of evolutionarily conserved transcription activators are held inactive in the cytoplasm through anchorage to the plasma membrane. The activation of receptor-associated JAK kinases after cytokine stimulation results in tyrosine phosphorylation of the STATS, their dimerisation and translocation to the nucleus where they activate target gene expression (Meyer and Vinkemeier, 2004). In reverse, retention of shuttling transcription factors in the nucleus, are regulated though binding to their cognate DNA elements, which favour their nuclear retention (Xu and Massague, 2004).

1.2.2.3 Post-translational regulation of transport signals

Post-translational modifications which include phosphorylation, ubiquitinylation, glycosylation, sumoylation and glutathionylation regulate the subcellular localisation of proteins (Schwoebel and Moore, 2000). The nuclear-cytoplasmic shuttling of the tumor suppressor p53 was shown to be regulated by phosphorylation and by ubiquitinylation. It was suggested that following DNA damage, specific serine residues located within the p53 NES become phosphorylated and this phosphorylation event blocks p53 nuclear export (Zhang and Xiong, 2001). In addition, ubiquitinylation of p53 in the nucleus by Hdm2 was shown to negatively regulate its transactivation activity, and enhance its nuclear export and degradation by exposing the NES (Tao and Levine, 1999). Glutathionylation, which will be further addressed in Section 1.2.1.4, is another post-translational modification that recently has been implicated in regulation of protein subcellular localisation (Shelton et al., 2005). One conclusive example has been provided for actin, where glutathionylation regulates its polymerisation and its translocation to the cell periphery (Wang et al., 2001).
Phosphorylation events have been shown to regulate the nuclear import and export of proteins by modulating the affinity of transporter molecules for NLS and NESs. One classic example of nuclear import regulation is the SV40 large T-antigen, where phosphorylation at Ser$^{111/112}$, flanking the NLS, enhances the affinity of IMPα/β1 binding by 100-fold, thereby increasing nuclear import by 50-fold (Poon and Jans, 2005). On the other hand, phosphorylation can also act in an inhibitory manner as described for the yeast protein Hog1p, where phosphorylation at Thr$^{174}$ and Tyr$^{176}$ mask its NES, inhibiting recognition by the exportin1 molecule and leading to its nuclear retention (Ferrigno et al., 1998). Transcription factors are additional family of proteins whose subcellular localisation and function are largely regulated by phosphorylation events (reviewed in Jans et al., 2000).

1.2.2.3.1 Redox regulation of transport signals

The nuclear translocation of proteins can also be regulated through reversible oxidative modifications of redox-sensitive cysteine residues leading to masking, or enhancing, the availability of transport signals. The *S. Cerevisiae* Yap-1 protein is a transcription factor essential for the yeast response to oxidative stress, (Kuge et al., 1997) whose activity is induced by H$_2$O$_2$ and other oxidants (Hirata et al., 1994; Kuge and Jones, 1994) and regulated through redox modifications. Yap-1 is restricted to the cytoplasm by continuous nuclear export by CRM1 (Kuge et al., 1998; Yan et al., 1998). Upon activation by oxidative stress Yap-1 accumulates in the nucleus where it induces the expression of stress-response genes (Gasch et al., 2000; Kuge et al., 1997). This is regulated through an oxidation induced reversible disulfide bond formation within a cysteine rich domain that flanks the Yap-1 NES, inhibiting the interaction with CRM1 and thus promoting its nuclear retention (Delaunay et al., 2000; Kuge et al., 2001; Kuge et al., 1998; Yan et al., 1998). Oxidation of other cysteine residues were also shown to play a role in the prolonged nuclear retention of Yap-1 (Kuge et al., 2001). Recently, structural studies of Yap-1 provided direct evidence that oxidation induces a conformational change that leads to masking of the NES, whereas reduction promotes an unstructured protein conformation with an exposed NES (Wood et al., 2004). The intracellular localisation of a large number of proteins is mediated through redox control (Liu et al., 2005; Nose, 2005) nevertheless, the Yap-1 model represents one of the most complete and best understood examples of redox regulation of protein localisation and function.
1.3 REDOX REGULATION AND OXIDATIVE STRESS IN MAMMALIAN CELLS

Reactive oxygen species (ROS) are continuously produced in cells as a consequence of aerobic life. During normal cellular metabolism by-products of the respiratory chain or other oxidation reactions, such as hydrogen peroxide \((H_2O_2)\), superoxide anions \((O_2^-)\), hydroxyl radicals \((\cdotOH)\) and nitric oxide \((NO)\) are being generated and metabolised by the cellular redox controlling systems. At low, physiological concentrations ROS function as regulatory molecules and control a variety of cellular processes such as the immune response, cell proliferation, cell-cell adhesion, inflammation, metabolism, aging and cell death (Finkel, 2003; Sen, 1998). On the other hand, excessive production of ROS, or insufficient levels of antioxidant defence, lead to the unfavourable condition defined as oxidative stress, during which severe damage can be caused to proteins, nucleic acids, lipids and other macromolecules often leading to cell death (Klatt and Lamas, 2000; Sies, 1991). This appears to be a major factor in aging, and has been implicated in numerous diseases such as Alzheimer’s, diabetes, and cancer (Aliev et al., 2002; Berlett and Stadtman, 1997; Kovacic and Jacintho, 2001). Thus, the difference between the concepts of ‘redox regulation’ and ‘oxidative stress’ lays in the amount of oxidants generated and redox regulation entails oxido-reductive processes that do not result in cell death or malfunctioning (Ghezzi, 2005).

Oxidation-reduction (redox) based regulation of signal transduction and gene expression appears to be a fundamental regulatory mechanism in cell biology. Redox regulation is carried out by the electron flow through side-chain, reactive -SH groups of cysteine residues, which also serve as redox sensing centres (Linke and Jakob, 2003; Sen, 1998). This mechanism is believed to account for most of the major redox driven signal transduction. Most intracellular protein thiol groups are protected against oxidation by the highly reducing environment inside the cell. However, in their oxidised state, cysteines can form disulfide bonds stabilising the native protein conformation and are normally found buried in hydrophobic pockets. Consequently, only accessible protein thiol groups with high thiol-disulfide oxidation potentials are
likely to be redox sensitive (Fiser and Simon, 2000). Such activities have been described for members of families such as transcription factors, antioxidants, cytokines, kinases, phosphatases and others (Filomeni et al., 2005; Liu et al., 2005; Rudolph, 2005). However, it is important to note and distinguish that redox regulated proteins fall into two distinct categories. The first class includes the redox-active enzymes such as thioredoxins and glutaredoxins that use thiol disulfide exchange reactions in their catalytic cycle to interact with other proteins and to transfer redox equivalents. The second class contains the proteins that use highly reactive cysteines to sense changes in the cellular redox potential and that undergo reversible disulfide bond formation to alter their conformation and most importantly, their activity (Linke and Jakob, 2003).

Markers for oxidative stress have been identified and divided into three categories: firstly, the formation of modified molecules by ROS reactions; secondly, the consumption or activation of antioxidant molecules or enzymes; and thirdly, the induction and inhibition of separate pools of transcription factors (Toyokuni, 1998). The reaction between ROS and DNA induces strand breaks, DNA-protein cross links and base modifications. The most prominent and well characterised marker for oxidative DNA damage is the 8-oxoguanine (8-oxoG) which induces a G:C to T:A transversions at DNA replication (Kasai et al., 1991; Kohda et al., 1987). The second group of markers for oxidative stress, also referred to phase II enzymes, include the antioxidant thiol-disulfide oxidoreductases such as glutaredoxins, glutathione reductases, thioredoxin and thioredoxin reductases, catalase and superoxide dismutase and importantly, the well known physiological redox buffer the tripeptide Glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) and ascorbate. These systems act in concert to combat the effects of the oxidative stress (Ghezzi, 2005). The third category, which is regulated by and thus closely associated with members of the second category includes redox-sensitive transcription factors such as NF-κB, SP1, HIF, HSF c-myc, c-fos and c-jun that are activated to induce gene expression of antioxidant defense and repair systems to ultimately restore the intracellular redox homeostasis (Finkel, 2003; Sen, 1998; Toyokuni, 1998).
1.3.1 REDOX-DEPENDENT MODIFICATIONS OF PROTEIN THIOLS

Cell signalling employs a variety of post-translational modifications of effector proteins, some transient and others sustained. Among these alterations are oxidative protein-sulfhydryl modifications which are induced by ROS. Protein cysteines are oxidised to form different products including the reversible intra-protein disulfides (S-S), inter-protein disulfides (S-S), glutathione-mixed disulfides (S-SG), S-nitrosothiols (S-NO), sulphenic acids (S-OH), or to the irreversible forms of sulphinic (S-O$_2$H) and sulphonic acids (S-O$_3$H) (Green and Paget, 2004; Klatt and Lamas, 2000). Protein disulfides and glutathione mixed disulfides are however, the predominant and best characterised thiol redox modification within mammalian cells.

1.3.1.1 S-nitrosylation

S-nitrosylation, the attachment of nitric oxide (NO) to protein thiols, is a fairly recently discovered cysteine modification which is generated through reactions with reactive nitrogen species (RNS) (Stamler, 1994; Stamler and Hausladen, 1998). S-nitrosylation is a rather unstable modification that readily can be removed non-enzymatically by ascorbate (Liu et al., 1998) or enzymatically by glutaredoxin and thioredoxin (Zai et al., 1999). Currently, S-nitrosylation is established as an important mechanism in NO signalling, and regulates the function of a growing list of proteins including caspases (Mannick et al., 1999), thioredoxin (Haendeler et al., 2002) and ryanodine receptors (Eu et al., 1999). The process of S-nitrosylation under physiological conditions is not well understood, however, an enzymatically catalysed reaction mechanism with thioredoxin as a central player has been suggested (Mitchell and Marletta, 2005).

1.3.1.2 Sulphenic acids

Sulphenic acid modifications in proteins (R-SOH) are generally unstable under physiological conditions and can easily become oxidised to the more stable but irreversible states of sulphinic and sulphonic acids. R-SOH modifications have been identified in protein tyrosine phosphatases-1B (PTP-1B) (Denu and Tanner, 1998;
Lee et al., 1998) and the insulin receptor kinase (Schmid et al., 1999) and shown to modulate their activity, and they can also be formed as intermediates in the catalytic redox cycles of enzymes (Claiborne et al., 2001; Schroder et al., 2000). R-SOH modifications can be reduced non-enzymatically by GSH, or enzymatically by Trx or peroxiredoxin (Prx) but cannot be directly reduced by glutaredoxin (Biteau et al., 2003; Claiborne et al., 1999).

1.3.1.3 Disulfide bond formation

For many years, studies of disulfide bonds focused on their roles in protein folding processes and in the structural stability of secreted proteins (Darby and Creighton, 1995; Wedemeyer et al., 2002). Currently, reversible disulphide bond formation is a well established mechanism by which cells modulate enzyme activity and function, and transduce oxidative stress signals to modulate transcriptional and post-transcriptional processes (Linke and Jakob, 2003).

Under normal conditions, disulfide bond formation occurs primarily in the oxidising environment of the endoplasmic reticulum (ER) of eukaryotic cells. The reducing intracellular environment does generally not support the existence of disulfide bonds. This is because the vast majority of protein cysteine residues have a pKa >8.0 and remain protonated (-SH) in the reducing environment of the cytoplasm (Cumming et al., 2004; Rietsch and Beckwith, 1998). However, certain redox sensitive proteins contain cysteine residues that exist as thiolate anions (-S¯) at physiological pH due to a lowering of their pKa values by charge interactions with neighbouring amino acids, and are thus more susceptible to oxidation (Cumming et al., 2004). The number of proteins that are regulated through reversible disulfide bond formation is constantly growing. Many well characterised models of such regulation exist and some examples include the prokaryotic transcription factor and oxidative stress response regulator OxyR, the molecular chaperone and heat shock protein Hsp33, the negative regulator of the antioxidant defense RsrA and the yeast oxidative stress response transcription factor Yap-1 (Linke and Jakob, 2003; Liu et al., 2005). Reversible disulfide bond formation is also a major regulator of signal transduction and gene expression in mammalian cells. Well characterised examples of mammalian transcription factors whose DNA binding activity is reliant on the redox status of critical cysteine residues
include AP1, NFκ-B, NF1, Nrf 2 and p53 (Liu et al., 2005). The importance of cysteine based regulation is becoming increasingly evident also for other classes of proteins such as signal transduction protein phosphatases and tumour suppressor proteins (Liu et al., 2005).

As these events are very dynamic and sensitive to oxidation which easily occurs upon disruption of cellular membranes, one struggle that the redox field has faced is the existence of reliable methods to study these events in vivo. However, novel experimental tools and techniques for studying redox events in vivo have recently been developed which should enable more straightforward approaches. Therefore, it is most likely that many more substrates of redox regulation await discovery in the near future.

1.3.1.4 Protein S-glutathionylation

Cellular redox status is also dependent on the balance between the levels of reduced and oxidised redox buffer couples such as reduced glutathione (GSH)/oxidised glutathione (GSSG), NADPH/NADP and NADH/NAD. The predominant intracellular redox buffer is GSH, which in most cells is present in concentrations in the millimolar range. In a resting cell, the ratio between GSH and its oxidised counterpart GSSG exceeds 100:1 (Ostergaard et al., 2004), however, upon oxidative stress this ratio can decrease to values of 10:1 (Ault and Lawrence, 2003).

As a result of increased ROS production, protein cysteine residues also form reversible mixed disulfides with GSH, a process that is referred to as S-glutathionylation (Schuppe-Koistinen et al., 1994). Glutathionylation is believed to play a significant role in the buffering of oxidative stress and in preventing proteins from oxidation to the more detrimental, irreversible states. More recently, glutathionylation has also been recognised as a major mediator of signal transduction during physiological conditions mediated by the ROS produced in the normal metabolism (Cotgreave and Gerdes, 1998; Fratelli et al., 2005; O'Brian and Chu, 2005). Moreover, glutathionylation has been implicated in stabilisation of extracellular proteins, regulation of enzyme activity and ROS-mediated inhibition of protein synthesis (Latour et al., 1999; Park and Thomas, 1989; Qiao et al., 2001).
GSH-mixed disulfides are generally formed non-enzymatically, through thiol-disulfide exchange reactions between protein thiols and GSSG or, from reactions between existing R-SOH and R-SNO with GSH (Klatt and Lamas, 2000; Schuppe-Koistinen et al., 1994; Stamler, 1994), and are induced by changes in redox potential in the vicinity of the redox sensitive thiol. Removal of GSH or de-glutathiolation is predominantly achieved by enzymatic cleavage of the disulfide bond involving the action of glutaredoxins, and less efficiently by thioredoxin or through non-enzymatic reduction. Several reports have indicated that glutaredoxin can act in the reverse direction and catalyse GSH-mixed disulfides formation (Lind et al., 1998; Lundstrom-Ljung et al., 1999; Starke et al., 2003). It has therefore been speculated that bidirectional glutathionylation/de-glutathionylation processes may have wider regulatory roles in the post-translational regulation of protein function, however further evidence is yet required.

1.3.2 THIOL-DISULFIDE REDOX SYSTEMS

As previously mentioned, a wide variety of proteins are regulated by redox mechanisms. This can be at the level of function, subcellular localisation and activation, however, they are all controlled by oxidoreductases with catalytic activities that function based on alteration in the redox potentials. The two major thiol-disulfide systems are the thioredoxin and the glutaredoxin system.

1.3.2.1 The thioredoxin system

The thioredoxin system is composed of thioredoxin (Trx), thioredoxin reductase (TR) and NADPH (Holmgren and Bjornstedt, 1995). Trx is a small (12 kDa) ubiquitous protein that contains the di-cysteine active site motif CXXC, in a structure called the thioredoxin fold (Holmgren et al., 1975; Martin, 1995). Mammalian cells contain two Trxs, one cytoplasmic and nuclear, Trx1, and one targeted to the mitochondria, Trx2 (Pekkari and Holmgren, 2004; Spyrou et al., 1997). TR is member of a family of selenoenzymes that will be described in detail in Section 1.3.2.1.2. Trxs catalyse the reduction of disulfides in substrate proteins at the expense of a disulfide bond
formation within their active site. Oxidised Trx is subsequently reduced by TR, using electrons from NADPH, thus being recycled and ready for another reaction. This mechanism is schematically presented in Figure 1.2.

![Figure 1.2 Reduction of protein disulfides by the Thioredoxin system.](image)

As the main thiol-disulfide reductase, the thioredoxin system controls a remarkably wide range of biological processes many of them vital such as DNA synthesis, transcriptional regulation, regulation of apoptosis and activation of the immune system (Arner and Holmgren, 2000; Nakamura, 2005).

### 1.3.2.2 The glutaredoxin system

The second main redox thiol-disulfide reducing system is the glutaredoxin system, which consists of GSH, glutathione reductase (GR), glutaredoxin (Grx) and NADPH (Fernandes and Holmgren, 2004). Grxs are similar to Trxs in that they also contain a CXXC active site motif, are low molecular weight proteins and have general thiol disulfide oxido-reductase activities that can reduce protein disulfides (Holmgren et al., 2005). However, distinguishing features between Grxs and Trxs are the strong preference of Grxs for GSH-mixed disulfides over protein-disulfides as substrates, and their ability to use reducing equivalents from GSH (Luthman and Holmgren, 1982a; Padilla et al., 1995). Several mammalian Grxs have been identified, the cytosolic Grx1, and the mitochondrial specific enzyme Grx2 which is also alternatively spliced to generate different protein isoforms (Holmgren et al., 2005). The mechanism by which Grxs reduce GSH-mixed disulfides is different to the mechanism employed by Trxs and Grxs when reducing protein disulfides, and is referred to as the monothiol mechanism. This employs a nucleophilic attack by an N-
terminal reactive cysteine towards the GSH moiety of the GSH-mixed target, formation of a Grx-SG intermediate which is finally reduced by GSH (Figure 1.3) (Bushweller et al., 1992).

**Figure 1.3 Reduction of GSH-mixed disulfides by the Glutaredoxin system.** GSH-mixed disulfides are reduced by Grx via the monothiol mechanism. This involves an attack of a Grx reactive cysteine residue with the GSH moiety of the substrate, formation of Grx-SG intermediate and recovery by reaction with GSH. (Courtesy of Catrine Johansson, Biochemistry Department, Karolinska Institute)

Reversible protein glutathionylation has been in the past few years realised as an increasingly important regulatory mechanism in mammalian cells, beyond what was originally believed to be a protective effect during oxidative stress. Examples of proteins whose activity is regulated by glutathionylation include PTP1B, actin, protein kinases A and C, PKA and PKC, annexin II, and nuclear factor 1 NF1 (reviewed in Shelton et al., 2005).

**1.4 SELENIUM AND SELENOPROTEINS**

**1.4.1 SELENIUM**

Selenium (Se), named after the moon, *selene* in Greek, by its discoverer Swedish chemist Berzelius in 1817, is well established as an essential trace mineral of fundamental importance to human health (Rayman, 2000). Selenium is known primarily for its antioxidant properties, and its chemical properties are similar to those of sulphur as both elements are found in the same periodic group. However, selenium is ~6 times more reactive. In biological systems selenium enters the food chain through plants, by uptake from the soil, and becomes converted to organic forms such as selenomethionine. In animal cells, selenomethionine is converted to the amino acid selenocysteine (Sec), the most prevailing biologically active form of selenium, and the analogue of cysteine (Cys). The difference between the two amino acids is that at physiological pH, the selenol group of Sec exists in the more reactive, ionized form, whereas the thiol group of Cys is protonated and less reactive (Stadtman, 1996).
addition, selenium compounds are metabolised to more reduced states in biological systems, whereas sulphur compounds become more oxidised. These differences are proposed to account for selenium compounds being more effective antioxidants and more potent cancer preventive agents than their sulphur analogues (Ip and Ganther, 1992; Whanger, 2004).

Selenium exerts its biological functions largely through its presence in selenoproteins, where it becomes incorporated co-translationally as the amino acid Sec, but also low molecular weight Se-containing compounds such as methylselenic acid, methylselenocysteine and selenomethionine, which have proven efficient as cancer preventing agents (Ganther, 1999; Ip et al., 2000; Whanger, 2004). In functionally characterised selenoenzymes, Sec is part of the catalytic group in the active site and is directly involved in redox reactions (Zhong and Holmgren, 2000). The functions of many selenoprotein families are not yet elucidated, nevertheless, it is clear that at least one selenoprotein is essential for life as elimination of the tRNA\textsuperscript{Sec} gene in mice causes early embryonic lethality (Bosl et al., 1997).

The physiological effects of selenium are strictly concentration dependent (Wu et al., 2005). In the nanomolar range, selenium compounds have antioxidant effects protecting the cell against oxidative stress through their presence within selenoenzymes such as TR and glutathione peroxidase (GPx). In contrast, at higher concentration when selenoprotein synthesis has reached its maximum, inorganic selenium compounds may accumulate and redox cycle with intracellular thiols leading to oxidative stress, and thus having a complete opposite biological action (Figure 1.4).
Figure 1.4 Biological effects of selenium at various concentrations. The effects of selenium on the organism are concentration dependent. Se is an essential trace mineral required for life. In the nanomolar range Se has beneficial antioxidant effects. In higher doses it can have negative effects on the cellular environment by promoting oxidation and inactivation of proteins. Extremely high doses of Se are toxic and can have detrimental effects.

1.4.1.1 Selenium and health

A growing body of evidence has established that selenium deficiency has adverse consequences for disease susceptibility and the maintenance of optimal health reviewed in (Rayman, 2000). Selenium is required for the functioning of the immune system, and deficiency is linked to the occurrence, virulence or disease progression of some viral infections. In particular, selenium appears to be a key nutrient in counteracting the development of virulence, inhibiting HIV progression to AIDS and decreasing mortality from HIV-related causes (Rayman, 2000). Selenium is essential for reproduction. In males it is required for testosterone biosynthesis and the formation and development of spermatozoa. Low serum selenium levels have been detected in women who experienced recurrent miscarriages. Thyroid function is as well dependent on selenium availability, as thyroid hormone metabolism is reliant on the activity of the selenoproteins iodothyronine deiodinases (DIOs). Selenium-deficient rats showed a decreased production of the T3 hormone in the liver, highlighting the importance of an adequate selenium supply with regard to hormone metabolism (Arthur et al., 1990; Behne et al., 1990). In addition, combined Se and iodine deficiency leads to a severe form of myxoedematous cretinism indicating a link in the metabolism of these two essential trace elements (Zimmermann and Kohrle, 2002).
A large number of health conditions have been linked to selenium status (Rayman, 2000). For example, selenium deficiency-diseases in animals have been identified in livestock in countries with low selenium content in soil, causing symptoms such as growth depression (ill-thrift), reproductive impairment and a myopathy of heart and skeletal muscle, called white-muscle disease (Osame et al., 1990). Severe selenium deficiency in humans leads to Keshan disease, a cardiomyopathy lethal if not treated, but fully preventable with dietary selenium (Reeves et al., 1989). Kashin-Becks disease, a deforming arthritis is another example where selenium deficiency is a contributing factor. Both disorders are prevalent in selenium deficient areas in China. It is however not known whether these diseases are linked to the loss of function of a particular selenoprotein (Kohrl et al., 2000).

In higher doses, selenium compounds can be extremely toxic and acute intoxication gives symptoms such as garlic odour breath, tachycardia, emesis, diarrhea, dyspnea and pulmonary oedema that may lead to death. The main signs of chronic selenosis are loss of hair and brittle fingernails (Tinggi, 2003; Yang and Zhou, 1994).

As selenium behaves both as an antioxidant and anti-inflammatory agent, it is not surprising that it has been found beneficial in oxidative stress or inflammatory conditions such as cardiovascular disease and arthritis. These effects have been linked to the GPx group of antioxidant enzymes which contain selenium in their active site (Brigelius-Flohe et al., 2003).

Selenium has for many years been known to possess anticarcinogenic properties. Clinical trials have shown that dietary supplementation with selenium resulted in a significant reduction in cancer mortality and overall cancer incidence (Clark et al., 1996). Such cancers included lung, prostate and colon cancers. Epidemiological studies have also found a link between low plasma selenium levels and increased incidence of liver cancers (Yu et al., 1999).

Several epidemiological studies have also provided evidence of an inverse correlation between adequate selenium intake and cancer incidence and mortality (Rayman, 2000). The precise molecular mechanisms behind the cancer preventive effects of selenium have so far remained elusive; however, a great deal of experimental
evidence has made it arguable that selenium acts as a tumour preventive agent by increasing the activity/and or the selenium saturation of selenoproteins (Ganther, 1999).

1.4.2 SELENOPROTEINS: OVERVIEW AND EVOLUTIONARY PERSPECTIVE

The functions and beneficial effects of selenium as an essential nutrient are largely attributed to its presence within selenoproteins, where it is co-translationally inserted as part of the 21st amino acid Selenocysteine (Sec). Selenoprotein synthesis represents a unique mechanism in that Sec is encoded by UGA, which normally serves as termination codon. Consequently, the translation machinery needs to be capable of distinguishing between “Sec” and “Stop”. Indeed, a number of cis- and trans- acting factors, conserved through evolution, have been identified and shown essential for this re-coding event (addressed in detail below) (Caban and Copeland, 2006; Driscoll and Copeland, 2003; Small-Howard and Berry, 2005). The universal determinant of Sec codon read-through is the Sec Insertion Sequence (SECIS) element, a secondary structure in the 3′-untranslated regions of selenoprotein mRNAs, found in all lineages of life (Low and Berry, 1996; Thanbichler and Bock, 2002). While the mechanism of Sec incorporation in prokaryotes is now well understood (Bock, 2000), the eukaryotic counterpart still has some parts of the puzzle missing.

The dual function of the UGA codon has until recently made selenoprotein identification a challenge; consequently, selenoproteins are almost universally mis-annotated in sequence databases (Zhang et al., 2005). However, computational approaches based on searches for SECIS elements and evolutionary conservation of Cys-containing homologs, combined with the completion of genome sequencing projects has in the past few years enabled the characterisation of selenoproteomes among species leading to identification of novel selenoprotein families, and expanding the understanding of selenocysteine evolution (Castellano et al., 2001; Castellano et al., 2004; Kryukov et al., 2003; Kryukov and Gladyshev, 2004; Lescure et al., 1999).
The characterisation of the prokaryotic selenoproteome using the dataset from the largest prokaryotic sequencing project - the microbial population of the Sargasso Sea (Venter et al., 2004), was recently conducted (Zhang et al., 2005). In this study, 101 novel selenoprotein genes, belonging to 15 families were identified, most of them containing domains of proposed redox-based functions. The dataset analysis also revealed that some selenoprotein genes, previously considered of eukaryotic origin as based on their known functions, were present in bacteria suggesting not only a larger overlap between the prokaryotic and eukaryotic selenoproteomes than previously thought, but also a functional diversity between the same selenoprotein in different lineages of life. For example, prokaryotic homologs of deiodinases, which function in thyroid hormone metabolism, were identified in the dataset implying that deiodinases serve other functions in bacteria than in higher eukaryotes.

Between the three kingdoms of life bacteria, eucarya and archaea, thirty selenoprotein families have been identified (Castellano et al., 2005; Kryukov et al., 2003; Kryukov and Gladyshev, 2004; Zhang et al., 2005). Selenoproteomes among species are generally small, the largest one containing less than 30 proteins, possibly due to the limited Se supply in nature (Castellano et al., 2005). An overview of the distribution of selenoproteins in eukaryotes is presented in Figure 1.5. Amongst organisms in which the selenoproteome has been delineated, fish seem to have the highest selenoprotein repertoire, with 30 individual selenoproteins identified, followed by humans with 25 selenoproteins discovered (Castellano et al., 2005; Castellano et al., 2004; Kryukov et al., 2003). Drosophila genome studies have revealed existence of three selenoproteins (Castellano et al., 2001; Martin-Romero et al., 2001), whereas C. Elegans contain only one selenoprotein, a thioredoxin reductase (Gladyshev et al., 1999a; Taskov et al., 2005). S. Cerevisiae and S. Pombe do not possess any selenoproteins, however they do contain orthologous proteins in which Cys is present in the Sec position (Kryukov et al., 2003). For most selenoprotein families, Sec/Cys interconversion is commonly observed across the species (Figure 1.5) (Castellano et al., 2005).
Figure 1.5 Distribution of eukaryotic selenoprotein families and Cys-containing homologs in eukaryotes. Computational approaches were used to screen multiple eukaryotic genomes to compile an overview of Sec conservation among species and to identify and characterise selenoprotein families. Sec-containing proteins are shown in red, Cys-containing proteins are shown in green and unknown proteins are shown in yellow. Open boxes represent absent proteins in the genome (Castellano et al., 2005).
Only a few selenoprotein families have been functionally characterised. These include glutathione peroxidases, thioredoxin reductases and methionine sulfoxide reductases which are involved in antioxidant protection and cellular redox regulation, and iodothyronine deiodinases that catalyse thyroid hormone metabolism (Schomburg et al., 2004; Stillwell and Berry, 2005). For the remaining selenoprotein families, the functions remain elusive. GPx4/PHGPx has been attributed a structural role as part of the midpiece of the mature spermatozoa, linking the mechanical instability of the mitochondrial midpiece observed in selenium deficiency to the GPx 4 protein (Castellano et al., 2005; Ursini et al., 1999). The fish-specific SelJ selenoprotein shows significant similarity to the jellyfish crystallin proteins and displays a homogenous expression in the eye lens of zebrafish suggesting a potential role as a structural crystallin (Castellano et al., 2005). SelP is another selenoprotein with a non-enzymatic function that is required for transport and storage of selenium (Schweizer et al., 2005).

Characterisation of selenoproteomes has laid the foundation for what from now on may prove to be the greatest challenge in the selenium field: the functional characterisation of these proteins, elucidating their evolutionary purpose and their involvement in the etiology of disease.

1.4.2.1 The human selenoproteome

The mammalian selenoproteome was recently characterised (Kryukov et al., 2003). Computational analysis of the newly sequenced human genome identified seven new previously unknown selenoprotein genes, thus compiling the human selenoproteome to 25 individual selenoproteins contained within 17 families. Families of human selenoproteins for which multiple genes have been identified include: glutathione peroxidases (5 genes), thioredoxin reductases (3 genes) and iodothyronine deiodinases (3 genes). The remaining selenoprotein families were identified only as individual genes and have been annotated in alphabetical order and include: the 15 kDa selenoprotein/ Sel15, SelH, SelI, SelK, SelM, SelN, SelO, SelP/SepP, SelR, SelS, SPS2, SelT, SelV and SelW. Selenoprotein families that have been functionally characterised have been the topic of a number of reviewes (Behne and Kyriakopoulos,
2001; Gromer et al., 2005; Hatfield and Gladyshev, 2002; Schomburg et al., 2004) and are only briefly discussed below.

1.4.2.1 Glutathione peroxidases

Glutathione peroxidase (GPx) was the first mammalian protein shown to incorporate Sec in its catalytic site (Forstrom et al., 1978). GPxs are well known for catalysing the reduction of hydrogen peroxide and organic hydroperoxides, thus protecting the cells from oxidative damage (Behne and Kyriakopoulos, 2001). Within the GPx family 5 members exist: the classical or cytosolic cGPx (GPx1), a gastro-intestinal specific GI-GPx (GPx2), a phospholipid hydroperoxide PHGPx (GPx4), a secreted protein found in plasma pGPx (GPx3) and a sperm nuclei specific snGPx (GPx6). These isozymes differ in their tissue distributions and their substrate specificity for peroxide degradation (Arthur, 2000; Brigelius-Flohe et al., 2002). Mouse knock-out models of the cGPx gene showed normal development, however, when challenged with oxidative stress causing agents, knock-outs were significantly more sensitive to damage than wild-type controls suggesting that the protective effects of cGPx are of particular importance when the organism is exposed to exogenous stress (Cheng et al., 1998). Unlike other GPxs, PHGPx can directly reduce phospholipid- and cholesterol-hydroperoxides, is also involved in arachidonic acid metabolism. Moreover, PHGPx serves as a structural protein during sperm maturation, a finding that provides the molecular explanation for the link between selenium deficiency and male infertility (Pfeifer et al., 2001; Ursini et al., 1999). PHGPx gene knock out in mouse models produced an embryonic lethal phenotype (Imai et al., 2003; Yant et al., 2003) whereas GPx1 and GPx2 knock out mice appeared normal under standard growth conditions (Beck et al., 1998; Esworthy et al., 2000). The GI-GPx, expressed in the gastrointestinal tract, is thought to provide a barrier against hydroperoxides derived form the diet (Wingler and Brigelius-Flohe, 1999). Similarly, the pGPx is suggested to act as a peroxidase in the plasma, in concert with the thioredoxin reductase system (Soderberg et al., 2000). It was recently reported that allelic loss of the cGPx gene is a common event in cancer development; in particular cancers of the head and neck, breast, lung and colon showed loss of heterozygosity (LOH) related to the GPx gene locus (Hu et al., 2005).
Mammalian TRs are a family of ubiquitously expressed homodimeric flavoenzymes containing an FAD domain, an NADPH binding site and a Sec residue in the catalytic domain which is indispensable for their enzymatic activity (Gladyshev et al., 1996; Luthman and Holmgren, 1982b; Zhong et al., 2000; Zhong et al., 1998; Zhong and Holmgren, 2000). Three mammalian TR selenoenzymes have been identified: the cytosolic enzyme TR1 (Tamura and Stadtman, 1996), the mitochondrial enzyme TR2 (Lee et al., 1999; Miranda-Vizuete et al., 1999) and a testis specific enzyme TGR/TR3 that also possesses GR activity (Sun et al., 1999). Moreover, alternative splicing mechanisms within these enzymes have been reported (Rundlof et al., 2000, Rundlof et al., 2004, Osborne et al., Sun et al., 1999, Lescure et al., 1999, Sun, 2001; Lescure, 1999) suggesting the existence of an even greater variety of thioredoxin reductase species.

TR is the only enzyme known to catalyse the NADPH-dependent reduction of oxidised thioredoxin, which in turn is a central factor in cellular redox regulation (Arner and Holmgren, 2000). Many cellular processes are therefore reliant on the activity of these enzymes (Nakamura, 2005). TR and Trx act in concert to catalyses the reduction of protein disulfides such as ribonucleotide reductase- an enzyme essential for DNA synthesis, and thioredoxin peroxidase a critical enzyme in the defence against oxidative stress. The TR system is also responsible for regulation of gene expression via redox control of transcription factors, cellular growth and immune response activation (Arner and Holmgren, 2000; Mustacich and Powis, 2000; Nakamura, 2005). The redox sensitive Sec residue within TR may also act as a cellular redox sensor and regulator of cell signalling in response to elevated levels of reactive oxygen species (ROS) (Sun et al., 1999). TR2 is also involved in control of mitochondrial redox processes and was shown to reduce cytochrome c, possibly playing a role apoptosis signalling (Nalvarte et al., 2004). A recent study also showed that induction of mutant TR2 affected the expression of several cell cycle proteins and increased the progression rate of cells from G1 to S phase, implicating TR2 in cell proliferation (Kim et al., 2003).
Targeted disruption of either TR1 or TR2 genes resulted in an embryonic lethal phenotype. TR1 null embryos were affected primarily by compromised cell proliferation, whereas TR2 null embryos suffered from severe anaemia and improper heart development (Conrad et al., 2004; Jakupoglu et al., 2005).

1.4.2.1.3 Iodothyronine deiodinases

The iodothyronine deiodinases (DIOs) connect selenium biology to thyroid hormone metabolism. The family of DIOs consist of three, differentially distributed, functionally distinct selenoenzymes that are responsible for catalysing the activation and inactivation of the thyroid hormone thyroxine and its metabolites. DIO1 catalyses the deiodination of thyroxine (T4) to the biologically active form triiodothyronine (T3), as well as it inactivates T3 and T4 and regulates the elimination of byproducts of these reactions (Kohrle et al., 2000). These hormones regulate various metabolic processes and are indispensable for the normal development of the fetal brain. DIO2 and DIO3 possess similar functions and they are predominantly involved in tissues specific regulation of hormone metabolism (Behne and Kyriakopoulos, 2001).

1.4.2.1.4 Selenophosphate synthetase 2

Selenophosphate synthetase 2 (SPS2) is interesting in that it represents a unique example of a selenoprotein’s involvement in Sec biosynthesis, as being part of its own autoregulatory loop. SPS2 catalyses the reaction of selenide with AMP to yield selenophosphate, which is a precursor in the mechanism of Sec production (Guimaraes et al., 1996).

1.4.2.1.5 Selenoprotein P

Selenoprotein P (SePP, SelP), is a secreted selenoprotein and the second major selenoprotein in plasma after GPx3. It is estimated that more than 60% of the plasma selenium is found within SelP (Read et al., 1990). SelP is different from all other selenoproteins as it incorporates multiple Sec residues per protein molecule, with 9 Sec residues in the human protein and 17 residues in zebrafish (Kryukov et al., 2003).
Although highly probable, an enzymatic activity for SelP has not been identified yet, its role being implicated in transport and delivery of selenium to remote tissues. Consistent with these suggestions, SelP knock-out studies confirmed a disturbance in tissue distribution of Se (Hill et al., 2003; Schomburg et al., 2003; Schweizer et al., 2005) that also led to neurological and growth defects, and infertility in male mice (Schomburg et al., 2004). Some evidence for a link between down-regulation of SelP and prostate cancer progression has also been reported (Calvo et al., 2002).

1.4.2.1.6 Functionally uncharacterised selenoproteins

At least nine families of selenoproteins exist for which a definite function has not yet been attributed. A possible role in redox function, due to the presence of Sec within putative catalytic domains such as the C-XX-Sec within SelW and SelT has been suggested, however, direct evidence is still lacking (Carlson et al., 2005). For some of these proteins differences in expression among tissues, as well as distribution among different subcellular compartments have been detected, suggesting that their functions might be linked to their localisation (Korotkov et al., 2001; Korotkov et al., 2002). Moreover, a hierarchy in the synthesis of individual selenoproteins in various tissues is observed upon Se repletion after Se deprivation, indicative of difference in the requirement and biological roles among selenoprotein families in different tissues (Driscoll and Copeland, 2003; Hatfield and Gladyshev, 2002; Schomburg et al., 2004).

Several links between selenoproteins and disease have been reported. For instance the Sep15 and GPx1 have both been frequently linked to LOH associated with tumour progression, suggesting potential tumour suppressor roles for these selenoproteins (Cheung et al., 1999; Hu et al., 2005). Protein levels of Sep15 were also reduced substantially in prostate cancer cell lines and hepatocellular carcinomas (Kumaraswamy et al., 2000). SelN is the only selenoprotein so far linked to an inherited disease. Mutations in SelN have been described in a form of rigid spine muscular dystrophy; however, the underlying molecular mechanisms are not well understood (Moghadaszadeh et al., 2001).
Years of elaborate future studies will progressively unveil the functions and biological significance of these families of proteins, as well as their involvement in disease cause, onset, progression and most importantly prevention.

### 1.4.3 SELENOPROTEIN SYNTHESIS

Selenoprotein synthesis is an evolutionary conserved process, nevertheless, major differences in the mechanisms between prokaryotes, eukaryotes and archaea do exist. The common feature to all organisms is the requirement of Sec codon, UGA, its specific tRNA\(^{Sec}\) and the SECIS element (Caban and Copeland, 2006). Several Sec-specific translation factors in prokaryotes and eukaryotes have been identified and the contribution of some of these to selenoprotein synthesis has been delineated. These features and factors as well as the mechanisms of Sec insertion in bacteria and eukaryotes are discussed below.

#### 1.4.3.1 Selenocysteine

Selenocysteine, the 21\(^{st}\) amino acid in the genetic code, is a naturally occurring amino acid translated into proteins by reading of the UGA codon (Lee et al., 1989). Incorporation of Sec into polypeptide chains is supported by the selenocysteyl-tRNA\((\text{Ser})\), tRNA\([\text{Ser}]^{\text{Sec}}\) (Lee et al., 1989). Structurally, Sec is identical to Cys, except for the presence of selenium instead of sulphur (Figure 1.6) which ascribes Sec more reactive properties.
Figure 1.6 Schematic representations of the amino acids cysteine and selenocysteine. The sulfur within the cysteine residue and the selenium within selenocysteine are shown in red.

In mammalian cells Sec is synthesised from serine and it occurs on its own tRNA through the conversion of seryl-tRNA(Ser) via phosphoseryl-tRNA(Ser) to selenocysteyl-tRNA(Ser) (Lee et al., 1990). Interestingly, the tRNA[^Ser]Sec has a dual function, firstly, it serves as a backbone molecule upon which Sec is synthesized and secondly, it incorporates Sec at distinct UGA codons (Lee et al., 1990; Lee et al., 1989).

A mammalian kinase that specifically phosphorylates the seryl-tRNA(Ser) in the reaction was recently identified (Carlson et al., 2004). The SectRNA[^Ser]Sec controls the expression of all selenoproteins, a model that has not been reported for any other tRNA molecules. Sec biosynthesis, which in turn is controlled by the availability of Se, regulates selenoprotein synthesis in an autocrine mechanism (Hatfield and Gladyshev, 2002). Two Sec tRNA[^Ser]Sec isoforms occur in higher vertebrates, a methylated form and an un-methylated form, and interestingly, they appear to differentially regulate the translation of selenoproteins. The methylated form controls the synthesis stress related selenoproteins such as GPx1, whereas the un-methylated form governs over synthesis of housekeeping selenoproteins such as TR (Carlson et al., 2005).
1.4.3.2 SECIS elements

The major determinant for decoding of UGA as Sec is the SECIS element, an RNA stem-loop structure located downstream of the UGA codon (Low and Berry, 1996). The location of the SECIS element in relation to the UGA codon that it re-codes constitutes one of the main differences between the prokaryotic and the eukaryotic mechanism of Sec incorporation. In prokaryotes, it is located immediately after the UGA-Sec codon (Zinoni et al., 1990) whereas in eukaryotes it is situated in the 3′ untranslated region (UTR) (Berry et al., 1991), in some cases kilobases apart from the UGA (Buettner et al., 1998). The sequences and structures of SECIS elements differ among prokaryotes and eukaryotes, however, they are well conserved within each lineage (Hoffmann and Berry, 2005). The SECIS elements in archaea differ structurally from those of both prokaryotes and eukaryotes and at least one has been found located in the 5′-UTR (Wilting et al., 1997).

1.4.3.2.1 The eukaryotic SECIS element

Eukaryotic SECIS elements have low sequence similarities however, their secondary structures are highly conserved and contain consensus sequences that direct Sec incorporation (Walczak et al., 1996). Several distinct eukaryotic SECIS elements shown to support Sec incorporation have been identified (Kryukov et al., 2003) and they possess the following defining features: an apical loop with a conserved AAR motif with two consecutive unpaired AA residues, or two CC residues as identified in the SelM and SelO SECIS elements (Korotkov et al., 2002; Kryukov et al., 2003) and a stem structure located 11 to 12 nucleotides apart from the AA motif, which holds the conserved AUGA motif referred to as the SECIS core, and an A residue that precedes the SECIS core. The SECIS core base pairs with the GA motif in a non-Watson-Crick manner as depicted in Figure1.7 (Berry et al., 1997; Walczak et al., 1996). Figure 1.7 A displays a schematic representation of the conserved feature and Figure 1.7 B shows examples of SECIS elements within the newly identified selenoproteins. A single SECIS element is sufficient to dictate Sec incorporation in all selenoproteins except for SelP, where the incorporation of multiple Sec residues/molecule is directed by the concerted action of two SECIS elements (Caban and Copeland, 2006).
Figure 1.7 The eukaryotic SECIS element. (A) The structure of the eukaryotic SECIS element is schematically drawn showing the two helixes, the apical and internal loops and the SECIS-core. The location of the conserved nucleotides: the AA doublet in the apical loop, and the AUGA-GA non-Watson-Crick base-pairs in the SECIS core. (B) Examples of SECIS elements from different human selenoproteins. (Kryukov et al., 2003)

As mentioned above, the unique features of the SECIS element and its specificity for Sec decoding has been an essential tool in the identification of selenoproteins in genomes across species. Similarly, as intact SECIS elements are required for synthesis of functional selenoproteins, these motifs need also to be considered when searching for disease-linked mutations within selenoprotein genes.

1.4.3.3 Sec-decoding protein factors

Apart from the cis-acting components the UGA-Sec and the SECIS element, and the trans-acting Sec-tRNA^{Sec} molecule, Sec decoding requires the cooperative action of several protein factors. In prokaryotes, this is achieved by the multifunctional protein SelB, a GTP-dependent elongation factor, specific for Sec incorporation (Forchhammer et al., 1989; Forchhammer et al., 1990). SelB interacts with the SECIS element and also facilitates the delivery of the Sec-tRNA^{Sec} to the ribosomal A site. Recently, Sel B was shown to play an additional role in regulating selenoprotein synthesis in response to selenium levels (Thanbichler and Bock, 2002).

In mammals, the mechanism of Sec decoding is more complex and less well understood. The function of SelB has been proposed to be divided between two protein factors: the SECIS binding protein 2, SBP2 (Copeland and Driscoll, 1999;
Copeland et al., 2000) and the Sec specific elongation factor mSelB/eEFSec (Tujebajeva et al., 2000; Fagegaltier et al., 2000) and for several years these were the only known protein factors involved in the co-translational incorporation of Sec. In the past year however, three additional proteins that appear to function in the Sec decoding machinery were identified. These include the ribosomal protein L30 (Chavatte et al., 2005), the 43 kDa RNA-binding protein, Secp43, and the soluble liver antigen, SLA (Xu et al., 2005). Moreover, new aspects of regulation of “old” factors (Kinzy et al., 2005), as well as novel molecular interactions between protein factors have been discovered recently (Small-Howard and Berry, 2005). These aspects are discussed below. Since SBP2 is the topic of investigation in this thesis, it is discussed in detail in Section 1.3.4.

1.4.3.3.1 The Sec-specific elongation factor eEFSec

The mammalian eEFSec protein displays sequence and domain similarity to the general translation elongation factor EF1A (Tujebajeva et al., 2000). eEFSec was shown to interact directly and specifically with both isoforms of the Sec-tRNA\textsubscript{Sec}, and when co-transfected with SBP2 in human cell lines, the two proteins co-immunoprecipitated in complex with each other (Fagegaltier et al., 2000; Tujebajeva et al., 2000). The SBP2-binding region was mapped to the C-terminal portion of eEFSec, and Sec-tRNA\textsubscript{Sec} was found necessary for complex formation (Zavacki et al., 2003). Sec-tRNA\textsubscript{Sec} also had a stabilising effect on eEFSec protein levels (Zavacki et al., 2003). The eEFSec-binding region within SBP2 has so far not been defined.

Even though eEFSec does not contain an RNA binding domain, recombinantly expressed protein was shown to bind to the SECIS element \textit{in vitro} (Tujebajeva et al., 2000). However, the binding was not affected by mutations to the SECIS core, suggesting the possibility of a different binding site or a non specific binding. In a different report, eEFSec was shown unable to directly bind to the SECIS element, leaving this issue in need of further investigations (Lescure et al., 2002).
1.4.3.3.2 The SECp43 and the SLA protein factors

Among the latest addition to the selenoprotein synthesis machinery are the SECp43 and the SLA proteins (Xu et al., 2005). These proteins were previously identified as putative Sec incorporation factors as proposed by their association with the Sec-tRNA\textsuperscript{Sec} (Ding and Grabowski, 1999; Gelpi et al., 1992). Recently, when transiently expressed in cells these proteins were shown to co-immunoprecipitate in complex with Sec-tRNA\textsuperscript{Sec} and depletion of either SECp43 or SLA resulted in decreased binding of the remaining protein to the Sec-tRNA\textsuperscript{Sec} (Xu et al., 2005). When co-expressed in cells, it appeared that SECp43 was able to sequester SLA from the cytoplasm to the nuclear compartment. Depletion of SECp43 resulted in decreased levels of methylation of the Sec-tRNA\textsuperscript{Sec} and decreased GPx1 expression and the combined depletion of both proteins caused a general decrease in selenoprotein synthesis (Xu et al., 2005). These findings implicate additional levels of regulation of selenoprotein synthesis by novel protein factors, however further studies are required in order to completely elucidate their functions.

1.4.3.3.3 Ribosomal L30 protein

L30 belongs to the ribosomal L7Ae family of proteins and contains an L30-type of RNA binding motif (Moore et al., 2004). This feature of L30 is shared with SBP2, although the sequence identity between the two proteins is only 20% (Chavatte et al., 2005). L30 was previously shown to exist as an essential component of the ribosome and to function in autoregulation of its own splicing and translation (Vilardell et al., 2000). Recent molecular and biochemical characterisation of L30 revealed that it is also a component of the UGA-Sec recoding machinery. L30 binds directly to SECIS elements \textit{in vivo} and \textit{in vitro} and stimulates Sec incorporation in transfected cells, an effect that was further enhanced by co-transfection with SBP2. Biochemical assays demonstrated that L30 and SBP2 compete for SECIS binding and are able to efficiently displace each other from the SECIS element. However, L30 was proposed to preferentially bind a conformational altered SECIS element that may be induced by the release of the Sec-tRNA\textsuperscript{Sec} whereas SBP2 has higher affinity for the open SECIS conformation (Chavatte et al., 2005). Based on these lines of evidence a model was proposed suggesting that L30 may function in displacing SBP2 from the SECIS
element, and upon its own binding inducing a conformational change in the SECIS that would allow delivery of the eEFSec/Sec-tRNA\textsuperscript{Sec} complex to the ribosomal A site. However, the model does not agree with previous reports that demonstrated a stable association of SBP2 with the ribosomes rather than the SECIS element. Nevertheless, these findings appear to suggest that ribosomal protein L30 may play an important role in the molecular events that occur at the UGA decoding, \textit{in vivo}.

1.4.4 SECIS BINDING PROTEIN 2, SBP2

The best characterised of the known Sec incorporation factors is the SECIS binding protein SBP2. SBP2 was isolated and cloned from rat testicular extracts based on its high binding affinity for SECIS elements (Copeland and Driscoll, 1999). SBP2 binding activity was detected in a large molecular mass complex of ~500 kDa which is indicative of its existence in a multiprotein complex in cells. To date, the only characterised SBP2 binding partners are ribosome and eEFSec (Zavacki et al., 2003). At sequence level, SBP2 is not homologous to other any known protein, however, it contains an L30 RNA binding domain shared with several ribosomal proteins, a yeast omnipotent suppressor of translation termination (SUP1), the eukaryotic translation termination release factor 1 (eRF-1) and the 15.5 kD/Snu13p protein (Allmang et al., 2002; Caban and Copeland, 2006; Copeland et al., 2001). Rat SBP2 was shown to bind SECIS elements \textit{in vivo} and the SBP2 binding region within the SECIS element was mapped to the AUGA consensus sequence within the SECIS core (see fig 1.5) (Copeland et al., 2000). Reticulocyte lysates depleted of SBP2 were shown to lack selenoprotein translation activity, whereas upon repletion with SBP2, translation was restored (Copeland et al., 2000). These experiments established a requirement of SBP2 for Sec incorporation \textit{in vitro}; however an equivalent role for SBP2 \textit{in vivo} has not yet been reported.

SBP2 is ubiquitously expressed among tissues and may be encoded by as many as three distinct transcripts: two larger transcripts of ~4 and ~3.5 kb generally detected in tissues, and a smaller transcript ~2.5 kb only detected in testis where it was also the most abundantly expressed of the three transcripts (Copeland et al., 2000). Protein expression levels in tissues or cell lines have however not been reported.
The human SBP2 homolog has also been cloned, and as expected, human SBP2 had similar *in vitro* SECIS binding activity to the rat protein. Interestingly, SBP2-SECIS interactions *in vitro* were stimulated by the addition of eEFsec, suggesting that eEFsec may stabilise the SBP2-SECIS complex possibly by inducing a structural change within SBP2 (Lescure et al., 2002).

### 1.4.4.1 SBP2 structure and RNA binding properties

Human SBP2 is an 854 amino acids (846 for rat SBP2) long protein, with a predicted pI of 8.3 which renders it a slightly basic protein. The predicted SBP2 molecular weight is 95.8 kDa, however, the rat protein was shown to migrate close to 120 kDa during electrophoresis (Copeland et al., 2000). This discrepancy was attributed to a portion of the SBP2 sequence, overlapping with the RNA-binding domain that was proposed to cause a specific structure formation, or modifications, within this region (Copeland et al., 2001).

Several domains have been identified and some of them characterised within SBP2: an L30-type RNA binding domain (RBD) (Allmang et al., 2002; Copeland et al., 2001), a lysine rich region predicted, but not confirmed, to be a nuclear localisation signal (NLS) (Copeland et al., 2000), a functional domain dispensable for SECIS binding but required for Sec incorporation activity (Copeland et al., 2001), and a ribosomal interaction domain that overlaps with the functional and the RNA binding domains but has not been precisely defined (Copeland et al., 2001; Kinzy et al., 2005). An overview of SBP2 structure and the location of these domains are presented in Figure1.8.
Figure 1.8 Structure and domains of human SBP2. An overview of predicted and functionally characterised domains of SBP2 is schematically presented. The putative NLS, the functional domain, the SECIS binding domain encompassing the L30-RNA binding motif and the minimal functional protein with their corresponding amino acid location within the human protein are shown. The structure of the L30 RNA binding motif as determined for the ribosomal protein L30 is shown in the lower panel. Yellow indicates the three anti-parallel $\beta$-sheets that form the RNA binding platform. Pink indicates two $\alpha$-helices required for the structural stability of the overall fold. The structure is available at the Brookhaven database IBYY.pdb. This image was generated using the rasmol program.

Intriguingly, *in vitro* and *in vivo* structure-function studies have clearly demonstrated that the first N-terminal $\sim$400 amino acids are dispensable for the SBP2 Sec incorporation function. The only predicted motif within this region is the NLS, suggesting the possibility of SBP2 having additional nuclear functions (Driscoll and Copeland, 2003). The functionality of the NLS as well as its role in regulating SBP2 function however, remains to be demonstrated.
Studies on the SBP2 RNA binding properties have shown that the core L30 RBD was not sufficient for SBP2 SECIS binding, rather an extended sequence was required (Copeland et al., 2001). Several amino acid residues required for SECIS binding have been identified and these were mapped to Gly$^{676}$, Glu$^{679}$, Glu$^{699}$ and Arg$^{731}$, positions within the human protein (Allmang et al., 2002). SBP2 RNA binding properties were shown to be redox state dependent as treatment of SBP2 with oxidising agents completely abrogated RNA binding, which was reversed by reducing agents. Moreover, treatment of SBP2 with the thiol modifying agent N-ethylmaleimide (NEM) also completely eliminated RNA binding, (Copeland and Driscoll, 1999) further demonstrating that free cysteine residues are required for RNA binding (Copeland and Driscoll, 1999). On the contrary, point mutation to one cysteine residue, C$^{691}$, had almost no effect on SBP2 RNA binding activity and had a minor effect on Sec incorporation (Copeland et al., 2001). However, several other cysteine residues are present within the RNA binding domain, which may play a more significant role in SBP2 RNA binding, but this remains to be investigated.

### 1.4.4.2 Subcellular localisation of SBP2

SBP2 was initially described as a cytoplasmic protein based on its isolation from cytoplasmic extracts (Copeland et al., 2000). Using glycerol gradients it was later demonstrated that transiently expressed SBP2 was stably, and directly associated with ribosomes, and that the interaction was mediated by the 28S rRNA subunit (Copeland et al., 2001). Interestingly, SBP2 did not seem to simultaneously associate with the ribosome and the SECIS element, as excess SECIS element addition was found to effectively shift SBP2 off the ribosomes (Copeland et al., 2001; Kinzy et al., 2005). Immunofluorescence analysis of transiently expressed SBP2 also demonstrated a cytoplasmic localisation of the protein. These observations may seem intriguing with regards to the predicted strong NLS within the N-terminal SBP2 region. Taken together, these observations may suggest that the steady state SBP2 localisation is within the ribosomes, but the possibility of SBP2 being able to enter and transit through the nucleus cannot be completely excluded.
1.4.5 PROPOSED MODELS FOR SEC INCORPORATION

The incorporation of Sec into selenoproteins requires decoding of the UGA codon, and is likely to involve competition between translation termination and Sec insertion. This task is accomplished by the concerted action of the several known factors described above and additional participants that most likely await discovery. A comprehensive view of the mechanism that directs this event is at present lacking. However, based on the existing evidence, two distinct models have been proposed. In the first model, steady state SBP2 is depicted as bound to the SECIS element in selenoprotein mRNA upon their synthesis in the nucleus and prior to their arrival to the ribosomal sites for translation, based on predicted NLSs found in both SBP2 and eEFSec (Tujebajeva et al., 2000). SBP2 is thus proposed to act in recruiting the eEFSec/SectRNA^[Ser]Sec/Secp43/SLA complex to the SECIS element and facilitate its delivery to the ribosomal A site (Small-Howard and Berry, 2005) (Figure 1.9). Experimental evidence that entirely supports this model, for example SBP2 being able to localise to the nuclear compartment is currently lacking. The second, and so far the best experimentally supported model proposes that SBP2 exclusively resides at the ribosomes and only binds to the SECIS element when the ribosome arrives at the UGA site. SBP2 is then temporarily released from the ribosome permitting loading of the SectRNA^[Ser]Sec at the UGA codon which in turn results in a structural change in the SECIS element that favours binding by the L30 ribosomal protein. SBP2 would thus be replaced by L30 and return to its location at the ribosome (Caban and Copeland, 2006; Kinzy et al., 2005). A collective body of experimental evidence indeed supports this model. Furthermore, the lack of direct and comprehensive evidence in favour of the former model is also naturally supportive of the later.
Figure 1.9 Current proposed models for Sec incorporation. Two models for Sec incorporation are currently proposed in the literature. In the first model, SBP2 is proposed to bind to the SECIS element prior to its arrival at the ribosomes as depicted in the upper panel (Berry, 2005). In the second model, SBP2 is proposed to stay bound to the ribosome and interact with the SECIS element during translation as shown in the lower panel (Caban and Copeland, 2006).

As a clear picture of the intricate process of selenoprotein synthesis is lacking, and until more evidence is provided, both models must be taken into consideration and remain valid. Indeed, it is more likely that rather than being mutually exclusive, the models may prove to somewhat complement each other. In either case, SBP2 has so far shown to play a central role in this process, therefore further insight into its regulation, in particular at the level of its subcellular localisation should help clarify the currently obscure picture.
1.5 PROJECT AIMS

The combined efforts of several laboratories has over the past few years led to considerable progress being made in understanding the mechanism of selenoprotein biosynthesis in mammalian cells. Very recently, several novel factors have been identified and work has been initiated in elucidating their roles in this mechanism. In parallel, a number of recent discoveries have been made for the “older” players, one of them being SBP2. Nevertheless, the picture is far from being completely understood. One particular aspect of selenoprotein biosynthesis that is not well characterised includes its regulation.

The overall aim of this thesis was to further characterise the human SBP2 gene and protein, and to investigate its regulation in order to better understand its Sec incorporation function. The specific aims of this project are listed below.

(1) Alternative splicing represents a functionally important and major regulatory pathway of gene expression and ultimately protein function. Recent genomic studies have indicated that at least 60% of mammalian genes are subject to alternative splicing. To date, this level of regulation has not been reported for any of the factors involved in Sec incorporation. Therefore, one aim of this project was to establish whether human SBP2 is subject to alternative splicing, and to identify any possible resulting protein isoforms. Experimental and computer-based approaches were employed to conduct an in depth characterisation of SBP2 regulation by alternative splicing.

(2) The topic of SBP2 localisation in cells has been subject of some controversy. A putative NLS within the amino-terminal SBP2 protein sequence was previously identified, but not characterised and generally, SBP2 is depicted as a ribosomal protein. The aspect of SBP2 subcellular localisation is closely related to its Sec incorporation function, this is an important but unclarified issue in the field. Elucidating the SBP2 subcellular localisation patterns constituted the major focus of investigation in this project. Structure-function approaches including truncation and point mutation analysis were initially
employed to identify and characterise motifs involved in regulation of SBP2 subcellular localisation.

(3) Protein function is usually controlled at several levels, one being the regulation by subcellular localisation. Post translational modifications can induce protein translocation within subcellular compartments. Redox regulation by cysteine residue modifications is one such mechanism. The third aim of this project was to determine the requirement of SBP2 for selenoprotein synthesis \textit{in vivo}, to characterise how redox control mechanisms impact on SBP2 subcellular localisation and function, and to delineate what cellular redox systems may contribute to these levels of regulation. \textit{In vivo} assays, siRNA technology and \textit{in vitro} biochemical approaches were combined to address these aspects.
CHAPTER 2.

MATERIALS AND METHODS
2.1 MATERIALS

2.1.1 REAGENTS

Table 2.1 lists reagents employed in this study. Solutions were prepared using deionised water from a Millipore MilliQ Ultrapure water system with a resistance of 18 mΩ/cm. Where required solutions were sterilised by autoclaving at 120°C, 101.3 kPa for 20 min or were filter sterilised through a 0.22 µm Millipore filter.

TABLE 2.1 SUPPLIERS OF REAGENTS UTILISED IN THIS STUDY

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<th>Supplier</th>
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2.1.2 CELL LINES

The cell lines used throughout the project are listed below. Cell lines were routinely screened for Mycoplasma contamination using the DAPI staining method.

293T human embryonic kidney cell line transformed with the SV40 T-antigen. 293T cells possess tumorigenic properties in nude mice. This cell line is an adherent type cell line.

HeLa a human cervical carcinoma cell line with adherent growth properties. It expresses low levels of p53 and normal levels of pRb (retinoblastoma suppressor). P53 and Rb are non-functional in this line due to inactivation by E6 and E7 proteins of human papilloma virus.

COS7 a monkey (Cercopithecus aethiops, African green) kidney fibroblast-like cell line derived by transformation with SV40 antigen. This cell line is an adherent type cell line.

MCF7 a hypertriploid to hypotetraploid human breast epithelial adenocarcinoma that contains a wild type p53 gene and is estrogen receptor positive. MCF7 cells have adherent growth properties.

HCC1937 This cell line was initiated from a primary ductal breast carcinoma from a 23 years old Caucasian female in 1995 and is homozygous for the BRCA1 5382C mutation (insertion C at nucleotide 5382). The cell line has an acquired mutation of p53 with wild type allele loss; an acquired homozygous deletion of the PTEN gene, and loss of heterozygosity at multiple loci known to be involved in the pathogenesis of breast cancer. The cell line grows as large adherent epithelial cells.
SVCT an SV40 transformed, normal breast epithelial cell line. This cell line was kindly provided by Dr. Melissa Brown (University of Queensland, Brisbane, Australia).

2.1.3 ANTIBODIES

Primary and secondary antibodies used throughout this study are outlined in tables 2.1 and 2.2 respectively.

**TABLE 2.2 PRIMARY ANTIBODIES**

W: Western Blotting; IF: Immunofluorescence; IP: Immunoprecipitation

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<td>Mouse anti-PDI</td>
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<td>Molecular Probes</td>
</tr>
<tr>
<td>Rabbit anti-γtubulin</td>
<td>W 1:1000</td>
<td>Sigma</td>
</tr>
<tr>
<td>Rabbit anti-GFP</td>
<td>W 1:5000</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td></td>
<td>IP: 1µg/ml</td>
<td></td>
</tr>
</tbody>
</table>
TABLE 2.3 SECONDARY ANTIBODIES AND STAINING DYES

W: Western Blotting; IF: Immunofluorescence

<table>
<thead>
<tr>
<th>Secondary antibody/ Staining dyes</th>
<th>Dilution used</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-rabbit horse radish peroxidase (HRP)</td>
<td>W 1:8000</td>
<td>Sigma</td>
</tr>
<tr>
<td>Rabbit anti-mouse HRP</td>
<td>W 1:3000</td>
<td>Caltag Labs</td>
</tr>
<tr>
<td>Goat anti-rabbit FITC</td>
<td>IF 1:200</td>
<td>Zymed Labs. Inc.</td>
</tr>
<tr>
<td>Goat anti-rabbit Texas Red</td>
<td>IF 1:200</td>
<td>Zymed Labs. Inc.</td>
</tr>
<tr>
<td>Streptavidin HRP</td>
<td>W 1:1000</td>
<td>DAKO</td>
</tr>
<tr>
<td>DAPI</td>
<td>IF 1:1000</td>
<td>Molecular Probes</td>
</tr>
</tbody>
</table>

2.1.4 OLIGONUCLEOTIDES

Oligonucleotides used in this study were purchased from Proligo, Lismore, NSW, Australia and are listed in Table 2.4.

TABLE 2.4 OLIGONUCLEOTIDES USED IN CONSTRUCTION OF SBP2 EXPRESSION PLASMIDS. Restriction enzyme sites are indicated in italics.

<table>
<thead>
<tr>
<th>Oligonucleotide name</th>
<th>Sequence (5′ → 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLS_M2,3_FWD</td>
<td>GCCTCAAGAAAGAATAAGCAGCAAGAAAGAAATCTAC</td>
</tr>
<tr>
<td>NLS_M2,3_REV</td>
<td>GTAGATTTTTCTTTGCTGCTTTATTCTTTCTTGAGGC</td>
</tr>
<tr>
<td>NES1FWD</td>
<td>GATCAGATGCTTCTAGTTAAGAAGTGATGCTTGTGTTACC, GACCTACTCAAAGAACCTGTCGTTTTCCAAGAC</td>
</tr>
<tr>
<td>NES1REV</td>
<td>GATCGGTTCCTGAAACGGACCAGTTTCTTTGAGTAGGTCGG, TAACACAAACCATCCACTTTACTAAGCATCT</td>
</tr>
<tr>
<td>NES2FWD</td>
<td>GATCTCAACACCTGAAGCTCAAAAAACTGAAATGTGT, CATTATTTCCTCC</td>
</tr>
<tr>
<td>NES2REV</td>
<td>GATCGGGAGAAATAATGACACATTTTGAGTTTTTTGAGCTCAGGTTTGA</td>
</tr>
<tr>
<td>NES3FWD</td>
<td>GATCGCGGTCGGCTGGATGACCTTTTGCACACAATTATTTG</td>
</tr>
<tr>
<td>Oligonucleotide name</td>
<td>Sequence (5′ → 3′)</td>
</tr>
<tr>
<td>--------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>NES3REV</td>
<td>GATCATAATCAATAATTGTGTGCAAAAGTGTATCCAGCCACCCG</td>
</tr>
<tr>
<td>NES4FWD</td>
<td>GATCGAAAGCTCTGGGGGGCAGTTTTGAATAAGGCAGTTCCTGTCAGTGTGGTGGGATCTTCAGCTAT</td>
</tr>
<tr>
<td>NES4REV</td>
<td>GATCATAGCTGAAGATCCCCACCACACTGACAGGAAACTGCCTTATTTCAAAGTCGCCCCAGAGCTTTGAC</td>
</tr>
<tr>
<td>NES5FWD</td>
<td>GATCAGGATCAGTTCCACAAAGATGGTGAGCTGACAGTGCGCGCGCA</td>
</tr>
<tr>
<td>NES5REV</td>
<td>GATCTCGGCCCCACACTGTCAGCTCAACCCTTGTGGAACGTATCCT</td>
</tr>
<tr>
<td>FLFWD</td>
<td>GGCATGGCGTCGGAGGGG</td>
</tr>
<tr>
<td>ISO2FWD</td>
<td>GAAAGCGAGGCAGAAAT</td>
</tr>
<tr>
<td>ISO3FWD</td>
<td>GTGACAGAATGTTTACTC</td>
</tr>
<tr>
<td>ISO2/3REV</td>
<td>GTCCAGTGTTGTAATTC</td>
</tr>
<tr>
<td>β-Actin FWD</td>
<td>CGTGACATTAAGGAGAAGCTGTGC</td>
</tr>
<tr>
<td>β-Actin-REV</td>
<td>CTCAAGAGAGAATGATCCTT</td>
</tr>
<tr>
<td>FLM-FWD</td>
<td>GGAATTCGCGCATGGTTAGAGTCCTCAGAAG</td>
</tr>
<tr>
<td>SBP2-REV</td>
<td>CGCCTCGAGGGGCTAAATTCAAATCATCATATT</td>
</tr>
<tr>
<td>FLN-FWD</td>
<td>GGAATTCCGCGCATGGCGTCGGAGG</td>
</tr>
<tr>
<td>FLN-REV</td>
<td>CTGTCAAAACTTTGCTGATCAGTAAC</td>
</tr>
<tr>
<td>SDMC6SFWD</td>
<td>GATTCAGGGATTACTCCAGCCAGAGTGCTTAG</td>
</tr>
<tr>
<td>SDMC6SREV</td>
<td>CTAAGCATTCTGGGTGAATCCCTGAGATC</td>
</tr>
<tr>
<td>SDMC7SFWD</td>
<td>GAGGTGGATGCTTCTGTATCCGACCTACTC</td>
</tr>
<tr>
<td>SDMC7SREV</td>
<td>GAGTAGGTCGGTAACGAGACATCCACTTC</td>
</tr>
<tr>
<td>SDMC8SFWD</td>
<td>GCTCAAAAAACTGAAATCTGTATCTTCTTACCAGTACTC</td>
</tr>
<tr>
<td>SDMC8SREV</td>
<td>GGGAGAAATAATGACAGATTTTCAGTTTTTGTAGC</td>
</tr>
<tr>
<td>SDMC9SFWD</td>
<td>CATTATITCTCCAAACTCTGTGAGATACAGTGC</td>
</tr>
<tr>
<td>SDMC9SREV</td>
<td>GACTGTATCTTCTCAGAGTTGGGAGAAATAATG</td>
</tr>
<tr>
<td>SDMC10SFWD</td>
<td>CAATTATAGATTAGCTGCTGACGCAACATTC</td>
</tr>
<tr>
<td>SDMC10SREV</td>
<td>GGAATGTTCTGCTCAGGGCATATAATCATATAG</td>
</tr>
</tbody>
</table>
2.1.5 STOCK SOLUTIONS

**Luria Broth (LB)**
10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl in 1L ddH₂O.

**LB Agar**
15 g bacto-agar in 1L LB.

**2-YT medium**
15 g bacto-tryptone, 5 g bacto-yeast extract, 5 g NaCl in 1L ddH₂O

**TAE (1x)**
40 mM Tris-acetate (pH8.3), 1mM diaminoethanetetra-acetic acid disodium salt (EDTA)

**TE (1x)**
10 mM Tris-HCl (pH8.0), 1 mM EDTA

**PBS**
(pH7.4), 137 mM NaCl, 2.6 mM KCl, 10.1 mM Na₂HPO₄

**PBST**
0.1% Tween-20 in PBS

**Running buffer (10x)**
30g Tris-base, 144g glycine, 10g SDS in 1L ddH₂O

**Protein loading buffer**
1.52% Tris-base, 20% glycerol, 2% SDS, 2% β-mercaptoethanol, 0.001% bromophenol blue (pH 6.8)

**Transfer buffer (10x)**
30g Tris-base, 60g glycine dissolved in 1L ddH₂O

20% methanol was added to the 1x buffer prior to use

**Blocking solution (10%)**
10g Skim milk powder in 100 ml PBST

**Stripping buffer**
Tris-HCl 30 mM, SDS 5%, β-mercaptoethanol 0.5%
2.2 METHODS

2.2.1 BACTERIAL CULTURE

The concentrations for the antibiotics used for plasmid propagation in bacterial cultures are listed in Table 2.3.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloramphenicol(Cm)</td>
<td>34 mg/ml</td>
</tr>
<tr>
<td>Kanamycin (Kan)</td>
<td>50 mg/ml</td>
</tr>
</tbody>
</table>

2.2.1.1 Overnight bacterial cultures

2 ml LB medium containing the appropriate antibiotic was inoculated with either a single colony from an LB agar plate or 5 µl from a bacterial glycerol stock and incubated at 37°C for 16 hrs in a shaker, at 220 rpm.

2.2.1.2 Freezing bacterial cultures

Bacterial glycerol stocks were prepared by mixing 200 µl of sterile 80% glycerol with 800 µl of overnight grown bacterial culture in a cryovial. Samples were snap-frozen on dry ice and stored at -70°C.

2.2.1.3 Preparation of DH5α competent cells

Competent *E.Coli* strain DH5α cells were prepared by adding 8 ml of overnight culture into 500 ml of LB broth (containing 10 mM MgSO₄, 10 mM MgCl₂). The
cells were grown in a shaking incubator at 37°C until they reached an OD_{600} of 0.4-0.5. The cultures were then aliquoted into 250 ml centrifuge tubes (JA-20, Beckman), chilled on ice for 15 min and pelleted by centrifugation for 15 min at 2500 rpm at 4°C. The resultant pellet was resuspended in 80 ml solution 1 (100 mM RbCl, 50 mM MnCl₂, 30 mM KAc, 10 mM CaCl₂, 15% glycerol, adjusted to pH 5.8), left on ice for 15 min, and followed by centrifugation as before. The pellets were then resuspended in solution 2 (50 mM MOPS buffer, 10 mM RbCl, 40 mM CaCl₂, 15% glycerol, adjusted to pH 6.8) and incubated on ice for 15 min. Aliquots of cells (0.2 ml) were transferred to sterile 0.5 ml microcentrifuge tubes and snap frozen on dry ice and stored at -70°C for later use.

2.2.1.4 Heat shock transformation of DH5α cells

Heat shock competent *E. Coli* DH5α cells were transformed by mixing plasmid DNA, or ligation reaction (20-50 ng, 2-5 µl), and competent cells to a final volume of 50 µl and incubating on ice for 20 minutes. Cells were heat shocked in a water bath at 42°C for 90 sec, returned to ice for 5 min, diluted in 1 ml LB medium and incubated on a shaker at 37°C for 1 hr. 50µl and 200µl of the broth was plated on LB agar plates containing antibiotics appropriate for selection (100 µg/ml of ampicillin or 50 µg/ml kanamycin) and incubated at 37°C for 16 hr.

2.2.2 PREPARATION OF PLASMID DNA

2.2.2.1 Mini-preps

Plasmid DNA was prepared by using the Qiaprep Spin Miniprep kit (Qiagen) using the buffers supplied by the manufacturer. A 2 ml overnight bacterial culture (see Section 2.2.1.1) was centrifuged at 4500 rpm for 5 min and the bacterial cell pellet was resuspended in 250µl of Buffer P1. 250µl buffer P2 was added to the cell suspension and mixed by inverting the tube 4-6 times to allow the cells to lyse. Buffer N3 (350 µl) was added to precipitate the cell debris and this was removed by centrifuging at 13000 rpm for 10 mins. The supernatant was applied to the QIAprep
spin column, incubated at room temperature for 1 min and centrifuged at 13000 rpm for 1 min. The column was then washed with 0.5ml Buffer PB and then with 0.75ml Buffer PE. After discarding the eluant the column was centrifuged for one additional minute. The column was transferred to a new 1.5 ml eppendorf tube and the DNA was eluted by adding 55 µl of Buffer EB (10 mM Tris-Cl, pH 8.5), incubating 1 min and centrifuging 1 min 13000 rpm.

2.2.2.2 Maxi-preps

Plasmid DNA was prepared in large amounts by a modification of the alkaline lysis and cesium chloride/ethidium bromide gradient centrifugation method (Sambrook et al., 1989). A 350 ml overnight bacterial culture was transferred into a 500 ml sterile centrifuge bottle (Nalgene). The bacteria were pelleted by centrifugation at 6370 g for 15 min at 4°C (Beckman J2-M1, JA-10 rotor). The pellet was resuspended in 20 ml of solution I (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA; pH 8.0), 40 ml of freshly made solution II (0.2 M NaOH, 1% SDS) was added, mixed gently and allowed to stand on ice until clear (usually 10-15 min), which indicated that complete lysis had occurred. 60 ml of solution III (3 M potassium acetate) was added, mixed gently and incubated on ice for a further 5 min. Cellular debris was precipitated by centrifugation at 3840 g, 20 min at 4°C (Beckman J2-M1, JA-14 rotor). The supernatant was poured into a clean 250 ml centrifuge bottle (through sterile muslin to filter out any loose debris) containing an equal volume of ice cold isopropanol. The solution was mixed, incubated on ice for 30 min and centrifuged at 12400 g at 4°C for 20 min (Beckman J2-M1, JA-14 rotor) to pellet the DNA. The supernatant was poured off, the DNA pellet air dried for approximately 10 min, resuspended in 2.25 ml of TE (10 mM TrisCl-1mM EDTA) buffer and transferred to a 10 ml centrifuge tube containing 2.75 g cesium chloride. The mixture was resuspended by gentle vortex, mixed with 125µl ethidium bromide solution (10 mg/ml), incubated at room temperature for 5 min and centrifuged at 800 g (Beckman J-6M/E) to pellet debris. The cleared supernatant was then transferred to Beckman Quick-Seal ultracentrifuge tube. The tube was topped up and carefully balanced with 1.1 g/ml CsCl in TE solution and heat sealed. The solution was centrifuged at 227,000 g (Beckman TL-100) for 16 hr at 21°C. After centrifugation the DNA band was removed with a
hypodermic needle and transferred to a microfuge tube. Ethidium bromide was removed by extracting a minimum of 3 times with an equal volume of isopropanol at room temperature. DNA was precipitated by addition of 2 volumes of water and 2 volumes of 100 % ethanol and gentle rocking at room temperature for 30 min. The DNA was pelleted by centrifuging at maximum speed for 10 min in a bench-top centrifuge. The pellet was washed with 500µl 70% ethanol, re-centrifuged and supernatant removed, dried for 5-10 min in a vacuum desiccator and resuspended in 100µL 10 mM Tris pH 8.0. The concentration of the DNA was determined as described in Section 2.2.2.3 and the integrity of the plasmid DNA checked by restriction digest and agarose gel electrophoresis as described in Sections and 2.2.3.6 2.2.3.2, respectively.

2.2.2.3 Quantification of DNA

The concentration of plasmid DNA was determined by measuring the absorbance at wave lengths of 260 and 280 nm in a spectrophotometer (Biomate 3, ThermoSpectronic). For the determination of DNA concentration a 1:50 dilution was used for miniprep DNA and 1: 200 or higher for maxiprep DNA. The samples were diluted in ddH₂O and the optical density (OD) determined with a wavelength of 260 nm. 1 OD reading was taken to equal 50 µg/ml DNA at this wavelength. An A₂₆₀:A₂₈₀ ratio of 1.8-2.0 indicated highly pure DNA with minimal protein contamination.

2.2.3 METHODS USED IN PLASMID CONSTRUCTION

2.2.3.1 Amplification of DNA by PCR

Amplification of specific DNA sequences by the Polymerase Chain Reaction (PCR) (Saiki et al., 1988) was performed on a Biorad i-cycler™. PCR amplifications were performed in 50µl reactions in thin walled PCR tubes, under the following conditions, unless otherwise stated: 2.5 mM MgCl₂, 1x polymerase specific buffer, 125 µM dNTPs, 100-400 ng plasmid DNA, 1-2.5 U of polymerase (Taq, Tth plus or Pfu Turbo), 50 pmoles of each primer or 150 ng of each primer when Pfu Turbo was used.
Non-specific amplification was monitored by the inclusion of negative controls containing all reaction components except template. A gradient PCR was first set up to determine the optimal primer annealing temperature. Cycling conditions were: denaturation of DNA at 94°C for 1 min, annealing at 60°C for 30 s, extension at 72°C for 1 min/kb of template to be amplified for 25 cycles. PCR products were confirmed by electrophoresis of an aliquot of the reaction (typically 5µl) on a TAE/ Ethidium bromide agarose gel as described in Section 2.2.3.2. The remainder of the reaction was purified by the Qiagen PCR purification kit as instructed by manufacturer.

2.2.3.2 Resolving DNA on agarose gel

Electrophoresis of PCR products or plasmid DNA was carried out at room temperature using 1-1.5% (w/v) agarose gels with appropriate molecular weight markers 1 kb DNA ladder (Life technologies). Gels were run at 80-100 V for 90 min in an electrophoresis tank containing TAE buffer system. 1% agarose gels were prepared by adding 0.5g of agarose to 50ml of 1x TAE buffer, and the solution boiled in a microwave until the agarose was completely dissolved. After the solution was cooled to approximately 50°C, 2µl of ethidium bromide (10 mg/ml) was added and the solution was poured into a tray. Once set, the wells were loaded with the DNA samples containing 1x loading buffer. The gels were visualized under UV illumination.

2.2.3.3 Purification of DNA from agarose gels

DNA fragments were purified from an agarose gel using the QIAquick Gel Extraction kit (Qiagen) using the buffers supplied by the manufacturer. The DNA fragment of interest was excised from the gel and placed in a pre-weighed eppendorf tube. The gel slice was dissolved in buffer QG (three times the gel volume) then incubated at 50°C for 10 min with regular vortexing. After the gel was dissolved, one gel volume of isopropanol was added, transferred to a QIAquick spin column in a 2 ml collection tube and centrifuged 1 min at 13000 rpm. The eluant was discarded; Buffer QG (0.5 ml) was added to the column and centrifuged for 1 min. The column was washed again by adding Buffer PE (0.75 ml) and centrifuged for 1 min. The eluant was
discarded and the column was centrifuged again for 1 min. The DNA was eluted by adding 30 µl of Buffer EB (10 mM Tris-HCl, pH 8.5) to the column, incubated 1 min and centrifuged 1 min. The DNA was stored at 4°C.

2.2.3.4 Annealing complementary oligonucleotides

Complementary oligonucleotides were annealed by incubating 1 µg of each oligonucleotide in annealing buffer (100mM potassium acetate, 30mM HEPES-KOH pH 7.4, 2mM Mg-acetate) at 95 °C for 4 min, at 70°C for 10 min then allowed to slowly cool down to 4°C over a period of 1.5 h.

2.2.3.5 Phosphorylation of 5’ DNA ends

Annealed complementary oligonucleotides were 5’ end phosphorylated in T4 polynucleotide kinase buffer containing ATP at 37°C for 1 h using T4 polynucleotide kinase (New England Biolabs). PNK was subsequently heat inactivated at 70°C for 10 min.

2.2.3.6 Restriction digest

Plasmid DNA (1-2 µg) or purified PCR products were digested in the appropriate 1x buffer (supplied by the manufacturer) with the required restriction enzyme (1-10 U) in a 20 µl reaction. The reactions were incubated at the recommended optimal temperature in a water bath for 2-3 hr. Once digests were completed, enzymes were inactivated either by heating, or through electrophoresis on agarose gels. Complete digestion was confirmed by running 5 µl of the reaction on an agarose gel (see Section 2.2.3.2).
2.2.3.7 Dephosphorylation of 5′ DNA ends

Following DNA digestion, the 5′ phosphate groups of vector DNA were hydrolysed by incubation with 10 U/µg DNA of calf intestinal phosphatase (CIP) (New England Biolabs) in the buffer provided by manufacturer at 37°C for 1 hr.

2.2.3.8 Ligation

Ligation of inserts into plasmid DNA were carried out in 20µl reactions containing 3:1 molar ratio quantities of insert and vector DNA, 1x T4 DNA ligase buffer (Gibco BRL) and 1U T4 DNA Ligase (Gibco BRL). Ligations were incubated for a minimum of 2 hr at room temperature or 12-16 hr at 14°C. The amounts of insert DNA to be used for each ligation were calculated using the following formula, where the amount of vector DNA was first chosen:

\[
\frac{(\text{Vector DNA}) \text{ ng} \times (\text{insert size}) \text{ kb}}{(\text{Vector size}) \text{ kb}} \times \frac{3}{1} = \text{insert DNA (ng)}
\]

2.2.3.9 PCR screening for recombinant clones

Screening for clones containing the desired insert was performed using the PCR screening method as follows. A number of colonies, 10-20, depending on the transformation efficiency, were picked, inoculated in 2 ml LB media containing the appropriate antibiotic for selection and grown at 37°C with shaking for 16 hr. Following incubation 2 x 20µl of each of the incubated cultures was aseptically removed and spun for 5 min at 5000 rpm. The pellets were resuspended in 10µl ddH2O and boiled in a microwave on the maximum setting (1000 W) for 2 min. Boiled samples were spun for 5 min at 1800 rpm. The supernatant was collected and 8µl was used as DNA template in a 20µl PCR reaction mix containing 2µl 1.25 mM dNTPs, 2µl 25mM MgCl2, 2µl 10x Taq polymerase buffer, 50 pmoles of each primer specific to the insert of interest, 0.1µl (1 U) Taq polymerase and 3.9µl sterile ddH2O. A master mix was first prepared to ensure equal distribution of reagents and to avoid
pipetting errors. Amplification of DNA was performed by the PCR as follows: denaturing at 94°C for 1 min, annealing 60°C for 1 min and extension at 72°C for 1.5 min for a total of 25 cycles. The reactions were then electrophoresed on an agarose gel as described in Section 2.2.3.2 and products were visualised under UV illumination.

2.2.3.10 DNA sequencing

A Thermal i-Cycler™ (BioRad) was utilised for the sequencing PCR reactions. Sequencing was performed using the ABI Prism dye terminator cycle sequencing reaction kit according to the manufacturer’s instructions. 200 ng of plasmid DNA was used as a template in a 10µl reaction containing 4µl Big Dye Terminator Mix and 1.6 pmol of primer. The sequencing PCR parameters were as follows: 96°C for 30 sec denaturing, 50°C 15 s annealing, and 60°C for 4 min extension, for 25 cycles. Following the PCR reaction, unincorporated Big Dye Terminators were removed from the sample as described below. The sequencing reaction was transferred to a sterile microfuge tube, 50µl 100% isopropanol was added and incubated at room temperature for no longer than 15 min to precipitate the extension products. Following precipitation, the sample was centrifuged for 20 min at 13000 rpm and the supernatant discarded. The pellet was rinsed in 70% ethanol (room temperature) and centrifuged for another 5 min at 13000 rpm. The pellet was then dried in a vacuum desiccator for 5 min and stored at -20°C until the sequencing was performed. Sequencings were performed at the Queensland Institute of Medical Research, QLD Australia.

2.2.3.11 Site-directed mutagenesis

The QuickChange Site-directed Mutagenesis Kit (Stratagene) was used to introduce point mutations into the specified plasmids. Briefly, PCR reactions were performed containing 75 ng of template plasmid DNA, 125 ng of each forward and reverse primers containing the desired mutations, 4U of Pfu Turbo proofreading polymerase enzyme and buffers as instructed by the manufacturer in a final volume of 50µl. The
extension time for the PCR reaction was 1.5 min/kb of template to be amplified. Following PCR, the methylated parental DNA template was digested with 10U of the DpnI restriction enzyme. 5 µl of the reaction was electrophoresed on agarose gel prior to propagation into competent cells (described in Section 2.2.3).

2.2.4 CELL CULTURE

2.2.4.1 Propagation of cell lines

Cells were maintained at 37°C in a 5% CO₂ incubator and cultured in RPMI 1640 medium supplemented with 3% fetal bovine serum (GIBCO-BRL) and 7% serum supreme (Cambrex), 1% L-glutamine, 100 U/ml penicillin and streptomycin. Cell cultures were grown and maintained at 37°C in an atmosphere of 5% CO₂ in air, in a humidified incubator. Confluent cells were split by first removing the old medium, rinsing with an equal volume of phosphate buffered saline (PBS), rinsing with versene and treated with 1ml trypsin in PBS at 37°C/ 5% CO₂ for 2-5 min. After incubation, cells were resuspended in medium, and for further propagation the cells were seeded into a fresh culture flask at the desired concentration or onto sterile glass coverslips for immunofluorescence.

2.2.4.2 Cell treatments

Cells were exposed to 5, 10 or 20 J/cm² ultraviolet type A irradiation (UVA) in PBS using a closely spaced array of five UVA-340 sunlamps (Q-Panel, Co). PBS was removed and cells were incubated in preheated complete medium, at 37°C at 5%CO₂ for 2 hrs. Control cells were processed in parallel by keeping in PBS for the same amount of time as UV-treatments.

H₂O₂ (Merck, Germany) was freshly diluted in sterile ddH₂O from a 5 M stock to yield a 100 mM stock. H₂O₂ was added from this stock, to serum free media preheated to 37°C at a final concentration of 300 µM, unless otherwise indicated.
Cells were rinsed in PBS once and H₂O₂ containing media was applied for 2 hours, or as indicated. Cells treated in serum free medium were used as controls for all treatments.

Sodium selenite (Na₂SeO₃) (Merck) was dissolved in sterile ddH₂O to yield a 100 mM stock solution. Prior to each treatment, selenite was diluted into serum free media preheated to 37°C to the concentration indicated and applied to cells for 2 hours unless otherwise stated.

Diamide (Sigma) was dissolve in ddH₂O as a 1 M stock and stored at -20°C. Diamide was applied to cells in serum free media for 2 hrs unless otherwise indicated.

The nuclear export inhibitor Leptomycin B (LMB) was kindly supplied by Dr. M. Yoshida (Department of Biotechnology, University of Tokyo, Japan) as a 10 µg/ml stock solution in ethanol and added to culture medium at a final concentration of 2 ng/ml.

Actinomycin D (Act. D) (ICN) was dissolved in ethanol at a 2.5 mg/ml stock. To inhibit nuclear import Act. D was used at a final concentration of 5 µg/ml. Cycloheximide (CHX) (Sigma) was added in combination with Act D to block de novo protein synthesis from a 100mg/ml stock solution in DMSO to a final concentration of 15 µg/ml. Act D was also used in separate experiments to block RNA synthesis and for this purpose Act D was used at a final concentration of 0.2 µg/ml.

Osmotic stress was imposed by treating cells with sorbitol (Sigma) in serum free media at a final concentration of 1 M. A 5 M stock was prepared and sterilised by autoclaving. Cells were rinsed in PBS once and sorbitol-containing media preheated to 37°C was added and incubation was conducted for similar length of time as indicated for H₂O₂ treatment.
Cells were incubated with the JNK-specific inhibitor SP600125 (Calbiochem) in serum free media at a final concentration of 50 µM for 1 hr prior to H2O2 treatment. A 10 mM stock was prepared in DMSO and stored at -20°C. Cells were incubated with the p38 kinase-specific inhibitor SB203580 (Calbiochem) in serum free media at a final concentration of 10 µM for 30 min prior to H2O2 treatment.

### 2.2.4.3 Transfections by electroporation

For western blot purposes, plasmids were transfected into 293T or HeLa cells by electroporation. Cells were detached from flasks as described in Section 2.2.4.1 and pelleted by centrifugation at 1000 rpm for 5 min. For each electroporation, $5 \times 10^6$ cells were resuspended in a total volume of 300µl fresh medium, gently mixed with 10 µg of plasmid DNA and transferred to a 4mm gap cuvette (BioRad). Cells were electroporated immediately at 240 V with two pulses for 10 msec each, using an Electro SquarePorator TM ECM 830 (BTX Genetronics Inc). Cells were then resuspended in 700 µl of media and transferred to 500 ml cell culture flasks with 30 ml of fresh medium and incubated for 24-48 hrs unless otherwise stated. Transfection efficiency was monitored by seeding 1 ml of GFP-electroporated cells from the large flask onto a 40mm dish with 2 ml of fresh medium. 24-48 hours later cells were visualised for GFP expression on a Leica inverted fluorescent microscope, and fluorescent cells were counted and compared to the total number of cells per field. A minimum of 5 fields were counted.

### 2.2.4.4 Transfections by cationic lipids

For fluorescence microscopy COS7 cells were grown on coverslips in 6 well plates until ~60-70% confluence was reached. Plasmid DNA was transfected into cells using the FuGene6 reagent (Roche). DNA (1µg/well) was diluted in 100 µl OptiMem serum free medium (Gibco) and 3 µl of FuGene6 reagent was added. Complexes were allowed to form by incubating at room temperature for 20 min. Cells on coverslips were rinsed in PBS once and complexes were added dropwise, followed by addition of 2 ml of OptiMem medium and incubation at 37°C, 5% CO2 for 6 hours.
Transfection medium was then replaced by complete RPMI medium and incubations were continued for 24-48 hours. All manipulations were performed in a laminar flow hood.

2.2.4.5 GFP-fluorescence microscopy

Prior to fixation, medium was removed and cells were washed twice with PBS. Cells were then fixed with 1 ml of 4 % paraformaldehyde in PBS for 30 min at room temperature, and washed twice in PBS. Nuclei were stained with the DNA dye 4’,6-diamidino-2-phenylindole (DAPI) diluted in PBS for 20 min at room temperature. Following DAPI staining cells were washed twice in PBS and mounted in Mowiol (ICN) containing 0.6% DABCO (Diazobicyclo-octane) and allowed to set overnight at room temperature prior to visualisation. Fluorescent images were captured using a Leica inverted fluorescent microscope. Figures were processed using AdobePhotoshop 6.0.

2.2.4.6 Cell counting

Localisation of SBP2 was determined by counting and scoring cells according to their fluorescent intensity as nuclear (N), equal nuclear/cytoplasmic (N/C) or cytoplasmic (C). Minimum 200 cells were counted per sample.

2.2.4.7 Cell lysis

Transfected or untransfected cells grown to 80-90 % confluence were harvested by scraping in the growth medium. The suspension was centrifuged at 1000 rpm for 10 min at 4°C, washed briefly with 10 mL 1xPBS, and re-spun as above. Total cell extracts were prepared by resuspending cell pellets in universal immunoprecipitation buffer (UIP) (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 25 mM β-glycerophosphate, 0.2% Triton X-100, and 0.3% NP40) supplemented with 25 mM sodium fluoride, 25 mM sodium orthovandate, and protease inhibitor cocktail (Sigma). Lysis was conducted at 4°C for 30 min on a rotating wheel and lysates were subsequently centrifuged at 13000 rpm for 20 min to remove cell debris.
The supernatant was collected and lysates were stored for long term at -70°C and for short term at -20°C.

2.2.4.8 Immunoprecipitation

Cells were lysed as described in Section 2.2.4.5. 1-2 mg of total cell lysate was pre-cleared by incubation with 30µl of Protein A-Sepharose as 50% slurry (Pharmacia Biotech, Sweden) for 1 hr at 4°C with rotation. Following incubation, beads were pelleted by centrifugation at 3000g for 5 min at 4°C and lysate was transferred to a new centrifuge tube. 1 µg of the appropriate antibody was added to the pre-cleared lysates incubated on a rotating wheel at 4°C for 2 hours. A mixture of 20µl of Protein G-Sepharose (Pharmacia Biotech, Sweden) beads and 30µl Protein A-Sepharose (Pharmacia Biotech, Sweden) beads were then added to the tubes and incubation was continued overnight, on the rotating wheel at 4°C. Immune complexes collected on beads were pelleted by centrifugation at 3000g for 5 min at 4°C. The supernatant was removed and saved for quantitation of proteins by western blotting. Beads were then washed 4 times with 1 ml of lysis buffer, and an equal bead volume of 2x SDS PAGE sample buffer was added to the tubes.

2.2.4.9 Metabolic labelling of cells with $^{75}$Se and $^{35}$S

For $^{75}$Se labelling of untransfected cells, cells were seeded into 10 cm dishes and grown for 24 hrs to reach ~60% confluence. Where indicated, treatments were performed prior to labelling as described in the referred Section. If transfections were conducted prior to labelling, cells were electroporated with the indicated plasmids as described in Section 2.2.4.3 and incubated for 24 hrs prior to labelling. For siRNA experiments cells were electroporated and incubated for ~48 hrs. Cells were then detached and plated onto new dishes and grown for another 24 hrs to yield maximum SBP2 siRNA effect and $^{75}$Se was added. $^{75}$Se was obtained as [$^{75}$Se] selenious acid from the Research Reactor Facility (University of Missouri, Columbia) at a 30µCi/µl stock, and a 1:10 dilution in ddH$_2$O was prepared and used as stock for the cell labelling experiments. The half life of the $^{75}$Se isotope is 120 days, which was taken
into consideration and volumes were adjusted accordingly during the course of the
experiments. The required volume of $^{75}\text{Se}$ equivalent to 10µCi radioactivity per dish
was removed from the 1:10 diluted stock and diluted further into 200 µl of sterile PBS
prior to addition to the cell culture media, also supplemented with non-radioactive
sodium selenite (50 nM). The media was then dispersed equally among the treated
and untreated dishes and incubation was conducted for 24 hrs unless otherwise
indicated. At the end of incubation, cells were harvested and lysed as described in
Section 2.2.4.7. Samples were separated on 10-15% SDS-PAGE gels. $^{75}\text{Se}$-Labeled
proteins were visualized on SDS-PAGE gels by autoradiography.

For $^{35}\text{S}$ labelling cells were treated with LMB (2ng/ml) for 16 hrs. Following
treatment, media was changed to cysteine and methionine free RPMI media
containing dialysed fetal calf serum 1 hr prior to addition of $^{35}\text{S}$ (Promix, Amersham).
Labelling was conducted for 24 hrs in the presence or absence of LMB (2ng/ml).
Samples were separated on 10% SDS-NuPAGE gels (Novex) and $^{35}\text{S}$-labeled proteins
were visualized by autoradiography.

2.2.4.10 Antibody affinity purification

Polyclonal antibodies against SBP2 were affinity purified from rabbit sera by
chromatography through GST-coupled CNBr-activated Sepharose-4B columns
(Pharmacia) to remove antibodies against GST, followed by chromatography of the
unbound fraction on CNBr-activated Sepharose-4B columns bound with SBP2-fusion
protein used for immunisation. Antibodies were eluted with TRIS-glycine buffer (pH
2.6) and dialysed against PBS. The final antibody concentration obtained was
1mg/ml.
2.2.5 METHODS FOR PROTEIN ANALYSIS

2.2.5.1 Determination of protein concentration

Protein concentrations were determined using the BioRad dye-binding assay as per manufacturer’s instructions. Briefly, 2µl of sample was pipetted into an eppendorf tube containing 798µl of ddH₂O and 200µl of dye reagent was added to each tube. The mixture was incubated at room temperature for at least 5 min and the optical density of each sample was measured at 595 nm of visible light by using a spectrophotometer. The optical density of 2, 4, 6, 8, 10 µg/ml of bovine serum albumin (BSA) were measured in parallel to generate a standard curve. Sample concentrations were extrapolated from the BSA standard curve.

2.2.5.2 Resolving proteins on SDS-PAGE

SDS polyacrylamide gel electrophoresis (PAGE) was performed as described in (Ausubel et al., 1999; Laemmlli, 1970). Samples for electrophoresis were prepared by diluting in 5 × sample buffer with DTT at a final concentration of 100 mM, heating for 5 min at 95°C, then a brief spin at 13 000 rpm to remove any particulate mater. For non-reducing SDS-PAGE, DTT was omitted in the loading buffer. Polyacrylamide gels were prepared with 8% acrylamide for the separating gel and with 5% acrylamide for the stacking gel of an acrylamide/bis-acrylamide ratio of 29:1 (BioRad). Gels were run in a Mini-protean II cell (Bio-Rad, USA) at 15 mA through the stacking gel and at 25 mA through the separating gel in 1 × running buffer. Prestained molecular weight markers used were from Fermentas or BioRad.

2.2.5.3 Western blotting

Following SDS-PAGE, gels were pre-equilibrated in transfer buffer for a minimum of 10 mins before the proteins were transferred onto a PVDF membrane (Millipore) in high molecular weight transfer buffer (40mM Tris, 40 mM glycine (pH 9.4), 0.1% SDS, and 20% methanol) at 4°C for 2 hours at 100 Volts using the BioRad minigel
apparatus. Following transfer, membranes were blocked in a solution of 10% non-fat dry milk in PBS-0.1% Tween 20 (Sigma) (PBST) for at least 1 hr at room temperature. Primary detection antibody was diluted in 10% non-fat dry milk in PBST and incubated overnight at 4°C. Membranes was then washed 3 times for 15 min in PBST and incubated for 1 hr at room temperature with horseradish peroxidase conjugated secondary antibodies diluted in 10% non-fat dry milk in PBST. Finally membranes were washed 3 times for 15 min in PBST and developed for 1 min in fresh chemiluminescence reagent (Pierce) and exposed to X-ray film, or developed using a Fuji imaging system.

2.2.5.4 Membrane stripping

Membranes were stored in PBST until they were stripped by incubating in 25 ml of stripping buffer (see stock solution list) for 30 min at 55°C. Membranes were then washed 3 × in PBST before being blocked and re-probed.
CHAPTER 3.

IDENTIFICATION OF ALTERNATIVELY SPliced HUMAN SBP2 ISOFORMS
3.1 INTRODUCTION

Selenocysteine, the 21st naturally occurring amino acid, represents a remarkable example of a dual role for the UGA codon most commonly used for translation termination (Lee et al., 1989). Decoding of UGA as Sec leads to its co-translational incorporation into selenoproteins by a mechanism that employs several evolutionarily conserved cis- and trans-acting factors (Driscoll and Copeland, 2003). In addition to the UGA codon, a second cis-acting determinant is found in the selenocysteine insertion sequence (SECIS) element located within the 3’ UTR of selenoproteins mRNA (Berry et al., 1991; Berry et al., 1993). The SECIS element is recognised and bound by the SECIS binding protein 2 (SBP2) (Copeland and Driscoll, 1999) which is believed to recruit the Sec specific elongation factor (eEFSec)-tRNA[^Ser]Sec complex to the ribosome (Tujebajeva et al., 2000). The eukaryotic Sec incorporation mechanism is slowly becoming deciphered, however, a complete understanding is currently still lacking.

Based on in vitro evidence, SBP2 was proposed to be essential for selenoprotein synthesis, yet a complete characterisation of this protein has not been conducted. For this reason, the first aim of this project was to assemble the full length human SBP2 cDNA. As it became apparent that multiple transcripts were present in cells, I set out to investigate the possible regulation of the human SBP2 gene by alternative splicing.

Alternative splicing, the process by which multiple mRNA variants can be generated from a single pre-mRNA by joining of different combinations of splice sites, is believed to occur in 60-80% of human genes (reviewed in Matlin et al., 2005), providing a significant contribution to protein diversity beyond that encoded in the genome sequence. Pre-mRNA splicing occurs based on the presence of both cis- and trans- components. Cis components are consensus sequences that define the exon-intron boundaries within a gene. In most cases, splice sites conform to consensus sequences at each end of the intron: GT at the 5’ end and AG at the 3’ end (Miriami et al., 2004), however, non-consensus splice sites have also been described (Mount, 2000; Thanaraj and Clark, 2001). Pre-mRNA splicing takes place within the spliceosome, a large macromolecular complex composed of five small ribonucleoprotein particles (snRNPs) (U1, U2, U4, U5 and U6) and several associated
non-snRNP proteins (Sanford and Caceres, 2004). The major class of non-snRNPs are the serine arginine rich SR-proteins which contain one or two RNA-binding domains and a C-terminal serine-arginine rich domain (RS domain) (Jurica and Moore, 2003). The spliceosome assembly onto each intron is a highly dynamic process and splicing reactions occur in a series of complex spatial and temporal conformational rearrangements leading to the formation of the spliced mRNA.

It is often not possible to make a clear distinction between ‘constitutive’ and ‘alternative’ splicing elements and factors, rather, the relative frequency with which an exon is selected depends on a combination of events dictated by the participation of positively and negatively acting factors in a precise combination and relative ratios, specific to a cell type, developmental stage or signalling pathway (Ladd and Cooper, 2002). Such elements include the strength of the splice sites (Eperon et al., 1993; Huh and Hynes, 1993), the relative enrichment of regulatory elements such as exon splicing enhancers (ESEs) or exon splicing silencers (ESSs) (Wang et al., 2004), (Zhang and Chasin, 2004) and the combinatorial binding of the basal splicing factors to the template pre-mRNA (Mount, 2000). Mabon and co-workers recently showed that distinct combinations of splicing factors are recruited to sites of alternatively spliced transcripts in intact cells, and that both protein-protein and protein-RNA interactions are required for the differential recruitment (Mabon and Misteli, 2005). Most alternative splicing events affect the coding sequence and it is believed that half of these alter the reading frame (Clark and Thanaraj, 2002) and a third lead to nonsense mediated decay of the RNA product (Lewis et al., 2003).

Defects in alternative splicing and generation of aberrant ratios of mRNA transcripts from a single gene are now also recognised as major contributors to human disease and it is estimated that at least 15 % of the mutations that cause hereditary disease affect pre-mRNA splicing (Faustino and Cooper, 2003). Examples of disease coupled to alternative splicing include cystic fibrosis, Alzheimer’s disease, spinal muscle atrophy, tyrosinemia type I, acute intermittent porphyria, beta-thalassemia and Hutchinson-Gilford progeria syndrome (Eriksson et al., 2003). In addition, cancer associated splice variants have been identified for genes such as EGFR (Wikstrand et al., 1995), CD44 (Naor et al., 2002), NER (Saito et al., 1997) and BRCA1 (Orban and
Olah, 2003) and a genome wide analysis of human ESTs found strong evidence for cancer specific splice variants in 316 human genes (Xu and Lee, 2003).

Very recently, it was reported that inherited mutations in the SBP2 gene were the likely cause of an abnormal thyroid hormone metabolism with growth retardation symptoms in their carriers (Dumitrescu et al., 2005). Interestingly, one of the mutations identified appears to create an alternative donor splice site within exon XII of the SBP2 gene which produces a transcript with a 26 bp intron retention. This abnormal splicing event abrogates the normal reading frame to produce a putative truncated protein. Since the mutation was located upstream of the SBP2 functional and RNA binding domains the affected individuals were estimated to produce only 24% normal transcript. The phenotype observed was caused by a deficient Sec incorporation mechanism manifested through lack of functional thyronine deiodinase 2 (DIO2), however, other selenoprotein levels were also affected. These findings represent not only the first link between SBP2 and a human disease, but also the first direct example of Sec incorporation defect described in humans providing new insight into the consequences of SBP2 deficiency. As mentioned by the authors, these defects represent the early (childhood) phenotype of SBP2 deficiency, however, additional manifestation with increasing age such as cancer predisposition due to impaired antioxidant protection, or decreased fertility due to lack of selenoprotein PHGPx, are not to be excluded.

The regulation of SBP2 by alternative splicing may have significant implications for its function. The data presented in this chapter provides an experimental and computational based investigation of the splicing events that occur within the SBP2 gene and reports the identification of at least one additional, alternatively spliced SBP2 isoform.
3.2 MATERIALS AND METHODS

3.2.1 Generation of full length SBP2

Full length SBP2 cDNA was synthesised in two stages. A partial human SBP2 cDNA (AL136881) lacking the 5′ end was obtained as a kind gift of the German Cancer Research Centre, Berlin, Germany. This sequence was sub-cloned into the EcoRI/SalI sites of the EGFP-N1 vector (Clontech) using the FLM-FWD primer 5′GGAATTCGGCATGGTTAGAGTCCTCAGAAG3′ and the SBP2-REV primer 5′CGCCTCGAGGGGCTAAATTCAAATTCATCATTTT3′. Restriction enzyme sites within primers are indicated in italics. The missing 5′ sequence, as cloned by (Lescure et al., 2002) (NM024077), was generated by PCR using the FLN-FWD primer 5′GGAATTCCGCGCATGGCGTCGGAGG3′ and the FLN-REV primer 5′CTGTCAAAACTTTTGTGATCATACG3′ using Pfu polymerase. cDNA was synthesized from 293T cells by first extracting mRNA using the RINAEasy kit (Qiagen) followed by amplification with Superscript reverse transcriptase (Invitrogen) and oligo dTs, as directed by the manufacturers. Resulting products were sub-cloned into the existing partial SBP2-EGFP-N1 plasmid, using the EcoRI/BclI sites, to obtain full length SBP2-GFP. The integrity of plasmids was verified by sequencing.

3.2.2 Sequence analysis and bioinformatics

The GenBank EST and non-redundant databases were searched for SBP2 specific sequences using the herein identified PCR products as queries. Intron-exon boundaries were inspected manually by comparing to the SBP2 gene sequence available at the USCS Genome Bioinformatics site, http://genome.ucsc.edu. Protein characteristics and domain predictions were analysed with the PSORT II program (http://psort.ims.u-tokyo.ac.jp).

3.2.3 RT-PCR of SBP2 transcript variants in breast cancer cell lines

For RT-PCR analysis of SBP2 isoforms expression in breast cancer cell lines (purchased form ATCC) (SKBR3, T47D, ZR75-1, BT20, MDA-MB231, MCF7,
BT474, BT483, 21MT-1, 21MT-2, NB88A, BC312) 1 µg of total RNA (kindly provided by Dr. Jeremy Arnold, Queensland Institute of Medical Research, QLD, Australia) was reverse transcribed using Superscript reverse transcriptase (Invitrogen) and oligo dT primers, as directed by the manufacturer. To avoid DNA contamination, samples were treated with Dnase I (New England Biolabs) before each reverse transcription. PCRs were carried out using the ISO2/3 reverse primer (designed to anneal within exon V) 5’GTCCAGTGTGGTAAATTC3’. Forward primers, designed to span over the splicing junctions (indicated by |) were used as follows: for SBP2 (exon 1) FLFWD: 5’GGCATGGCGTCGGAGGGG3’, for SBP2_A2 (spliced exon I to IIIa) ISO2FWD: 5’GAAAGCGAG|GCAGAAAAT3’ and for mtSBP2 (spliced exon II to IIIb) ISO3FWD: 5’GTGACAGA|AATGTTTACTC3’ (See Appendix 1 for exon boundaries). β-actin primers forward: 5’CGTGACATTAAGGAGAAGCTGTGC3’ and reverse: 5’CTCAGGAGGAGCAATGTCTT3’ were used as control. Products were resolved on 1.5 % agarose gels.

3.2.4 Northern Blotting

An SBP2 probe for northern blot hybridization was prepared by PCR of full length SBP2 using the SBP2-GFP construct as template. A GAPDH probe to serve as loading control was kindly provided by Dr. Jeremy Arnold (Queensland Institute of Medical Research, QLD, Australia). Probes were [α-32P]-dCTP labelled using the MegaPrime kit (Amersham). A commercial membrane containing polyA+ RNA from multiple human tissues (spleen, thymus, prostate, testis, ovary, small intestine, colon, peripheral blood monocyte) (Clontech) and a membrane containing polyA+ RNA from various cancer cell lines (T-cell leukaemia, Burkitt’s lymphomas type 1, 2 and 3, breast, cervical adeno-, epidermal, uterine and diploid lung carcinomas, osteosarcoma) (Ambion) were sequentially hybridised with the α-32P-labelled SBP2 and GAPDH probes. Hybridisations were carried out for two hours in ExpressHyb solution (Clontech) at 65°C in accordance with the protocol provided by the manufacturer.
3.2.6 Immunoprecipitations and proteomics analysis

293T cells were maintained as described in Section 2.2.4.1 and lysed as described in Section 2.2.4.5. Protein concentrations were measured by the Bradford method as described in Section 2.2.5.1. Immunoprecipitations of SBP2 for mass spectrometry analysis were carried out as described in Section 2.2.4.6 from 30 mg of 293T total cell lysates using 30µg of anti SBP2 antibodies and 150 µl of Protein G-Sepharose beads (Pharmacia). Western blots were carried out as described in Section 2.2.4.7. SBP2 was detected with affinity purified rabbit polyclonal antibodies raised in house, at 1:2000 dilution in 10% blotto. For mass spectromtry analysis immune complexes were washed 5 times in 5 ml of UIP lysis buffer then separated by SDS-PAGE, stained with Colloidal Coomassie Blue and bands of interest were excised. Excised bands were analysed at the Proteomics Analysis Centre, Karolinska Institute, Stockholm, Sweden by in-gel digestion with Trypsin, followed by DE-Pro MALDI mass spectrometry using a Voyager 6083 System (Applied Biosystems). Peptide masses obtained from the MALDI-TOF analysis were analysed against the NCBI protein database using the MS-Fit tool at http://prospector.ucsf.edu/.
3.3 RESULTS

3.3.1 Assembly of human SBP2 cDNA and identification of 5′-end splice variants

In order to obtain the human SBP2 encoding sequence, a BLAST search against the NCBI nucleotide database was performed using the previously identified rat SBP2 cDNA (Copeland et al., 2000). A matching complete SBP2 cDNA, with 84% homology to rat SBP2 was identified, and obtained as a kind gift from the German Cancer Research Centre (DKFZ), Berlin, Germany. Soon after, the cloning of the human SBP2 gene was reported by (Lescure et al., 2002) and it became evident that the two sequences displayed differences in the 5′-region, and the clone obtained from the DKFZ centre was lacking some of the 5′ sequence. In order to assemble the correct full length human SBP2 cDNA, a primer pair complementary to the missing region was designed and used in reverse transcription PCR (RT-PCR) on cDNA obtained from 293 T cells. These primers generated two individual PCR products (Figure 3.1), both of which were further analysed by sequencing.

![Figure 3.1. PCR amplification of SBP2 5′-region. SBP2 5′-region was amplified from cDNA obtained from 293T cells and resolved on a 1% agarose gel. The different lanes show the products amplified at annealing temperatures in a gradient ranging from 55°C to 65°C. A indicates the expected ~300 base pair product, and B points to the additional product (~250) simultaneously amplified.](image)

Sequence analysis revealed the existence of several different SBP2 transcripts, arising from alternative splicing events within the 5′-UTR. Although not a major aim of this PhD project, this data was further analysed and complemented with an extensive GenBank database search to verify these results. This search identified several EST clones that supported these results, and additional EST sequences encoding additional splice variants not detected in the RT-PCR reactions. The human SBP2 gene spans a 43 kb region on chromosome 9q22.1 and the transcript encoding full length SBP2 is comprised of 17 exons, which are herein indicated in roman numerals. Exon
organisation of human SBP2 is presented in Appendix 1. The splice variants identified are listed in Table 3.1 along with their tissue of origin, corresponding accession numbers, and the proposed nomenclature. Sequences representing complete SBP2 open reading frames (ORFs) were also identified and are listed in the last column of the table. Combined, the data led to identification of nine SBP2 transcript variants produced by different alternative splicing events.
TABLE 3. HUMAN SBP2 5’-END SPLICE VARIANTS. The SBP2 gene is comprised of 17 exons. Alternative splice variants within the 5’-end (up to and including exon X) of SBP2 identified experimentally and by EST database search are summarized and listed according to the nomenclature proposed in the text. The exon combination, ESTs tissue of origin with corresponding GenBank accession numbers, or complete mRNAs are listed. ESTs arising from tumors or cancer derived cell lines are listed in separate columns. (ES=embryonic stem)

<table>
<thead>
<tr>
<th>SBP2 Transcript Nomenclature</th>
<th>Exon combination (17 exons in total)</th>
<th>Number of ESTs</th>
<th>Non-cancerous tissue of origin ESTs</th>
<th>Primary tumors ESTs</th>
<th>Cancer cell lines ESTs</th>
<th>Complete mRNAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. <strong>SBP2</strong> (full length)</td>
<td>I,II,III,IV,V,VI, VII...</td>
<td>28</td>
<td>Fetal brain (AL534644) Testis(BG722344), (BI460721), (BG722857), (BG772706), (BI561413), (BG772785), (CD171759) Pooled colon, kidney, stomach (BI765648) Stomach SNU-1 cell line (BM835609) Placenta (CB988930), (CF994706) Pancreas (BG777010), (BQ631642) Liver (AV654623) Pooled (AI769091) Brain medulla(B1292681) Liver Cho-CK cell line (CB121625) Liver SCK cell line (CB118677, CB123294)</td>
<td>Retinoblastoma (BU154465)</td>
<td>Amelanotic melanoma (BM557450) (BM810842) Lung epidermoid carcinoma (BQ398269) Lymphoma cell line (BQ50011) Osteosarcoma cell line (BG113716) Amelanotic melanoma (BM557450)</td>
<td>AF090608 AX746532 BC036109 AF380995 NM024077</td>
</tr>
<tr>
<td>2. <strong>SBP2</strong>&lt;sub&gt;A2&lt;/sub&gt;</td>
<td>I-IIIa,IV,V...</td>
<td>2</td>
<td>HEK 293T cell line ES cell line (CV811081)</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
</tr>
<tr>
<td>3. <strong>SBP2</strong>&lt;sub&gt;A3&lt;/sub&gt;</td>
<td>I,II,IV,V...</td>
<td>1</td>
<td>Not found</td>
<td>Genitourinary tract 2 pooled high-grade transitional cell tumors (AM630547)</td>
<td>Not found</td>
<td>Not found</td>
</tr>
<tr>
<td>4. <strong>mtSBP2</strong></td>
<td>I,II,IIIb,IV, V,VI...</td>
<td>11</td>
<td>Pooled glandular (CB956376) Pancreas(BM315064) ES cell line H9 (CN256608), (CN256604) ES cell line BGO1 (CX870404) T-lymphocytes (CR994587) Liver Cho-CK cell line (CB121625)</td>
<td>Retinoblastoma (BE252177) Placenta choriocarcinoma (BG481218)</td>
<td>Lymphoma cell line (BQ49808) Osteosarcoma cell line (BG113716)</td>
<td>AL136881</td>
</tr>
<tr>
<td>5. <strong>mtSBP2b</strong></td>
<td>I,II,IIb,IV, V,VI-VIIc...</td>
<td>2</td>
<td>Uterus (AI127999)</td>
<td>Retinoblastoma (BC001189)TRD</td>
<td>Not found</td>
<td>Not found</td>
</tr>
<tr>
<td>6. Not known</td>
<td>...VI-VIIb...</td>
<td>1</td>
<td>Brain (AB208940)</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
</tr>
<tr>
<td>7. Not known</td>
<td>...VI-VIIe... (TRAD)</td>
<td>5</td>
<td>ES cell line (CN256603), (CX787609)</td>
<td>Squamous cell carcinoma (BP352801) Large cell carcinoma (BQ430224)</td>
<td>Ovary teratocarcinoma cell line (BUB50141)</td>
<td>Not found</td>
</tr>
<tr>
<td>8. <strong>SBP2</strong>&lt;sub&gt;2&lt;/sub&gt;</td>
<td>...VI-VIIe... (TRAD)</td>
<td>2</td>
<td>Pancreas (BU069273) ES cell line (DN602260)FL</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
</tr>
<tr>
<td>9. Not known</td>
<td>...VIII-X...</td>
<td>1</td>
<td>Retinal pigment epithelium (CA389610)</td>
<td>Not found</td>
<td>Not found</td>
<td>Not found</td>
</tr>
</tbody>
</table>

80
3.3.2 Intron-exon splice site boundaries

The *SBP2* genomic sequence covering the 5′-end at http://genome.ucsc.edu (July 2003 assembly) was used to analyse the exon/intron composition and splicing patterns for the identified *SBP2* transcript variants. Genomic sequences at the intron-exon splice site boundaries followed splicing consensus (Mount, 1982) for most of the splice variants, however, a few deviations were noted. The majority of exons had one donor and one acceptor splice site, however, it became evident that some exons had several donor or acceptor splice sites, which will be indicated by $a$, $b$ or $c$. The summary of the alternative splicing events, including the intron/exon boundaries are presented in Table 3.2 and will be described individually herein. The majority of the ESTs identified in the database search corresponded to the full length *SBP2* transcript containing all exons within the 5′-region (Table 3.1, row 1).

Exon II was found to be excluded in transcript variants identified experimentally and confirmed by one additional EST. Both these transcripts were detected in embryonic derived cell lines (Table 3.1, row 2).

Exon III was the first exon detected to have two alternative acceptor sites, denoted IIIa and IIIb. Splicing to the alternative acceptor sites within exon III occurred from either exon I or exon II (Table 3.2, and Table 3.1, rows 2 and 4). The splicing event from exon II to the alternative acceptor site at IIIb seemed to be the most common alternative splicing event within the *SBP2* gene, as indicated by the large number of corresponding ESTs originating from both normal and cancer derived tissues and cell lines (Table 3.1, row 4).

The most complex splicing pattern noted was at the exon VI-VII splice junction. Exon VI contained two alternative donor sites and was spliced at three different acceptor sites within exon VII (Table 3.2). Except for the acceptor splice site at the VIIa, which generates the full length transcript, two alternative sites were identified. The most unusual acceptor splice site was the VIIb site, only found in one EST (Table 3.1, row 6). Several ESTs were found that supported splicing into the VIIc site (Table 3.1, rows 5, 7 and 8). Interestingly, the VIIc splice site appeared to have two alternative acceptor sites which created two different amino acid sequences at the splice junction: either TRD or
TRAD, depending on the splice site used. These alternative splicing events seemed not to be directed by any of the identified upstream splicing pattern.

Finally, one EST lacking exon IX, with alternative splicing between exon VIII and X was detected, however, the upstream exon combination remains unclear (Table 3.1, row 9). This also appeared to be the last alternative splicing site within the SBP2 transcript, as no further splice variants were detected for the remaining downstream exons despite the presence of a large number of ESTs covering the 3′-region. The genomic sequence covering the 3′-end used to define the exon intron boundaries was at GenBank accession number AL929575.24.
| Donor Exon (nt) | Acceptor Exon (nt) | Intro nb size (kb) | EXON/intron/EXON<sup>c</sup> (splice site consensus: ...AG|gurgu...y|nyyagG...) |
|----------------|--------------------|--------------------|-------------------------------------------------|
| I (93)         | II (146)           | 1.04               | _AGCCAG| _guaagguucauacuuucucau | GGCAUC... |
| I (93)         | *IIIa (121)        | 6.81               | _AGCCAG| _guaagguucauacuuucucau | GCAGAA... |
| II (146)       | *IIIa (121)        | 5.63               | _GACAGA| _guauguucauacuuucucau | GCAGAA... |
| II (146)       | *IIIb (129)        | 5.63               | _GACAGA| _guauguucauacuuucucau | AAUGUU... |
| II (146)       | IV (91)            | 5.63               | _GACAGA| _guauguucauaauucuucugcau | AAGAAA |
| III (250)      | IV (91)            | 0.20               | _UUURAG| _gugagguucauacuuucucau | AAGAAA... |
| IV (91)        | V (227)            | 2.67               | _AAUCAG| _guaaaaaaauuuuuuuuauacugu | AUGGUU... |
| V (227)        | VI (78)            | 4.02               | _UCACAG| _gugagguucauacuuucucau | GUGUAA... |
| VI (78)        | *VIIa (209)        | 1.53               | _CAACAG| _guaaaaaaauuuuguuuacuuucugacu | AGUUAU... |
| VI (78)        | *VIIb (173)        | 1.53               | _CAACAG| _guaaaaaaauuuuguuuacuuucugacu | AGUUAU... |
| VI (78)        | *VIIc (95)         | 1.53               | _CAACAG| _guaaaaaaauuuuguuuacuuucugacu | AGUUAU... |
| **VI (77)      | *VIIc (93)         | 1.53               | _CAACAG| _guaaaaaaauuuuguuuacuuucugacu | AGUUAU... |
| VIII (123)     | X (132)            | 2.76               | _AUGUAA| _guaagucauuuucauacuuucucau | GUAAYA... |

<sup>a</sup>The donor and acceptor exons are shown with their sizes in nucleotides in brackets.

<sup>b</sup>Intron sizes resulting from the indicated splicing events are given in kilobases.

<sup>c</sup>Boundaries of splice sites as identified from the genomic DNA sequence compared to the consensus of splice site motifs (Matlin et al., 2005). Consensus nucleotides at the 5′ and 3′ splice sites are indicated in bold letters, and the splice site indicated with a vertical line. The polypyrimidine stretch is shown in italics. Deviations from the consensus are underlined.

* Indicates exons with alternative acceptor sites.

** Indicates exons with alternative donor sites.

### 3.3.3 Alternative SBP2 open reading frames and protein isoforms

The transcript variants were further investigated for their potential to produce protein isoforms. Several complete open reading frames (ORFs) containing all 17 exons in consecutive order were present in the GenBank database (Accession numbers are
listed in table 3.1). Analysis of the nucleotide sequences of the splice variants described above revealed the existence of multiple ORFs, putatively encoding different protein isoforms. Exon combinations within the 5′-end of SBP2 and the resulting protein isoforms are schematically presented in Figure 3.2.

The ORF including all 17 exons produces the protein described as full length SBP2 (Lescure et al., 2002), consisting of 854 amino acids with a predicted molecular weight of 95.5 kDa. Translation of this isoforms is initiated at the first ATG codon within exon I (Met 1) (Figure 3.2). This variant (SBP2) was clearly the most abundant in the EST database, with at least twenty-eight ESTs and five complete mRNAs identified.

![Figure 3.2 Schematic representation of the human SBP2 5′-region transcript variants](image)

- Indicates the ATG used for translation initiation
- Indicates splice variants identified experimentally
- The ATG start codon (Met 1b) within exon II is generated by a reading frame shift to a -1 frame relative to the reading frame of start codon in exon I
- MW calculated following cleavage of Mitochondrial Targeting Sequence

**Figure 3.2 Schematic representation of the human SBP2 5′-region transcript variants.** Genomic location of the first seven SBP2 exons indicated with different colours and roman numerals is shown in the top Section. Transcript variants containing different exon combinations are shown schematically. Translation start sites are indicated by arrows, the nomenclature for the encoded protein isoforms, start codon number and the predicted molecular weight are indicated to the right of each transcript.

One of the variants experimentally identified lacked exon II, and was spliced from exon I to exon IIIa, which disrupted the reading frame and led to a premature termination codon. The first in frame ATG start codon after this splicing event was therefore Met 69 in exon IIIa, potentially producing an isoform with a predicted
molecular weight of 88 kDa, designated SBP2_Δ2. This splice variant was supported by one additional EST in the GenBank database.

A transcript variant lacking exon III, designated SBP2_Δ3, was identified in one EST. This splicing event lead to loss of the reading frame initiating in exon I, and the first in frame putative start codon was identified within exon IV (Met 233). This variant would generate a protein isoform of 69.2 kDa.

A second splice variant identified experimentally, confirmed by a large number of ESTs and the cDNA obtained form the DKFZ center (AL136881) was generated by splicing between exon II and the alternative acceptor site at exon IIIb. Analysis of this nucleotide sequence revealed that an ORF including all the subsequent exons was created through use of an alternative reading frame within exon II relative to the reading frame starting in exon I (Figure 3.3). This frame-shift encoded an SBP2 isoform with a different N-terminal protein sequence. Analysis of this sequence using the domain predicting program PSORT II (Horton and Nakai, 1997), predicted a mitochondrial targeting signal (MTS), putatively generating a mitochondrial-targeted SBP2 isoform, hence designated mtSBP2. The predicted molecular weight of this isoform was 85.9 kDa. However, as the mitochondrial import pathway for proteins with a leader sequence involves cleavage of the MTS upon entry into the mitochondrial matrix (Truscott et al., 2003), the resulting mitochondrial mtSBP2 isoform would have a molecular weight of 79.4 kDa. A similar transcript variant identified by several ESTs included a second splicing event between exon VI and the alternative acceptor site VIIc, putatively creating a second mitochondrial isoform, mtSBP2b with a predicted molecular weight of 82.0 kDa including the MTS, and 75.5 kDa after cleavage of the MTS. The existence of a mitochondrial SBP2 isoform may represent an important finding as it raises the intriguing possibility of SBP2 having additional cellular function in the mitochondrial compartment. This will be addressed further in Chapter 4.
Figure 3.3 Alternative reading frame use within exon II of human *SBP2*. A reading frame shift within exon II (arrow) generates an N-terminal amino acid sequence encoding a putative mitochondrial targeting signal (underlined). Exon I and II are indicated and the splicing site shown by a vertical line. Frame 1 indicates the frame used to generate the full length SBP2 starting at Met 1, and Frame 3 contains the frame shift to encode the mitochondrial isoform, mtSBP2.

3.3.4 Expression of SBP2 transcript variants in breast cancer cell lines

Expression of the *SBP2*, *SBP2Δ2* and *mtSBP2* splice variants was further investigated in various breast cancer derived cell lines by RT-PCR, using primers designed to overlap the alternative splice junctions specific for each of the transcripts. A normal mammary epithelial cell line (Bre80-1) was used as reference for comparison of possible differences in mRNA levels of the individual transcripts in normal and cancer derived samples. RNA from breast cancer cell lines was kindly provided by Dr. Jeremy Arnold (The Queensland Institute of Medical Research, Australia). Primers specific to actin were used in parallel reactions as an internal reference to standardise the cDNA loading between samples. The results confirmed expression of the three transcript variants in the majority of cell lines (Figure 3.4). Variations in levels of expressions of the same transcripts among the various cell lines, as well as the different transcripts within the same cell line were noted, which could be indicative of differential regulation of expression for each individual transcript. However, in order to determine a putative link between the expression of the various transcripts in breast cancer cell lines, further studies using more accurate quantitation methods need to be employed. Although only speculative, potentially, expression of SBP2 transcripts or protein isoforms may correlate to cellular redox status, selenium levels, selenoprotein requirement or occur in a tissue specific manner.
These questions need to be addressed by further studies, outside the scope of this thesis.

![Figure 3.4](image-url)

**Figure 3.4 Expression of **$SBP2$** transcript variants in breast cancer cell lines.**
Expression of $SBP2$ transcript variants was analysed by RT-PCR using primers specific to $SBP2$, $SBP2_{A2}$ and $mtSBP2$ and cDNA from breast cancer cell lines as indicated. A normal mammary epithelial cell line (Bre80-1) was used as reference. Primers specific to actin were used in parallel reactions as internal loading reference.

3.3.5 Analysis of $SBP2$ mRNA expression by Northern blot

The expression of $SBP2$ mRNA was further analysed by northern blotting. Northern blots were performed using two commercial membranes, one containing poly A$^+$ RNA from multiple human tissues, and one containing poly A$^+$ RNA from various cancer cell lines. The membranes were hybridised with a $^{32}$P labelled probe of full length $SBP2$. The predicted size of the complete $SBP2$ encoding mRNA is 3.6 kb. Consistent with the results presented by (Lescure et al., 2002), a predominant ~4 kb mRNA was detected in all tissues. The amount of $SBP2$ mRNA expression showed variations between tissues, with relatively high levels in spleen, prostate and ovary and significantly higher levels in testis (Figure 3.5A). In addition, two larger transcripts and one smaller transcript were detected in testis. The smaller transcripts may represent alternative splice variants, however, it is not clear whether the larger transcripts are caused by non specific hybridisation of the probe, or whether they represent additional $SBP2$ transcript variants so far not identified. Transcripts of similar size (4 and 3.5 kb) to human $SBP2$ were previously reported for rat $SBP2$, which also expressed a testis specific 2.5 kb transcript (Copeland et al., 2000),
suggesting that multiple SBP2 transcripts may arise due to alternative splicing of SBP2 mRNA.

Figure 3.5 Northern blot analysis of human SBP2 mRNA expression. A full length $^{32}$P-labelled SBP2 probe was hybridised on membranes containing polyA+ RNA from various human tissues (A) or cancer cell lines (B). (A) Autoradiography shows one predominant ~ 4 kb mRNA transcript in all tissues and three additional transcripts in testis indicated by * in the right panel following a shorter exposure time. SBP2 mRNA appears significantly over-expressed in testis. (B) SBP2 mRNA levels vary among different cancer cell lines, a shorter transcript (*) is detected in some lanes. GAPDH was used as loading control on both membranes.

SBP2 expression levels showed large variations among the different cancer cell lines, and smaller transcript variants were detected in some cancers with high SBP2
expression (Figure 3.5B). Although, highest SBP2 expression was detected in leukaemia and lymphomas, lanes 1, 2, 4 and 7 which are known to express several selenoproteins (Gladyshev et al., 1999b; Chow et al., 2001; Spyrou et al., 1996) and relatively low SBP2 transcript levels were detected in epidermal, uterine, lung and cervical carcinomas, and osteosarcoma, it is difficult to rationalise this data as matched normal samples were not available. GAPDH was used as reference for mRNA loading.

3.3.6 Analysis of expressed SBP2 isoforms

Existence of multiple transcripts may not always correlate with protein expression. Rather, alternatively spliced transcripts may play other regulatory roles, such as acting as ‘buffers’ in controlling the availability of regulatory factors to control the production of one particular transcript (Lewis et al., 2003). In order to investigate the expression of SBP2 protein isoforms in cells western blotting was initially employed. The anti-SBP2 antibodies generally show high specificity for a 120 kDa protein which is the predicted molecular weight of full length SBP2 based on its electrophoretic mobility in SDS-PAGE (Copeland et al., 2000). The antibodies do recognise additional bands that could potentially represent SBP2 isoforms (see below). However, as polyclonal antibodies are often found to recognise multiple proteins non-specifically, this method was considered unreliable for use in identification of SBP2 isoforms. Therefore, immunoprecipitation combined with mass spectrometry analysis were employed.

Large scale immunoprecipitations using affinity purified anti-SBP2 antibodies were carried out in cell lysates from 293T cells, where the various spliced transcripts were initially detected. As the different isoforms were found to vary in the N-terminal region, N-terminal sequencing was employed (performed by the Karolinska Institute Proteomics Analysis Centre) to analyse several bands immunoprecipitated and transferred to PVDF membranes (data not shown). The results showed that the proteins were N-terminally blocked and could not be amino acid sequenced, a common phenomenon observed in cellular proteins.
A second approach was undertaken using MALDI-TOF mass spectrometry. Large scale immunoprecipitations were conducted and proteins were resolved by SDS-PAGE and stained by coomassie staining. Eight bands were excised from the coomassie stained gels, and submitted for MALDI-TOF mass spectrometry analysis (performed by the Karolinska Institute Proteomics Analysis Centre) (data not shown). A fraction of the immunoprecipitation reaction from the experiment was analysed by western blot as shown in Figure 3.6, indicating some of the bands excised. This was performed in order to compare the MALDI-TOF results to bands generally recognised by the anti-SBP2 antibodies on western blots, to identify SBP2 alternate splice variants. As shown in Figure 3.6, three major bands are detected in cell lysates and immunoprecipitations. Several of the bands excised from coomassie stained gels were not recognised by the anti-SBP2 antibodies on western blots. These bands could either represent specific SBP2 interacting proteins or non-specific bands precipitated with the antibodies.

The MALDI-TOF analysis provided several peptide masses for each of the bands submitted, and these were analysed using bioinformatics. Masses matching to the human SBP2 protein were detected within two of the bands excised. Band indicated as no.2 in Figure 3.6 contained the predicted full length 120 kDa SBP2, as expected. Interestingly, an additional band (no.3 in Figure 3.6) at ~75 kDa was identified as SBP2, suggesting that at least one alternatively spliced isoform is translated into protein in 293T cells. The data obtained was however insufficient to determine which particular isoform the band corresponded to, nevertheless, it represents an important finding that implies that at least one alternatively spliced isoform is being expressed in cells and indicates a more complex regulation of SBP2 than previously thought. Band indicated as no. 1 in Figure 3.6 at ~180 kDa is commonly detected by the anti-SBP2 antibodies on western blots and in immunoprecipitations. This band did however not contain any SBP2 matching peptides. The band was identified as early endosome antigen 1 (EEA1). Computational alignment of the SBP2 sequence used as an antigen to generate SBP2 antibodies with the EEA1 amino acid sequence did not reveal any significant homology between the polypeptides, therefore the reason for cross-reactivity with this protein remains unclear.
Additional proteins were identified within the other bands excised from the coomassie stained gel, which likely represent proteins found in complex with SBP2. Like SBP2, most of these proteins were involved in RNA metabolism, and they included members of the eukaryotic translation initiation complex, eIF2C1, eIF2C3 and eIF3/p110, translational control protein 80 (TCP80) and the ATP-dependent RNA helicase 1. Evidently, further investigations are required in order to confirm and determine the functional relevance of these putative interactions, however these experiments are beyond the scope of this project and need to be addressed separately.

Figure 3.6 Western blot of immunoprecipitated SBP2 bands subjected to MALDI-TOF mass spectrometry. SBP2 was immunoprecipitated from 293T total cell lysates using anti-SBP2 antibodies. (A) 1/10th of the reaction was resolved on SDS-PAGE transferred to a PVDF membrane and western blotted for SBP2. The bands excised from the corresponding coomassie stained gels and also detected by western blot and are indicated as 1, 2, and 3. Band indicated by * only appeared on the western blot. (B) A sample of total cell lysate was electrophoresed in parallel to compare the bands recognised by the anti-SBP2 antibodies with the mass spectrometry results. SBP2 was identified in bands 2 and 3.
3.4 DISCUSSION

The data presented in this chapter represents the first reported investigations into the regulation of the human SBP2 gene by alternative splicing. Experimental and computer based approaches were combined in order to perform a detailed analysis of the splicing events that occur within the SBP2 gene. Several transcript variants resulting from alternative splicing events within the 5′ region of the SBP2 gene were indeed identified. It was noted that alternative splicing was restricted to the 5′-region of the SBP2 gene comprising exons I-X. The 3′-region of the SBP2 gene including exons X-XVII, did not demonstrate any alternative splicing events, which was concluded from the presence of a large number of ESTs containing the complete exon set (X-XVII). The SBP2 gene is comprised of 17 exons that yield a 2562 bp cDNA encoding an 854 amino acid polypeptide. Using an in vitro system, it was demonstrated that the N-terminal portion (aa 1-399) of SBP2 is dispensable for Sec incorporation (Copeland et al., 2000), in fact, the only region required is contained within aa 399-777 representing less than half of the entire protein. At the gene level, this region is encoded within exons IX-XVI. Two domains required for SBP2 function in in vitro systems and in transfected cells have so far been reported: the L30-type RNA binding domain which resides within exons XIV and XV, and a functional domain specifically required for Sec insertion but not RNA binding which is encompassed by exons IX-XI (Copeland et al., 2001; Mehta et al., 2004). Additional SBP2 motifs previously reported include a putative NLS (Copeland and Driscoll, 1999) which is found in exon VIII, and a ribosome binding domain that has not been entirely defined but encoded within exons IX-XVI (Copeland et al., 2001; Kinzy et al., 2005). One central, yet unanswered question regards the relevance of the N-terminal half of the SBP2 protein. From the splicing point of view, it was interesting to note that the vast majority of detected splicing events occurred within the 5′ region encoding the Sec-dispensable part of the protein. When alternative splicing events were present, they provoked pre-mature stop codons and ORF alterations, potentially promoting translation from downstream Met codons. In these cases, proteins with variable N-termini would be produced.

Attempts to identify alternative SBP2 protein isoforms in cells, demonstrated that at least one smaller isoform with an electrophoresis migration at ~75 kDa was expressed
in 293T cells. The identity of the isoform remains unclear and needs to be investigated further. Whether the majority of alternative transcripts give rise to translated protein products in cells remains to be established, as does any relevance of alternatively spliced SBP2 N-terminal isoforms during translation of target transcripts. Although previously reported data provided evidence that suggests that this region lacks functionality in terms of Sec insertion, the experimental approach using an in vitro translation system where SBP2 is incubated with already transcribed SECIS-containing mRNA in rabbit reticulocyte lysates (Mehta et al., 2004) is far from what would occur in vivo. For example, it is possible that the Sec incorporation process in vivo may require the association of SBP2 with the SECIS-mRNA upon transcription within the nucleus, consequently requiring the presence of the NLS domain encoded by exon VIII. Interestingly, no splice variants lacking exon VIII were detected, which would fit some models proposed in Chapter 1. Alternatively, the variable N-termini regions may well function in sequestering specific protein factors that affect translation of particular SECIS elements. The human selenoproteome consists of 25 different selenoproteins (Kryukov et al., 2003), but the SECIS core element and the binding site of SBP2 are well conserved (Section 1.3.3.2.1). As a result, Sec incorporation as seen from the point of view of SECIS element binding would not require much variability within the SBP2 RNA binding region which could explain the lack of splicing events observed. In contrast, regulation of expression of any particular selenoprotein would be expected to involve unique or specific factors that may serve to dictate the binding of SBP2 to their mRNAs, thus it is possible that a variable N-terminal domain of SBP2 could function in recruitment of such factors. Alternatively, the N-terminal domain may not be involved in the Sec incorporation mechanism but may participate in other, yet to be identified cellular functions.

It was also noted that alternative splicing events are not restricted to humans; rather, they seemed evolutionary conserved. A number of splicing events identical to ones occurring within human SBP2 were detected in other species such as monkeys, rodents, calf, and dog (data not shown), but also species specific events were observed that did not correlate to ones detected in humans. A comparison of transcript variants expression in normal vs. cancer derived tissues or cell lines did not show a clear link with particular transcript variants.
Although the exact mechanisms that control use of alternative splice sites are mostly unknown, it is well documented that alternative splicing is regulated by a variety of factors and at multiple levels, such as: intrinsic regulatory elements located within the gene, the availability, concentration and activity of regulatory splicing proteins, tissue or cell specific splicing factors, developmental stage regulated splicing and general or stress related signalling pathways (Black, 2003; Matlin et al., 2005; Stamm, 2002). Such factors and levels of regulation may also control alternative splicing events within the SBP2 gene. Further elaborate studies, not covered in this thesis, need to be performed to address these issues in order to understand fully the regulation of functions of SBP2 by alternative splicing.
CHAPTER 4.

SUBCELLULAR LOCALISATION OF SBP2
4.1 INTRODUCTION

The spatial separation of mRNA synthesis from translation in the eukaryotic system relies on efficient transport across the nuclear envelope. The majority of proteins destined for import into the nucleus are bound in the cytoplasm by members of the Importin-α/-β family of proteins. This requires the presence of a conserved signal sequence, the lysine-rich NLS, within the cargo protein. Transport in the opposite direction is mediated by the nuclear export receptor CRM1, which binds to leucine-rich NESs (Fornerod et al., 1997; Stade et al., 1997). Translocation in both directions takes place through the nuclear pore and is controlled by the Ran-GTP/GDP gradient across the nuclear envelope.

Many cellular proteins continuously traffic between the nuclear and cytoplasmic compartments and these are usually referred to as shuttling proteins. Some shuttling proteins contain intrinsic NLS and NES motifs, others may translocate in complex with NLS/NES containing binding partners and small proteins can passively diffuse through the nuclear pore. Thus, the intracellular localisation of a protein at any one time largely depends on the accessibility of its nuclear transport signal to its cognate receptor, which in turn can be modulated by changes in the intracellular conditions.

As mentioned in the Introduction, a lysine-rich stretch of amino acids resembling a putative NLS was identified within the central region of the SBP2 protein sequence. It was therefore suggested that SBP2 may utilise this motif to enter the nucleus to associate with selenoprotein encoding mRNAs and facilitate their transport to the cytoplasm for translation (Copeland et al., 2000). However, when the subcellular localisation of endogenous SBP2 was investigated it was found that SBP2 localised predominantly to ribosomes within the cytoplasm (Copeland et al., 2001), suggesting that the NLS does not appear to be a dominant motif. Although the above findings depicted SBP2 as a ribosomal protein, the possibility of SBP2 being able to shuttle between the nucleus and the ribosomes in accordance with the proposed model, was not rejected. Moreover, the region of the SBP2 protein that harbours the putative NLS was shown to be dispensable for Sec incorporation assayed using an in vitro model system, a situation that does not resemble what may occur in vivo. The possibility of
the NLS playing an indirect but important role in the regulation of SBP2 function could not be excluded. Therefore, a detailed investigation of the subcellular localisation of SBP2 with a particular emphasis on defining the functionality of the predicted NLS and additional motifs that may be involved in this process was undertaken.

As presented in Chapter 3, SBP2 is subjected to alternative splicing events and at least one additional isoform appears to be expressed in cells. One particular isoform was predicted to possess a potential mitochondrial targeting signal, which implies that SBP2 may have a function in the mitochondrial compartment. If a mitochondrial isoform was indeed expressed in cells, it would most certainly constitute an intriguing, but significant finding which may expand the SBP2 functional repertoire.

In this chapter, the subcellular localisation of full length and two alternatively spliced SBP2 isoforms were investigated. The predicted NLS was assessed for its functionality and several leucine-rich putative NESs were identified and characterised for their role in SBP2 trafficking between the nucleus and the cytoplasm. The role of these motifs in the regulation of Sec incorporation was also addressed.
4.2 MATERIALS AND METHODS

4.2.1 Plasmid construction

The subcloning of full length SBP2 and isoforms into the GFP vector was described in Section 3.2.1. The deletion construct referred to as NLS-GFP was generated by restriction enzyme digest of the complete 5′-end from the SBP2-GFP vector using the EcoR1 and BclI sites as described in Section 2.2.3.6. The fragment was subsequently ligated into the EcoR1/BclI digested pre-existing NLS-GFP vector (L.V. Papp, Honours dissertation thesis, Griffith University) which lacked some of the 5′ sequence, as described in Section 2.2.3.8. The resulting NLS-GFP construct encompassed bp 1-1751 corresponding to aa 1-583. The NES-GFP was previously assembled (L.V. Papp, Honours dissertation thesis, Griffith University) and encompassed bp 1752-2562, aa 584-854. Mutations in the putative NLS were introduced into the SBP2-GFP and the NLS-GFP constructs as described in Section 2.2.3.11 using the NLS-M-2,3 forward and NLS-M-2,3 reverse primers outlined in Table 2.4. For the in vivo NES assay, double-stranded oligonucleotides (outlined in Table 2.4, named NES1-5 FWD and REV) encoding the amino acid sequence of candidate SBP2 NESs, were annealed and phosphorylated as described in Sections 2.2.3.4 and 2.2.3.5 respectively, and cloned into the BamHI site of the pRev(1.4)-GFP vector (Henderson and Eleftheriou, 2000) by standard procedures described in Section 2.2.3. Constructs were subsequently screened for orientation by sequencing (2.2.3.10).

4.2.2 Subcellular localisation of SBP2-containing GFP reporter constructs

Subcellular localisation of GFP-tagged SBP2 isoforms was studied in COS7 cells by fluorescence microscopy. Cells were transfected with SBP2-GFP, SBP2Δ2-GFP and mtSBP2-GFP plasmid DNA as described in Section 2.2.4.4 and processed as described in Section 2.2.4.5. For western blot purposes plasmids were transfected by electroporation as described in Section 2.2.4.3 and analysed as described in Sections 2.2.4.6, and 2.2.5.
4.2.3 Immunofluorescence microscopy

Cells were grown on coverslips in 6 well plates for 24-36 hours to reach ~70-80 % confluency. Cells were subsequently washed with PBS $1 \times 5$ min at room temperature, fixed in 4% paraformaldehyde (PFA) in PBS for 30 min and washed twice in PBS as above. Cells were permeabilised in 0.2% Triton X-100/PBS for 15 minutes, washed 2 × in PBS and subsequently blocked in 3% goat serum in PBS for 1 hr. The blocking solution was removed and cells were incubated with primary antibodies diluted in blocking solution as described in Table 2.1, overnight at 4°C. After washing 3 × 15 min with PBS, cells were incubated with FITC- or CY3-conjugated secondary antibodies (Table 2.2) for 1 hr at room temperature. Finally, coverslips were washed with PBS $3 \times 5$ min and mounted onto microscope slides with Mowiol containing 0.6% DABCO (Diazobicyclo-octane) and allowed to set overnight at room temperature prior to visualising on a microscope. Staining for mitochondria was carried out by adding MitoTracker (Molecular probes) in culture medium at 37°C/5%CO₂ for 30 min prior fixing cells.

4.2.4 Subcellular fractionation

Subcellular fractions were prepared by a differential centrifugation method (Antonsson et al., 2001). $5 \times 10^6$ 293T cells grown in 15 cm diameter culture dishes were scraped into phosphate buffered saline containing 1 mM EDTA and centrifuged at 750g for 10 min, washed and suspended in mitochondrial buffer (MB) (210mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM Hepes-NaOH, pH 7.5) complemented with protease inhibitors. Cells were disrupted by passing through a 25G1 needle five times followed by centrifugation at 2000 × g for 5 min. The supernatant was saved and pellet was resuspended in MB. The breakage procedure was repeated four times. The combined supernatants were centrifuged at 2000 × g for 5 min to remove nuclei and unbroken cells. The supernatant was subsequently centrifuged at 13 000 × g for 10 min. This supernatant was kept and centrifuged at 100 000 rpm for 30 min to yield the microsomal pellet and centrifuged again at 100 000 rpm for 16 hrs to yield the free cytosolic ribosomes. The mitochondrial containing pellet was washed 3 × by suspending in MB and centrifuging at 13 000 ×
for 10 min. Pellets were lysed in UIP lysis buffer (Section 2.2.4.6) and protein concentration was determined by the Bradford assay (Section 2.2.5.1). Alternatively subcellular fractionation was performed using the Q-Proteome Cell Compartment Kit (Qiagen) as instructed by the manufacturer.

4.2.5 In vivo NES assay

Analysis of candidate SBP2 NESs was carried out as described by (Henderson and Eleftheriou, 2000). Briefly, Rev1.4-GFP-fusion proteins containing SBP2-NES sequences were expressed in COS7 cells and their subcellular distribution was analysed by fluorescence microscopy. Where indicated cells were treated with LMB, Act.D and cycloheximide as described in Section 2.2.4.2.

4.2.6 Metabolic labelling of HeLa cells with $^{75}$Se and $^{35}$S

HeLa cells were plated onto 10 cm dishes and incubated for 24 hours, followed by a 16 hr incubation in the presence of LMB (2 ng/ml). 10 μCi $^{75}$Se was added to each dish in fresh medium supplemented with 50 nM sodium selenite and labelling was conducted in the presence of LMB for 24 hrs. For $^{35}$S labelling RPMI medium lacking methionine and containing dialysed serum was used and 100μCi of $^{35}$S was added to each dish. Cells were harvested and lysed in UIP buffer. Proteins were resolved by SDS-PAGE and analysed by autoradiography. Densitometry of radioactive signals was performed using the MultiGauge v2.3 software.
4.3 RESULTS

4.3.1 Subcellular localisation of GFP-fused SBP2 isoforms

As described in Section 3.3, several alternatively spliced variants were identified during sub-cloning of full length SBP2 into the GFP expression vector. The plasmids generated thus contained the full length SBP2 (SBP2-GFP), splice variant SBP2_Δ2 (SBP2_Δ2-GFP) and the predicted mitochondrial isoform mtSBP2 (mtSBP2-GFP). To initiate the studies of SBP2 subcellular localisation, these GFP fusions constructs were transiently transfected in COS7 cells and the distribution of the expressed proteins was investigated by fluorescence microscopy. It was observed that the SBP2-GFP and SBP2_Δ2-GFP proteins displayed a predominantly cytoplasmic localisation. Some cells did however display an equal nuclear-cytoplasmic fluorescence, which was more frequent in the cells expressing the SBP2_Δ2-GFP variant (Figure 4.1 A left and middle panels). In order to compare these differences, cells were counted and scored for nuclear (N), nuclear/cytoplasmic (N/C) or cytoplasmic (C) fluorescence. In repeated experiments, it was found that cells expressing full length SBP2 displayed a predominantly cytoplasmic fluorescence in >85% of cells. However, cells expressing the SBP2_Δ2 isoform were found to display an equal nuclear-cytoplasmic distribution to a larger extent (~40%) compared to full length SBP2 (~15%). Entirely nuclear fluorescence was observed in a small proportion of cells for both isoforms, and again the SBP2_Δ2 isoform displayed nuclear fluorescence in a larger proportion of cells (10-20%) than the full length SBP2 (3-5%). Western blot analysis of cells expressing the two isoforms confirmed the production of full length fusion proteins, as determined by their migration at the predicted molecular weight, ~150 kDa for SBP2-GFP and ~130 kDa for SBP2_Δ2-GFP (Figure 4.1 D).
Figure 4.1 Subcellular localisation of GFP-tagged SBP2 isoforms in COS7 cells. GFP-tagged SBP2 and SBP2_Δ2 isoforms were transiently expressed in COS7 cells and their subcellular distribution analysed by fluorescence microscopy. Hoechst DNA stain (blue) indicates the nuclei arrows point to transfected cells. Images were acquired at a 60 x magnification. (A) Fluorescence microscopy shows that SBP2-GFP is localised predominantly within the cytoplasm of cells. (B) An example of SBP2_Δ2-GFP protein displaying an equal nuclear/cytoplasmic distribution. (C) Fluorescent cells were counted and scored based on the fluorescent intensity as either nuclear (N), nuclear-cytoplasmic (N/C) or cytoplasmic. Graphs represent mean cell counts from two independent experiments in which the full length SBP2-GFP (top graph) and the SBP2_Δ2-GFP (bottom graph) were expressed. (D) Western blot analysis using anti-SBP2 antibodies confirmed the expression of full length fusion proteins. SBP-GFP (Met 1) migrates at ~150 kDa and SBP2_Δ2-GFP (Met 69) at ~130 kDa. A faster migrating band was detected in transfected cells, likely representing a protein expressed from an internal downstream Methionine (Met 233) present in both constructs. Untransfected cells are shown in the middle lane. Endogenous SBP2 is detected at 120 kDa in all lanes. A non-specific band was detected at 95 kDa.
Interestingly, the predicted mitochondrial isoform mtSBP2-GFP demonstrated a staining pattern that resembles mitochondria (Figure 4.2 A). In order to confirm that this was indeed the case, mtSBP2-GFP transfected cells were stained with the mitochondrial marker Mitotracker. As shown in Figure 3.1 D, the mtSBP2-GFP protein co-localised with Mitotracker as indicated by the yellow colour in overlayed images, demonstrating that the mitochondrial targeting sequence (MTS) encoded by the alternative frame within exon II, is a functional motif. This was an exciting finding which opens up the possibility that a mitochondrial-targeted SBP2 isoform may be expressed in cells.

Figure 4.2. Subcellular localisation of GFP-tagged mitochondrial SBP2 isoform. The predicted mitochondrial SBP2 isoform mtSBP2-GFP was transiently expressed in COS7 cells and its subcellular distribution was analysed by fluorescence microscopy. (A) mtSBP2-GFP displayed a mitochondria-resembling fluorescence. (B) Western blot analysis using anti-GFP antibodies shows the expression of mtSBP2 and the SBP2Δ2-GFP isoforms. (C) MtSBP2-GFP mitochondrial localisation was confirmed by co-staining with the mitochondrial marker Mitotracker (red) as indicated by the yellow staining in the right panel.
4.3.2 Subcellular localisation of endogenous SBP2 in cells

Immunofluorescence microscopy was subsequently employed to investigate the subcellular localisation of endogenous SBP2 in cells. Several cell lines including HCC1937, MCF7 and SVCT were immuno-stained with affinity purified anti-SBP2 antibodies described in Section 2.2.4.10. As shown in Figure 4.3 A, a strong perinuclear staining pattern was observed in all cells stained with the anti-SBP2 antibodies whereas the pre-immune serum showed an even staining throughout the cell. To define the perinuclear staining pattern, COS7 cells were co-stained with a marker for the ER, anti-protein disulfide isomerase (PDI) as it was thought that the staining may represent the SBP2 localisation at the ribosomes on the ER membrane. The mitochondrial marker Mitotracker was also used in parallel experiments. Surprisingly, co-staining was observed in cells treated with Mitotracker (Figure 4.3 B, top panels), but not in the cells stained with the ER marker (bottom panels), as shown in the merged images in the right panels. The observed staining pattern did not agree with the proposed ribosomal localisation of SBP2 (Copeland et al., 2001), however, it did support the evidence for the existence of a mitochondrial targeted SBP2 isoform. It was previously shown that SBP2 is present in cells in a large multiprotein and ribosomal complex (Copeland and Driscoll, 1999; Copeland et al., 2001). It is therefore possible that the SBP2 antibody recognition site is blocked by protein/ribosome interactions, thus making ribosomal SBP2 undetectable by immunostaining. Whether the mitochondrial staining is caused by the existence of a mitochondrial SBP2 isoform can not be concluded from these experiments as non-specific recognition of additional mitochondrial proteins by the SBP2 antibodies cannot be ruled out. Therefore, this method was not used in further studies of SBP2 subcellular localisation.
Figure 4.3 Immunofluorescence microscopy of endogenous SBP2. Immunofluorescence microscopy using affinity purified anti-SBP2 antibodies and FITC-conjugated secondary antibodies, was performed in HCC1937, MCF7 and SVCT cell lines. Images were acquired at 60x magnification. (A) Predominantly mitochondrial staining detected by the anti SBP2 antibodies (left panels). Pre-immune serum was used as control which showed an even cytoplasmic staining. (B) SBP2 co-stained with the mitochondrial marker Mitotracker (top panels) but not with the ER marker PDI (bottom panels).

To further investigate the subcellular localisation of endogenous SBP2 and to address the expression of a mitochondrial-targeted isoform, organelle fractions were prepared from 293T cells, known to express at least one additional SBP2 isoform (Section 3.3.6), using a differential centrifugation method. The fractions yielded included: nuclei, mitochondria, microsomes (ER and Golgi), ribosomes and cytosol. Protein extracts from these subcellular compartments were resolved by SDS-PAGE and
western blotted for SBP2. As previously reported (Copeland et al., 2001; Kinzy et al., 2005), the largest pool of full length SBP2 was detected in the ribosomal fraction (Figure 4.4). In contrast to previous data, SBP2 was also detected in the microsomal fraction, which likely represents SBP2 within the ER-bound ribosomes, the nuclear fraction and the cytosol. The presence of SBP2 in the nuclear fraction was an interesting finding that is consistent with the presence of a predicted NLS within the SBP2 amino acid sequence. Interestingly, a protein of the size predicted for the mitochondrial isoform of SBP2 identified by mass spectrometry (Chapter 3, Figure 3.6) was detected in the mitochondrial fraction (Figure 4.4). The HSP60 protein was used as marker for the fractionation experiment showing the cleaved, mitochondrial imported protein in the mitochondrial fraction. The nuclear fraction appeared to contain a slight mitochondrial contamination, possibly caused by the presence of intact cells that may have been pelleted in the first centrifugation step. The band detected in the mitochondrial fraction could represent a mitochondrial-targeted SBP2 isoform, however, factors such as antibody non-specificity for a protein enriched in the mitochondrial fraction cannot be ruled out, and therefore the data remains solely indicative. Additional bands were detected in some of the fractions by the anti-SBP2 antibodies upon a longer exposure of the membrane (Figure 4.4 bottom panel). Similarly, these bands could represent alternatively spliced SBP2 isoforms as indicated in the figure, or non-specific bands detected by the antibodies. Expression of alternatively spliced SBP2 isoforms putatively targeted to different subcellular compartments indeed represents an exciting and important finding that needs to be further investigated in order to completely elucidate SBP2 function. For the remaining part of this thesis, the work presented will elaborate on the characterisation of only the full length SBP2.
Figure 4.4 Subcellular distribution of endogenous SBP2 in 293T cells. Localisation of SBP2 and putative isoforms was investigated by subcellular fractionation in 293T cells. Western blots show that several bands are being detected by the anti-SBP2 antibodies, as indicated by arrows in the top and bottom panels. Full length SBP2 was predominantly present within the ribosomal fraction, but also in the ER, nuclear and cytosolic fractions. A smaller protein was detected within the mitochondrial fraction. Antibodies against HSP60 were used as reference for the fractions. The full length and mitochondrial cleaved HSP60 isoforms are indicated (middle panel).
4.3.3 SBP2 shuttles between the nucleus and the cytoplasm in a CRM1 dependent manner

A preliminary study of SBP2 subcellular localisation revealed that transiently expressed SBP2-GFP relocated from the cytoplasm to the nucleus in response to LMB treatment (L.V. Papp, Honours dissertation thesis, Griffith University). These results indicated that SBP2 may shuttle between the nucleus and the cytoplasm, in agreement with the proposed hypothesis. Because the SBP2 cDNA previously used was incomplete, the experiments were repeated using the SBP2-GFP construct engineered in Section 3.3.1. As expected, LMB treatment of COS7 cells induced the translocation of SBP2-GFP from the cytoplasm to the nucleus (Figure 4.5 A). GFP alone was not affected by LMB treatment (Figure 4.5 B). These results were confirmed in repeated experiments and are summarised in the graphs shown in Figure 4.5 C. The graphs represent counts of cells scored for nuclear, nuclear/cytoplasmic or cytoplasmic fluorescence following a 2 hr LMB treatment. The fast kinetics of nuclear accumulation suggested that over-expressed SBP2-GFP continuously shuttles between the nucleus and the cytoplasm. However, rapid nuclear export mediated by CRM1 confines the steady state SBP2-GFP levels to the cytoplasm. The results thus indicated that the NLS may indeed be a functional domain and in addition that SBP2 may also contain a functional NES that mediates its nuclear export.

The study was extended to analyse the effect of LMB on endogenous SBP2 localisation. Subcellular fractionation of 293T cells subjected to a 2 hr LMB treatment followed by analysis by western blotting revealed no increase in nuclear SBP2 levels (data not shown) which was in contrast to what was observed for SBP2-GFP. One explanation for this lack of effect could be the existence of a separate nuclear and ribosomal pool of SBP2 in cells, in which case SBP2 would not be shuttling. Alternatively, endogenous SBP2 may not continuously shuttle between the ribosomes and the nucleus in a similar fashion as observed for SBP2-GFP; rather, its translocation to the nuclear compartment may only occur upon induction by certain stimuli or, may be taking place immediately after its synthesis. In the event that the second proposal was correct, the 2 hrs LMB treatment time would not have been sufficient to detect SBP2 nuclear accumulation. To test this possibility, 293T cells were treated with LMB for 16 hrs and subcellular fractionation was performed using
the Q-Proteome cell compartment Kit (Qiagen). To ensure the LMB treatment was effective, western blotting for the Cdc25c protein, previously shown to accumulate in the nuclei of cells exposed to LMB treatment (Graves et al., 2001), was conducted in parallel. As shown in Figure 4.5 D, a 16 hr LMB treatment led to accumulation of both SBP2 (sometimes two bands of SBP2 were seen on Western blots) and Cdc25c suggesting that SBP2 does translocate through the nuclear compartment. Surprisingly, SBP2 was not detected in any other fractions, suggesting that SBP2 was not present in the soluble cytosolic fraction. Gamma-Tubulin was used as cytosolic marker, which ensured that the nuclear fraction was not contaminated by cytosolic proteins. In order to assess the reciprocal decrease of SBP2 levels from the ribosomes, the differential centrifugation method was employed and the levels of SBP2 in the nuclear and ribosomal fractions were compared by western blotting. As shown in Figure 4.5 E, a 16 hr LMB treatment caused an increase in the SBP2 nuclear pool as well as a simultaneous decrease in the ribosomal SBP2, suggesting that endogenous SBP2 does transit through the nuclear compartment and that it utilises the CRM1 pathway for nuclear exit. Total SBP2 protein levels were examined which showed that the LMB treatment did not alter the total cellular SBP2 pool (Figure 4.5 F bottom panel). The longer time required for endogenous SBP2 to accumulate in the nucleus compared to over-expressed SBP2, suggests that relocation occurs in response to signalling events, rather than continuous shuttling. From these results, two equally possible mechanisms can be suggested: SBP2 may shuttle between ribosomes and the nucleus in response to signalling events or secondly, SBP2 may enter the nucleus immediately after its synthesis and subsequently relocate to ribosomes. According to the second possibility, the change in localisation of SBP2 would only occur once the existing pool of SBP2 is degraded and freshly synthesised SBP2 is available for export by CRM1.
Figure 4.5 SBP2 shuttles between the nucleus and the cytoplasm via the CRM1 pathway. (A) COS7 cells were transiently transfected with SBP2-GFP and either left untreated (-LMB) or treated (+LMB) with Leptomycin B (2ng/ml) for 2 hrs. (B) GFP alone was processed in parallel as a control. Images were acquired at 40x magnification. (C) Graphical representation of the percentage cells showing changes in distribution of SBP2-GFP upon LMB treatment. Fluorescent cells were scored as either nuclear (N), cytoplasmic (C) or nuclear/cytoplasmic (N/C). Values represent the mean of three independent experiments. Minimum 200 cells were counted per experiment. DAPI stained nuclei are shown in blue and arrows indicate transfected cells. (D) Subcellular fractionation using the Q-Proteome cell compartment kit showed nuclear accumulation of SBP2 in response to a 16 hr LMB treatment. Cdc25c was used as a positive control for the LMB experiment and γ-tubulin, a marker for the cytosol, was used to verify the purity of the nuclear fraction. (E) Subcellular fractionation using the differential centrifugation method of untreated and LMB treated HeLa cells showed nuclear accumulation and a reciprocal ribosomal decrease of endogenous SBP2 in response to LMB treatment. PCNA was used as nuclear marker and Actin was used as ribosomal marker.
4.3.4 SBP2 contains a functional NLS

The ability of SBP2 to translocate between the nucleus and the ribosomes not only suggested that the putative NLS may be a functional motif but also implicated the requirement of a CRM1-dependent NES to direct its export from the nucleus. A detailed SBP2 amino acid sequence analysis, assisted by Dr. Beric Henderson (Westmead Millennium Institute for Medical Research, Sydney) was performed and revealed the existence of five putative NESs, reminiscent of the HIV Rev protein NES (Wen et al., 1995) within the carboxy-region of SBP2 protein.

To address the role of these motifs in regulating SBP2 subcellular localisation two SBP2 deletion-constructs were generated as GFP-fusions: the N-terminal portion of SBP2 encompassing the predicted NLS (382KKK385), and the C-terminal region containing the putative NESs (L.V. Papp, Honours dissertation thesis, Griffith University). However, the N-terminal SBP2 sequence used at the time was incomplete and therefore required re-assembling and analysis. The constructs prepared, were named NLS-GFP (aa1-583) and NES-GFP (aa584-854) and are schematically presented in Figure 4.6 which also indicates the location of the motifs within the SBP2 protein.
Figure 4.6 SBP2 deletion constructs generated to characterise the NLS and NES motifs. The structure and the location of motifs within SBP2 are shown in the top panel. The NLS-GFP deletion construct was generated to contain the predicted NLS. The NES-GFP construct contains the putative NESs. Both sequences were sub-cloned N-terminal to the GFP reporter protein.

The subcellular distribution of the newly assembled NLS-fusion protein was analysed by fluorescence microscopy in transiently transfected COS7 cells. In contrast to the full length SBP2-GFP protein the NLS-GFP deletion protein was localised entirely within the nuclei of COS7 cells (Figure 4.7 A), suggesting that the NLS may be a functional motif.
Figure 4.7 SBP2 contains a functional NLS motif. COS 7 cells were transiently transfected with the wt \(^{382}\text{KKKK}^{385}\) (NLS-GFP WT) or mutant \(^{382}\text{KAAK}^{385}\) NLS (NLS-GFP M2,3) in either the NLS-GFP deletion construct (A) and (B) respectively, or in the full length SBP2-GFP construct (C) and (D). Representative microscopy images of the localisation of the expressed proteins are shown. (A) NLS-GFP wt is localised within the nucleus whereas the mutant NLS-GFP M 2,3 displays an equal nuclear cytoplasmic fluorescence (B). (C) SBP2-GFP efficiently accumulated in the nucleus after 2 hrs of LMB treatment. (D) This effect was inhibited by the mutations introduced to the NLS (SBP2-GFP M 2,3). (E) Graph represents the results from two independent experiments where cells displaying exclusive nuclear localisation of the wild type NLS (NLS wt) or mutant NLS (NLS mut) SBP2-GFP protein following 2 or 4 hours of LMB treatment were counted.

As described in Section 1.1.1.1.2, pathways independent of NLS by which proteins gain nuclear access are commonly observed. To ensure that nuclear localisation of the NLS-GFP protein was dependent on the intrinsic NLS \(^{382}\text{KKKK}^{385}\), site directed mutagenesis was employed and the two core lysine residues, \(K^{383}\) and \(K^{384}\) were mutated to alanines (NLS-GFP M 2,3). As shown in Figure 4.7 B, inactivation of the NLS induced a partial shift of the protein towards the cytoplasm and the majority of cells displayed an equal nuclear/cytoplasmic fluorescence, suggesting that the SBP2
NLS is a functional motif, capable of mediating SBP2 nuclear localisation. The mutations were also engineered in the SBP2-GFP construct to test their effect in context of full length SBP2 protein. Because the SBP2-GFP protein was already cytoplasmic, cells expressing either the wt or the NLS mutant SBP2-GFP were treated with LMB and the efficiency of nuclear accumulation was monitored at different time points after treatment. As depicted in Figure 4.7 C and D, at 2 hrs after LMB the mutant protein had only partially translocated to the nucleus whereas the wt counterpart showed significantly higher nuclear localisation. This was also the case at 4 hrs post treatment. A detailed quantitation of cells expressing NLS wt or NLS mutant full length SBP2-GFP in two independent experiments, at two hours or four hrs of LMB treatment is represented in the graph Figure 4.7 E showing that 75% of the wt SBP2-GFP expressing cells showed exclusively nuclear localisation. In contrast, only 30% of cells expressing mutant SBP2-GFP showed exclusive nuclear localisation, confirming that the amino-terminal $^{382}$KKKK$^{385}$ sequence is indeed a functional NLS that facilitates nuclear translocation of SBP2.

4.3.5 Mapping the carboxy-terminal SBP2 NES

The localisation of the NES-GFP fragment was investigated in a previous study (L.V. Papp, Honours dissertation thesis, Griffith University) and was shown to reside within the cytoplasm of COS7 cells as presented in Figure 4.8 left panel. To ensure that cytoplasmic localisation arose from active nuclear export by CRM1 rather than lack of functional NLS, cells expressing the NES-GFP deletion protein were treated with LMB. Interestingly, the treatment induced nuclear accumulation of the NES-GFP protein (Figure 4.8 right panel), suggesting that an active NES was present within this region. The nuclear localisation of this fragment could be facilitated by a cryptic NLS exposed in the truncated protein, or by a piggy-back mechanism through association with an NLS-containing binding partner.
Figure 4.8 Subcellular localisation of the carboxy-terminal SBP2 region. COS7 cells were transiently transfected with the SBP2 NES-GFP truncation construct and its subcellular localisation was investigated in untreated cells and in cells treated with LMB. The NES-GFP truncation construct was shown to relocate from the cytoplasm to the nucleus in response to LMB treatment.

Figure 4.8 Subcellular localisation of the carboxy-terminal SBP2 region. COS7 cells were transiently transfected with the SBP2 NES-GFP truncation construct and its subcellular localisation was investigated in untreated cells and in cells treated with LMB. The NES-GFP truncation construct was shown to relocate from the cytoplasm to the nucleus in response to LMB treatment.

The nuclear accumulation of the NES-GFP in response to LMB treatment suggested that this fragment shuttles between the nucleus and the cytoplasm due to the presence of an active NES. The amino acid analysis of SBP2 identified five candidate NES sequences within this region. Therefore, the next aim was to identify which, if any of these putative NES are functional.

In order to test their potential export activity, a previously described in vivo NES assay was employed (Henderson and Eleftheriou, 2000). In brief, this assay employs a construct containing a nuclear export deficient HIV-Rev protein fused to the GFP reporter protein (Rev1.4-GFP). This fusion protein localises within the nuclei of cells, due to a deficient NES. Functional NESs can thus be identified based on their ability to promote nuclear export of Rev1.4 protein when inserted between the Rev1.4 and GFP moieties. Rev1.4-GFP constructs containing the five candidate SBP2 NES sequences were generated and tested for export activity in transiently transfected COS7 cells. As the Rev1.4 protein contains a strong NLS that may not always be overcome by a weak NES, cells were treated with actinomycin D (Act.D) to block nuclear import. Cycloheximide was added in co-treated samples to inhibit de novo protein synthesis during the time course of the experiment to ensure that cytoplasmic fluorescence was caused by nuclear export and not by newly synthesised protein.
LMB was added to test the specificity of the NES for CRM1 nuclear export. Candidate NESs tested and their efficiency in the export assay are outlined in Table 4.1, and representative images from the experiment are shown in Figure 4.9.

**Table 4.1 Candidate NESs tested in the nuclear export assay.** Five putative NES sequences were identified within SBP2. The amino acids that putatively constitute the NESs are indicated in bold.

<table>
<thead>
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<th>Candidate NES</th>
<th>Export activity</th>
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<tr>
<td>NES 1* Q&lt;sup&gt;634&lt;/sup&gt;MLSKEVDACVT&lt;sup&gt;657&lt;/sup&gt;LI&lt;sup&gt;657&lt;/sup&gt;LVR&lt;sup&gt;657&lt;/sup&gt;FQD</td>
<td>+++</td>
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<tr>
<td>NES 2 L&lt;sup&gt;680&lt;/sup&gt;KKHK&lt;sup&gt;717&lt;/sup&gt;LKKLVIL&lt;sup&gt;717&lt;/sup&gt;</td>
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<td>NES 3 Q&lt;sup&gt;711&lt;/sup&gt;R&lt;sup&gt;731&lt;/sup&gt;ALGRS&lt;sup&gt;747&lt;/sup&gt;LNKA&lt;sup&gt;752&lt;/sup&gt;V&lt;sup&gt;752&lt;/sup&gt;FVSVGV&lt;sup&gt;752&lt;/sup&gt;GFSY</td>
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<td>NES 4 Q&lt;sup&gt;756&lt;/sup&gt;DQH&lt;sup&gt;795&lt;/sup&gt;KMVELT&lt;sup&gt;795&lt;/sup&gt;VAA</td>
<td>+</td>
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<tr>
<td>NES 5* Q&lt;sup&gt;756&lt;/sup&gt;DQHKMVELTVAAR&lt;sup&gt;770&lt;/sup&gt;</td>
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In line with the results from LMB treatment of both full length SBP2 and deletion constructs, two of the five SBP2 NES sequences tested were found to possess nuclear export activity. The sequence depicted as NES1, Q<sup>634</sup>MLSKEVDACVT<sup>657</sup>LVRFQD<sup>657</sup>, had the ability to partially shift the Rev1.4 protein to the cytoplasm in the absence of Act. D treatment (Figure 4.9, second row, left column), an effect that was greatly enhanced in treated cells (Figure 4.9 second row, middle column). The NES5 sequence, Q<sup>756</sup>DQHKMVELTVAAR<sup>770</sup>, was able to partially shift the Rev1.4 protein to the cytoplasm mainly after Act. D treatment (Figure 4.9, bottom row, middle column), which suggested that this sequence may represent a weaker, albeit functional NES. LMB treatment abrogated the cytoplasmic localisation of both NESs, confirming their CRM1 specificity (Figure 4.9 right column). Additional sequences tested that did not display NES activity are also presented in Figure 4.9. These results suggest that SBP2 may contain two functional CRM1-dependent NESs.
Figure 4.9 Mapping of functional SBP2 NESs. The activity of five candidate SBP2 NES sequences was tested using an in vivo export assay. NESs were subcloned in the export deficient Rev1.4-GFP vector and analysed for their capacity to shift the nuclear Rev1.4 protein to the cytoplasm. Act.D was used to block nuclear import in order to identify weak NESs. LMB was used to ensure CRM1 specificity for the identified NESs. NES1 and NES5 were identified as functional SBP2 NESs.
Interestingly, these NESs are located on both sides of the RNA binding domain, raising the possibility that their availability for CRM1 binding may be related to conformational changes possibly caused by interactions with target mRNA. To test this possibility, cells expressing SBP2-GFP were treated with Act. D to inhibit mRNA synthesis and SBP2-GFP localisation was monitored during 24 hrs. If SBP2 is exported from the nucleus in complex with mRNA, Act.D treatment would lead to its retention in the nucleus as no selenoprotein transcripts would be available. As shown in Figure 4.10, Act. D treatment for as long as 20 hours did not lead to nuclear retention of NES-GFP or SBP2-GFP (not shown), suggesting that RNA binding is not a prerequisite for nuclear export of over-expressed SBP2. I was unable to replicate the effect of Act. D treatment on localisation of endogenous SBP2 since the anti-SBP2 antibodies do not work for immunostaining. These results may therefore not reflect the situation for endogenous SBP2, and do therefore not exclude the possibility that the role of SBP2 in the nucleus is to bind to mRNA and facilitate its transport to the cytoplasm.

**Figure 4.10 Actinomycin D treatment of NES-GFP.** COS7 cells were transiently transfected with the NES-GFP deletion construct. Cells were subsequently treated with Act.D (0.2 µg/ml) and incubated for 24 hrs. Cells were fixed at 2, 4, 8 and 20 hrs after Act.D addition. Representative images show that Act.D did not alter the cytoplasmic localisation of the NES-GFP protein. Images were acquired at a 40x magnification.
4.3.6 The role of SBP2 subcellular localisation in controlling Sec incorporation

As shown in Figure 4.5, LMB treatment caused a significant decrease in the ribosomal pool of SBP2, simultaneously increasing the nuclear pool. To investigate the effect of SBP2 localisation on selenoprotein synthesis, HeLa cells were pre-treated with LMB for 16 hrs to deplete SBP2 from the ribosomes and Sec incorporation was monitored by $^{75}$Se labelling for 24 hrs in the presence of LMB. Incorporation of Sec was analysed by comparing the radioactive signals between lanes, relative to the gel loading by coomassie staining. Because the gel loading between the lanes was slightly different, densitometry was performed to assess the differences in signal intensity. The values were normalised against the gel loading. Interestingly, cells treated with LMB exhibited a ~2.3 fold reduction in $^{75}$Se radioactive labelling than untreated control cells (Figure 4.11 A), indicating that selenoprotein synthesis was compromised. It is tempting to speculate that the effect observed was caused by the decrease in the ribosomal SBP2 pool. However, because LMB affects trafficking of many other proteins that traffic via the CRM1 pathway including general translation factors such as eIF2β, eIF4A1, eIF2Bε, eIF5, eEF2 and eRF1 (Bohnsack et al., 2002), it was therefore planned to study the effect of LMB on global protein synthesis. For this purpose, the above mentioned experiment was repeated using $^{35}$S Methionine for labelling of HeLa cells. Virtually no difference in global protein synthesis was observed in LMB treated cells compared to untreated controls (Figure 4.11 B). Some proteins however, displayed an increased synthesis in response to the treatment. Collectively, these results suggested that the decrease in selenoprotein synthesis was specific and may have been caused by the translocation of SBP2 from the ribosomes to the nucleus. Nevertheless, the effect observed may not be solely attributed to the lack of ribosomal SBP2 as LMB may affect additional Sec incorporation factors, a possibility that remains to be investigated.
Figure 4.11 Effect of LMB treatment on selenoprotein synthesis. HeLa cells were treated with LMB 2ng/ml for 16 hrs. (A) Selenoprotein synthesis was investigated by $^{75}$Se labelling during a period of 24 hrs. (B) Global protein synthesis was investigated in parallel by $^{35}$S labelling. Autoradiographs of untreated and LMB treated cells show a 2.3 fold decrease in selenoprotein synthesis in response to LMB treatment (A), whereas global protein synthesis was virtually not affected or 1.2 fold up-regulated (B). Analysis of radioactive signal intensity and coomassie staining was performed by densitometry and values were normalised to the gel loading.
4.4 DISCUSSION

Based on the existing evidence two models for the role of SBP2 in Sec incorporation have been proposed in the literature. In one model SBP2 was depicted as an exclusively ribosomal protein (Kinzy et al., 2005) while in the second, although experimental evidence was lacking, SBP2 was proposed to bind SECIS elements in the nucleus and facilitate their transport to the ribosomes (Small-Howard and Berry, 2005). The work presented in this chapter has investigated the subcellular localisation of over-expressed and endogenous SBP2, characterised motifs that control its localisation to different subcellular compartments and addressed the role they play in SBP2 Sec incorporation function. In agreement with previous reports (Copeland et al., 2001), the data showed that SBP2 is predominantly localised within the ribosomal fraction of cells. In addition, novel evidence was provided that indicated that SBP2 subcellular localisation is not solely restricted to the ribosomes, rather, that SBP2 shuttles between the nucleus and the ribosomes. The ability of SBP2 to shuttle was shown to be facilitated by a conserved, centrally located NLS, and at least by one, if not two, carboxy-terminal NES motifs. Functional studies on the involvement of these motifs in SBP2 Sec incorporation function suggested that the NES motif/s may be required for SBP2 function, as inhibition of nuclear export by LMB treatment led to a decreased selenoprotein synthesis which correlated with an increased nuclear SBP2 pool. These results are in support of a requirement for SBP2 in the cytoplasm in order to maintain efficient selenoprotein synthesis; however the role of additional factors regulated by nuclear export cannot be excluded.

In one of the recently proposed models (Caban and Copeland, 2006) it was suggested that SBP2 remains stably bound to the ribosomes during several rounds of translation, except during the Sec delivery to the ribosomal A-site when SBP2 is released and the eEFSec/tRNA^Sec complex is loaded and incorporation of Sec takes places. The data provided in this chapter indicated that SBP2 may shuttle between the ribosomes and the nucleus however, the trigger that mediates its release from the ribosomes and/or nuclear localisation currently remains unknown. It is possible that its release from the ribosome during the process of Sec incorporation may induce the exposure of its NLS and trigger its nuclear translocation. A conformational change within the nucleus may lead to exposure of the dominant NES which in turn would trigger its rapid export to
the cytoplasm mediated by CRM1. Alternatively, nuclear localisation of SBP2 may be triggered by signalling events due to changes in the intracellular conditions.

A second question that remains open for investigations is the relevance of SBP2 nuclear translocation. mRNAs are generally exported from the nucleus as ribonucleoprotein complexes including RNA binding proteins and transport factors (Zenklusen and Stutz, 2001). Because translation of selenoproteins requires unique factors, the transport of their cognate mRNAs from the nucleus may involve interactions with some of these factors prior to their arrival at the ribosomal sites. The results presented herein have demonstrated that SBP2 contains NLS and NES motifs to perform this function, consequently its shuttling properties may be involved in transport of mRNA and be directly linked to its Sec incorporation function. Interestingly, the functional NESs identified are located in close proximity to the RNA binding domain and within the Sec incorporation functional domain, raising the possibility that their availability for CRM1 binding may be related to the conformational changes caused by the binding of SBP2 to SECIS elements. Nevertheless, inhibition of transcription using Act.D for up to 24 hours did not lead to nuclear retention of over-expressed SBP2-GFP, suggesting that RNA binding is not a prerequisite for its nuclear export. An alternative possibility is that SBP2 nuclear localisation may serve additional functions, possibly unrelated to Sec incorporation.

From the attempts to identify alternatively spliced SBP2 protein isoforms in cells, it can be concluded that at least one isoform, possibly the one destined for the mitochondria is expressed in 293T cells. On western blots, the 120 kDa full length SBP2 was often, but not always detected as a doublet and it is not clear whether the bands represent a post-translationally modified protein or yet another alternatively spliced variant. When over-expressed as a GFP fusion protein, the SBP2_∆2 isoform displayed a different pattern of localisation in cells with more frequent nuclear localisation compared to the full length protein. It is possible that the variable N-terminal regions may play a role in regulating SBP2 subcellular localisation, with some isoforms being more easily targeted to the nucleus than others.
Another significant and interesting finding presented in this chapter was the identification of an SBP2 isoform capable of localising to the mitochondria. This finding was supported by several lines of evidence. Firstly, the cDNA predicted to encode a mitochondrial targeting signal through use of an alternative reading frame within exon II, was indeed localised to the mitochondrial compartment when over-expressed as a GFP fusion. Secondly, a protein of similar molecular weight as predicted for the mtSBP2 following cleavage of the MTS was recognised by the anti-SBP2 antibodies on western blots of mitochondrial extracts which is in agreement with a similar size protein identified as SBP2 by mass-spectrometry analysis of bands immunoprecipitated with SBP2 antibodies, presented in Chapter 2. Thirdly, the results were further supported by immuno-staining with affinity purified anti-SBP2 antibodies and co-localisation with a mitochondrial marker. An important yet puzzling question arising from these findings is the significance of a mitochondrial targeted SBP2 isoform. As the only known function assigned to SBP2 to date is Sec incorporation, the most obvious reason for the presence of SBP2 in the mitochondria would be the existence of selenoproteins encoded by the mitochondrial genome that would require the presence of SBP2 for translation or transport of these mRNAs to the ribosomes. A bioinformatics based method was employed to test this hypothesis and the mitochondrial genome was screened for presence of SECIS elements using the SECISearch 2.0 program (Kryukov et al., 2003). This analysis did however not identify any strong SECIS elements within the mitochondrial genome, suggesting that the mitochondrial targeting of SBP2 may not directly involve SECIS binding. It is tempting to speculate that a mitochondrial SBP2 isoform may serve a function unrelated to Sec insertion, however, for the time being this remains elusive.

In summary, the results presented in this chapter have indicated that SBP2 shuttles between the nucleus and the ribosomes. This shuttling activity of SBP2 is mediated by intrinsic NLS and NES motifs. Its translocation to the nuclear compartment suggested that SBP2 may perform an additional function which may involve transport of SECIS containing mRNAs, however this remains speculative. SBP2 nuclear export was shown to be directed by dominant NES motifs and facilitated via the CRM1 export pathway. The NES was indicated to be required for Sec incorporation as depletion of SBP2 from the ribosomes and simultaneous nuclear accumulation resulted in decreased selenoprotein synthesis. The expression of alternatively spliced SBP2
isoforms in cells targeted to different subcellular compartments, also represent novel and essential findings. These findings will serve as a platform for further elaborate studies that will ultimately lead to a complete understanding of the regulation of SBP2 expression and function.
CHAPTER 5.

REDOX REGULATION OF SBP2
5.1 INTRODUCTION

Organisms growing in the presence of oxygen are continuously exposed to oxidative challenge caused by reactive oxygen species (ROS) generated either endogenously through oxygen metabolism, or exogenously from environmental stress. Changes in the intracellular redox state lead to modifications of reactive -SH groups within cysteine residues, which in turn affect protein function, interactions and subcellular localisation. Upon oxidation, cysteine thiols can be modified to form inter- or intra-molecular disulfide bonds, or become S-glutathionylated to form glutathione-mixed disulfides (R-SSG), as described in detail in Section 1.2.1. These modifications are believed to represent the two major intracellular mechanisms of reversible cysteine modifications, not only during oxidative stress conditions, but also during normal cell signalling (Fratelli et al., 2005; Shelton et al., 2005).

Some of the functionally characterised selenoproteins such as TR and GPx are enzymes that are directly involved in redox processes. TR, as part of the thioredoxin system, plays a central role in maintaining the intracellular redox homeostasis by reducing oxidized substrates; it modulates the function, activity and localisation of proteins and governs over cellular processes such as cell proliferation and apoptosis (Powis and Montfort, 2001). On the other hand, GPx functions as a direct antioxidant by disposing of, and preventing the accumulation of peroxides, thus protecting the cell against oxidative damage (Dringen et al., 2005). Consequently, these enzymes are regulated in response to oxidative stress by changes in activity, induction of their expression or changes in their subcellular localisation (Hattori et al., 2005; Hirota et al., 1997; Karimpour et al., 2002).

Since SBP2 is a central component of the selenoprotein synthesis machinery and thus controls the production of redox-active selenoproteins, it is likely to play an essential role in the oxidative stress response pathway. Therefore, I sought to investigate the regulation of SBP2 in response to oxidative stress, and the impact oxidative stress may have on its function in translation of selenoproteins. This was conducted using both in vivo assays and in vitro biochemical approaches. Moreover, the requirement of SBP2 for Sec incorporation has only been demonstrated using in vitro systems. Use of small interfering RNAs (siRNAs) is now a well established technology that is being
widely used to specifically silence target genes in mammalian cells in order to study their function (Elbashir et al., 2001). This technique was employed to deplete SBP2 in cells, and the role of SBP2 in Sec incorporation \textit{in vivo} was evaluated.
5.2 MATERIALS AND METHODS

5.2.1 Cell transfections and treatments

For fluorescent microscopy analysis COS7 cells were transfected with the indicated plasmids as described in Section 2.2.4.4. Wild type and cysteine mutant SBP2-GFP plasmids were transfected into 293T cells by electroporation as described in Section 2.2.4.3. Cell treatments were performed as described in Section 2.2.4.2, at the concentrations specified for each experiment. Cells on coverslips were processed as described in Section 2.2.4.5. Cell counting was performed as described in Section 2.2.4.6.

5.2.2 Determination of the SBP2 redox state in vivo

293T cells were plated onto 15 cm diameter dishes and grown for 24 hrs to reach ~80% confluence. The different oxidants were applied to cells as described in Section 2.2.4.2. At the end of treatment, Iodoacetamide (IAM) was added to dishes, at a final concentration of 30 mM for 20 min prior to harvest to irreversibly alkylate all free thiols \textit{in vivo}. Cells were then washed twice in PBS and lysed in UDP buffer (Section 2.2.4.7). Residual IAM was removed by passing lysates through a G25-desalting column (Amersham). Lysates were incubated with 3.5 mM DTT at room temperature for 30 min to reduce any \textit{in vivo} formed disulfide bonds and subsequently passed through a G25 column to remove DTT. Newly reduced thiols were alkylated with Biotin-conjugated IAM (BIAM), N-(biotinoyl)-N′-(iodoacetyl)ethylene diamine (Molecular Probes) at 37°C for 30 min. SBP2 was subsequently immunoprecipitated from lysates as described in Section 2.2.4.8, resolved by SDS-PAGE and transferred to PVDF membrane as described in Sections 2.2.5.2 and 2.2.5.3 respectively. Membranes were firstly probed with HRP-conjugated Streptavidin (Molecular Probes) to detect BIAM-alkylated protein, stripped as described in Section 2.2.5.4, and re-probed with anti-SBP2 antibodies for total SBP2.
5.2.3 $^{75}\text{Se}$ labelling in HeLa cells

HeLa cells were labelled with $^{75}\text{Se}$ as described in Section 2.2.4.9. Densitometry analysis of radioactive signals was performed using the MultiGauge v2.3 software.

5.2.4 Generation of construct for recombinant expression of carboxy-terminal SBP2

The carboxy-terminal region of SBP2 (bp 1752-2562, aa 584-854) was amplified from the full length SBP2-GFP plasmid using primers NESpET24aFWD 5′GGGAATTCCATATGGACGAACTGATCTCCACTC and pET24aREV 5′ GGCCT CGAGTAAATCTAAAATTCATTTGAGAGG engineered to contain the XhoI and Ndel restriction enzyme sites (shown in italics). PfuTurbo proofreading polymerase (Invitrogen) was used in PCR reactions, as described in Section 2.2.3.1. The fragment was ligated into the XhoI and Ndel digested, His(6)-containing pET24a vector (Novagen) as described in Section 2.2.3.6 and 2.2.3.8 to create the construct named NES-His(6). The integrity of construct was confirmed by sequencing (2.2.3.10).

5.2.5 Expression on NES-His(6) in E.Coli

The NES-His(6) construct was transformed into E. coli BL21(DE3)-pRIL strain (Clonetech) and a stationary overnight-culture (in LBKan/Cm) was used to inoculate (1:100 dilution) 20 L of 2-YT-medium containing 50 mg/L Kanamycin and 34 mg/l Chloramphenicol in a fermenter (Microferm enter, New Brunswick). The cells were grown at 37°C to an OD$_{600}$=1.0, then induced with 1mM IPTG and the temperature was lowered to 30°C. Protein expression was carried out for 3 hrs. The bacterial cell pellet (~50 g) was cracked at -70°C using a French press (Hydraulic press HP-20) and stored at -80°C overnight.

5.2.6 Purification of recombinant NES-His(6)

The pellet thawed and resuspended in ~250ml lysis buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole, pH 7.5) including 1mM PMSF and 1x protease
inhibitor cocktail (Roche). The lysate was sonicated at 70% output on ice until appropriate viscosity was reached in the presence of DNase (10 µg/ml) to obtain efficient lysis. The lysate was cleared by centrifugation for 30 min, 4°C, 9000 rpm (SS34 rotor, Sorvall). 12 ml of Ni-NTA slurry, pre-equilibrated in lysis buffer was added to the cleared lysate and the sample was incubated at 4°C for 1 hr on a shaker to bind the His-tagged protein. Subsequently the slurry was transferred to a column, washed with 200 ml wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 7.5) and NES-His(6) was eluted with a linear gradient of imidazole using a salt bridge (from 20 mM to 250 mM imidazole within 100 ml) at a flow rate of ~1.25 ml/min using a peristaltic pump (P-3, Amersham). 1 ml fractions were collected and assessed for their purity. For further purification of Ni-NTA purified NES-His(6), the fraction containing the NES-His(6) proteins were pooled, concentrated to 1.5 ml and applied to a column (height: 1m, total volume: 250 ml) containing Sephadex G-75 (Amersham Biosciences). NES-His(6) was separated from contaminating protein at a flow rate of 0.2 ml/min using a peristaltic pump P-50 (Amersham Pharmacia). As running buffer 50 mM NaH₂PO₄, 300 mM NaCl, pH 7.5 was used. Fractions were collected using the LKB-SuperFrac-System (Amersham Pharmacia).

5.2.7 Determination of free thiols content and reduction of oxidised NES-His(6) by Trx in vitro

The number of free thiols in the oxidised and reduced NES-His(6) proteins were determined by DTNB assay. Thiols were determined with dithionitrobenzoate (DTNB) in a total reaction volume of 200 µl containing 1 µM of NES-His(6) with a control containing only buffer. Enzyme was reduced by incubation with 1mM DTT in a total volume of 45 µl at room temperature for 5 min, after which the enzyme was denatured by adding 150 µl of 8 M guanidine hydrochloride (Gdn·HCl) in 50 mM Hepes and 2 mM EDTA, pH 7.6. The thiol content was determined after addition of 1.58 mM DTNB by absorbance at 412 nm recorded in an Ultrospec 3000 UV/Visible spectrophotometer (Amersham Pharmacia). Measurements were made anaerobically with 1-cm cuvettes covered with rubber septa. The absorbance at 412 nm of each sample was recorded against the relevant control solution in an Ultrospec 3000 UV/Visible spectrophotometer (Amersham Pharmacia). The thiol content was
calculated by using an extinction coefficient of thionitrobenzoate (TNB) at 412 nm [14.15 mM\(^{-1}\)·cm\(^{-1}\) in buffer and 13.7 mM\(^{-1}\)·cm\(^{-1}\) in 6 M Gdn-HCl].

Kinetics of reduction of oxidized NES-His(6) by Trx were measured in a master mix containing 200 µM NADPH, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.1 mg/ml BSA, 50 nM TR, NES-His(6) and Trx at concentrations indicated. The absorbance was followed at 340 nm for 5 minutes in an Ultrospec 3000 UV/Visible spectrophotometer (Amersham Pharmacia).

5.2.8 Preparation of glutathionylated NES-His(6)

78 µM NES-His(6) was reduced with 10 mM DTT at 37°C for 30 min, then DTT was removed by passing through a Sephadex G-25 (Amersham Pharmacia) desalting column. The reduced protein was incubated with 100 mM GSSG at 37°C for 2 hours, and passed through Sephadex G-25 column to remove unbound GSSG.

5.2.9 In vitro reduction of NES-His(6) by human Grx 1 and Grx 2

Recombinant human Grx 1 and Grx 2 was kindly provided by Dr. Catrine Johansson (Department of Biochemistry, Karolinska Institute, Sweden). Recombinant TR was kindly provided by Mr. Olle Rengby (Department of Biochemistry, Karolinska Institute, Sweden). Reduction of NES-His(6)-S-SG by human Grx 2, independent of GSH, was analysed in a mixture containing 200 µM NADPH, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.1 mg/ml BSA, 50 nM TR and 5 µM of Grx2. 10 µM NES-His(6)-S-SG or NES-His(6) were added to both reference and sample cuvettes and the activity was determined from the decrease in absorbance at 340 nm during a period of 5 minutes in an Ultrospec 3000 UV/Visible spectrophotometer (Amersham Pharmacia). For comparison of Grx2 efficiency, GSSG was used in parallel reactions at 10 µM and 20 µM. For kinetics using Grx1 the following master mix was used: 200 µM NADPH, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM GSSG, 0.1 mg/ml BSA, 6 µg/ml GR and 10 µM NES-His(6) or NES-His(6)-S-SG. The absorbance was followed at 340 nm in an Ultrospec 3000 UV/Visible spectrophotometer (Amersham Pharmacia) for 5 minutes. Master mix without Grx 1 or Grx 2 were used as reference.
5.2.11 Fluorescence emission spectra

DTT-reduced Trx (0.1 μM) in nitrogen-equilibrated TE buffer was mixed with oxidized NES-His(6) (0.1 μM). A thermostatic SPEX-FluoroMax spectrofluorometer was employed to measure the fluorescence as previously described (Zhong et al., 2000). Excitation of fluorescence was at 290 nm and emission spectra from 300 to 500 nm were recorded. Emission at 340 nm was followed to record the rate of oxidation of Trx-(SH)2 by NES-His(6). The fluorescence spectra of oxidized Trx were obtained by subtracting the value of oxidized NES-His(6) alone from the value of the mixture of reduced Trx with oxidized NES-His(6).

5.2.12 Mass spectrometry of glutathionylated NES-His(6)

Glutathionylated NES-His(6) was prepared as described above and denatured in 6 M Guanidinium hydrochloride at 37°C. Denatured protein was diluted 10 times with 10 mM ammonium bicarbonate, pH 7.6 and cleaved by Endoproteinase Glu-C (Roche) for 2 hours. Proteins were analysed at the Proteomics Analysis Centre, Karolinska Institute, Stockholm, Sweden by MALDI-TOF mass spectrometry using a Voyager 6083 System (Applied Biosystems).

5.2.13 Generation of cysteine-mutant SBP2-GFP constructs

Cysteine residues C633, C644, C691, C698 and C719 were mutated to serines in the SBP2-GFP construct by side directed mutagenesis as described in Section 2.2.3.11. Mutations were confirmed by sequencing (2.2.3.10). The primers used are listed in Table 5.1 the nucleotides mutated are underlined in the forward primer.
TABLE 5.1 PRIMERS USED TO ENGINEER CYSTEINE MUTATIONS INTO THE SBP2-GFP CONSTRUCT. The primers used to engineer mutations into SBP2 cysteine residues C633, C644, C691, C698 and C719 are listed and the nucleotide mutated is underlined. All cysteines were mutated to serines.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence 5′→3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDMC633SFWD</td>
<td>GATTCAGGGATTACTCAGCCAGATGCTTTAG</td>
</tr>
<tr>
<td>SDMC633SREV</td>
<td>CTAGACATCTGGCTGGAGTAATCCCTGAATC</td>
</tr>
<tr>
<td>SDMC644SFWD</td>
<td>GAAGTGGATGCTTCTGTACACCACTACTC</td>
</tr>
<tr>
<td>SDMC644SREV</td>
<td>GAGTAGGTCGGTAACAGAACAGATCCACTTC</td>
</tr>
<tr>
<td>SDMC691SFWD</td>
<td>GCTCAAATAACTGAAATCTGTCATTATTTCTCCC</td>
</tr>
<tr>
<td>SDMC691SREV</td>
<td>GGGAGAAATAATGACAGATTTTCTTTTGAGC</td>
</tr>
<tr>
<td>SDMC698SFWD</td>
<td>CATTATTTCTCCCAACTCTGAGAAGATACAGTC</td>
</tr>
<tr>
<td>SDMC698SREV</td>
<td>GACGTGATCTTTCTACGATTTGGGAGAAATAATG</td>
</tr>
<tr>
<td>SDMC719SFWD</td>
<td>CAATTATGTTATATGCTTCTGAGCAGAACCATTCC</td>
</tr>
<tr>
<td>SDMC719SREV</td>
<td>GGAATGTTCTGCTCAGAGGCTATAATCAATAATAG</td>
</tr>
</tbody>
</table>

5.2.14 siRNA mediated depletion of SBP2

The following 21 nt sequence: AAAATCAGCTAGGCTTCACA (complementary to nt 516-537 within SBP2 cDNA, see also Appendix 1) was used to deplete SBP2 expression by siRNA. The sequence was expressed in cells using the vector system pSUPER (a gift of Dr. Reuven Agami, the Netherlands Cancer Institute, Amsterdam), which directs the synthesis of the siRNAs in mammalian cells (Brummelkamp et al., 2002). A 21 nt sequence complementary to GFP (not present in cells) was used as control. Plasmids containing the siRNA sequences were transfected into cells by electroporation as described in Section 2.2.4.3 and incubated for 72 hrs to achieve maximal depletion of SBP2 protein.

5.2.15 Cell viability and proliferation.

Cell viability was analysed using the XTT based Cell Proliferation Kit II (Roche). HeLa cells were transfected with siSBP2-pSuper or siGFP-pSuper plasmids and 48 hrs after transfections cells were detached and plated at a density of $4 \times 10^4$ cells/well.
in 96 micro-well plates, and allowed to grow in the normal growth medium for 24 hrs as described in Section 2.2.4.1. H$_2$O$_2$ and sodium selenite treatments were prepared as described in Section 2.2.4.2 and applied to cells in 96 micro-well plates in a serial dilution and incubation was conducted for another 20 hrs. The XTT reagent (50 µl/well) was added, and plates were read at 475 nm in a Benchmark Plus microplate reader at 3, 6 and 20 hrs of XTT incubation. Data points were plotted using the Microsoft Excel program and analysed for statistical significance using a One-Way ANOVA test.
5.3 RESULTS

5.3.1 SBP2 accumulates in the nucleus in response to oxidative stress

The regulation of SBP2 in response to oxidative stress was first investigated at the level of protein expression. Oxidative stress was induced by treating 293T cells with H$_2$O$_2$ for 24 hrs. Cells were harvested at several time points during this period and SBP2 protein levels were analysed by western blotting. The data showed that SBP2 protein levels remained unchanged at all time points investigated during the course of treatment (data not shown). Similar results were obtained using sodium selenite as an oxidative stress causing agent (data not shown). These results suggested that SBP2 may not be regulated at the level of translation in response to oxidative stress.

Data presented in Chapter 4 demonstrated that SBP2 subcellular localisation is dynamic and not restricted to ribosomal sites as previously thought, and is regulated at the level of nuclear export. To study signalling events regulating SBP2 in cultured cells, the effects of different stress inducing agents on subcellular localisation of SBP2 was investigated. To induce oxidative stress, COS7 cells expressing full length SBP2-GFP were treated with H$_2$O$_2$ at concentrations ranging from 10µM to 1 mM and type-A UV (UVA) irradiation doses of 5, 10 and 20 J/m$^2$ and the localisation of SBP2 was monitored. Interestingly, both H$_2$O$_2$ and UVA treatments induced a relocation of SBP2-GFP from the cytoplasm to the nucleus, and the effect was observed at H$_2$O$_2$ concentrations of 100 µM or higher, and at all UVA doses used. Figure 5.1 A, shows examples of SBP2-GFP nuclear localisation in response to H$_2$O$_2$ (1 mM) treatment and UVA irradiation (10 J/m$^2$). A detailed quantitation of the cells expressing SBP2-GFP (Figure 5.1 B) showed that oxidative stress treatment significantly increased the proportion of cells with nuclear and nuclear/cytoplasmic SBP2-GFP and decreased the proportion of cells with cytoplasmic SBP2-GFP. The results presented represent the mean of cell counts from three independent experiments.
Figure 5.1 Nuclear accumulation of SBP2-GFP in response to oxidative stress. (A) COS7 cells were transiently transfected with SBP2-GFP and the subcellular localisation of the expressed protein was investigated in untreated cells or cells treated with H$_2$O$_2$ (1 mM) or exposed to UVA irradiation (10 J/cm$^2$). Nuclei were stained with DAPI. Images were acquired with a 60x magnification lens. (B) Graphical representation of percentage of cells displaying predominantly nuclear (N), nuclear/cytoplasmic (N/C) or cytoplasmic (C) SBP2-GFP fluorescence, as mean of three independent experiments. (C) Graphical representation of changes in SBP2 subcellular localisation in response to H$_2$O$_2$ (100 µM) treatment over a 24 hour period. (D) Osmotic stress induced by treatment with sorbitol (1M) did not affect the subcellular localisation of SBP2-GFP in COS7 cells. Arrows point to the cells expressing SBP2-GFP stained with DAPI. Images were acquired using a 40x magnification lens.

To determine if the nuclear accumulation of SBP2 occurred in a reversible manner, cells were treated with 100 µM H$_2$O$_2$ for 2 hrs, a dose that was sufficient to trigger nuclear accumulation of SBP2-GFP but did not induce cell death as determined by the presence of intact nuclei in more than 90% cells using DAPI staining, and SBP2-GFP localisation was monitored over a 24 hr period after release from H$_2$O$_2$. Cells were counted and scored for nuclear, nuclear/cytoplasmic or cytoplasmic fluorescence at several time points following treatment. Nuclear localisation of SBP2-GFP appeared to be maximal at 4 hrs after treatment, and at later time points (12 and 24hrs) cells with mainly cytoplasmic fluorescence were observed (Figure 5.1 C), suggesting that
nuclear accumulation of SBP2-GFP in response to oxidative stress occurred in a reversible manner.

To verify whether nuclear accumulation of SBP2-GFP was a global response to stress, COS7 cells expressing SBP2-GFP were treated with sorbitol, to provoke osmotic stress (Fleming et al., 2000) and SBP2-GFP localisation was observed. Osmotic stress applied for up to four hours did not lead to nuclear accumulation of SBP2-GFP (Figure 5.1 D), suggesting that nuclear accumulation of SBP2 is specifically triggered after oxidative stress.

These were interesting and exciting results that suggested that SBP2 may be subject to redox regulation by translocation to different subcellular compartments. It was next postulated that nuclear accumulation of SBP2-GFP could be triggered in at least two different ways: by phosphorylation via the stress induced MAPK pathway, or by direct oxidation of critical cysteine residues. To test the former model, SBP2-GFP transfected COS7 cells were treated with either the stress activated protein kinase (SAPK/p38) specific inhibitor SB203580 (Badger et al., 1996), or with the Janus kinase (JNK) specific inhibitor SP600125 (Bennett et al., 2001) prior to H$_2$O$_2$ treatment. If phosphorylation by these kinases was the trigger for translocation of SBP2 to the nucleus, inhibition of their activity should prevent its nuclear translocation in response to oxidative stress. As shown in Figure 5.2 A and B, neither inhibitors prevented nuclear accumulation of SBP2-GFP. Therefore, phosphorylation by p38 or the JNK protein kinases, does not seem to be the trigger of SBP2-GFP nuclear accumulation in response to H$_2$O$_2$ treatment. Moreover, oxidative stress treatments did not induce SBP2 or SBP2-GFP electrophoresis mobility shift (data not shown), which often is observed when proteins undergo phosphorylation, further suggesting that phosphorylation events are not the likely cause of nuclear accumulation of SBP2-GFP.
Figure 5.2 JNK- and p38 kinase-independent nuclear accumulation of SBP2-GFP. COS7 cells were transfected with SBP2-GFP and treated with either the JNK specific inhibitor SP600125 (A), or the p38 kinase specific inhibitor SB203580 (B) prior to incubation with H$_2$O$_2$ (100 µM). Representative images show that SBP2-GFP accumulates in the nucleus in spite of inhibition of the JNK and p38 kinase pathways. Arrows point to the cells expressing SBP2-GFP stained with DAPI. Images were acquired using a 40x magnification lens.

5.3.2 In vivo oxidation of endogenous SBP2

To investigate the effect of oxidative stress on endogenous SBP2 protein and to address the hypothesis that its nuclear accumulation may be caused by direct cysteine oxidation, the redox state of SBP2 was investigated in untreated cells and in cells exposed to oxidative stress. As described in Section 1.2, during normal conditions the reducing intracellular environment maintains cysteine residues in a reduced state.
However, during oxidative stress imposition, redox sensitive cysteine residues become oxidised to various states, delineated in Section 1.2.1. Detection of such changes in vivo can be analysed using the two-step alkylation assay. This assay was employed to detect a potentially oxidised form of SBP2. 293 T cells were treated with several oxidising agents. These included H₂O₂, a relatively mild oxidant produced physiologically and implicated in both oxidative stress and cell signalling; diamide, a thiol cross-linking agent, and sodium selenite (Na₂SeO₃). At high concentrations selenite causes oxidative stress by reacting with protein cysteine and selenocysteine residues leading to increased production of ROS and depletion of the protective form of reduced GSH with a concurrent increase in oxidised form of GSSG (Shen et al., 1999). Following treatments and prior to harvest, cells were incubated with Iodoacetamide (IAM) to irreversibly bind all cysteines present in their thiol form. Subsequently, protein lysates were reduced with DTT and incubated with biotin-conjugated IAM (BIAM) in order to detect newly reduced cysteines, which was indicative of their presence in an oxidised state in vivo. SBP2 was immunoprecipitated from treated and untreated cell lysates and its redox state was analysed by western blotting using streptavidin, which only recognises BIAM alkylated SBP2. As expected and shown in Figure 5.3 A lane 1 in unstressed cells, the entire SBP2 pool was present in a reduced state, as indicated by the absence of any BIAM-conjugated SBP2. Interestingly, all oxidants used induced oxidation of SBP2 in vivo as determined by the presence of BIAM-conjugated SBP2 in protein samples obtained from treated cells (Figure 5.3 A, lanes 2, 3, and 4). The treatments caused varying levels of oxidation of SBP2, the greatest effect being observed in selenite treated samples. This may reflect the strong oxidising properties of selenite manifested through the depletion of both the buffering GSH pool and the antioxidant enzymes (Shen et al., 1999). Importantly, in vivo oxidation of SBP2 occurred at similar time points after stress treatment as its nuclear accumulation, further supporting the hypothesis of redox regulated nuclear accumulation of SBP2.

Another method commonly used to analyse redox modifications within proteins, is SDS-PAGE under non-reducing conditions combined with western blotting which reveals oxidation-induced mobility changes due to charge differences within redox sensitive proteins. This method was employed to analyse any potential oxidation induced mobility changes within SBP2 following treatment of 293 T cells with
selenite. Non-reducing SDS-PAGE revealed the formation of oxidation-induced mobility alterations of SBP2, noticeable by the appearance of an additional band in samples treated with selenite at a concentration of 100 µM or higher (Figure 5.3 B, top panel). A cross-reactive band detected on the same blot did not display any similar changes, suggesting that the modifications are specific to SBP2. Importantly, this shift was eliminated by reducing conditions (Figure 5.3 B, bottom panel) further suggesting that the mobility change was caused by direct oxidation of the protein. The poor detection of SBP2 in samples treated with the highest concentration of selenite treatment (Figure 5.3 B, top panels last lane) may be a result of masking of antibody epitope in a highly oxidised protein. The other possibility is that high concentration of selenite causes degradation of SBP2 since total SBP2 is reduced but not cross-reactive protein at this concentration (Figure 5.3 B, bands in 1000 µM lanes, non-reducing and reducing). Collectively, these results suggested that direct redox modification indeed occur within SBP2 in vivo.

**Figure 5.3** SBP2 undergoes cysteine oxidation in response to oxidative stress in vivo. (A) 293T cells were treated with 1mM of H$_2$O$_2$, diamide or sodium selenite for 2 hours and redox status of SBP2 was analysed by a two step alkylation method. Biotinylated IAM (BIAM) was used as the second alkylation agent. SBP2 was immunoprecipitated and BIAM-conjugated SBP2, indicative of oxidised protein in vivo was detected using HRP conjugated streptavidin (top panel). The membrane was re-probed with anti-SBP2 antibodies to detect total SBP2 (bottom panel). (B) 293 T cells were treated with various concentrations of sodium selenite and SBP2 was analysed by non-reducing and reducing SDS-PAGE. Selenite treatment caused altered mobility of SBP2, which can only be detected during electrophoresis on non-reducing SDS-PAGE gels.
5.3.2 The carboxy-terminal region of SBP2 mediates its nuclear retention in response to oxidative stress

In order to delineate the mechanisms involved in redox regulation of SBP2, an in silico approach was initially undertaken. SBP2 protein sequence was subjected to analysis by the cys-redox server available at http://manaslu.aecom.yu.edu/cysredox.html, which predicts redox sensitive cysteine residues within proteins (Fiser and Simon, 2000). Four cysteines, C$_{633}$, C$_{644}$, C$_{691}$ and C$_{698}$ with putative redox properties were predicted using this method and they were all located within the carboxy-terminal region of SBP2. Further analysis of SBP2 amino acid sequence revealed that SBP2 contains 12 cysteines in total, and that seven of these, including the ones predicted in silico, are present in a cluster within the carboxy-terminal region. Interestingly, this cluster overlaps with the RNA binding domain and the identified NESs, and may therefore constitute a redox-sensitive cysteine rich domain (named CRD). This is reminiscent of the CRD identified within the Yap-1 transcription factor which was shown to be directly involved in regulating Yap-1 transactivation function in response to oxidative stress (Kuge et al., 1997). The location of the CRD and other cysteine residues within the SBP2 amino acid sequence is schematically presented in Figure 5.4. Potentially, the redox state of this domain may play a regulatory role in SBP2 localisation and function, and was investigated further.
Figure 5.4 Schematic representation of SBP2 cysteine rich domain (CRD). Cysteine residues and their distribution within SBP2 are schematically represented; cysteines within the CRD domain are shown in bold (top). The amino acid sequence of carboxy-terminal SBP2 region is presented (bottom). The RNA binding domain is indicated in blue italics, the two NESs identified in this study are underlined and cysteines within the predicted CRD are shown in red.

To explore the role of this domain in regulation of SBP2, its contribution to the nuclear sequestration of SBP2 in response to oxidative stress was investigated. This was conducted using the NES-GFP construct (see Chapter 4, Figure 4.6) which encompasses the CRD domain as well as the RNA binding domain and the identified NESs. COS7 cells, transiently transfected with the NES-GFP construct were treated with various concentrations of H$_2$O$_2$ and the distribution of NES-GFP was investigated at different time points after treatment. As shown in Figure 5.5 A, treatment with H$_2$O$_2$ (1 mM) caused nuclear accumulation of the otherwise cytoplasmic NES-GFP protein. Similar changes were induced by lower H$_2$O$_2$ concentrations and, as observed for full length SBP2-GFP, the nuclear accumulation of NES-GFP was reversible. The graphs in Figure 5.5 B represent cell counts from two independent experiments where cells expressing NES-GFP were scored for nuclear, cytoplasmic or equal nuclear/cytoplasmic fluorescence. Sodium selenite was
also used in parallel experiments and was also able to induce the nuclear accumulation of NES-GFP, however, the effect was most prominent at a higher concentration than observed with H₂O₂ (Figure 5.5 C). The reason for this may be that the nuclear accumulation of NES-GFP was an indirect effect, caused by elevated H₂O₂ levels due to inactivation of antioxidant systems by selenite. The selenite concentration that induced nuclear accumulation of NES-GFP correlated with the concentration shown to cause oxidation of endogenous SBP2 \textit{in vivo} (see Figure 5.3 A and B).

Collectively, these results suggested that the CRD may indeed contain redox sensitive cysteine residues that are involved in regulating the subcellular localisation of SBP2. The location of cysteine 644 (C₆₄⁴) within the ‘stronger’ of the two NESs (see Figure 5.4) identified in Section 4.3.5, and the presence of the CRD in close proximity to both NESs suggests that this domain may be mediating nuclear retention of SBP2 during oxidising conditions, by masking the NES via disulfide bond formation and inhibiting export by CRM1. If this model was correct it would imply that SBP2 requires free, reduced cysteines in order to undergo nuclear export.
Figure 5.5 NES-GFP accumulates in the nucleus in response to oxidative stress. COS7 cells expressing the NES-GFP were treated with H$_2$O$_2$ (200 µM or 1 mM) or sodium selenite for 2 hrs and the subcellular distribution of the NES-GFP protein was analysed. (A) Representative images show that the protein accumulated in the nucleus in response to H$_2$O$_2$ treatment (1 mM). (B) The subcellular localisation of the NES-GFP protein was monitored at several time points after stress imposition, and cells were counted and scored for nuclear (N), cytoplasmic (C) or equal nuclear/cytoplasmic (N/C) fluorescence. Minimum 200 cells were counted for each sample. Graphs show the distribution of the protein in response to treatment of cells with H$_2$O$_2$ at different time points during 24 hrs. (C) Subcellular distribution of NES-GFP was analysed after treatment of cells with various concentrations of sodium selenite for 2 hrs.
To test this hypothesis, cells expressing NES-GFP were treated *in vivo* with the irreversible cysteine alkylation IAM and localisation of the protein was monitored at various time points. Treatments including H$_2$O$_2$, LMB and sorbitol were performed in parallel experiments to compare the kinetics of nuclear accumulation of the NES-GFP protein. As shown in Figure 5.6 A, a 2 hr treatment with IAM efficiently induced nuclear accumulation of NES-GFP, confirming that nuclear export requires the presence of free cysteine residues. As previously shown for full length SBP2-GFP, osmotic stress did not induce any changes in NES-GFP subcellular localisation. Among the compounds tested, LMB was the most efficient in triggering the nuclear accumulation of NES-GFP as a 2 hr treatment led to a complete effect (Figure 5.6 B). This indicated that the NES-GFP protein is in continuous traffic between the nucleus and the cytoplasm, and becomes trapped in the nucleus upon inhibition of CRM1. IAM was also an efficient nuclear trigger of NES-GFP however, complete nuclear localisation was observed only after 4 hrs of treatment. The results obtained are in agreement with the proposed hypothesis that free cysteines are required to facilitate CRM1 dependent nuclear export of SBP2. H$_2$O$_2$ treatment did not yield complete nuclear accumulation of NES-GFP, or SBP2-GFP (Figure 5.1 B), such as that induced by LMB treatment. The stress treatments are likely to affect cells to varying degrees, dependent not only on their stage during the cell cycle, but also on the amount of antioxidant systems present at the time of treatment, which can show individual variations among an unsynchronised cell population. As the oxidation events occurring within the NES-GFP protein are reversible and given that the cells have a residual antioxidant protection upon stress induction, a pool of the oxidised protein would be immediately reduced in the nucleus and exported to its cytoplasmic location. Collectively, these results suggest that cysteine residues within the CRD are directly involved in regulation of SBP2 nuclear export and are required in a reduced state in order to undergo nuclear export.
Figure 5.6 CRD cysteine residues in their reduced state are required for nuclear export of NES-GFP. COS 7 cells expressing the NES-GFP protein were treated with: H₂O₂ (200 µM), IAM (1 mM), LMB (2 ng/ml) or with sorbitol (1 M) to induce osmotic stress. (A) Representative images of the subcellular localisation of the protein in response to treatments as indicated. Arrows indicate cells expressing NES-GFP stained with DAPI (blue) (B) Graphical representation of cells scored in two independent experiments showing the distribution of NES-GFP in response to the various treatments. Minimum 200 cells were counted for each treatment.

5.3.3 Redox properties of recombinant carboxy-terminal SBP2 region

The C-terminal region of SBP2 appears to contain additional regulatory features to the previously identified RNA binding domain and functional domain (Copeland et al., 2000; Copeland et al., 2001) (see Figure 1.5). The two novel motifs identified in this study, the NES and the CRD were shown to regulate the subcellular localisation of SBP2, and as implicated in Chapter 4, Figure 4.6 the NES appears to play a role in
SBP2 Sec incorporation function. Redox dependent modifications of cysteines within the CRD were shown to affect the localisation of over-expressed SBP2-GFP and NES-GFP protein, possibly by masking the availability of the NES for CRM1 binding, which represents a novel mechanism of SBP2 regulation. Characterising the nature of these cysteine modifications should provide clues to how SBP2 is regulated in vivo.

To assess direct redox-dependent modifications of SBP2, a biochemical approach was undertaken. The C-terminal region of SBP2 represented by the NES-GFP construct used in the studies above, was expressed and purified recombinantly as a 37 kDa (6)His-tagged fusion (named NES-His(6)). Sufficient yields of pure protein were obtained (Figure 5.7) to conduct a detailed biochemical characterisation.

Non-reducing SDS-PAGE and thiol-specific alkylation with the fluorescent probe 5-Iodoacetofluorescein (5-IAF) revealed the presence of NES-His(6) oxidised as monomer, characterised by a faster migration than the reduced monomer (Figure 5.8 A, left panel). Likewise, a dimer was determined by migration of the protein at double the molecular weight, and oligomers representing highly oxidised aggregated protein (Figure 5.8 A, left panel). The absence of fluorescent signal in the absence of reductant confirmed the existence of the NES-His(6) protein in an oxidised state (Figure 5.8 A, right panel lane 1′). Reduction with DTT or the physiological reductant
GSH resulted in complete alkylation of the protein as indicated by the strong fluorescent labelling and the electrophoretic mobility shift (Figure 5.8 A, right panel).

The redox state of NES-His(6) was quantitatively determined in vitro using the DTNB assay (Holmgren, 1984) (Figure 5.8 B). Without prior reduction, one cysteine in its reduced, thiol state was detected within the NES-His(6), suggesting that the other cysteines were present in oxidised states such as disulfide linkages or sulphenic acids. Upon reduction with DTT, followed by immediate processing in aerobic solutions, five free thiols were detected. Incubation of the protein in aerobic solutions for as short as 1-2 min, led to rapid re-oxidation to the fully oxidised form. Detection of completely reduced protein with all seven cysteines present in their thiol form was only achieved when preparations were conducted under anaerobic conditions. These results suggested that the NES-His(6) protein is an extremely redox sensitive protein.

Dimerisation of NES-His(6) was further confirmed by electrospray ionization mass spectrometry ESI MS/MS (Figure 5.8 C) which demonstrated that in the presence of DTT, most of the protein was detected in the monomeric form (31693 Da). However, in the absence of reductant the dimeric form (63385 Da) prevailed. Dimerisation of SBP2 is currently an issue of controversy. In early studies using recombinant proteins dimerisation of SBP2 was detected and it was proposed that functional SBP2 may bind RNA in both monomeric and homodimeric forms (Lescure et al., 2002) (Allmang et al., 2002). More recent studies indicated that dimerisation of SBP2 was an artefact caused by the tag used to purify the protein (Kinzy et al., 2005). The data presented here, clearly demonstrates that the carboxy-terminal region of SBP2 does form dimers in the absence of reductant, and implies that SBP2 has the potential to dimerise during conditions of oxidative stress. Whether this occurs in vivo, and whether it has any implications on SBP2 Sec incorporation function remains to be investigated.
Figure 5.8 Redox sensitivity of the SBP2 carboxy-terminal region. (A) The redox state of recombinant NES-His(6) was analysed by alkylation of non-reduced, and DTT- or GSH- reduced proteins with the fluorescent thiol alkylationator 5-IAF, and subsequent non-reducing SDS-PAGE. Total protein was visualised by coomassie staining (left panel) and alkylated proteins by UV light (right panel). The different redox forms of the NES-His(6) protein are indicated. 5-IAF fluorescence was only observed when proteins were reduced prior to alkylation (lanes 2' and 3' compared to lane 1'). (B) Quantitative analysis of free thiols in reducing and oxidising conditions using the DTNB assay. (C) Electrospray Ionization mass spectrometry showing the peak representing the monomer NES-His(6) in the presence of reductant, and the peak representing the dimer in the absence of reductant.

5.3.4 Oxidised NES-His(6) is a substrate of the thioredoxin system

The data presented above demonstrated that SBP2 contains redox sensitive cysteines within the CRD that upon oxidation, form intra- and inter-molecular disulfide bonds.
Experiments using cell systems presented in Section 5.3.2 demonstrated that SBP2 undergoes oxidation in response to increased levels of ROS *in vivo*. Within the cell, disulfide bonds are reversed predominantly by the thioredoxin system, described in Section 1.2.2.1. Therefore, substrate specificity of oxidised NES-His(6) for the thioredoxin system was investigated *in vitro*. Using the NADPH oxidation assay (Holmgren, 1984), it was determined that oxidised NES-His(6) is not a direct substrate of TR (data not shown). Interestingly, oxidised NES-His(6) at various concentrations was efficiently reduced by recombinantly purified human Trx, as shown in a comparative assay with insulin, one of the best Trx substrates described to date. The results of three independent kinetic measurement experiments are graphically represented in Figure 5.9 A.
Figure 5.9 Redox sensitive cysteine residues within carboxy-terminal SBP2 act as a substrate of the thioredoxin system. (A) Graphical representation of a comparative Trx activity assay using insulin, NES-His(6) or GSSG as substrates. The graphs represent the average absorbance change at 340 nm, in three independent experiments, during the initial 1 min of reduction of oxidised substrates by Trx. Reactions were measured in a master mix containing 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.1 mg/ml BSA, 50 nM TR, 200 µM NADPH and 5 µM Trx. (B) Direct interactions between Trx and NES-His(6) are shown by changes in fluorescence emission spectra of reduced Trx (solid line) upon incubation with oxidised NES-His(6) (dashed line).

When enzymes react with their substrates, conformational changes are created which can be detected by measuring the emission spectra in the UV range, if the protein contains tryptophan residues. Trx from E.Coli contains one tryptophan residue which enables the analysis of conformational changes within the protein using a fluorescent UV spectrophotometer. To confirm direct molecular interactions between SBP2 and Trx, reduced E.Coli Trx was incubated with oxidised NES-His(6) and the fluorescent emission spectra was measured. As demonstrated in Figure 5.9 B, incubation of Trx
(E.Coli) with NES-His(6) induced a conformational change, indicated by the change in fluorescent emission spectra of Trx, providing evidence of direct molecular interactions between the two proteins. These results identified SBP2 as a novel substrate of the thioredoxin system and suggested that SBP2 redox status \textit{in vivo} may be modulated by the thioredoxin system.

5.3.5 SBP2 forms GSH-mixed disulfides and is a substrate of the glutaredoxin system

S-glutathionylation is a second major form of oxidative modification that proteins undergo not only during oxidative stress but also as a regulatory mechanism during normal metabolism (see Section 1.2.1.4). Therefore, it was sought to investigate whether SBP2 can be modified by S-glutathionylation.

In order to address this, NES-His(6) was first reduced with DTT and immediately incubated with glutathione disulfide (GSSG), or IAM and analysed by non-reducing SDS-PAGE and western blotting. Interestingly, upon incubation with GSSG, approximately half the total NES-His(6) protein displayed a slower electrophoresis migration, similar to the IAM alkylated protein (Figure 5.10 A, lanes 4 and 3 respectively), suggesting that a glutathionylated form of NES-His(6) was present. This was confirmed by western blotting with anti-GSH antibodies (Figure 5.10 B), suggesting that glutathionylation may provide additional means of redox regulation of SBP2.

Mass spectrometry analysis was next employed to map the glutathionylated cysteine residues. MS/MS analysis by nano-ESI-Q-TOF demonstrated the existence of NES-His(6) linked with one or two GSH moieties (data not shown). Subsequent MALDI-TOF analysis of endoproteinase-Glu-C-digested GSSG-oxidised NES-His(6) identified two glutathionylation sites, shown in Figure 5.10 C. One of the GSH-linked cysteines were identified within the peptide containing both C691 and C698, therefore the exact residue remains unknown. The second glutathionylated residue was identified at C803.
Figure 5.10 SBP2 forms glutathione mixed disulfides on distinct cysteine residues. NES-His(6) was reduced with DTT prior to incubation with oxidised glutathione (GSSG) or IAM. Complexes were resolved by non-reducing SDS-PAGE. (A) Coomassie stained gel shows the altered migration of GSSG and IAM treated samples and the presence of oxidised monomer and dimer. (B) Western blot analysis using anti-His antibodies (top panel) shows the different redox forms of NES-His(6) protein. Anti-GSH (bottom panel) confirmed the presence of glutathionylated NES-His(6)-S-SG. (C) MALDI-TOF analysis of endoproteinase-Glu-C digested GSSG-oxidised NES-His(6) identified two GSH bound cysteines. One site was identified within the peptide encompassing C<sup>691</sup> and C<sup>698</sup>, and the second cysteine was mapped to C<sup>803</sup>. Peptide sequences and their molecular masses containing the glutathionylated cysteines are outlined in bottom panels.

The second essential cellular redox controlling system is the glutaredoxin system, described in Section 1.2.2.2, which preferentially removes GSH moieties from glutathionylated protein substrates (Fernandes and Holmgren, 2004). To test whether human glutaredoxins 1 and 2 (Grx1 and Grx2) were able to enzymatically reduce GSH-SBP2 mixed disulfides, enzyme kinetic measurements were performed using NES-His(6) as substrate. These experiments demonstrated that GSSG-oxidised NES-
His(6) was ~5 fold more efficiently reduced by human Grx1, than oxidised NES-His(6) (Figure 5.11 A). Interestingly, the mitochondrial enzyme Grx2, independent of GSH and GR, using reducing equivalents from NADPH via thioredoxin reductase was also able to efficiently reduce the NES-His(6)-GSH-mixed disulfides (Figure 5.11 B). This may be of relevance in terms of the mitochondrial targeted SBP2 isoform described in Chapter 3.

Attempts to determine whether SBP2 undergoes glutathionylation in vivo were unsuccessful due to technical difficulties, and due to time constraints the difficulties were not conclusively resolved and this question therefore remains the subject of future investigations. However, this study did identify SBP2 as a novel substrate for human Grx 1 and Grx 2, and suggested that glutathionylation may have an in vivo significance in regulation of SBP2 function and/or act as a protective mechanism, preventing irreversible cysteine modifications during oxidative stress imposition.
Figure 5.11 SBP2 is a substrate of human Grx 1 and Grx 2. (A) NES-His(6) was incubated with GSSG and used as a substrate for recombinant human Grx1 in enzymatic reactions. Reduction of glutathionylated NES-His(6) was measured in a master mix containing 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.1 mg/ml BSA, 6 µg/ml glutathione reductase, 200 µM NADPH, 10 µM NES-His(6) and 12 nM or 600 nM hGrx1. Oxidised NES-His(6) was used in parallel reactions for comparison. The absorbance was followed at 340 nm for 10 min. A master mix without Grx1 was used as the reference. (B) To test substrate specificity of recombinant human Grx 2 for glutathionylated NES-His(6), reactions were performed in a master mix containing 200 µM NADPH, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.1 mg/ml BSA, 50 nM TR and 5 µM of Grx2. Oxidised NES-His(6) and GSSG were used in parallel reaction for comparison. The absorbance was followed at 340 nm.

To further identify the contribution of individual cysteine residues within the CRD of SBP2 on its nuclear localisation and function, cysteines within SBP2 (C633, C644, C691, C698 and C719) were singly mutated to serines resulting in five mutated SBP2-GFP constructs. Subcellular localisation studies of the expressed mutant proteins in response to oxidative stress did not reveal any clear differences in nuclear
accumulation compared to wild type protein in preliminary experiments (data not shown). Due to time limitations, a detailed study of the localisation of these mutants was not conducted. However, the constructs were used in other studies.

A previous report showed that over-expression of SBP2 resulted in a four fold increase in selenoprotein synthesis in HEK 293 cells (Low et al., 2000) and therefore this method was considered suitable for addressing the effect of cysteine mutations on SBP2 Sec incorporation function. Wild type SBP2-GFP and the individual cysteine mutants were expressed in 293T cells and their effect on selenoprotein synthesis was analysed by $^{75}$Se labelling. Contrary to the previous study and as shown in Figure 5.12 it was found that overexpression of SBP2-GFP did not increase synthesis of endogenous selenoproteins. Moreover, this experiment did not reveal any clues as to the relevance of these cysteine residues in SBP2 function. The variations observed on the autoradiograph are due to differences in loading between the lanes as indicated by the coomassie stained gel.

![Figure 5.12 Effect of SBP2 CRD cysteine residues on Sec incorporation in 293T cells.](image)

293T cells were transiently transfected with SBP2-GFP, GFP (control) and SBP2-GFP cysteine mutants as indicated. $^{75}$Se labelling was performed for 24 hrs and Sec incorporation was analysed by autoradiography and compared to the coomassie stained gel to determine loading levels. As demonstrated, no significant differences in amounts of $^{75}$Se labelled proteins were detected between wild type, mutants and GFP control.
5.3.6 Decreased selenoprotein synthesis is response to oxidative stress

Eukaryotic cells have the ability to adapt their gene expression to changes in the intracellular environment caused by a variety of stimuli including oxidative stress. The oxidative stress response in human cells has been widely investigated at the transcriptional level in yeast and mammalian cell systems. Recently, microarray technology has been used to analyse the gene expression profile of a variety of human cells in response to various stress treatments, including oxidative stress. The data emerged has been somewhat conflicting in terms of transcriptional regulation of antioxidant genes, including selenoprotein genes, as some studies detected no changes (Murray et al., 2004), others reported an induction of gene expression (Li et al., 2002) whereas a third group found a decrease of gene expression (Weigel et al., 2002). These discrepancies were most likely due to variations in conditions, oxidant treatments, cell lines and time points used. Moreover, emerging evidence suggests that transcription and translation in response to stress do not always correlate (Holcik and Sonenberg, 2005).

In order to address the effects of redox modifications of SBP2 on its Sec incorporation function, selenoprotein synthesis was investigated in HeLa cells in response to H$_2$O$_2$ treatment. The conditions of the experiment were chosen according to the gene expression profile study described by (Murray et al., 2004) in order to compare the transcriptional and translational responses. Metabolic labelling with $^{75}$Se was used to monitor selenoprotein synthesis during a period of 24 hrs in HeLa cells subjected to oxidative stress by treatment with H$_2$O$_2$. The translational response was monitored at 8, 16 and 24 hrs post stress imposition. Interestingly, selenoprotein synthesis appeared to decrease in stressed cells, as shown by less $^{75}$Se incorporation compared to untreated controls at the time points tested (Figure 5.13), suggesting that oxidative stress treatment had an inhibitory effect on selenoprotein synthesis. This was a somewhat unexpected result as according to the general belief, an induction rather than a decrease of antioxidant enzymes expression should occur during stress conditions. To compare the observed translational response to data from earlier reports at the transcriptional level, the microarray database from (Murray et al., 2004) was checked for the effects of H$_2$O$_2$ stress on expression of selenoprotein genes. Twelve selenoprotein genes were included on the microarray chip, and none of these
transcripts were significantly altered, neither up- or down-regulated, in the H₂O₂ treated HeLa cells, suggesting that the decrease in ⁷⁵Se incorporation observed was most likely caused by inhibition at the translational level. One feasible explanation for this effect could be a compromised selenoprotein synthesis due to sequestration of SBP2 in the nucleus due to redox modifications described in this chapter.

![Image of Figure 5.13](image-url)

**Figure 5.13 Decreased selenoprotein synthesis in HeLa cells in response to H₂O₂ treatment.** HeLa cells were treated with H₂O₂ (300µM) for 1 hr. Fresh media containing ⁷⁵Se (10µCi/10cm dish) was added to untreated and treated cells and labelling was performed for the indicated amounts of time. (A) ⁷⁵Se incorporation visualised by autoradiography after separation of proteins on SDS-PAGE, shows a decreased selenoprotein synthesis in H₂O₂ treated cells at the time points tested. Coomassie blue staining of the gel shows the loading between lanes. (B) Graphical representation of the decrease as a fold difference, in radioactive signal intensity between untreated and H₂O₂ treated samples as determined by densitometry. The graph shows differences in the regulation of individual selenoprotein synthesis (10 kDa-60 kDa) following H₂O₂ induced oxidative stress.
5.3.7 SBP2 is required for Sec incorporation in vivo

Several lines of evidence from *in vitro* and cell culture models have indicated SBP2 as the central regulator of selenoprotein synthesis. However, conclusive evidence *in vivo* has so far not been provided. Recently, using siRNA technology, two novel factors, the Secp43 and the SLA proteins, were shown to be required for selenoprotein biosynthesis (Xu et al., 2005) in cell lines. To directly assess the function of SBP2, the cellular phenotype of SBP2-depleted cells using siRNA was characterised.

The potential physiological role of SBP2 in the cellular response to oxidative stress, was investigated by a comparative analysis of cell survival following H$_2$O$_2$ and selenite exposure in SBP2-depleted (transfected with SBP2 siRNA) and control cells (transfected with GFP siRNA). Only a partial knock-down of SBP2 (~50%) was achieved in cells (Figure 5.14 A, bottom panels), despite high transfection efficiencies suggesting either a negative selection against SBP2 depletion, or that SBP2 mRNA is protected against the siRNA effect. Partial depletion of SBP2 in HeLa cells had no effect on cellular viability when propagated under normal conditions. On the other hand, H$_2$O$_2$ and sodium selenite stimulated a dose-dependent decrease in cell survival, however, SBP2-depleted cells exhibited significantly greater sensitivity to H$_2$O$_2$ and selenite than control cells as evidenced by reduced survival at most dose points (Figure 5.14 A). In order to investigate the possible contribution of selenoprotein synthesis to enhanced sensitivity of SBP2-depleted cells to oxidative stress, we compared the effect of H$_2$O$_2$ on Sec incorporation in SBP2-depleted and control cells by $^{75}$Se labelling. We found a modest decrease in Sec incorporation in untreated, SBP2-depleted HeLa cells (Figure 5.14 B, lanes 1 and 3) with a ~1.5 fold decrease into the low molecular weight selenoproteins and ~1.3 fold decrease into thioredoxin reductases at 55 kDa, correlating with the partial effect of siRNA on SBP2 levels. As demonstrated earlier in this chapter (see Figure 5.13) a general decrease in selenoprotein synthesis was observed at several time points during an oxidative stress time course experiment, suggesting a compromised translation process. Importantly, H$_2$O$_2$ treatment combined with siRNA mediated depletion of SBP2 showed a significant decrease in Sec incorporation (Figure 5.14 B, lanes 2 and 4), with a ~3.5 fold decrease in $^{75}$Se labelling of the low molecular weight selenoproteins, and a ~1.8 fold decrease in the 55 kDa selenoproteins, providing direct evidence for the
requirement of SBP2 for Sec incorporation \textit{in vivo}. The increased sensitivity towards oxidative injury in SBP2-depleted cells may therefore arise as a direct consequence of the decreased selenoprotein synthesis capacity in these cells, suggesting a vital role of SBP2 in the protection against oxidative stress.

![Figure 5.14](image)

**Figure 5.14** SBP2 is required for incorporation of $^{75}$Se into selenoproteins \textit{in vivo}. (A, B) HeLa cells were transfected with SBP2 siRNA or GFP siRNA as control. (A) Cell viability assay was employed to measure the sensitivity of SBP2-depleted or control cells to oxidative stress caused by H$_2$O$_2$ or sodium selenite. (B) Immunoblot analysis using anti SBP2 antibodies shows reduced SBP2 protein levels in the SBP2-siRNA but not GFP-siRNA transfected cells. (C) Sec incorporation was analysed by $^{75}$Se labelling with or without prior treatment of cells with H$_2$O$_2$ in SBP2 depleted or control cells (top panels). Coomassie staining shows the gel loading. Graphical representations of signal intensity for the 55 kDa selenoproteins (top graph) and the low molecular weight selenoproteins (10 kDa-25 kDa) (bottom graph) as analysed by densitometry.
5.4 DISCUSSION

Data presented in Chapter 4 demonstrated that the subcellular localisation of SBP2 is not restricted to the ribosomes as previously proposed, but that SBP2 also transits through the nuclear compartment. The regulation of SBP2 localisation within cells was shown to occur at the level of nuclear export, and to be facilitated by CRM1 and intrinsic NES motifs. In this chapter, studies were undertaken to elucidate the molecular events that are involved in the regulation of SBP2 subcellular localisation and function. Three novel findings were made: firstly, that SBP2 is subject to redox regulation; secondly, that SBP2 is required for selenoprotein synthesis in vivo and thirdly, that translation of selenoproteins is inhibited by oxidative stress.

Oxidative stress specifically induced the relocation of over expressed SBP2 from the cytoplasm to the nucleus, due to direct cysteine oxidation. A novel regulatory domain, the CRD, was identified within the carboxy-terminal region of SBP2 and was shown to direct the nuclear accumulation of SBP2 in response to oxidative stress (Figure 5.6). A well characterised model of redox regulation with striking similarities to SBP2 has been demonstrated for the S. Cerevisiae transcription factor Yap-1. Yap-1 controls the activation of gene expression in response to oxidative stress (Kuge et al., 1997). Similar to what was observed for SBP2, Yap-1 protein expression does not increase following stress, rather, the regulation of its activity is controlled by changes in subcellular localisation. In unstressed cells, Yap-1 is localised within the cytoplasm of cells due to constitutive export by CRM1, however, oxidative stress triggers nuclear accumulation of the protein (Kuge et al., 1997; Kuge et al., 1998). Interestingly, this is mediated by several conserved cysteine residues within a CRD (Kuge et al., 2001), that upon oxidation form disulfide bonds that mask the availability of an NES embedded within this domain leading to retention of the protein in the nucleus (Wood et al., 2004). Thus, cysteine residues appear to serve as redox sensors that regulate the availability of the NES, and consequently the localisation and activity of the Yap-1 protein. Evidence presented herein suggests that similar mechanism may be involved in regulation of SBP2. In support of this, SBP2 also contains NESs embedded within the CRD, shuttles via the CRM1 pathway and upon oxidation it accumulates within the nuclei of cells. Furthermore, in vivo IAM alkylation induced the complete nuclear accumulation of SBP2, probably by
inhibiting its interactions with CRM1, directly demonstrating the requirement of free cysteine residues for its nuclear export.

Biochemical characterisation of cysteine modifications encompassed within the CRD showed that SBP2 forms intra- and inter- molecular disulfide bonds, as well as glutathione-mixed disulfides which were mapped to positions C^{691} or C^{698}, and C^{803}. These modifications were efficiently reversed \textit{in vitro} by the two main redox systems: the thioredoxin and the glutaredoxin system. As the major disulfide reductase system, the thioredoxin system has a wide range of protein substrates, and interestingly, thioredoxin also accumulates in the nuclei of cells in response to stress (Hirota et al., 1997; Karimpour et al., 2002). It is possible that nuclear export of SBP2 depends on the reducing capacity of these two systems in the nucleus, a reasonable possibility since members of both systems are localised within the nuclear compartment (Holmgren et al., 2005).

S-glutathionylation is emerging as an important regulatory mechanism of protein function not only during oxidative stress, but also during normal cell signalling (Ghezzi, 2005). Nevertheless, one major role of S-glutathionylation is proposed to be the protection of protein -SH groups from irreversible oxidation during oxidative stress (Shelton et al., 2005). \textit{H}_2\text{O}_2 and diamide treatments induced a 14-19 fold increase in glutathionylation of proteins in cell lines (Cumming et al., 2004) and in yeast, \textit{H}_2\text{O}_2 treatment caused a rapid but reversible inhibition of protein synthesis via glutathionylation of translation factors (Shenton and Grant, 2003). Similar results were reported in hepatocytes using \textit{tert}-butyl hydroperoxide (tBOOH) as oxidative stress agent (Latour et al., 1999). Although glutathionylation of SBP2 was not successfully demonstrated \textit{in vivo}, SBP2 was shown to be a substrate of both human Grx1 and Grx2 \textit{in vitro}. It is therefore tempting to speculate that glutathionylation, by introducing a steric hindrance, may have an inhibitory effect on SBP2 Sec incorporation function.

It was previously indicated that SBP2 RNA binding activity is redox sensitive, and required reduced cysteines (Copeland and Driscoll, 1999). In this chapter, evidence was provided to show that SBP2 also requires reduced cysteine residues for nuclear export (Figure 5.6). In order to link the redox status of SBP2 to its function, the
synthesis of selenoproteins was investigated in cells exposed to oxidative stress. As several selenoproteins are directly involved in the antioxidant defence mechanism and detoxification of ROS, it was expected that selenoprotein expression would be up-regulated in response to oxidative stress. Contrary to expectations, a clear reduction in selenoprotein synthesis after oxidative stress was observed (Figures 5.13 and 5.14). Analysis of data from a previous study (Murray et al., 2004) revealed no changes in mRNA transcripts of 12 selenoprotein genes during similar stress conditions, suggesting that the transcriptional response was not affected. Therefore, it is likely that reduced protein levels observed herein are due to inhibition at the level of translation. In accordance with these observations, the growing body of evidence suggests that global protein translation is reduced in response to most types of cellular stresses, allowing the cells to conserve resources and to initiate a reconfiguration of gene expression to effectively manage stress conditions (Holcik and Sonenberg, 2005; Wek et al., 2006). These events are mainly regulated through inhibitory phosphorylation of the global initiation factor 2-α (eIF2-α) (Dunand-Sauthier et al., 2005; Fernandez et al., 2001). Simultaneously, a switch to the cap-independent, internal ribosomal entry site (IRES)-mediated translation occurs to allow production of a select set of proteins required for cell survival, proliferation or death, depending on the severity of the stress (Fernandez et al., 2001; Nevins et al., 2003). Transient stress conditions (such as used in this study) were shown to favour the translation of pro-survival IRES elements such as present in the mRNA of anti-apoptotic XIAP, whereas severe stress supports translation of pro-death IRES elements such as the pro-apoptotic molecule Apaf-1 (Nevins et al., 2003). To date, no selenoprotein mRNAs have been shown to contain IRES elements within their mRNAs. It is thus possible that their decreased translation may reflect the absence of such elements. Moreover, since Sec is an extremely redox sensitive amino acid, it seems feasible for its translation to be restrained by oxidising conditions. It would be interesting to find out how tRNA[^Ser]Sec biosynthesis is affected by oxidative stress, as tRNA[^Ser]Sec availability is believed to be a main regulatory point in selenoprotein synthesis (Hatfield and Gladyshev, 2002).

Collectively, evidence presented in this chapter strongly indicates that translational inhibition of selenoprotein synthesis during oxidative stress is mediated through sequestration of SBP2 from the ribosomes into the nuclear compartment. SBP2
nuclear retention appeared to be mediated by oxidised cysteines leading to masking of the NES in a manner that interferes with CRM1 binding. Alternatively, SBP2 may form complex with a protein in the nuclear compartment which may lead to its nuclear retention by masking of the NES.

Earlier studies have proposed SBP2 as central for selenoprotein synthesis, however, direct in vivo evidence had not been provided. Partial depletion of SBP2 in cells using siRNA led to an equivalent decrease in selenoprotein production. Importantly, the effect was greatly enhanced by oxidative stress, under which conditions, oxidation of the remaining SBP2 pool may have caused its recruitment from the cytoplasm to the nucleus, as observed for over-expressed SBP2-GFP. Preliminary data showed a decrease in endogenous SBP2 protein levels in the ribosomal fraction after oxidative stress (data not shown), indicating that oxidation may directly affect its ribosomal localisation and negatively regulate its Sec incorporation function. Accordingly, several translation factors have been reported to form inactivating disulfide linkages within cells exposed to oxidative stress and these include elongation factors EF-1-α1, EF-1-γ, EF-2, initiation factor eIF-2-γ, cysteinyl-RNA synthase and others (Cumming et al., 2004). In yeast, several translation and RNA splicing factors were found down-regulated in response to H\textsubscript{2}O\textsubscript{2} treatment and a transient decrease in gene expression was observed for up to six hours post stress imposition (Pocsi et al., 2005) suggesting a redox dependent regulation of protein synthesis. Although several examples of translation factors shown to undergo oxidative modifications in response to oxidative stress have been reported, the direct molecular mechanisms that control these events have not been reported. In that respect, SBP2 may represent the first example of a translation factor shown to be directly inactivated by cysteine oxidation. The data presented in this chapter supports a model where during oxidative stress, selenoprotein synthesis is inhibited by sequestration of SBP2 from its active, reduced form at the ribosomes to an inactive oxidised form within the nucleus. Sequentially, its re-activation would require the action of the two major redox systems, the thioredoxin and glutaredoxin systems.
CHAPTER 6.

SUMMARY AND GENERAL DISCUSSION
6.1 SUMMARY AND GENERAL DISCUSSION

Oxidative injury plays a critical role in the pathogenesis of many diseases including cancer. Organisms growing in the presence of oxygen have evolved a cellular defence system comprised of antioxidant proteins that helps them deal with oxidative stress. These proteins convey the oxidative stress alarm swiftly and precisely to numerous pathways across the cell, and some of these include members of the selenoprotein family. In humans, this family consists of 25 members, many of which have not been functionally characterised. Among the best characterised selenoproteins are TR and GPx, which are central enzymes in redox regulation of cellular signalling and antioxidant defence. Selenium compounds in various forms are cancer preventive agents and their anticarcinogenic properties are believed to be attributed largely to the presence of Se as an essential component of selenoproteins. Selenium is incorporated into these proteins in the form of Sec. Translational incorporation of Sec occurs via a highly specialised mechanism that involves decoding of the UGA, which normally serves as a termination codon. The translational apparatus must therefore distinguish which function to perform at each UGA codon in the cell. Among the factors involved in decoding the UGA codon, SBP2 appears to be the key regulator of selenoprotein synthesis as demonstrated by its requirement for translational incorporation of Sec into selenoproteins in in vitro systems.

In spite of the progress that has been made in delineating the mechanism of Sec insertion in mammalian systems, a number of central questions remain yet to be answered. In particular, the molecular events that regulate this process in its entirety and also the individual role played by its components. The objective of this thesis was to broaden our understanding of the mechanism that involves selenoprotein synthesis by expanding the characterisation of one of the known factors, human SBP2. Collectively, the findings of this thesis implicate multiple levels of regulation of SBP2 function by alternative splicing, subcellular localisation and redox control.

In Chapter 3, it was discovered that SBP2 is subject to alternative splicing. In total, nine SBP2 transcript variants were identified by experimental and computational approaches, all differing in the 5’-region. On the contrary, it appeared that the 3’-region, which displays the highest level of evolutionary conservation and
encompasses the functional and RNA binding domains, was devoid of alternate splicing events. This was not surprising, and reflects the importance of the integrity within this region for SBP2 function.

The complex splicing pattern within the 5′-region revealed the existence of five distinct, potential N-terminal domains, one of which contains a mitochondrial targeting sequence. Interestingly, studies using a GFP-SBP2 reporter construct of this particular isoform demonstrated that the MTS is capable of targeting the SBP2 isoform to the mitochondria. Several lines of evidence from proteomics approaches and studies in cell systems supported the existence of a mitochondrial localised endogenous SBP2 isoform, therefore designated as mtSBP2. An interesting feature of this particular isoform is its translation through a -1 reading frame-shift within an exon common to the full length and several other SBP2 splice variants. A programmed ribosomal frame-shift (PRF) is frequently observed in viral mRNAs, which encode polycistronic messages that direct ribosomes to synthesize polyproteins (Plant et al., 2004). In eukaryotes, and in particular in mammals, this mechanism of post transcriptional regulation is a rare event and in most cases, due to generation of premature termination codons, leads to mRNA degradation through the nonsense mediated decay (NMD) pathway (Culbertson and Leeds, 2003; Plant et al., 2004). Nevertheless, an increasing number of eukaryotic genes have been demonstrated to be translated through a programmed ribosomal frame shift mechanism. For instance, a programmed +1 frame-shift was shown to direct translation of several S. Cerevisiae proteins: actin binding protein 140 kDa (ABP140) (Asakura et al., 1998), telomerase-associated protein EST 3 (Morris and Lundblad, 1997) and the ciliate telomerase-associated protein p43 (Aigner et al., 2000). A -1 frame-shift, such as the one observed for mtSBP2, appears to be more common in viral mRNA (Brierley and Dos Ramos, 2005), and only two examples have so far been provided in mammals, which include the mouse embryonal differentiation regulated protein (Edr) (Shigemoto et al., 2001) and the human para-neoplastic Ma3 antigen (Wills et al., 2006). The regulation of the -1PRF mechanism has been well characterised in viral genomes and requires multiple cis-acting features that include a so called “shifty, or slippery site”, a linker region and an appropriately positioned mRNA structural feature that acts to enhance the level of frame-shifting (Plant et al., 2003; Wills et al., 2006). The shift to the -1 frame involves detachment of tRNAs in both the A and P sites and re-attaching to
mRNA at the two overlapping -1 frame codons. Well characterises viral sequences that direct -1 frame shifting are generally referred to as pseudoknots which are RNA elements with stem loop structures (similar to the SECIS element described in this thesis) located 6-8 nt downstream of the shifty site (Brierley et al., 1989; ten Dam et al., 1990). The primary sequence of the slippery site and its placement in relation to the incoming translational reading frame is critical and must contain a consensus sequence: X XXY YYZ where X must be a stretch of three identical nucleotides, Y is either AAA or UUU and Z is A, C or U (Plant et al., 2003).

Primary nucleotide sequence analysis did not reveal the presence of such sequence at the frame shift site or nearby within SBP2. However, further analysis using the recently reported bioinformatics approach (Wills et al., 2006) to identify putative secondary elements in the mRNA of SBP2 will help delineate whether this mechanism is involved in directing translation of the mtSBP2 isoform in a -1 frame shift. Alternative mechanism for expression of this isoform could involve its transcription from an alternative promoter, potentially located within downstream introns. If this was the case, the frame shift mechanism would no longer apply, as an alternative transcription start site would be present and translation would be directed in a “zero” frame. Multiple promoters with different transcription factor binding sites within the 5'-UTR and downstream SBP2 introns were predicted using bioinformatics tools (data not shown). Some of the binding sites predicted included housekeeping transcription factors such as SP1, SP3, Oct1. An NFκB binding site, which was specific to one of the alternative promoter, was also predicted. Features such as alternative promoters and different transcription factor binding sites may therefore represent potential regulatory elements of not only the mtSBP2 isoform but also of additional alternatively spliced variants. This remains to be experimentally verified in future studies. Recent examples of alternative promoters regulating expression of multiple protein isoforms include the tumour suppressor p53 and p53-related genes p73 and p63 (Bourdon et al., 2005) which were differentially expressed in breast tumours compared to normal tissues, directly linking the regulation by alternative splicing to the p53 tumour suppressor function. Future elaborate studies at both transcriptional and translational levels are required in order to delineate what mechanisms regulate the alternative splicing events within the human SBP2 gene.
The most interesting aspect of these splicing events is their relevance in terms of SBP2 function, if expressed as protein. To date, Sec incorporation is the only known function of SBP2 and interestingly, the variant N-terminal region that harbours the splicing events appeared to be dispensable for Sec incorporation in \textit{in vitro} systems. Therefore, the specific functions of the different protein isoforms represents a major aspect that needs to be studied in depth. As mentioned previously, the existence of alternative SBP2 N-termini may serve as regulatory domains for protein-protein interactions that may act in promoting the translation of specific selenoproteins. Alternatively, protein variants may be involved in regulating the function of SBP2 in different subcellular compartments. As identified in this study and discussed further below, an additional level of regulation of SBP2 at subcellular localisation was discovered. In Chapter 4, over-expressed SBP2\textsubscript{Δ2} isoform appeared to display a more frequent nuclear localisation than full length SBP2, suggesting a potential involvement of variant N-termini in directing SBP2 localisation to different subcellular compartments. However, further studies need be initially performed in order to determine which of the additional transcripts encode proteins in cellular systems.

Probably the most intriguing finding that emerges from the studies of alternative splicing was the identification of a mitochondrial SBP2 isoform. This finding in turn raised the question of what functional relevance may be attributed to a mitochondrial targeted SBP2 isoform. Since mitochondria originate from a prokaryotic lineage of life, they have circular DNA genomes and also encode their own components of the translation apparatus such as rRNA and tRNA, as well as mRNAs for proteins that are synthesised within the organelle. Therefore, it is quite unlikely that the mitochondrial function of SBP2 is directly related to translation. Moreover, the mitochondrial genetic code usage differs from the universal code and here, UGA encodes tryptophan, further supporting the lack of evidence for the existence of a mitochondrial encoded selenoprotein gene. However, if Sec insertion components such as SBP2, or other universal translation factors are transported to mitochondria and these organelles use a eukaryotic translation mechanism, it would be a major discovery with impact outside of the selenoprotein field.
It can therefore be concluded that the most likely role of SBP2 in the mitochondria is to perform a Sec-unrelated function. Mitochondria are the cellular sites of oxidative phosphorylation and energy production, and perform multiple functions including calcium buffering and regulation of apoptosis (Chen and Chan, 2005). In addition, mitochondria are central to both oxidative damage and redox signalling within the cell, and are also under constant oxidative stress due to the generation of high levels of oxygen metabolites. As a result, most mitochondrial proteins contain redox sensitive thiols that are modified by redox mechanisms including disulfide bond formation and glutathionylation (Hurd et al., 2005b). Concurrently, mitochondria contain redox controlling systems to reverse such modifications and combat excess levels of ROS. These systems include the mitochondrial specific Trx and Grx 2 systems, and the mitochondrial pool of the glutathione system (Holmgren et al., 2005; Hurd et al., 2005a). As demonstrated in Chapter 5, SBP2 is a redox sensitive protein that contains a cysteine rich domain with several potentially exposed thiols that become modified to disulfide bonds and form glutathione-mixed disulfides in response to oxidative stress. Since the full length and mitochondrial isoforms are identical within this region, the modifications apply to both proteins. Interestingly, the CRD was efficiently reduced by the mitochondrial enzyme Grx 2 using reducing equivalents from both the TR and GR systems. These findings further strengthen the evidence of a functional relevance of a mitochondrial targeted SBP2 isoform. In that respect, it is tempting to speculate that SBP2 may serve an additional function in the mitochondria, potentially in redox sensing and signalling.

At present, two models that describe the role of SBP2 in translation of selenoproteins have been proposed. In one model SBP2 is proposed to remain stably associated with ribosomes during multiple rounds of translation and only slightly dissociate during the Sec delivery to the ribosomal A site (Caban and Copeland, 2006). In the second model, SBP2 is proposed to translocate to the nuclear compartment to potentially associate with SECIS-containing mRNA and/or protein factors and to deliver mRNA to the ribosome for translation (Small-Howard and Berry, 2005). The major part of this thesis, included in Chapters 4 and 5, investigated in detail the subcellular localisation of over expressed and endogenous SBP2, its regulation in response to different stimuli and its role in controlling the Sec incorporation function of SBP2. Interestingly, the data obtained support both of the proposed models and shows that
SBP2 subcellular localisation is not solely confined to ribosomal sites, rather, that SBP2 shuttles between the nucleus and the cytoplasm in a motif-regulated manner. In addition, it was demonstrated for the first time that SBP2 is required for Sec incorporation \textit{in vivo} and that lack of SBP2 renders cells more sensitive to oxidative injury. This was most likely caused by a depletion of antioxidant selenoproteins due to a decreased capacity of their translation.

Initial experiments verified that the putative SBP2 NLS was functional and that it enabled the trafficking of both over-expressed and endogenous SBP2 through the nuclear compartment. In addition, the presence of two functional NESs within the carboxy-region of SBP2 and their dependence for the CRM1 nuclear export pathway was demonstrated. Treatment of cells with LMB reduced both the ribosomal localisation of SBP2 and selenoprotein synthesis without affecting the efficiency of non-selenoprotein translation, suggesting that nuclear export of SBP2 is required for Sec incorporation.

The specific mechanism that triggers nuclear translocation of SBP2 remains at present unclear. One possibility is that SBP2 is directed to the nuclear compartment immediately after its synthesis, due to exposure of its NLS. Alternatively, SBP2 may be directed to the nucleus upon its release from the ribosome during delivery of Sec. A recent study demonstrated that L30 protein and SBP2 compete for binding to SECIS elements (Chavatte et al., 2005). It is therefore possible that L30 replaces SBP2 from the SECIS element and the ribosome which may trigger its nuclear translocation. For the time being these possibilities remain speculative.

A central question that arises from these findings is the functional relevance of SBP2 nuclear localisation. mRNAs are generally exported from the nucleus as ribonucleoprotein complexes including RNA binding proteins and transport factors (Zenklusen and Stutz, 2001). Because translation of selenoproteins requires unique factors, the transport of their cognate mRNAs from the nucleus may involve interactions with some of these factors prior to their arrival at the ribosomal sites. SBP2 contains functional NLS and NES motifs to perform this function, consequently its shuttling properties may be involved in transport of mRNA and be directly linked to its Sec incorporation function. Interestingly, these NESs are located on both sides
of the RNA binding domain, raising the possibility that their availability for CRM1 binding may be related to the conformational changes caused by the binding of SBP2 to SECIS elements. Alternatively, its nuclear localisation may serve additional functions, possibly unrelated to Sec incorporation. Elucidating the nuclear role of SBP2 would indeed be a very exciting finding.

Altered intracellular conditions lead to activation of signalling pathways and affect proteins at multiple levels, including activity, stability and subcellular localisation. The shuttling properties of SBP2 were tested in a number of stress-imposing conditions. Interestingly, oxidative stress causes oxidation of SBP2 in vivo, its relocation to the nuclear compartment and an inhibition of its function as was demonstrated by a decrease in selenoprotein synthesis during oxidising conditions. SBP2 RNA binding properties were previously shown to be inhibited in vitro by oxidation (Copeland and Driscoll, 1999). This is commonly observed in DNA and RNA binding proteins since cysteine residues are directly involved in protein-nucleic acid interactions. NFκB is one example where oxidation was shown to decrease its DNA binding activity in the nucleus (Toledano and Leonard, 1991). The iron regulatory protein 2 (IRP 2) is another example. IRP2 is an RNA binding protein that mediates the expression of proteins that participate in iron metabolism (Rouault and Klausner, 1997). Similar to SBP2, IRP 2 binds to iron response elements (IRE) within RNA stem-loop structures in the 5'- or 3'-untranslated region of mRNA. Oxidative modifications of cysteine residues within IRP2 were shown to negatively regulate its function by altering its structure and thus targeting the protein for proteasomal degradation (Kang et al., 2003). As demonstrated in Chapter 5, SBP2 contains a CRD, embedded within its RNA binding domain and its two functional NESs that directly regulates SBP2 subcellar localisation. Reduced cysteines are required for SBP2 nuclear export, most likely by affecting the availability of the NES for CRM1 binding, suggesting that the redox state of the protein regulates both its RNA binding abilities and its intracellular localisation. These findings thus identified a novel means of regulation of SBP2 by redox control.

A detailed biochemical characterisation of the CRD showed that SBP2 forms intra- and inter-molecular disulfides, as well as glutathione-mixed disulfides at positions C^{691} or C^{698}, and C^{803}. These modifications were efficiently reversed in vitro by the
two main redox systems: the thioredoxin and the glutaredoxin system. It is tempting to speculate that nuclear export of SBP2 and its location to the ribosomes is dependent on the reducing capacity of these two systems which also relocate to the nucleus in response to stress (Hirota et al., 1997; Hirota et al., 2000).

An investigation of selenoprotein synthesis during oxidative stress was conducted in order to link the localisation of SBP2 to its function. Contrary to expectations, a clear reduction in selenoprotein synthesis after oxidative stress was observed, indicating that translation ceased during oxidising conditions within the cytoplasm. In accordance with this, the growing body of evidence suggests that global protein translation is reduced in response to most types of cellular stresses, likely caused by inactivating disulfide bond formation within several translation factors. Simultaneously, a switch to IRES (internal ribosomal entry site) mediated translation occurs to allow translation of a select set of proteins required for cell survival (Cumming et al., 2004; Holcik and Sonenberg, 2005). Selenoproteins mRNA levels were shown to be unaffected by similar oxidative stress conditions in HeLa cells (Murray et al., 2004) suggesting that regulation occurs at the translational level. It is therefore proposed that SBP2’s Sec incorporation function is regulated by the redox state of the protein and its localisation to the ribosomes, providing for the first time evidence of a novel means of regulation of selenoprotein synthesis through redox regulation of SBP2.

Based on the data emerging from this thesis, a modified model for Sec incorporation is proposed. This model suggests that the steady state localisation of SBP2 is at the ribosomes. However, cell signalling events or altered intracellular conditions trigger SBP2 release from the ribosomes and its translocation to the nucleus, to potentially associate with SECIS containing mRNAs and facilitates their transport to the cytoplasm via the CRM1 pathway (Figure 6.1 A).
Figure 8. A proposed model for the regulation of SBP2 subcellular localisation and function through redox modifications. (A) In normal conditions the majority of the SBP2 pool is localised within the cytoplasm, at ribosomal sites where it functions as part of the Sec incorporation complex. Changes in the intracellular conditions or cell signalling cause the NLS-mediated nuclear translocation of SBP2. Rapid export mediated by the export protein CRM1 and the SBP2 NESs restores the cytoplasmic steady state levels of SBP2. (B) Oxidative stress induces modifications of SBP2 redox sensitive cysteines, potentially inducing a conformational change, disrupting its ribosomal localisation and triggering its nuclear translocation. Lack of sufficient reducing conditions in the nucleus maintain SBP2 in an oxidised state, masking the NES and inhibiting interactions with CRM1 thus leading to its nuclear retentions. (C) During the cell recovery phase, SBP2 is reduced by the thioredoxin and/or the glutaredoxin systems, leading to exposure of the NESs and interactions with CRM1, promoting export of SBP2 to the cytoplasm, re-assembly of translationally active ribosomal complexes and translation of selenoproteins.
During oxidative stress, modifications of redox sensitive cysteine residues within the CRD to disulfide linkages and glutathione mixed disulfides alter the conformation of SBP2 leading to nuclear retention potentially through masking of the NESs leading to an inhibition of selenoprotein synthesis during the oxidising conditions within the cytosol (Figure 6.1 B). During the cell recovery phase, SBP2 is reduced by the thioredoxin and/or glutaredoxin systems, facilitating its nuclear exit, its relocation to the ribosomes and re-initiation of selenoproteins translation (Figure 6.1 C).

The novel findings presented in this thesis most certainly will serve as a platform for future, more elaborate studies to understand the different aspects of SBP2 redox regulation, and their direct impact on selenoprotein synthesis. It is evident that regulation of SBP2 is more complicated than previously thought, and more likely requires multiple events such as disulfide bond formation and glutathionylation, as well as several pathways involving thioredoxin and glutaredoxin, all working in concert. As a future perspective, it would be interesting to elucidate the role of the nuclear pool of SBP2, the effect of oxidative modifications of SBP2 on its function and ultimately how these pathways can be targeted to modulate expression of selenoprotein synthesis.
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**APPENDIX**

**Human SBP2 cDNA sequence with exon boundaries**

The human SBP2 cDNA (NM 022077) sequence is provided. The exon boundaries are indicated by bars in green. Translation start Methionine residues are indicated in red. The nucleotide sequence towards which siRNA was targeted is shown in blue.

**Exon I**

1  C | CAAGCGAAC GGGCGCGTGC TGCCCTCCGGT GACCGGGCCT CCTCCGCGCC

**Exon II**

51  TGCGCGCATG GCCTGGAGGG GGGCGCGGGA GGGCGAAAGC GAG GGCATCA M A S E G P R E P E S E G I K

101  AGTTATACGC AGATGTCAA ACATTTGTCC CCAGATTGGC CGGCTCAAT L S A D V K P F V P R F A G L N

151  GYGGCAGTTGT TAGAGTCCTC AGAGACATGT GTCTTCGCGCA GCTCGACAC V A W L E S S E A C V F P S S A A

**Exon IIIa**

201  CACATATACT ATCGTTTGTTC AGGAACCACC AGTGACAGA|GCAGAAAATAT

251  ATACATGAGA CATGGGCTTT GGGCGTGCC CTTGTTACCC TCAGATATTA T E D M A F G A S T F P P Q Y L

301  TCCCTCTAGA TAATCTCTCA TCCATATGCC TATTTTCCTT ATACCTTGA S S E I T L H P Y A S P Y T L D

**Exon IIIb**

351  CTCCACACAG | AAATGTTTACT CAGTGCTGGG CTCCCAGTAT CTTTATACC T  Y Y P F V Q E F P V T E Q K I Y

401  AACCCAGTTG TTACCGAGGT TTTCAACAG TGAAGCATCG AAATGAGAAC P S C Y R G F Q T V K H R N E N

451  ACATGCCCTC TCCAGCAAGA ATGAAAGCT CTGTTTAAG|AAGAAAACCTA T C P L P Q E M K A L F K K K T Y

501  TGAATTACAA AAAAAAGTATG ATCAGGAAAA GTTTGAGAGT GAAAGGGCTG D E K K T Y D Q K F D S E R A D

551  ATGAAACTAT ATCATCTGAG ATAAAATCAG CTAGAGGTTC ACATCATTTG G  T  I  S  S  E  I  K  S  A  R  G  S H H L

**Exon IV**

601  TCCATTTCAG CTGAAATATG TTAGAATATCA G | AGTTTACCC ATAGCGAAC S I A E N S L K S D G Y H K R T

651  AGAACAGAAA TCGAGCAATCA TGGCAAAAAA TGATGTTCAC TCCAAACCTG D R K S R I A K N V S T S K P E

701  AGTGGTGAAT TCCACACCTTG ACGTTTACCC ATAGCGAAC S I A E N S L K S D G Y H K R T

751  ATATGTCAG AGATAACAGA GAAAGCCAGA TGGCGAGGTA CGGCTTCGTA N M S E I Q K K Q P K W G P V H S V

801  CTCCACACAG ATCGTTTACT CAGTGCTGGG CTCCCAGTAT CTTTATACC T  Y Y P F V Q E F P V T E Q K I Y

851  ATGAAACTAT ATCATCTGAG ATAAAATCAG CTAGAGGTTC ACATCATTTG S T D I S L L R E V V K P A A V L

**Exon V**

901  GCCACAACTT ATGAGATTTA ATACATCTTCA G | AGTTTACCC ATAGCGAAC S I A E N S L K S D G Y H K R T

951  ACCATGCTAT CTTGCCCCCGC C | AGACTG CACGAAACATCGTACGCCGCAG P M G Y V R Q T L S T E L S A A

1001  CCCCTAAACAA ATGAAAGTCA CAGATCAA ACATTCAGCA CTGCAAGACAG P K N V T S M I N L K T I A S S

**Exon VI**

1051  GC | AG | ATCCTA AAAATGTTAG ATACATCTTCA TCTGCAAGCT TATTTTCCTT A D P K N V S I P S E A L S S D

**Exon VIIa**

1101  GCCCGCCTTC TGCTCCAGC AAGACTG CAGAAACATCGTACGCCGCAG P M G Y V R Q T L S T E L S A A

1151  CCCCTAAACAA ATGAAAGTCA CAGATCAA ACATTCAGCA CTGCAAGACAG P K N V T S M I N L K T I A S S

**Exon VIIb**

1201  GCCCGCCTTC TGCTCCAGC AAGACTG CAGAAACATCGTACGCCGCAG P M G Y V R Q T L S T E L S A A

1251  CCCCTAAACAA ATGAAAGTCA CAGATCAA ACATTCAGCA CTGCAAGACAG P K N V T S M I N L K T I A S S

1301  GCCCGCCTTC TGCTCCAGC AAGACTG CAGAAACATCGTACGCCGCAG P M G Y V R Q T L S T E L S A A

1351  CCCCTAAACAA ATGAAAGTCA CAGATCAA ACATTCAGCA CTGCAAGACAG P K N V T S M I N L K T I A S S

1401  GCCCGCCTTC TGCTCCAGC AAGACTG CAGAAACATCGTACGCCGCAG P M G Y V R Q T L S T E L S A A

1451  CCCCTAAACAA ATGAAAGTCA CAGATCAA ACATTCAGCA CTGCAAGACAG P K N V T S M I N L K T I A S S

1501  GCCCGCCTTC TGCTCCAGC AAGACTG CAGAAACATCGTACGCCGCAG P M G Y V R Q T L S T E L S A A

1551  CCCCTAAACAA ATGAAAGTCA CAGATCAA ACATTCAGCA CTGCAAGACAG P K N V T S M I N L K T I A S S
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1101  TCCTTCTTAC  AACAAGAAAA  AACACATTAT  TCATCTTACC  CAAAAG  |  TCTA
  PSYNKKEHKIHHPKTQKSK

1151  AAGCATCACA  AGTGTAGTGAC  CTGGAGTTCA  AAGAAAAATC  TACATCAAAA  TATGAAGTCC  TGACAGTTCA
  KKKEKSTSKYTEVLTVQ

1201  AAGCATCACA  AGGTAGTGAC  CTTGAACAAA  ATGAAGCCTC  AAGAAAGAAT
  ASQGDLEQNEASRK

1251  AAGAAAAAGA  AAGAAAAATC  TACATCAAAA  TATGAAGTCC  TGACAGTTCA
  KKKEKSTSKYTEVLTVQ

Exon IX

1301  CATCTGAAAG  AAGAGCAGA  AGTACAGACC  CGAAATTTCA  ATCTAAGCAG
  SERRDRIETPKQSKQ

1351  AGGAGCTCCA  AGGATTGAA|GATGCCGAGGA  ATTTCCCAAC  CTGGCAGTTG
  EPPIEDAEFPELAV

1401  CATCTGAAAG  AAGAGACAGA  ATAGAGACAC  CGAAATTTCA  ATCTAAGCAG
  SERDERINPKFQSKQ

Exon X

1451  CAGCCACAG|GATAATTTTAA  AAATAATGTA  AAGAAGAGCC  AGCTTCCAGT
  QPQDNFKNNVKKSQLPV

1501  GTGCCAGTCC  TTTCCCAAAGA  ATGTGCATCA  GGGAGGAGAG  GCCGCCGAT
  VPVLSECKASCGERGRRM

1551  GAGAATAATG  AGAACCAGCCG  ACAATTCCTTT  AGAATCCAGCG  GCCCCACTGA
  SQMKTPHNPLDSSAPLM

1601  TGAAGAAGAG  GAGACAGAGG  GAGATCCCAAA  AGGCAAAGAGA  GCCAACTCCA
  KKKQKQREIPAKKPKTS

Exon XI

1651  CTGAAGAAG|ATTATTTTAA  AAATAATGTA  AAGAAGAGCC  AGCTTCCAGT
  LKIKILKERQERKQLQ

1701  AGAAGAATGCT  GTGATGTCAG  CTTTTACCAG  TGTAGACACA  CAAGATGGAG
  ENAVSPAFSTDQDGE

1751  AGAAGTGTGTTG  TGATGACCCCG  TTTCCGGAGGC  AGGCAGAGCT  GTCCAGGGAC
  GGDGQFPFPEQAELSGP

1801  GAGGAACTAGG  ATCTGACCCCG  CTGTCAGGGAG  AAAGCTCTGA
  EGMDIELISTPSVDEKSE

1851  AGGAGCCACCA  GCCACAGAGC  TCTAAGCCGAAGA  GCCAGGCCGCC  TCCACCTTGG
  EPPGETLQDRDTEASHLA

Exon XII

1901  CTCCCAATCA  CACACACCTCC  CCTAAGATGCC  ACAGCCGGCA  ATCTACCTCCA
  SPPHTTFPKIHSSRFFRD

1951  TACCTGAGCC  ATAGTTTCAAG  TAAAGAGATG  TATGTCTTGGT  TCAGCCGCTT
  YCSQMMLSKKEVDACVTDL

2001  ACTCAAGAAA  CTGGTCTCCGT  TTCAAGACCG  TATGTTACAGA  AAAGATGCCG
  LKELVRFQDRMRQKDPV

2051  TCAAGGGCCAA  GACAAACCTAG  CGAAGTTGTTG  TGGGGTGGAG  GAGGTTCCCT
  KAKTKRRLVLGLRELVL

2101  AAACACCTGA  AGCCTAAAAA  ACTGAAATGCT  GTGCTATTATTCTCCACATG
  KHLKLKKLKCVISPN

Exon XIII

2151  TGAAAGAATA  CAGTACAAAG  GTGGGCTGAGA  TGACTCTTTCTC  CACAAACTTA
  EKIQSKGLDLDTLHTII

2201  TGATTTAGCTGTGGGCTGAGA  AACACTTTCA  TGATGTTTTGG  TCTCAACCAGC
  DYZACEQNIFFVFALN

2251  AAGACTCTGG  GCCGCCCTTGG  GAAATAGGGCA  GTGCTCTTGGTCG  GTGGTGTTG
  KALKGRSLNKAVPVSVVG

Exon XIV

2301  GATCTCTACCC  TATGATGGGG  CCCAG|GATCAATGAGA  GTCTCAGGGAC
  IFSYDGADQDFHKMVEL

2351  TGACAGTGCC  GCCGCCAGAGA  GGTGACCATG  CCAAGCTGCAAGA  GAGATGACCA
  TVAARQAAYKTMLENVQ

2401  CAGGAGCTGG  TGAGAGAGCC  CAGCCTCAGCGGCACTTCCCA  GCCTACTCAC
```
Q E L V G E P R P Q A P P S L T

2451 ACAGGGCCCC AGCTGCCCTG CAGAAGATGG CCCCCCAGCC CTGAAAGAAA
Q G P S C P A E D G P P A L K E

Exon XVII

2501 AAGAAAGGCC ACACTACA TTGAATCTGGA AAAAACATCT GGAAGCATAC
E E P H Y I E I W K K H L E A Y

2551 AGTGGATGTA CCGTGGAGCT AGAAGAATCC TTGGAGGCTT CAACCTCTCA
S G C T L E L E E S L E A S T S Q

2601 AATGTGAAT TTGAATTTAT GAGAGTTCCT GCCTGTGTGT CTGTATTTTG
M M N L N L *

2651 GGTAAAGGGG GCGGGTCTGA AAAAGACTTT GGGGCTTTTT CTTCGTTTTT

2701 TCACTGCAAT GTAATTTTGT TAACTGTTGA ATCTGGAAT TGATCGACAT

2751 TAAAGGGCAC ATGAAAGCAGT GTCTGCAGGC GTTCAGTCG GCGGAGCCGT

2801 TTAAAGGCTA TCTGAGATGT CAGCTGTTAA TCTCTCTTAA AAGCCCTGGT

2851 ATACAGCTCT GCTTTCTGGA GCACACTACG GATCTGGAAA ATACTGGAAA

2901 ATGTGATACT TAGAATACCT TGGCTGCCTAA GGAACCTTCC TCTCCTATGC

2951 AGAAAGCTGT AGCCAGTGA GTGAGTTTTC AGAAAGCAGT GGGTGAAGCT

3001 CTGCCTGCTG GAGGCTGACG TGGGAGCCCA TTCTCTGCCG GCAACAGCAC

3051 CTGCCTGCTG GAGGCTGACG TGGGAGCCCA TTCTCTGCCG GCAACAGCAC

3101 AGCAAGGGG CTACAGGGAA AGGGCTTTTT CTCAGGGGAT GTAGCTTTTT

3151 TAAAAAGATT GGGGACATT GGGGATTTGC CTAAAAATTGAG CTTCAGAAGG

3201 AAAATGGTT TTCTAACCTG TGACTTTTTG AAGTGAATTA TTCTCTCTAG

3251 TTCTTATTCT TTAAAAGAAC AATGGTTATT GAAATACCTA GATTTGTTTG

3301 ATAATCAACA AATCTTCCCA TTTTCTACAG AATATATCTG AATGCTCTTT

3351 CTGCTCTCTT ACGGGAGGAC AGAACTGCTT TTCAATATTT CTCGTAAGT

3401 AAGAGGGCTT ATTTATTTTA AAATAGGAGT AATTATTAAA AAAAAAAA

3451 AAAAAA

203