THE ROLE OF THE TRANSCRIPTION FACTOR ETS-1 IN MITOCHONDRIA TO NUCLEUS SIGNALING

By

LEIGH A. WILSON, B.SC.

A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment Requirements

For the Degree

Doctor of Philosophy

McMaster University
© Copyright by Leigh A. Wilson, November 2005

Library and Archives Canada

Published Heritage Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et Archives Canada

Direction du Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 978-0-494-20414-6
Our file Notre référence
ISBN: 978-0-494-20414-6

NOTICE:
The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:
L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

THE ROLE OF ETS-1 IN MITOCHONDRIA TO NUCLEUS SIGNALING

DOCTOR OF PHILOSOPHY (2005) **McMaster University**

(Medical Sciences) **Hamilton, Ontario, Canada**

TITLE: The role of the transcription factor Ets-1 in mitochondria to nucleus signaling

AUTHOR: Leigh A. Wilson, B.Sc. (McGill University)

SUPERVISOR: Professor Gurmit Singh, Ph.D. (Dalhousie University)

NUMBER OF PAGES: xvi; 226

List of relevant publications and abstracts

Publications:

Wilson, L.A., Gemin, A., Espiritu, R. and Singh G. (2005). *ets-1* is transcriptionally up-regulated by H_2O_2 via an antioxidant response element. *FASEBJ* (in press).

Wilson, L.A., Yamamoto, H. and Singh, G. (2004). Role of the transcription factor Ets-1 in cisplatin resistance. *Mol. Can. Ther.* 3(7): 823-832.

Preston, T.J., Abadi, A., Wilson, L. and Singh, G. (2001). Mitochondrial contributions to cancer cell physiology: Potential for drug development. *Advanced Drug Delivery Reviews.* 49: 45-61.

Abstracts:

Leigh Wilson, Adam Gemin, Raissa Espiritu and Gurmit Singh (2004). ARE mediated regulation of the transcription factor Ets-1. Abstract presented at the Society for Free Radical Biology and Medicine 11th annual meeting (poster), November 2004 (St Thomas). Abstract published in Free Radical Biology and Medicine, 37 (S1) S74.

Leigh Wilson, Hirotaka Yamamoto and Gurmit Singh (2003). Ets-1: A target of mitochondrial signaling via reactive oxygen species. Abstract presented at the Society for Free Radical Biology and Medicine 10th annual meeting (presentation), November 2003 (Seattle). Abstract published in Free Radical Biology and Medicine, 34 (S) S76.

Leigh Wilson, Kenji Sakata and Gurmit Singh (2001). Ets-1: A mediator of nuclear-mitochondrial crosstalk. Abstract presented at Yeast as a model for mitochondria-related human disorders (presentation), August 2001 (Prague). Abstract published in Mitochondrion, 1 (2) 205-206.

Leigh Wilson, Kenji Sakata and Gurmit Singh (2001). The transcription factor Ets-1 functions in the regulation of mitochondria. Abstract presented at 92nd AACR Annual meeting (poster), April 2001 (New Orleans).

Abstract

Mitochondria are unique organelles within the cell in that they contain their own genome and in conjunction with the nucleus, are able to transcribe and translate genes encoding components of the electron transport chain (ETC). In order to do so, the mitochondria must communicate with the nucleus, via the production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), produced as a byproduct of aerobic respiration within the mitochondria. Mitochondrial signaling is proposed to be altered in cancer cells, where the mitochondria are frequently found to harbor mutations within their mitochondrial deoxyribonucleic acid (mtDNA) and to display altered functional characteristics. Investigating the downstream consequences of such mitochondrial alterations within cancer cells, specifically the 2008/C13* ovarian carcinoma cell model, was the purpose of this study. The C13* cell variants were derived from 2008 cells following 13 consecutive *cis*-platinum (II)-diammine dichloride (cisplatin) treatments; as well as displaying enhanced cisplatin resistance, C13* variants contain morphologically and functionally distinct mitochondria. The transcription factor E26 transformation specific-1 (Ets-1) was observed to be specifically up-regulated in C13* cells and was thus investigated as a possible nuclear target of mitochondrial generated H_2O_2.

Ets-1 is the prototype of the ETS transcription factor family and is known for its ability to regulate the expression of genes involved in extracellular matrix (ECM) degradation, migration and angiogenesis. Expression of this factor is increasingly associated with aggressive cancer and poor patient outcome, emphasizing the importance of elucidating the mechanisms of aberrant expression in tumor tissues. In this study,

C13* cell variants were demonstrated to produce greater amounts of ROS than the parental 2008 cells as well as to express higher levels of Ets-1 protein. Ets-1 protein levels were further shown to be up-regulated by exogenous H_2O_2. The *ets-1* promoter was investigated in order to delineate the elements which are involved in the H_2O_2 mediated up-regulation. The key promoter element involved in both basal and H_2O_2 induced promoter activity was localized to the previously characterized enhancer region and was identified as an antioxidant response element (ARE).

Consequences of Ets-1 up-regulation in 2008 cells were investigated to determine whether the H_2O_2 mediated up-regulation of this factor would have a feedback effect on the mitochondria, as may be expected for a gene target of mitochondrial initiated signaling. Gene array analysis, comparing the expression of genes in 2008 cells to that in 2008 cells over-expressing Ets-1 revealed that genes encoding antioxidant defense proteins were up-regulated in the Ets-1 over-expressing cells and that several genes encoding mitochondrial proteins were down-regulated, as were genes encoding metabolic enzymes involved in generating substrates for the ETC. The overall suggestion of array results was that Ets-1 over-expressing cells should display altered metabolism, favoring an alternate energy source over oxidative phosphorylation. Such a metabolic switch was confirmed, in that Ets-1 over-expressing cells were observed to be particularly sensitive to the inhibition of glycolysis and to consume significantly less oxygen than parental cells.

This cell model was also used to investigate the effects of Ets-1 expression on cisplatin resistance. The cisplatin resistant phenotype of C13* cell variants has been

proposed to be related to the altered mitochondria within these variants and thus it was hypothesized that the mitochondrial-mediated up-regulation of Ets-1 may be a component of this increased resistance. 2008 cells over-expressing Ets-1 were found to exhibit enhanced resistance to cisplatin, comparable to that of C13* cell variants. A second cell model of Ets-1 over-expression also displayed enhanced cisplatin resistance, demonstrating that this phenomenon was not cell specific.

Overall, this study has identified *ets-1* as a gene target of H_2O_2 via the ARE signaling pathway, a novel finding regarding the regulation of this transcription factor which has implications in cancer research as the aberrant expression of this factor in cancer cells has harmful consequences. Furthermore, novel targets of Ets-1 as well as functional consequences of the increased expression of this factor have been revealed. The altered metabolism of Ets-1 over-expressing cells as well as enhanced cisplatin resistance are further examples of roles of this factor which may promote increased tumor aggressiveness.

Acknowledgements

I would like to thank my supervisor, Dr. Gurmit Singh for the many years of guidance. Dr. Singh has provided me with the support and confidence needed to complete this work. I would also like to thank the members of my supervisory committee, Dr. Richard Austin, Dr. Mary Richardson and Dr. Richard Tozer for all of their encouragement, knowledge and direction. Studentships have been provided over the years from the natural sciences and engineering research council (NSERC), the Ontario graduate scholarship program (OGS) and from McMaster University (Douglas C. Russell scholarship, W.E Rawls memorial scholarship, Lee Nielson Roth award).

As for the students and staff at the Juravinski cancer center, I thank all of you for your help over the years and most importantly for your friendship, you made the long journey worth taking. Special thanks to those who helped me from the beginning- Tom Preston, Jane Barrett, Helga Duivenvoorden, Nishi Singh, Scott Su, Lisa Porter and Steve Innocente; to those who came on board in later years and have been important colleagues and friends- Adam Gemin, Erin Giles, Zeina Saikali, Andrea Hiltz, Stephanie Marra, Ian Hons; and of course those who have been with me all along- Arkan Abadi, Nisha Anand and Howard Cukier. I owe all of you, and many of the other staff and students at the center, thanks for years of support, fun, and friendship.

Most importantly, I would like to thank my family, my parents, Alex and John, Andrew, Jacob and Ella for rooting for me and providing unwavering love and support. Finally- to Mike dePass, thank you for your patience and love over this past year- couldn't have done it without you.

List of Abbreviations

2-DG	2-deoxy-D-glucose
ADP	Adenosine diphosphate
AML-1	Acute Myeloid Leukemia-1
Ant1	Adenine nucleotide translocator 1
AP-1	Activator protein- 1
AP-2	Activating enhancer binding protein-2
ARE	Antioxidant Response Element
ATM	Ataxia Telangiectasia Mutated protein
ATP	Adenosine triphosphate
ATP7A,7B	ATPase copper transporting alpha, beta polypeptides
BARD1	BRCA1 associated ring domain 1
BER	Base Excision Repair
bFGF	basic Fibroblast Growth Factor
Bfl-1	Bcl-2- related protein A1
BRCA1	Breast cancer 1,early onset susceptibility protein
CAMKII	Calmodulin dependent Kinase II
CBP	cAMP-responsive element binding protein (CBP) Binding Protein
CBPα2	CREB binding protein alpha 2
cDNA	Complementary DNA
ChIP	Chromatin Immunoprecipitation
CIAP	Calf intestine alkaline phosphatase
Cisplatin	*Cis*-platinum (II)-diammine dichloride
CMV	Cytomegalovirus immediate-early
CO_2	Carbon dioxide
CTR1	Copper Transporter 1
Cybrid	Trans-mitochondrial hybrid cell
Cyt b	Cytochrome b
DCFH-DA	2'7'- dichlorodihydrofluorescin diacetate
DEPC	Diethylpyrocarbonate
Dm	Median dose
DMEM	Dulbecco's modified Eagles' medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E.Coli	Escherichia Coli
EAPI/Daxx, EAPII	Ets-1 Associated Protein-1, 2
EBS	Ets binding sequence
ECM	Extracellular matrix
EDTA	Ethylenediamminetetraaceticacid
EGF	Epidermal Growth Factor
EMSA	Electrophoretic Mobility Shift Assay
EPO	Erythropoietin
ERCC1	Excision Repair Cross Complementation 1
ERF	Ets-2 Repressor Factor
Erk	Extracellular signal-related kinase

ETC	Electron Transport Chain
ETS	E26 Transformation Specific
FBS	Fetal Bovine Serum
GABP	GA Binding Protein
GADD153	Growth Arrest and DNA Damage inducible gene 153
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GST	Glutathione S-Transferase
H_2O_2	Hydrogen peroxide
HCF	Host Cell Factor
HIF	Hypoxia Inducible Factor
HNPCC	Hereditary Nonpolyposis Colorectal Carcinoma
HRE	Hypoxia Response Element
Hsp 60	Heat shock protein 60
IκB	Inhibitory Factor kappa B
IκK	Inhibitory Factor kappa B Kinase
IL	Interleukin
JNK	c-jun-NH_2-terminal kinase
Keap1	Kelch-like ECH associating protein 1
LacZ	Beta-D-galactosidase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated Protein Kinase
MCS	Multiple Cloning Site
MDR	Multi Drug Resistance
MEF2c	Member of the Myocyte Enhancer Factor 2
MEK-2	Mitogen-activated Protein Kinase Kinase- 2
MMP	Matrix Metalloprotease
mtDNA	Mitochondrial DNA
mtTFA	Mitochondrial Transcription Factor A
mtTFB	Mitochondrial Transcription Factor B
NAC	*N*-Acetyl-L-cysteine
ND1, ND4,ND5	NADH dehydrogenase subunits 1, 4, 5
NER	Nucleotide Excision Repair
NFκB	Nuclear Factor kappa B
NF-E2	Nuclear factor (erythroid derived) factor 2
NO	Nitric oxide
NQO1	NAD(P)H quinone reductase
NRF-1,2	Nuclear Respiratory Factors-1, 2
Nrf2	NF-E2 related factor 2
O_2	Oxygen
Oct-1,2	Octamer binding transcription factors 1, 2
Pax-5	Paired box gene-5
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor

PDGFR	PDGF receptor
PEA3	Polyoma Enhancer Activator-3
Per II	Peroxiredoxin II
PGC-1	Peroxisome-proliferator activated receptor γ Coactivator-1
PI	Phosphatidyl inositol
PIP3	PI 3,4,5 triphosphate
Pit-1	Pituitary-specific transcription factor-1
PKC	Protein Kinase C
PNT	Pointed
PTEN	Phosphatase and tensin homologue detected chromosome ten
PTHrP	Parathyroid Hormone related Protein
PTP	Protein Tyrosine Phosphatase
RAG-2	Recombination activating gene-2
RARE	Retinoic Acid Response Element
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RRE	Ras Responsive Element
rRNA	Ribosomal RNA
RT-PCR	Reverse Transcription- PCR
SDS	Sodium dodecyl sulfate
SO	Superoxide
SSAT	Spermidine/Spermine N'-Acetyl Transferase
SUMO	Small ubiquitin-like modifier
tBHQ	*Tert*-butylhydroquinone
TBST	Tris buffered saline plus Tween-20
TCA	Tricarboxylic acid
Tet	Tetracycline
TetR	Tetracycline repressor protein
TMPD	N,N,N',N'- tetramethyl-*p*-phenylene diamine
TNFα	Tumor Necrosis Factor alpha
TPA	12-O-tetradecanoyl-phorbol-13-acetate
tRNA	Transfer RNA
UCP-1	Uncoupling Protein-1
USF-1	Upstream transcription factor-1
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
VHL	Von Hippel-Lindau protein
wHTH	Winged Helix Turn Helix
XPA	Xeroderma Pigmentosum complementation group A

Table of Contents

1. Introduction **1**

1.1- The role of mitochondria in cancer **1**
1.1.1- Cancer associated mutations to mtDNA 2
1.1.2- Functional changes to cancer cell mitochondria 10

1.2- Mitochondria-generated H_2O_2 behaves as a signaling molecule **14**
1.2.1- Mitochondrial generated ROS 14
1.2.2- ROS as signaling molecules: effect on signaling pathways 19
1.2.3- ROS as signaling molecules: effect on transcription factors 23

1.3- Mitochondria-nucleus crosstalk **8**
1.3.1- Evidence for a nuclear response to mitochondrial stress 28
1.3.2- Mechanism of retrograde regulation 33
1.3.3- Regulation of HIF-1α by mitochondrial ROS: an example of crosstalk.... 39

1.4- The Ets-1 transcription factor **42**
1.4.1- Origin, structure, function 42
1.4.2- Regulation of Ets-1 activity 48
1.4.3- Transcriptional control of the ets-1 gene 54

1.5- Effect of Ets-1 overexpression: correlation with cancer **63**
1.5.1- Ets-1 expression and cancer 63
1.5.2- Ets-1 target genes: possible explanation for link to cancer 68

1.6- Use of an ovarian carcinoma, *cis*-platinum (II) diammine dichloride (cisplatin) resistance, model to study the effect of mitochondrial dysfunction on nuclear gene expression (retrograde regulation) **74**

1.7- Rationale, hypothesis and objectives **78**

2. Materials and Methods
Cell culture 80
Cell treatments 81
Plasmids 82
Construction of pcDNA3-*ets-1* expression vector 83
Construction of pcDNA3-*Nrf2* 86
Construction of full length *ets-1* promoter pGL3 vector, pGL3-*ets-1*-wildtype (wt) 87

Construction of ets-1 promoter truncation plasmids: pGL3-ets-1-1000 and pGL3-ets-1-570 88

Construction of mutant promoter plasmids: mutAP1, mutHRE and mutARE 92
Plasmid replication and purification 95
Stable transfection of inducible *ets-1* gene into 2008 cells and constitutively expressed *ets-1* into HT29 and ES-2 cells 97
Transient transfections and luciferase assays 98
Cell proliferation assay 99
Measurement of intracellular ROS 100
Preparation of whole cell lysates 101
Protein quantitation 101
Treatment of cell lysates with calf intestine alkaline phosphatase (CIAP) 102
Western blot analysis 102
Nuclear lysate preparation 103
Electrophoretic mobility shift assay 103
Oligonucleotide array analysis 105
Data analysis using Affymetrix software 105
Oxygen consumption assay 106
Statistical analysis and IC_{50} calculation 107

3. Results 108

3.1- Evaluation of the levels of intracellular ROS in the 2008 and C13* cell model as well as the expression of Ets-1 in these cells under basal conditions and following exposure to H_2O_2, a reducing agent and mitochondrial inhibitors (Objective 1) 108

3.1.1- Evaluation of intracellular ROS levels in 2008 and C13 cells* 108
3.1.2- Basal levels of Ets-1 protein in C13 and 2008 cells* 110
3.1.3- Regulation of Ets-1 protein levels by H_2O_2 110
3.1.4- Evaluation of the nature of the H_2O_2 mediated up-regulation of ets-1 113
3.1.5- Effect of a reducing agent on Ets-1 expression 114
3.1.6- Effect of mitochondrial inhibitors on Ets-1 expression 115

3.2- Evaluation of the response of the *ets-1* gene promoter to H_2O_2 and determination of the responsive region within this promoter (objective 2) 116

3.2.1- Basal activity of the ets-1 promoter 116
3.2.2- Evaluation of the transcriptional activity of the ets-1 promoter and certain promoter truncations following H_2O_2 treatment 117

3.3- Determination of the elements within the *ets-1* promoter responsible for the H_2O_2 mediated gene up-regulation and characterization of the protein complex which forms on these elements in a H_2O_2 dependent manner (objective 3)................... 120
3.3.1- ets-1 promoter mutants created to delineate H_2O_2 responsive element.... 120

3.3.2- Evaluation of basal and H_2O_2 inducible activity of ets-1 promoter mutants... 120
3.3.3- Characterization of the protein containing complex that binds to the ets-1 ARE in a H_2O_2 inducible fashion... 123

3.3.4- Expression of Nrf2 in 2008 cells... 127
3.3.5- Expression of HIF-1α in 2008 cells... 128

3.4- Evaluation of the effects of Ets-1 overexpression on mitochondrial function (objective 4).. 130
3.4.1- Creation of stable cell lines expressing increased levels of Ets-1.......... 130
3.4.2- Microarray analysis of stable cells overexpressing Ets-1......................... 133

3.4.3-Evaluation of glycolytic capability of stable ets-1 overexpressing cell lines 134

3.4.4- Evaluation of the O_2 consumption by Ets-1 over-expressing cells 140
3.4.5- Effect of Ets-1 overexpression on metabolic properties of ES-2 cells...... 140

3.5- Evaluation of the effect of increased Ets-1 expression on cisplatin resistance (objective 5) 142
3.5.1- Ets-1 is a cisplatin responsive gene... 142
3.5.2- Effect of increased Ets-1expression on the cisplatin resistance of 2008 cells... 145
3.5.3- Effect of overexpression of Ets-1 on the cisplatin resistance of a colorectal carcinoma cell line... 148

4. Discussion.. 151

4.4- H_2O_2 mediated up-regulation of *ets-1*.. 151
4.5- ARE within *ets-1* promoter is H_2O_2 responsive element 156
4.6- *ets-1* promoter ARE is bound by a Nrf2 containing complex......... 165
4.7- Ets-1 as a mediator of mitochondria-nucleus crosstalk................. 170
4.8- Ets-1 as a mediator of cisplatin resistance.................................. 182
4.9- Overall conclusions and significance....................................... 188
4.10- Future directions.. 193

5. References.. 195

6. Appendix... 224

List of figures

Figure 1: Mitochondrial alterations in cancer cells....................................15

Figure 2: Mechanisms of ROS mediated signaling....................................27

Figure 3: Examples of mitochondria to nucleus crosstalk............................43

Figure 4: Schematic diagram of Ets-1...46

Figure 5: Cloning strategy for *ets-1* promoter & *ets-1* promoter truncation constructs.. 91

Figure 6: Use of overlap extension mutagenesis to create site specific mutations in the *ets-1* promoter... 93

Figure 7: Levels of intracellular ROS in 2008 and C13* cells.....................109

Figure 8: Levels of Ets proteins in 2008 and C13* cells.............................111

Figure 9: Specific up-regulation of Ets-1 by H_2O_2 in 2008 and C13* cells......112

Figure 10: Transcriptional up-regulation of *ets-1* by H_2O_2.........................113

Figure 11: Ets-1 is down-regulated by a reducing agent............................114

Figure 12: Up-regulation of Ets-1 by mitochondrial stress in C13* cells........115

Figure 13: Basal and H_2O_2 inducible activity of *ets-1* promoter..................119

Figure 14: Construction and evaluation of *ets-1* promoter mutants............121

Figure 15: ARE mediates the inducibility of the *ets-1* promoter..................122

Figure 16: Gel shift analysis of protein-*ets-1* ARE complex.......................124

Figure 17: Gel shift analysis of *ets-1*, *NQO1* and mutARE complexes (competition assays)...126

Figure 18: Nrf2 expression in 2008 cytoplasmic and nuclear cell fractions.....128

Figure 19: HIF-1α expression in 2008 and C13* cells...............................129

Figure 20: Creation of Ets-1 inducible stable cell lines and evaluation of inducibility/stability...132

Figure 21: Effect of 2-DG on cell growth..138

Figure 22: Growth of cells in glucose free media.......................................139

Figure 23: Oxygen consumption by parental and Ets-1 overexpressing cells...141

Figure 24: Effect of 2-DG on cell growth of ES-2, Ets-1 overexpressing cells...143

Figure 25: Oxygen consumption by ES-2, Ets-1 overexpressing cells..............144

Figure 26: Up-regulation of Ets-1 by cisplatin treatment...............................145

Figure 27: Response of 2008 and C13* cells to cisplatin...............................146

Figure 28: Effect of overexpression of Ets-1 on cisplatin resistance of 2008 cells...147

Figure 29: Effect of overexpression of Ets-1 on cisplatin resistance of HT29 cells...149

Figure 30: Possible mechanisms for the down-regulation of GABP target genes by Ets-1..177

Figure 31: Summary of the role of Ets-1 as a mediator of mitochondria-nucleus crosstalk...192

List of tables

Table 1: Mutations to mtDNA found in cancer cells/tissues..............................4

Table 2: Association between Ets-1 expression and cancer..............................65

Table 3: Primers used in PCR reactions and plasmid construction...................86

Table 4: Genes differentially regulated in 2008 cells stably transfected with Ets-1...... 136

Table 5: Cisplatin IC_{50} values...150

Table 6: Further analysis of Ets-1 overexpression array (genes potentially involved in cisplatin resistance)..187

1. Introduction

1.1- The role of mitochondria in cancer

Mitochondria are distinct intracellular organelles, with an autonomous genome, cell surrounded by a double membrane, consisting of a smooth outer membrane and inner membrane folded into several cristae (Schatz, 1995). These organelles are the site of cellular respiration that is accomplished by oxidative phosphorylation. The respiratory process is performed by a series of proteins along the inner mitochondrial membrane termed the electron transport chain (ETC) (Schatz, 1995). Though the major contribution of mitochondria to the cell is considered to be the production of adenosine triphosphate (ATP) by oxidative phosphorylation, several other metabolic processes also take place within the mitochondria such as the tricarboxylic acid cycle (TCA), the urea cycle and fatty acid β oxidation (Carew & Huang, 2002). Mitochondria have also been recognized as central organelles in the process of programmed cell death, or apoptosis. Proteins involved in this process reside within the mitochondria and are released following apoptotic stimuli (Wang, 2001).

Close to 50 years ago, Otto Warburg first proposed that an injury to the mitochondria that leads to depressed ETC function, and a resulting defect in respiration, is an important step in carcinogenesis (Warburg, 1956). As a result of such an impairment, cancer cells become more reliant on glycolysis as a source of ATP, differing from normal cells which preferentially produce ATP via oxidative phosphorylation (Warburg, 1956). Since that time, Warburg's ideas have been supported by the observations of altered metabolism in numerous cancer cell types. In addition, several

other cancer associated mitochondrial defects have been reported including mutations to mitochondrial deoxyribonucleic acid (mtDNA), increased production of reactive oxygen species (ROS) and altered expression of mitochondrial enzymes involved in both glycolysis and oxidative phosphorylation (Carew & Huang, 2002). These observations indicate that mitochondria have the potential to play a pivotal role in the tumorigenic phenotype.

1.1.1- Cancer associated mutations to mtDNA.

The mitochondria of human cells possess a genome independent of that of the nucleus. The mitochondrial genome consists of approximately 16, 569 base pairs in a variable number of copies per cell (100-1000) and is organized in a double stranded circular molecule (Penta *et al.* 2001). This circular mtDNA molecule encodes 37 genes including 13 coding for components of the ETC, 22 transfer ribonucleic acids (tRNAs) and 2 ribosomal RNAs (rRNAs) required for the synthesis of these components (Chinnery & Turnbull, 1999). mtDNA also contains a non-coding region which includes three hypervariable regions and a displacement (D) loop; the D loop is the location of the origin of replication as well as transcription promoters (Penta *et al.* 2001). MtDNA has a mutation rate that exceeds that of nuclear DNA. Reasons proposed for this increased mutation rate include the lack of protective histone proteins, the fact the mtDNA does not contain introns and is in close proximity to damaging ROS generated via the ETC (Yakes & Van Houten, 1997). In addition, mtDNA lacks repair mechanisms that are readily available to nuclear DNA (Sawyer & Van Houten, 1999). It is thus not surprising that

mitochondria within cancer cells, subjected to DNA damaging carcinogens, frequently harbor mtDNA mutations (Penta *et al.* 2001).

Evidence of mtDNA alterations has been reported in a wide range of cancer types (summarized in table 1). The most frequent site of mtDNA mutations is within the D loop, (control region of mtDNA), where a mutational hot spot termed the D310 tract, a mononucleotide cytidine repeat at position 310, is frequently deleted or mutated (Carew & Huang, 2002; Penta *et al.* 2001). As the D loop is involved in replication, it can be hypothesized that a mutation here will have an effect on copy number. The frequent mutations to this region may be a consequence of the structure of this polycytidine stretch which appears to be more susceptible to mutation from DNA damaging agents and lags behind other DNA regions in terms of repair capacity (Mambo *et al.* 2003). This particular region was examined in a range of cancers using a PCR method to amplify a 109 base pair fragment containing the D310 site (Parella *et al.* 2003). Of 56 samples screened, including tumors of the bladder, breast, and cervix, 23% presented D310 abnormalities, with the highest frequency observed in bladder cancer (35%). However, occurrence of these mutations did not correlate to any clinical characteristics examined, drawing into question the functional consequence of such D loop mutations (Parrella *et al.* 2003). A breast cancer specific study identified the D310 region as a mutational hotspot in this form of cancer (Parrella *et al.* 2001). 19% of the specimens analyzed were found to have abnormalities, the majority of which were 1 or 2 base pair deletions or insertions within the D310 region (Parrella *et al.* 2001). Evaluation of the D loop region of mtDNA from these breast cancer samples again involved PCR amplification of the

Table 1: Mutations to mtDNA found in cancer cells/tissues

Type of Cancer	Region mutated	% samples tested with mutation(s)	Clinical consequences of mutation, comments	Reference
Breast	-D loop, ND1, ND2, ND5, Cyt b	-61	-none reported	-Parrella *et al.* 2001
	-D loop,16S rRNA, ND4, ATPase 6	-74	-none reported	-Tan *et al.* 2002
	-D loop	-15	-no correlations to clinical characteristics found	-Parella *et al.* 2003
Ovarian	-Dloop, 12S rRNA, 16S rRNA, Cyt b	-75	-none reported	-Liu *et al.* 2001
Colorectal	-12S rRNA, 16S rRNA, ND1, ND4L, ND5, Cyt b, COXI, COXII, COXIII	-70	-none reported	-Polyak *et al.* 1998
	-D loop, ND1, ND5	-44	-none reported	-Habano *et al.* 1999
	-D loop	-34	-no correlations to clinical characteristics found	-Guleng *et al.* 2005
	-D loop	-36	-overall survival of patients with mutations lower; loss of benefit of adjuvant therapy when mutation present	-Lievre *et al.* 2005
Gastric	-D loop, ND1, ND5, COXI	-44	-none reported	-Maximo *et al.* 2001
Hepatic	-D loop	-39	-decrease in mtDNA copy number in patients, particulary if harbored mutation (71%)	-Lee *et al.* 2004
	-D loop	-50	-significant up or down regulation of mtDNA content where mutation present; no correlation to clinical characteristics found	-Wong *et al.* 2004
Esophageal	-D loop	-40	-adenocarcinoma of Barretts's esophagus tested, no correlations reported	-Miyazono et al. 2002
	-D loop	-5	-none reported	-Hibi *et al.* 2001
	-D loop	-34	-no correlation to microsatellite instability	-Kumimoto *et al.* 2004
Pancreatic	-D loop, 12S rRNA, 16S rRNA, ND1, ND2, ND4, ND4L, ND5, ND6, Cyt b, COXI, COXII, COXIII, ATPase 6	-high % (exact not reported)	-increase in mtDNA mass in tumor cells versus normal pancreatic cells noted	-Jones *et al.* 2001
Cervical	-D loop	-15	-no correlation to clinical characteristics found	-Parrella *et al.* 2003
Endometrial	-D loop, 12S rRNA, 16S rRNA	-56	-none reported	-Liu *et al.* 2003
	-D loop	-16	-none reported	-Parella *et al.* 2003

Prostate	-D loop, 16S rRNA ND1, ND4	-19	-clinical status of patients with mutations indicates mutations early in disease progression	-Jeronimo *et al.* 2001
Thyroid	-D loop, 12S rRNA, 16S rRNA, tRNA: Asn-Arg-Leu, ND1, ND2, ND3, ND4, ND4L, ND5, ND6, Cyt b, COXI, COXII, COXIII, ATPase 6	-23	-Most mutations identified in complex I	-Yeh *et al.* 2000
	-ND2, ND4, ND5, ND6, tRNA Ser, COXIII	-37 in primary carcinomas; 25 in multinodular hyperplasias	-most mutations in complex I, complex I activity decreased in thyroid cancer cell lines	-Abu-Amero *et al.* 2005
	-D loop	-6.9	-no correlation to histological grade	-Tong *et al.* 2003
Brain	-Dloop, tRNA: Asp-Thr, COXI, COXII, ND4	-40	-medullablastoma investigated, patients with no detectable mutations at end of therapy continued disease free	-Wong *et al.* 2003
Bladder	-D loop	-25	-no correlations to clinical phenotypes found	-Parella *et al.* 2003
Gall bladder	-D loop	-38	-high % of normal and dysplastic epithelia also harbor mutations, mutations assumed to occur early in pathogenesis	-Tang *et al.* 2004
Non small cell lung cancer	-D loop	-35	-Mutation rate higher in late stage patients and in those with metastatic lesions; poorer prognosis when mutation present	-Matsuyama *et al.* 2003
Acute lympho-blastic leukemia	-tRNA Thr, ND5, COXII, ATPase 6	-37	-none reported	-Ivanova *et al.* 1998
	-D loop	-58	-none reported	-Grist *et al.* 2004
Head and neck	-D loop	-37	-probability of mutation increases with increasing severity of dysplasia	-Ha *et al.* 2002
Melanoma	-D loop	-45 tumor samples; 42 cell lines	-no correlations to clinical characteristics found	-Takeuchi *et al.* 2004
Renal	-D loop, ND2, ND4, ND5, tRNA Ala, mtTFI binding site, 16S rRNA	-78	-none reported	-Nagy *et al.* 2003

D310 region. This type of analysis will not detect any other mtDNA mutations and may not be indicative of the true percentage of tumors with mitochondrial abnormalities. To address this issue, the entire mitochondrial genomes of 18 tumor samples were sequenced. Mutations were found in 61% of the tumors sequenced, but not in adjacent normal tissue from the same individuals indicating that these mutations were somatic. Though the majority of mutations were found to be within other areas of the D loop, examples of mutations in the coding regions of NADH dehydrogenase subunits 1, 4 and 5 (ND1, ND4, ND5) as well as within cytochrome b (cyt b) were also observed (Parella *et al.* 2001).

The D loop was also found to be a hot spot for mutations to the mtDNA of colorectal cancers (Guleng *et al.* 2005). Tissue samples from 95 patients with colorectal cancers were analyzed for alterations within the D310 region as well as 3 other sites of monomeric repeats, all of which are considered to be sites of mtDNA microsatellite instability (i.e. sites where there are frequent changes in the length of a repetitive sequence). These 95 tissue samples were compared to mitochondria from corresponding blood samples as well as to 95 healthy controls. It was shown that 34% of the cancerous tissues contained mutations within the D310 region, whereas only 2% of normal controls showed differences. Again in this study, no link between clinical phenotypes and existence of D loop mutations were noted (Guleng *et al.* 2005).

Most studies on mtDNA mutations in cancer cells have been unable to correlate mutations to the D loop to clinical characteristics, however there are exceptions. One such study focusing on colorectal cancers reported that 36% of the 365 samples tested

contained mutations in the D310 region (Lievre *et al.* 2005). Though the existence of these mutations was not associated with tumor stage or microsatellite instability, the overall survival of patients with D loop mutations was found to be significantly lower than that of patients who did not harbor D loop mutations within their mtDNA. A role for the D loop in chemotherapy resistance was also concluded as adjuvant therapy for stage III patients was seen to be beneficial only if no D loop mutations were present (Lievre *et al.* 2005).

Mitochondrial D-loop mutations have also been associated with cancer progression. A study of pre-malignant lesions of the head and neck identified a link between D loop mutation and cancer progression (Ha *et al.* 2002). Of 137 isolated lesions, 37% had some form of alteration to the D310 region and it was noted that the probability of such an alteration increased with the severity of dysplasia indicating that much like nuclear DNA, mtDNA is subject to alterations early in the progression of head and neck cancers (Ha *et al.* 2002). In a prospective study done using tissue from 202 non-small cell lung cancer patients, 70% of the tissue samples screened were revealed to contain D loop mutations (Matsuyama *et al.* 2003). The mutation rate was higher in patients with late stage cancers, as well as in patients with metastases. Furthermore, patients at later stages of disease had poorer prognosis if they had D loop mutations within their mitochondrial DNA (Matsuyama *et al.* 2003).

Further examples of studies in which a high frequency of D loop mutations have been found in cancerous tissue include those examining hepatocellular carcinomas, acute leukemia, thyroid cancers, malignant melanoma, gall bladder carcinoma, esophageal

carcinoma, medulloblastoma, renal cell tumors and endometrial carcinomas (Wong *et al.* 2004; Lee *et al.* 2004; Grist *et al.* 2004; Takeuchi *et al.* 2004; Tong *et al.* 2003; Tang *et al.* 2004; Kumimoto *et al.* 2004; Wong *et al.* 2003; Nagy *et al.* 2003; Liu *et al.* 2003). In addition to mutations which involve the D loop, genes encoding components of the ETC as well as rRNA or tRNAs may be mutated. For example, in thyroid cancer the most common type of mtDNA mutation found is in genes encoding components of complex I and the activity of this ETC complex has been found to be defective in several different thyroid cancer cell lines (Abu-Amero *et al.* 2005)

Whether or not mutations to mtDNA mutations will lead to functional changes in the mitochondria of cancer cells underlying tumorigenesis is a contentious issue. The few studies that have found that such mutations have a negative effect on cancerous tissue have been cited, there are however many more which find no such correlation (summarized in table 1). In fact, some investigators do not accept that mtDNA mutations found in cancerous tissues are actually different in incidence to those found in the general population (Vega *et al.* 2004). A study examining mtDNA mutations in cancers of the central nervous system evaluated changes to the D loop region in tissue from 69 different patients- 39 % were found to have changes in tumor D loop sequences as compared to their blood, however all of the polymorphisms noted are also seen in the general population (Vega *et al.* 2004). These authors also claim that a thorough search of the literature indicated that other D loop polymorphisms reported in cancers are similarly found in the general population. Overall, this group has hypothesized that the mutations seen in cancer cell mtDNA arise as a result of random segregation during tumor

development and play no causal role in tumorigenesis (Vega *et al.* 2004). A similar conclusion was made by Rosson & Keshgegian who conducted a study of 15 breast cancer specimens that were separated from normal tissue by laser capture techniques (Rosson & Keshgegian, 2004). The hypervariable and D loop regions of isolated mtDNA from these tissues were evaluated by PCR amplification of the regions of interest. Findings indicate that though there is a high rate of mutation in cancerous tissue when compared to the accepted sequence of the mitochondrial genome, the same can be said for the normal tissue evaluated (Rosson & Keshgegian, 2004).

Many of these investigations fail to sequence the entire mitochondrial genome, exposing serious limitations to the conclusions that can be drawn. Though mutations to the D loop are frequent and potentially confer functional differences to the mitochondria, there are far fewer studies providing evidence for mutations to coding regions of mtDNA leaving gaps in the current understanding of mtDNA mutations in cancer. It is also very difficult to study the functional consequences of such mutations as no experimental protocols have yet been established which allow for the direct manipulation of mtDNA and/or gene expression.

One approach which has provided some evidence for a functional role of mtDNA mutations in tumorigenesis makes use of trans-mitochondrial hybrid cells (cybrids) (Petros *et al.* 2005). These cells are generated by depleting a cell line of mtDNA and fusing it with an enucleated cell. This approach was used in an attempt to answer whether mtDNA mutations contribute to tumorigenesis in prostate cells (Petros *et al.* 2005). The assumption by these investigators is that a mutation to a component of the

ETC will lead to inefficient electron transport, increased production of ROS and as a result, tumor promotion. In order to test this theory, mtDNA was deleted from a prostate cancer cell line and cells were fused with mitochondria harboring a mutation in the ATP6 gene, presumed to affect ETC function and ROS production. These cells were injected into nude mice, as were cybrids made with wild type mitochondria. It was noted that the average resulting tumor volume was significantly higher at every time point in the mice injected with cybrids containing defective mitochondria (Petros *et al.* 2005). Similar studies using HeLa cells devoid of mtDNA, fused to mitochondria with the same ATP6 gene mutation indicated that tumors derived from mutant cybrids were larger and grew faster than those from cells with wildtype mtDNA (Shidara *et al.* 2005). In this particular study an attempt to address the mechanism identified that the mutated mtDNA tended to suppress apoptosis (Shidara *et al.* 2005). Combining respiratory deficient mtDNA with nuclear DNA backgrounds does not always promote tumorigenicity however. No differences were found when such an experiment was performed in mouse fibroblasts indicating that the tumor phenotype likely relies on contributions from both mitochondrial and nuclear DNA (Akimoto *et al.* 2005). Obviously the issue of whether mtDNA mutations affect cancer cell promotion and progression is difficult to answer with the techniques currently available; however the high incidence of such changes to the mitochondria of cancer cells cannot be ignored and continues to be of interest to cancer researchers.

1.1.2- Functional changes to cancer cell mitochondria

Though they cannot be clearly related to mtDNA mutations, several functional changes to cancer cell mitochondria have been observed and further implicate this organelle in tumor formation. These alterations include increased production of ROS, decreased oxidative phosphorylation and a corresponding increase in glycolysis (Carew & Huang, 2002).

The increased production of ROS, including superoxide (SO) and hydrogen peroxide (H_2O_2) by cancer cells is likely a result of mitochondrial dysfunction. Four to five percent of the oxygen (O_2) consumed by oxidative phosphorylation in the mitochondria is converted to ROS under normal conditions, therefore defects to the electron transport system in cancer cells would likely result in greater than normal levels of ROS formation (Szatrowski & Nathan, 1991; Konstantinov *et al.* 1987). The elevation of ROS in several different cancer cell types is inhibitable by the specific H_2O_2 detoxifiying enzyme, catalase, implying that H_2O_2 is the particular ROS produced (Szatrowski & Nathan, 1991). There is also evidence that there is an imbalance of antioxidant enzymes in many cancer cell types which would add to the oxidative stress within the tumor environment (Oberley & Oberley, 1997). Evidence of such imbalances was noted in both mouse and human cancer cell lines which were generated to be highly tumorigenic as compared to matched, less malignant cell lines (Policastro *et al*, 2004). As well as displaying higher clonogenic capacity and anchorage independent growth, the more malignant cell lines had increased superoxide dismutase, (H_2O_2 generating), activity and decreased catalase and glutathione peroxidase, (H_2O_2 detoxifying), activities. The expected resulting increase in H_2O_2 production by these cell lines was confirmed, as was

a decrease in SO levels (Policastro *et al.* 2004). Similar imbalances in antioxidant enzymes were noted in melanoma cells as compared to melanocytes as well as in lung cancer cells versus normal lung cells (Picardo *et al.* 1996; Chung-man *et al.* 2001).

The metabolic changes seen in tumor cells can also be related to mitochondrial alterations. A higher glycolytic rate, noted in cancer cells of virtually all tissue types, is likely due to the increased expression of glycolytic enzymes as well as a decreased expression or activity of oxidative phosphorylation enzymes (Dang & Semenza, 1999). One key example of reduced expression of an enzyme involved in oxidative phosphorylation within cancer cells involves the deceased expression of ATP synthase β, a subunit of ATP synthase, which is the final complex of the ETC and catalyst for ATP synthesis. The expression of this enzyme has been evaluated in liver, kidney, colon, breast, gastric, lung and esophageal cancers and found to be drastically reduced as compared to normal tissues (Cuezva *et al.* 2002; Isidoro *et al.* 2004). In these studies, levels of heat shock protein 60 (Hsp 60), a general mitochondrial marker, were also evaluated as were glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels as an indication of glycolytic capability. The combined expression levels of these three proteins were used to establish a bioenergetic index of tissues studied (compromising ATP synthase β/Hsp 60 relative to GAPDH levels). A general decrease in mitochondrial biogenesis in liver carcinomas was observed as both mitochondrial proteins were reduced and the glycolytic enzyme level was increased (Cuezva *et al.* 2002). In all other cases, Hsp 60 levels were either not affected or increased in cancer tissue, a simultaneous decrease in ATP synthase β and typically an increase in GAPDH resulted in the

classification of cancer cells as having a decreased bioenergetic index as compared to normal tissue (Cuezva *et al.* 2002; Isidoro *et al.* 2004).

The evidence for the down-regulation of ATP synthase β in cancer cell mitochondria is most compelling, however other oxidative phosphorylation enzymes as well as metabolic enzymes which would promote this energy forming pathway are also down-regulated in cancer cells (Unwin *et al.* 2003; Pederson *et al.* 2002). One example is in renal cell carcinoma where a proteomic approach was used to identify proteins differentially expressed in carcinoma tissues as compared to matched normal tissue (Unwin *et al.* 2003). Changes confirmed in 6 different patients included an increase in several glycolytic enzymes in cancerous tissue and a downregulation of mitochondrial enzymes involved in oxidative phosphorylation as well as amino acid metabolism, the urea cycle and gluconeogenic enzymes (Unwin *et al.* 2003).

One of the key glycolytic enzymes whose expression is modified in cancerous tissue is that which catalyzes the first step of glycolysis, hexokinase (Pederson *et al.* 2002). This alteration may be related to mitochondrial changes within cancer cells since hexokinase II, an isoform typically expressed at low levels only in muscle and adipose tissue, is aberrantly localized to the mitochondria in several cancer cell lines (Pederson *et al.* 2002). This protein binds to the porin protein complex on the outer mitochondrial membrane which provides several advantages to the enzyme: easy access to ATP generated by oxidative phosphorylation, protection from proteolytic degradation and reduced sensitivity to endogenous inhibitors (Pederson *et al.* 2002). The mitochondrial localization of this enzyme also protects cells from apoptosis by inhibiting Bax induced

cytochrome c release and hence apoptosis (Pastorino *et al.* 2002). The up-regulation of hexokinase may be via transcriptional control as characterization of the promoter indicated that the promoter is activated by glucose, insulin, glucagons, p53 as well as hypoxia (Lee *et al.* 2003). This response appears to be mediated via a small region consisting of 4 Sp1 binding sites, a CREB binding site as well as an E2F binding site. Sp1 may be an important regulator as it has been shown to be phosphorylated in the presence of glucose (Lee *et al.* 2003). As well as promoter activation, gene amplification may also be involved in hexokinase II up-regulation within cancer tissues (Pederson *et al.* 2002).

With the number, and variability, of alterations seen in cancer cell mitochondria (summarized in figure 1), there are clearly reasons to evaluate more closely the role of mitochondria in tumorigenesis. This project has focused on the increased production of mitochondrial ROS by tumor cells and possible downstream effects on the nucleus.

1.2- Mitochondria-generated H_2O_2 behaves as a signaling molecule

1.2.1- Mitochondrial generated ROS

In the previous section, the increased production of ROS by tumor cells was described (Szatrowski & Nathan, 1991). This property may be linked to mitochondrial injury as the mitochondria are considered the major cellular source of ROS (Klaunig & Kamendulis, 2004). The production of ROS is an un-avoidable consequence of aerobic metabolism. Though the majority of O_2 consumed in the reaction will be reduced to water, an estimated 4 to 5% is converted to ROS (Klaunig & Kamendulis, 2004). This conversion occurs when electrons escape from the ETC and react with O_2 to produce

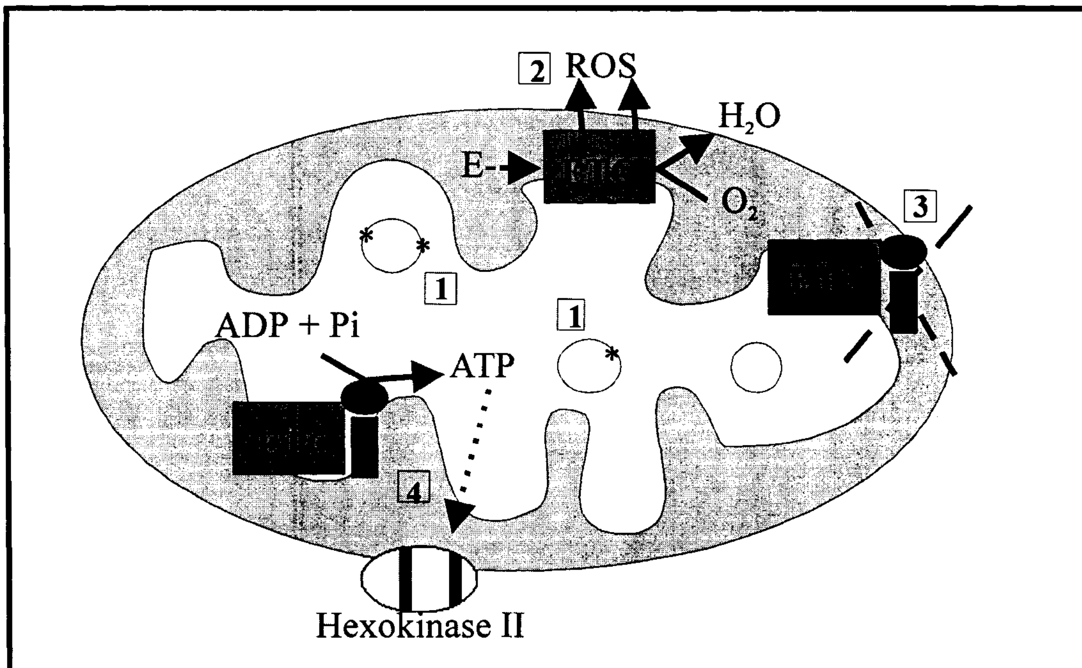


Figure 1: Mitochondrial alterations in cancer cells. Examples of alterations frequently observed in the mitochondria of cancer cells are outlined: 1) Mutations to circular mitochondrial genome frequently observed- particularly in control D loop region. 2) Elevated levels of ROS due to inefficient electron transfer along the ETC. 3) Down-regulation of ATPsynthaseβ, a subunit of the final enzyme involved in oxidative phosphorylation. 4) Translocation of hexokinase II, an enzyme involved in the first phase of glycolysis, to the outer mitohcondrial membrane where it has easy access to ATP generated by oxidative phosphorylation.

SO. SO is likely then converted to H_2O_2 in a reaction catalyzed by superoxide dismutase and a subsequent conversion to hydroxyl ion occurs in certain conditions (Barber & Harris, 1994; Betteridge, 2000). Though there are at least nine possible mitochondrial sites of electron leakage, and hence ROS production, the best described are complexes I and III of the ETC (Andreyev *et al.* 2005).

Complex I, NADH-ubiquinone oxidoreductase, is located within the inner mitochondrial membrane where it is able to oxidize NADH derived from the TCA and

utilizes coenzyme Q as an electron acceptor (Andreyev *et al.* 2005). Early studies indicated this complex is capable of generating SO in the presence of NADH (Cadenas *et al.* 1977). The generation of SO and other ROS from complex I is considered to be the most physiologically relevant site of ROS production via electron leakage (Liu *et al.* 2002; Sipos *et al.* 2003). By examining the production of H_2O_2 from rat liver isolated mitochondria in the presence of various respiratory substrates and inhibitors, it was demonstrated that succinate, a FAD- linked substrate which enters the ETC at complex II, causes the highest rate of H_2O_2 production in the absence of inhibitors (Liu *et al.* 2002). Although this was originally thought to be via electron leakage at complex III, it was noted that rotenone, a specific inhibitor of complex I, abolished succinate derived H_2O_2 production. It was therefore deduced that the succinate driven H_2O_2 production was due to reverse electron transfer in the absence of adenosine diphosphate (ADP), hence electrons transferred backwards along the ETC and electron leakage in this situation occurs at complex I (Liu *et al.* 2002). A later study using synaptic mitochondria confirmed that both inhibitors of complex I (rotenone) and complex III (antimycin) led to increases in H_2O_2 production, however the amount of inhibition of complex activity required to incite an increase in H_2O_2 was quite different (Sipos *et al.* 2003). Complex III could be inhibited by 70-80% before a noticeable increase in H_2O_2 was noted whereas a 16% decrease in activity of complex I was sufficient to induce a large increase in H_2O_2 production, again indicating that this site of ROS production is more physiologically relevant (Sipos *et al.* 2003). Increased production of ROS using these inhibitors was also confirmed in cardiomyocytes (Vanden Hoek *et al.* 1997).

The greater effect of complex I inhibition on H_2O_2 production can, however, be interpreted in different way- as an example of the importance of complex III as a ROS generation site (Chen *et al.* 2003B). Complex III, or ubiquinone:cytochrome c reductase, is the ETC site of coenzyme Q oxidation with cytochrome c as the electron acceptor. This complex is also generally accepted as a site of SO and subsequently H_2O_2 production (Andreyev *et al.* 2005). Findings using isolated rat heart mitochondria indicate that inhibiting complex III following administration of complex I substrates will induce H_2O_2 production, an effect lost if complex I activity is inhibited (Chen *et al.* 2003B). These data have been interpreted as indicating that a blockade of electron flow at complex I prevents ROS accumulation because the flow is unable to reach complex III, the major site of ROS production (Chen *et al.* 2003B). Regardless of the site of mitochondrial ROS generation, the effect of ETC inhibition on ROS generation is evident.

It is generally assumed that the effect of specific inhibitors of ETC complexes imitates the physiological consequence of mitochondrial injury, such as those seen in tumor cells; however it is difficult to demonstrate a direct correlation between such mitochondrial injuries and increased ROS experimentally. One model that attempted to address this issue involved the use of an adenine nucleotide translocator (*Ant1*) knockout mouse (Esposito *et al.* 1999). The product of *Ant1* catalyzes the exchange of ADP and ATP across the inner mitochondrial membrane; accordingly loss of this gene leads to a depletion of matrix ADP and a resulting inhibition of ATP synthase (Esposito *et al.* 1999). These mice display oxidative phosphorylation defects in several tissues where

Ant1 is typically expressed, and represent a model of mitochondrial injury. The generation of ROS by mitochondria from these mice was tested and found to be elevated, as was the expression of antioxidant defense enzymes, elevated in order to compensate for the increased oxidative stress. It was also observed that the mtDNA from these cells accumulated damage more rapidly than did mtDNA from wild type cells, indicating that increased ROS produced by damaged mitochondria will cause further mitochondrial injury perpetuating a vicious cycle (Esposito *et al.* 1999).

The mitochondria are by no means the only cellular source of ROS. Other identified sources include the endoplasmic reticulum where electrons are leaked from the NADPH cytochrome P450 reductase as well membrane bound xanthine oxidase, NADPH oxidase, lipooxygenase or cyclooxygenase enzymes (Kamata & Hirata, 1999). The levels of ROS within cells are also tightly controlled by antioxidant enzymes which have evolved to combat ROS toxicity, these include molecules such as glutathione and thioredoxin which act as redox buffering systems as well as enzymes capable of scavenging SO (superoxide dismutase) and H_2O_2 (catalase) (Pelicano *et al.* 2004; Martindale & Holbrook, 2002). If defense mechanisms are not adequate to counteract the production of ROS, as is often observed in cancer cells, the cells can be considered to be in a state of oxidative stress (Martindale & Holbrook, 2002). At high levels, several deleterious effects have been attributed to ROS, such as damage to DNA, protein and lipids. Low concentrations however, appear to have a mitogenic effect through various signaling pathways (Burdon, 1995 Pelicano *et al.* 2004; Martindale & Holbrook, 2002), thus the increased production of ROS from injured mitochondria within cancer cells may

activate cell proliferation leading to enhanced tumorigenesis (Szatrowski & Nathan, 1991).

1.2.2- ROS as signaling molecules: Effect on signaling pathways

The basic mechanism by which cells are induced to proliferate involves the interaction of growth factors with receptors on the cell membrane followed by the activation of a downstream protein tyrosine kinase, or a proximal membrane phospholipase, further leading to mitogen-activated protein kinase (MAPK) stimulation and finally the activation of proteins directly involved in proliferative control (Burdon, 1995). The mitogenic effect of SO and/or H_2O_2 has been observed in a range of cell types, where low concentrations of these species stimulate growth of cells such as hamster, rat, and human fibroblasts, as well as various mouse cell lines, human leukemia and smooth muscle cells (Burdon, 1995). In our lab, it was noted that the growth of a rat fibroblast cell line is inhibited by the scavenging of H_2O_2 with catalase, further implicating ROS in the proliferative response (Preston *et al.* 2001). The concept that H_2O_2 behaves as a growth factor is now accepted and mechanisms for this effect have been thoroughly investigated. Interactions with receptors, tyrosine and serine/threonine kinases, phosphatases as well as transcription factors have all been identified as components of this H_2O_2 mediated response (Kamata & Hirata, 1999; Lander, 1997; Burdon, 1995).

Evidence that H_2O_2 has a direct effect on proteins downstream of various receptors comes from studies indicating that growth factor administration actually induces a spike of H_2O_2 within cells which is necessary for receptor mediated effects.

This has been clearly demonstrated with the platelet derived growth factor (PDGF) receptor (PDGFR) (Sundaresan *et al.* 1995). Studies using vascular smooth muscle cells *in vitro* indicated that spikes in H_2O_2 levels occurred following PDGF addition, and that this increased H_2O_2 was required to initiate PDGF induced downstream events (Sundaresan *et al.* 1995). The role of H_2O_2 as an activator of the PDGFR was also demonstrated using *peroxiredoxin II* (*perII*) knockout mouse embryonic fibroblasts (Choi *et al.* 2005). PerII has a high affinity for H_2O_2 and is capable of eliminating this ROS produced in response to PDGF stimulation. Using knockout cells, it was demonstrated that in the absence of *perII*, PDGF stimulation caused a two-fold increase in H_2O_2 production as well as increased tyrosine phosphorylation of PDGFR as compared to wild type cells. Two distinct phosphorylation residues within the PDGFR specifically displayed enhanced phosphorylation leading to increased receptor kinase activity (Choi *et al.* 2005). H_2O_2 has also been noted to be capable of stimulating growth factor receptor transactivation and the subsequent activation of downstream events independent of the presence of the particular receptor ligand. This effect has been noted following H_2O_2 administration to the epidermal growth factor (EGF), PDGF and vascular endothelial growth factor (VEGF) receptors (Chen *et al.* 2004). Interestingly, this outcome appears to be dependent on mitochondrial function in that inhibition of the ETC abolishes H_2O_2-induced transactivation of target receptors. The suggestion is that ROS further stimulates the generation of mitochondrial ROS which directly manipulates growth factor receptors (Chen *et al.* 2004)

The main method by which H_2O_2 can affect target proteins involved in signaling pathways is likely via the oxidation and modification of cysteine residues within proteins. ROS can cause the formation of intra or inter-molecular disulfide bonds between cysteine residues leading to altered protein conformation which may then affect association with other proteins (Adler *et al.* 1999). The mechanism by which increased levels of H_2O_2 increase phosphorylation at PDGFR sites, for an example, appears to be via the oxidation and resulting inactivation of PDGF specific phosphatases within the membrane fraction of cells (Lee & Esselman, 2002). Generally, protein tyrosine phosphatases (PTPs) contain an essential catalytic cysteine residue subject to inhibition by H_2O_2 (Lee & Esselman, 2002).

The inhibition of specific phosphatases by H_2O_2-induced oxidation may also provide an explanation for the observed H_2O_2-induced activation of MAPK enzymes. The effect of H_2O_2 on MAPK enzymes was clearly demonstrated in a study done using mouse fibroblast cells (Guyton *et al.* 1996A). H_2O_2 treatment led to a rapid, transient phosphorylation of several cell proteins leading to an evaluation of cellular kinase activity. Kinase activity assays indicated that that the activity of MAPK family members c-jun-NH2 terminal kinase (JNK) and p38 kinase were increased by 3-5 fold following H_2O_2 treatment, while extracellular signal-related kinase (Erk) activity showed a striking 25 fold increase. These observations were further confirmed in a range of cell lines (Guyton *et al.* 1996A). These results have more recently been attributed to an inhibition of phosphatase activity. T cells treated with H_2O_2 display reduced activity of various T cell specific PTPs (CD45, SHP-1 and HePTP0). Ectopic expression of these

phosphatases revealed that SHP-1 was involved in inhibiting Erk and JNK phosphorylation whereas HePTP was involved in the inhibition of Erk and p38 phosphorylation in response to H_2O_2, implicating distinct roles for phosphatases in regulating the H_2O_2-induced activation of different MAPK enzymes (Lee and Esselman, 2002). A serine/threonine protein phosphatase (PP2A) has also been implicated as an H_2O_2 target involved in MAPK activation (Foley *et al.* 2004). In rat brain fractions, MAPK phosphatase activity was largely attributable to PP2A which was found to be reversibly inhibited by H_2O_2, providing another H_2O_2 target linked to MAPK activation (Foley *et al.* 2004).

The MAPK enzymes are not the only kinase targets activated by H_2O_2. Growth factor stimulation also leads to the activation of phosphatidyl inositol (PI) 3-kinase and a resulting production of PI 3,4,5-triphosphate (PIP3). PIP3 is then capable of activating various downstream targets such as the protein kinase Akt. A specific phosphatase, phosphatase and tensin homologue detected chromosome ten (PTEN), is involved in inhibiting PIP3 function (Kwon *et al.* 2004). It was recently shown that H_2O_2 produced by growth factor stimulated cells caused PIP3 production and Akt activation. In these same cells, a fraction of PTEN molecules were transiently oxidized causing enzyme inactivation (Kwon *et al.* 2004). This H_2O_2 mediated oxidation and inactivation of PTEN has also been demonstrated to occur by means of mitochondrial generated ROS (Connor *et al.* 2005). Cell lines engineered to overexpress mitochondrial superoxide dismutase, and thus produce elevated levels of H_2O_2, exhibited enhanced PTEN oxidation, resulting accumulation of PIP3 and activation of Akt. These effects were reversible following the

administration of catalase (Connor *et al.* 2005). Figure 2A depicts the described H_2O_2 signaling effects on kinase pathways.

1.2.3- ROS as signaling molecules: Effects on transcription factors

The above described effects of H_2O_2 on phosphatases which direct the activation of various signaling pathways is by no means inclusive but certainly provides an understanding of how H_2O_2 may ultimately lead to cellular proliferation and other downstream effects. Other H_2O_2 modification targets within the cell that may account for H_2O_2- induced proliferation include transcription factors. Signaling pathways activated by H_2O_2 ultimately effect the phosphorylation and activation of various transcription factors, but H_2O_2 can also directly oxidize and effect the DNA binding ability of various transcription factors. Examples include activator protein 1(AP-1), nuclear factor kappa B (NFκB) and p53.

NFκB has long been recognized as an oxidant sensitive transcription factor, however there are conflicting views on whether the overall effect of an oxidative environment will activate or inhibit activity of this factor. NFκB is an inducible transcription factor dimer made up of Rel proteins p65 and p50 and is involved in the activation of genes required for cell survival as well as differentiation, inflammation and growth (Chen *et al.* 2001). This factor is typically sequestered in the cytoplasm by an inhibitory complex, inhibitory factor kappa B (IκB), which may be dissociated via phosphorylation in response to H_2O_2. Dissociation of the IκB-NFκB complex reveals NFκB's nuclear localization signal, allowing nuclear localization and activation of NFκB target genes (Pahl, 1999). Phosphorylation of IκB is mediated by various IκB kinases,

such as inhibitory factor kappa kinase (IκK) which phosphorylates IκB on serine residues, resulting in ubiquitination and degradation (Martindale & Holbrook, 2002). The theory that NFκB activity is stimulated by H_2O_2 is supported by studies indicating that several antioxidant treatments block the activation of NFκB target genes (Nomura *et al.* 2000). An attempt to elucidate the exact mechanism of NFκB activation by H_2O_2 indicated that it was not through the expected serine phosphorylation and subsequent degradation of IκB, but through the activation of a Syk protein-tyrosine kinase which then led to tyrosine phosphorylation of IκBα (Takada *et al.* 2003). Phosphorylation at this site of IκB resulted in activation of NFκB by means of translocation to the nucleus without an accompanying degradation of IκB (Takada *et al.* 2003). Contradictory reports, indicating that ROS will in fact inhibit the activation of NFκB target genes are based mainly on the effect of oxidation on key cysteine residues within the NFκB DNA binding region required for DNA recognition. NFκB must be in a reduced form to bind DNA, thus oxidizing agents inhibit this binding activity (Matthews *et al.* 1993). The overall effect of an oxidizing environment on NFκB activity is therefore unclear- nuclear localization of this factor from the cytoplasm is favored following H_2O_2-induced phosphorylation events, however once inside the nucleus oxidation will inhibit DNA binding (Martindale & Holbrook, 2002)

AP-1 is another example of a redox sensitive transcription factor with many gene targets capable of inducing cell proliferation (Shaulin & Karin, 2001). AP-1 is a heterodimer made up of Jun and Fos protein family members. The phosphorylation of c-

Jun mediated by MAPK enzymes is a key event in AP-1 activation. Evidently the H_2O_2-induced activation of MAPK enzymes will lead to AP-1 induced proliferation (Klaunig & Kamendulis, 2004). The DNA binding of the c-Jun/c-Fos heterodimer is, however, negatively regulated by oxidation as a highly conserved cysteine residue modulates DNA binding (Abate *et al.* 1990). Therefore, much like the case of NFκB, the overall effect of an oxidizing environment on AP-1 is not obvious.

The tumor suppressor and transcription factor p53, a key player in regulating the expression of genes involved in growth arrest and/or cell death under conditions of cellular stress, is also affected by ROS levels (Martindale & Holbrook, 2002). p53 is another example of a transcription factor with a critical cysteine residue within its DNA binding domain, which is responsive to levels of ROS. Oxidation negatively affects the ability of p53 to bind DNA (Sun *et al.* 2003). However, the regulation of p53 and hence downstream targets is mainly via post-translational modifications such as phosphorylation (Colman *et al.* 2000). Genotoxic stresses, such as oxidative stress, tend to induce this phosphorylation which then stabilizes the protein and prevents nuclear export (Zhang & Xiong, 2001). H_2O_2 is capable of inducing p53 phosphorylation in endothelial cells, resulting in increased p53 levels within cells as well as an increase in the expression of a p53 downstream target (Chen *et al.* 2003A). Various inhibitors of kinases were used to identify the key enzyme involved in p53 phosphorylation under these circumstances and a DNA- dependent kinase [ataxia telangiectasia mutated protein (ATM) kinase], activated via the PDGFβ receptor was found to be uniquely involved (Chen *et al.* 2003A).

Figure 2: Mechanisms of ROS-mediated signaling. Examples of how ROS mediate signaling pathways are outlined: A) The effect of ROS on kinase pathways. 1) ROS is generated by cell membrane receptors following ligand binding. 2) ROS are also generated by mitochondria. 3) Mitochondrial generated ROS can directly activate receptors to produce further ROS, independent of ligand binding. 4) Intracellular ROS act to oxidize and inhibit MAPK specific PTPs which thus fail to dephosphorylate kinase enzymes such as Erk 1,2 which remain in active conformations. 5) Intracellular ROS oxidize and inactivate PTEN which fails to dephosphorylate Akt which remains in an active conformation. 6) Active kinase pathways lead to downstream gene expression changes resulting in enhanced proliferation and survival. B) The effect of ROS on transcription factors. 1) ROS are generated by mitochondria and bound receptors. 2) Intracellular ROS leads to the dissociation of NFκB from IκB and NFκB is free to translocate to the nucleus and activate target gene expression. 3,4) Intracellular ROS cause the phosphorylation of factors like c-Jun and p53 which are then activated to translocate to the nucleus and activate target gene expression. 5) Intranuclear ROS oxidize NFκB, c-Jun, c-Fos and p53 DNA binding residues and reduce DNA binding of these factors and as a result, down-regulates target gene expression.

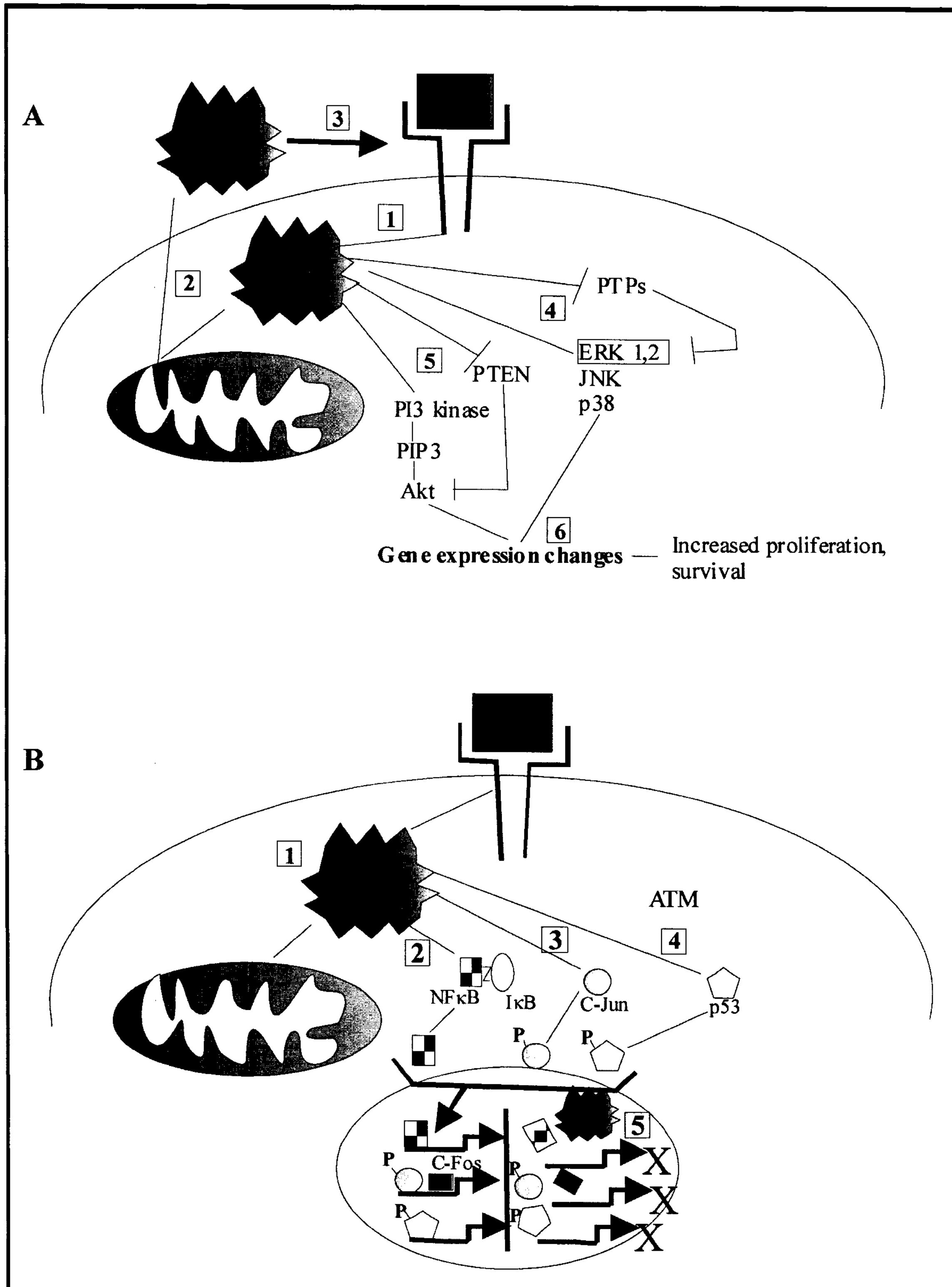


Figure 2: Mechanisms of ROS mediated signaling

Figure 2B summarizes the activation and/or inhibition of transcription factor activity by H_2O_2. Clearly, there are several lines of evidence describing H_2O_2 as a mitogenic signal, mediated by the activation of various signaling pathways. These effects of H_2O_2 no doubt play a part in tumorigenesis, as elevated levels of ROS have been noted within cancer cells (Szatrowski & Nathan, 1991). Other transcription factors not mentioned above, which are activated or repressed by H_2O_2, are those involved in feedback regulation of the mitochondria. These factors are affected by mitochondrial generated ROS and initiate a nuclear encoded mitochondrial response to this stress; in doing so they act as mediators of mitochondria-nucleus crosstalk. The importance of these factors is discussed in the following section.

1.3- Mitochondria-nucleus crosstalk

1.3.1- Evidence for a nuclear response to mitochondrial stress

Mitochondrial generated ROS, as well as other metabolites generated by this organelle, are proposed to behave as instigators of signaling pathways. One such pathway, where a mitochondria-originated signal induces changes in nuclear gene expression and overall mitochondrial and cell function, has been described in the context of mitochondrial biogenesis (Scarpulla, 2002). Though the mitochondria contain their own genome containing genes encoding several components of the ETC as well as 22 tRNAs and 2 rRNAs needed for gene transcription and translation, there is a requirement for coordination with nuclear encoded genes in order to generate a fully functional organelle. Nuclear genes involved in this process include those encoding several other ETC components as well as factors involved in mitochondrial DNA replication and

transcription (Scarpulla, 2002). Circumstances under which the cell may require an up or down-regulation of mitochondrial biogenesis, and therefore instigate communication between the mitochondria and nucleus, include responses to changes in energy demand encountered during exercise training, following thyroid hormone therapy, during oogenesis and adaptive thermogenesis (Williams *et al.* 1987; Pillar & Seitz, 1997; Ricquir & Bouillaud, 2000; Michaels *et al.* 1982). A compensatory response by the nucleus is also expected following damage to, or inhibition of, the mitochondria itself, as likely occurs in cancer cells. The pathway involving a mitochondrial response to stress, followed by a signal to the nucleus, initiating changes in gene expression is a communication pathway termed retrograde regulation (Butow & Avadhani, 2004). Evidence that such a pathway exists comes mainly from studies using mtDNA defective cell lines as well as specific inhibitors of ETC function (Butow & Avadhani, 2004).

Mt-DNA-less cells, referred to as rho^0 cells, are developed by long-term exposure to ethidium bromide which specifically inhibits mtDNA replication and transcription and therefore depletes cells of mtDNA (Wang & Morais, 1997). Changes in nuclear gene expression observed in these cells are attributed to the lack of mtDNA and stress signals that may have resulted from this state. Examples of genes which have been found to be up-regulated in these circumstances include *elongation factor 1 α*, *v-myc* and *β-actin* in an avian rho^0 cell model (Wang & Morais, 1997). Studies with this same model further identified a down-regulation of *mitogen- activated protein kinase kinase 2* (*MEK2)* in rho^0 cells as well as within cells rendered respiration deficient with the use of specific inhibitors, where the down-regulation appeared to be as a result of decreased mRNA

stability (Wang *et al.* 1997). Rho^0 cells made from human myeloid leukemia cells displayed changes in the expression of two key antioxidant enzymes involved in H_2O_2 metabolism; glutathione peroxidase and heme oxygenase 1 were both up-regulated indicating that oxidative stress may be induced by mtDNA depletion, leading to changes in nuclear gene expression which compensate for the altered redox status of the cell (Brambilla *et al.* 1997). Rho^0 HeLa cells were also used to investigate downstream events following mtDNA depletion (Seidel-Rogol & Shadel, 2002). Two key nuclear encoded factors involved in mtDNA replication and transcription, mtRNA polymerase and mitochondrial transcription factor A (mtTFA) were down-regulated following mtDNA depletion and levels began to rise as cells recovered in media without ethidium bromide, indicating that mtDNA replication and transcription machinery respond to levels of mtDNA within the cell (Seidel-Rogol & Shadel, 2002). Both rho^0 myocytes and rho^0 lung carcinoma cells exhibited an up-regulation of several genes implicated in tumor progression, such as *cathepsin L*, *TGF β* and *mouse melanoma antigen*- thus potentially connecting the process of retrograde regulation and tumor progression (Amuthan *et al.* 2001; Amuthan *et al.* 2002). Clear evidence for the occurrence of some form of mitochondria to nucleus communication in rho^0 breast cancer cells was obtained from microarray analysis comparing nuclear gene expression in a mtDNA-less variant of MDA-MB-435 breast cancer cells to that in parental cells. Several groups of genes were noted to be up- or down-regulated in the rho^0 variant including an up-regulation of genes such as *p19*, a cyclin-dependent kinase inhibitor involved in cell cycle arrest, transcription factors and signaling molecules such as *phospholipase c*, down-regulated

genes included those involved in fatty acid signaling (*phospholipase A2*) as well as *cytochrome p450* and many others (Delsite *et al.* 2002).

It should be noted that there is a lack of consistency in the specific genes which display altered regulation in rho^0 cells as compared to their parental cell lines due to differences in the nuclear backgrounds of cells. A study exemplifying this point compared gene expression changes in rho^0 cells created from both human osteosarcoma cells and retinal pigment cells to that in parental cells (Miceli & Jazwinski, 2005). A second model, used in this same study to examine gene expression changes as a result of mitochondrial injury, made use of fibroblast cells from an individual with a specific mitochondrial disease. Gene expression in this case was compared to that in fibroblasts from an unaffected sibling (Miceli & Jazwinski, 2005). Although common groups of genes were affected in all models of mitochondrial dysfunction, such as the up-regulation of genes encoding glycolytic enzymes, mitochondrial proteins as well as antioxidant defense proteins and the down-regulation of genes encoding products involved in lipid metabolism, specific genes affected differed in the respective cell lines. Alhough overall trends in response to mitochondrial stress were noted, it is difficult to pinpoint the specific genes involved (Miceli & Jazwinski, 2005).

Findings in rho^0 cells have been confirmed in cells made respiration deficient by ETC inhibitors such as antimycin A, which specifically inhibits complex III. Exposure to antimycin A caused an up-regulation of both mitochondrial and nuclear encoded ETC components indicating that changes to nuclear gene expression resulting from

mitochondrial stress are coordinated with changes to mitochondrial gene expression in models where mtDNA remains intact (Suzuki *et al.* 1998).

Observed changes in nuclear gene expression as a result of depletion of mtDNA or inhibition of ETC function indicate that there is a mitochondria-derived signal driving these changes. This may be in the form of ROS, produced as a result of inefficient electron transport. ROS are clearly capable of modulating signaling cascades and the inhibition of the ETC leads to the production of increased levels of ROS (Suzuki *et al.* 1998). The identification of genes up-regulated in rho^0 and respiratory deficient cells that are involved in antioxidant defense further implicates ROS as the stimulus for such responses (Brambilla *et al.* 1997; Miceli & Jazwinski, 2005). Another suggested mediator of mitochondrially-generated signals is increased calcium levels, generated from impaired mitochondria. An example of how an injury to the mitochondria could lead to increased cytoplasmic calcium levels, and eventually altered nuclear gene expression is as follows: a stress on the mitochondria such as mtDNA depletion leads to a disruption of the mitochondrial membrane potential followed by decreases in ATP levels which causes a resultant decrease in calcium uptake and calcium efflux and thus increased steady state concentrations of cytoplasmic calcium. Calcium then has several downstream targets such as calcium-dependent kinases, phosphatases and transcription factors which trigger nuclear gene expression changes (Biswas *et al.* 1999). This proposed role for calcium was confirmed in rho^0 myocytes and lung carcinoma cells, both of which displayed increased levels of cytoplasmic calcium and altered nuclear gene expression as compared to the parental cell lines (Biswas *et al.* 1999; Amuthan *et al.*

2002). Evidence that calcium may regulate mitochondrial biogenesis by altering nuclear gene expression comes from human granulosa cells; treatment with calcium in this model led to an increased expression of mtTFA as well as one nuclear and one mitochondrial encoded respiratory subunit (Yeh *et al.* 2005). Other mitochondria-generated signals include metabolites whose levels change as a result of inefficient mitochondrial functioning. Glucose, for an example, positively regulates the expression of mtTFA (Choi *et al.* 2004).

1.3.2- Mechanism of retrograde regulation

Regardless of the nature of a mitochondrial-generated signal, there is clearly a need for factors that will both regulate expression of both nuclear genes and mitochondrial genes if retrograde regulation is to effectively modulate mitochondrial biogenesis in response to environmental changes. Such a system exists in yeast and has been well described, particularly in the context of the regulation of the citrate synthase isoform *CIT2* gene (Butow & Avadhani, 2004). In the case of *CIT2*, expression is up-regulated in cells with dysregulated mitochondria; up-regulation involves the Rtg positive regulatory factors as well as a group of negative regulatory factors (Mks1, Lst8p, Bmh1p, Bmh2p). Rtg2p is the sensor of mitochondrial dysfunction, acts to dephosphorylate an Rtg1p, 3p complex and allows nuclear localization of this heterodimer and resulting activation of target genes with R box elements within their promoters. The existence of several negative regulatory factors adds complexity to the pathway (Butow & Avadhani, 2004).

No clear cut pathways for retrograde regulation has been determined in mammalian cells; however several factors are thought to be important in mediating a nuclear response to mitochondrial stress. For example, the nuclear respiratory factors, nuclear respiratory factor 1 and 2 (NRF-1 and NRF-2) as well as the co-activator peroxisome-proliferator activated receptor γ coactivator-1 (PGC-1) may regulate a coordinated nuclear and mitochondrial response given that these transcription factors regulate the expression of several nuclear genes encoding components of the ETC as well as factors involved in mediating mitochondrial DNA replication and transcription (Scarpulla, 2002).

NRF-1 was first identified following analysis of the *cytochrome c* promoter when a novel factor was found to bind to an enhancer region required for maximal promoter activity (Evans & Scarpulla, 1989). The factor binding this promoter element was recognized to be unique, generated from a single copy gene in vertebrates with no known family members. This novel protein binds its recognition site as a homodimer and acts as a transcription factor via a transactivation domain within its carboxy terminus (Virbasius *et al.* 1993). Though no similar human proteins have been identified, NRF-1 is related to developmental regulatory proteins in sea urchins and homologues in *Drosophila,* chicken, zebra fish and mouse have been identified (Scarpulla, 2002). Since the original identification of NRF-1 as a mediator of *cytochrome c* transcription, NRF-1 consensus binding sites have been identified in several other genes encoding either components of the ETC or factors involved in mitochondrial DNA replication and transcription- examples include subunits of *succinate dehydrogenase*, several *cytochrome oxidase*

subunits, *ATP synthase γ* and *c* subunits as well as mitochondrial transcription factors *mtTFA* and mitochondrial transcription factor B (*mtTFB*) and *mitochondrial ribosomal subunit S12* (Scarpulla, 2002).

The regulation of *mtTFA* as well as several nuclear encoded respiratory subunits indicates that NRF-1 may be an important factor in leading a coordinated response of the nucleus and the mitochondria to an environmental stress. NRF-1 appears to be a critical factor in early mouse embryo development since knocking out this gene was embryonic lethal between 3.5 and 6.5 days (Huo & Scarpulla, 2001). NRF-1 knockout blastocysts were confirmed to have significantly reduced mitochondrial DNA as compared to wildtype cells, furthering evidence for a key role of NRF-1 in directing mitochondrial DNA replication (Huo & Scarpulla, 2001).

A second respiratory factor, responsible for directing expression of several respiratory subunits was later identified when the NRF-1 independent promoter for the *cytochrome oxidase subunit IV* gene was analyzed (Virbasius & Scarpulla, 1991). Within this genes enhancer, several Sp1 binding sites are located in the 5' promoter region; however deletions of these sites had minimal effects on overall transcriptional activity. A repeated sequence was also localized to this area that was critical for activity and bound a novel nuclear factor. This repeated sequence corresponded to a core E26 transformation specific (ETS) binding sequence (EBS) and the newly identified NRF-2 was determined to be capable of binding a well characterized EBS as well as the EBS within the *cytochrome oxidase subunit IV* promoter, indicating that NRF-2 is in fact part of the ETS family (Virbasius & Scarpulla, 1991).

NRF-2 has since been identified as the human homologue of mouse GABP (GA binding protein) (Gugneja *et al.* 1995). GABP is unique among ETS proteins in that it is an obligate multimeric complex, consisting of α and β subunits that combine to form a heterotetramer. The α subunit contains the ETS DNA binding domain, and the β subunit contains ankyrin repeats involved in the interaction with the α subunit as well as the homodimerization of β subunits required for tetramer formation. Moreover, the transactivation domain lies in the β subunit (Rosmarin *et al.* 2004). GABP exists as a stable heterodimer in solution and the presence of adjacent ETS sites allows for stable heterotetramer formation (Chinenov *et al.* 2000). The list of GABP/NRF-2 regulated genes now includes several *cytochrome oxidase* subunits, *ATP synthase β, succinate dehydrogenase* subunits, *mtTFA* and *mtTFB* as well as *mitochondrial ribosomal S12*. Most NRF-2 regulated genes also contain an NRF-1 recognized site, however there are examples of uniquely NRF-2 regulated genes (Scarpulla, 2002). A GABPα knockout model indicates that loss of this gene is also embryonic lethal, indicating it is an essential factor and cannot be replaced by other ETS proteins (Ristevski *et al.* 2004). It should be noted that both NRF-1 and GABP/NRF-2 regulate a myriad of other genes besides those involved in respiratory chain function including enzymes involved in purine nucleotide biosynthesis, a chemokine receptor, neural and poliovirus receptors as well as myeloid specific genes, interleukin-1 (IL-1) receptor enhancer, IL-16 and the nicotine acetylcholine receptor (Scarpulla, 2002; Rosmarin *et al.* 2004).

The proposal that NRFs may be responsible for directing a coordinated nuclear and mitochondrial response to mitochondrial stress would indicate that expression and/or

activity of these factors must be responsive to mitochondrial generated signals. In the case of NRF-1, phosphorylation is required for translocation to the nucleus where a homodimer is formed on target gene promoters (Gugneja & Scarpulla, 1997). This phosphorylation may be induced by increased oxidative stress, likely generated by dysregulated mitochondria. Lipopolysaccharide (LPS) is an agent that depletes glutathione, stimulates ROS generation and has a deleterious effect on hepatic mitochondria (Suliman *et al.* 2003). LPS induced damage caused an increase in NRF-1 protein expression and binding activity in hepatic cells (Suliman *et al.* 2003). A proposed role for ROS in the LPS mediated NRF-1 stimulation was confirmed through the generation of rho^0 cells which displayed reduced NRF-1 levels following mtDNA depletion. Oxidant stimulation increased NRF-1 levels implying a mitochondrial ROS mediated regulation (Suliman *et al.* 2003). The induction of mitochondrial ROS by estrogen in several cell lines was also recently linked to an increased binding of NRF-1 to target gene promoters (Felty *et al.* 2005).

Mitochondrial generated ROS appear to have an opposite effect on NRF-2. Treatment of mouse fibroblasts with oxidizing agents led to a diminished binding of NRF-2 to target gene promoters, an effect rescued with antioxidant treatment. This loss of binding was also seen following administration of the oxidized form of glutathione. A cysteine residue within the DNA binding domain of NRF-2 was defined as the redox sensitive site (Martin *et al.* 1996). Though the two nuclear respiratory factors may react in opposite ways, they are both able to respond to mitochondrial stress in the form of ROS to direct changes to nuclear gene expression. Other regulators of NRF-2 activity

include depolarizing stimulation (KCl) in neurons, leading to an up-regulation of both GABP subunits, as well as thyroid hormone which transcriptionally up-regulates NRF-2 but not NRF-1 (Zhang & Wong-Riley, 2000; Rodriguez-Pena *et al.* 2002). Much like other ETS proteins, phosphorylation and protein partnerships also aid in regulating NRF-2/GABP target genes (Rosmarin *et al.* 2004).

It has recently become clear that the control of NRF target genes is also influenced by the activity of a co-activator, PGC-1. This co-activator was originally identified for its role in adaptive thermogenesis- the dissipation of energy in order to produce heat seen in the mitochondria of tissues such as brown fat and skeletal muscle (Puigserver *et al.* 1998). The uncoupling protein 1 (UCP-1) primarily mediates uncoupling of the mitochondria in this process. The activation of this gene is under the control of PGC-1 in conjunction with nuclear hormone receptors with which PGC-1 interacts (Yoon *et al.* 2001). The expression of PGC-1 was up-regulated in conditions favorable to mitochondrial uncoupling and overexpression of the protein in myoblasts remarkably increased mitochondrial biogenesis (Wu *et al.* 1999). This is likely due to a direct interaction with NRF-1, as such an interaction was noted and PGC-1 was found to transactivate NRF-1 target sites in the presence of functional NRF-1 (Weitzel *et al.* 2001). There may also be a link between the PGC-1 co-activator and NRF-2, though NRF-2 does not appear to bind PGC-1 directly, an interaction with host cell factor (HCF) which also associates with PGC-1 has been detected- indicating HCF may provide a bridge function between NRF-2 and PGC-1 (Scarpulla, 2002). PGC-1 may therefore be a

critical component of the mitochondria-nucleus communication pathway, responsible for sensing changes in signaling pathways and relaying these via interactions with NRFs.

1.3.3- Regulation of HIF-1α by mitochondrial ROS: an example of crosstalk

Though regulation of the NRFs by mitochondrial-generated signals has not been fully described, there is evidence that mitochondrial ROS are pivotal in directing another signaling pathway, the response to hypoxia. Hypoxia is a condition that arises when there is inadequate O_2 supplied to tissues to allow for normal biological processes (Shannon *et al.* 2003). In response to this state, hypoxia inducible genes are up-regulated and act to increase O_2 delivery to tissues via the increased expression of genes such as *erythropoietin* (*EPO*), which controls the production of O_2 carrying erythrocytes, and *VEGF* which stimulates neovascularization (Semenza & Wang, 1992; Forsythe *et al.* 1996). Proteins involved in anaerobic metabolism, such as those involved in glucose transport and glycolysis, are also up-regulated in order to provide alternate sources of energy in the low O_2 conditions (Semenza, 2003).

The hypoxic response is controlled by the Hypoxia Inducible Factor 1, (HIF-1). This factor is a heterodimer made up of an α and a β subunit; the β subunit is ubiquitously expressed whereas HIF-1α expression is tightly controlled (Semenza, 2003). It is the stability of the HIF-1α protein that is controlled by hypoxic conditions and hence provides a hypoxia-inducible mechanism to appropriately control target gene expression. Under normoxic conditions, HIF-1α is ubiquitinated and degraded by the proteasome (Huang *et al.* 1998). Degradation is regulated by an interaction with von Hippel-Lindau protein (VHL) that recognizes critical proline residues that are hydroxylated in normoxic

conditions. Under hypoxic conditions, the proline hydroxylase enzymes are incapable of sufficiently hydroxylating HIF-1α, it is therefore not recognized by VHL and accumulates (Ivan *et al.* 2001; Jaakkola *et al.* 2001). Mechanisms by which these proline hydroxylase enzymes sense O_2 concentration changes in order to elicit downstream effects remain to be clarified. A requirement for iron has been established (Jaakkola *et al.* 2001) and another likely factor involved is ROS.

Early studies on the hypoxic stabilization of HIF-1α identified the involvement of mitochondrial ROS in the O_2 sensing pathway (Semenza, 2003). Under hypoxic conditions it was observed that rho^0 cells, used as a model of mitochondrial dysfunction, were incapable of secreting EPO and that HIF-1α binding to promoters within these cells was abolished (Chandel *et al.* 1998). The proposed mechanism of mitochondria-mediated HIF-1α stabilization was via production of ROS as it was noted that control cells, containing normal levels of mtDNA, secreted increased levels of ROS in hypoxic conditions whereas rho^0 cells did not. Furthermore, an inhibition of mitochondria produced ROS in parental cells also blocked HIF-1α binding to DNA (Chandel *et al.* 1998). Inhibition of complex I with rotenone also prevented hypoxia-induced production of ROS; supporting the concept that hypoxia induces the production of ROS by the mitochondria specifically. Increases in H_2O_2 under hypoxic conditions from different ETC complexes indicated that complex III might be the key sensor of low O_2 conditions (Chandel *et al.* 2000). Another model of mitochondrial dysfunction, making use of cybrid cells created containing the mtDNA from a primate cell line with a human rho^0

nuclear background, which display less than 60% of normal complex I activity, also exhibited limited HIF-1α accumulation under hypoxic conditions (Agani *et al.* 2000).

The issue of whether mitochondrial produced ROS are involved in the stabilization of HIF-1α is, however, controversial. It has been suggested that a functional electron transport chain is not required to mediate the hypoxic stabilization of HIF-1α (Vaux *et al.* 2001; Srinivas *et al.* 2001). Rho^0 cells constructed from a variety of parental cells were tested for HIF-1α stabilization following exposure to hypoxia and were found to respond normally. Rotenone concentrations that inhibit respiration similarly had no affect on the hypoxic stabilization of HIF-1α, indicating that original observations may be specific to the type of rho^0 cells tested (Vaux *et al.* 2001; Srinivas *et al.* 2001). However, it has been suggested that the discrepancy in these studies was related to the O_2 level considered to be simulating hypoxic conditions rather than the cells used (Schroedl *et al.* 2002). Schroedl *et al.* (2002) demonstrated that in hypoxic conditions, or 1-2% O_2, rho^0 cells are incapable of accumulating HIF-1α, however, the protein appears to be stabilized in these cells under anoxic conditions [0% O_2]. These authors proposed that in anoxic conditions, not associated with increased production of ROS, proline hydroxylase enzymes cannot function as they require a minimum O_2 concentration for activity and thus HIF-1α accumulates regardless of the mitochondrial status. In hypoxic conditions however, there is a requirement for ROS to inhibit prolyl hydroxylase activity (Schroedl *et al.* 2002). More recent evidence indicates that it may not necessarily be the production of ROS by mitochondria that is imperative in regulating HIF-1α stabilization under hypoxic conditions, (Gong & Agani, 2005). It was observed that oligomycin, an inhibitor

of the ATP synthase complex, prevented HIF-1α expression in hypoxic tumor cells, as did inhibitors of complexes I, III and IV. However, the use of antioxidants indicated that this effect was not via increased ROS. Instead, authors suggest that inhibition of the ETC decreases O_2 consumption allowing for a redistribution of O_2 into the cytosol at sufficient concentrations to allow a maintained activity of prolyl hydroxylase enzymes (Gong & Agani, 2005).

A summary of the suggested mechanisms of mitochondria to nucleus signaling, via effects on nuclear respiratory factors as well as the HIF-1α pathway, are presented in figure 3. The studies described in this thesis have elucidated a further factor to be added to this list of proteins which respond to mitochondrial alterations within cells, the transcription factor Ets-1. The following sections describe this factor in detail.

1.4- The Ets-1 transcription factor

1.4.1- Origin, structure, function

Ets-1 is the prototype of a group of transcription factors that make up the ETS family (Graves & Petersen, 1998). The name ETS comes from the avian erythroblastosis virus, E26, where the *ets* sequence along with Δgag and *c-myb* were found to form a transforming gene. The newly found region was thus named E26 transformation specific sequence (Nunn *et al.* 1983). A cellular *ets* (c-*ets-1*) homologue was later identified (Watson *et al.* 1985). As proteins were identified with a homologous DNA binding domain, aptly named the ETS domain, they were grouped into the ETS family (Sharrocks, 2001). The *ets* gene family is found throughout all phyla tested of the metazoan lineage, including non-vertebrates such as *Drosophila melanogaster* and *C.*

Elegans. Genes within the vertebrate groups are best described and as of yet no *ets* genes have been identified in the yeast *Saccharomyces cerevisaie* (Graves & Petersen, 1998).

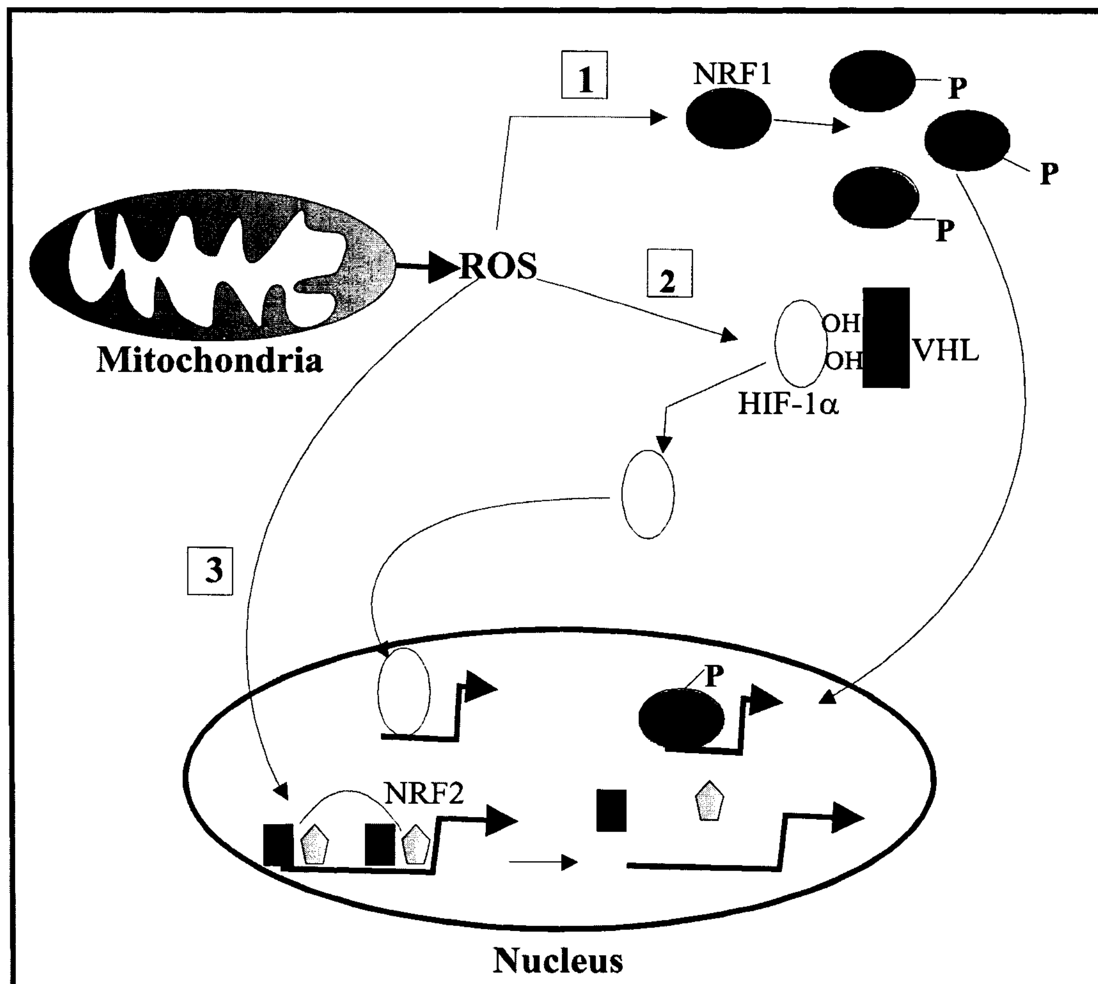


Figure 3: Examples of mitochondria to nucleus crosstalk. Mitochondrial generated ROS act as signaling molecules that can affect nuclear gene transcription through multiple pathways such as those outlined above. 1) Mitochondrial generated ROS lead to an up-regulation of NRF1 expression as well as phosphorylation which promotes nuclear translocation. As a result, NRF1 target genes, including several genes encoding mitochondrial proteins are up-regulated. 2) Mitochondrial generated ROS stabilize HIF-1α by inhibiting hydroxylation of proline residues and thus preventing recognition by VHL and subsequent degradation. Stabilized HIF-1α is then able to translocate to the nucleus and activate target genes involved in the hypoxic response. 3) ROS directly oxidize cysteine residues in the DNA binding domain of NRF-2 and thus inhibit DNA binding of this factor, decreasing target gene expression (including genes encoding mitochondrial proteins).

Genes within the *ets* family are further classified based on similarities within the ETS sequence as well as other functional domains. A recent attempt to describe the molecular phylogeny of this family defined a total of 13 subgroups- the diversification of the family clearly indicates an ancient origin. Furthermore, the conservation of Ets proteins since the beginning of metazoan life implies an importance for these proteins in basic cell functioning and embryonic development (Laudet *et al.* 1999).

The conserved component of the Ets family of proteins, the ETS domain can independently bind to DNA and these proteins have been identified to act as transcription factors, regulatory proteins that control the initiation of transcription of specific gene targets (Graves & Petersen, 1998). Though all Ets proteins share an identical core DNA binding site within promoters containing a GGAA/T element, interactions with other regulatory proteins, and components of the transcriptional machinery itself, as well as regulatory mechanisms induced by phosphorylation, all act to create target specificity for each Ets protein (Graves & Petersen, 1998).

The structure of Ets-1 has been well described, and various functions have been attributed to specific domains. The *ets-1* gene encodes eight exons which together comprise the human p51 Ets-1 protein. The sequence of the full-length protein is highly conserved among species, whereas a p42 variant found in mice, rats and humans lacks exon VII (Dittmer, 2003). The protein can be subdivided into 6 domains, the DNA binding ETS domain, the Pointed domain, transactivation domain as well as three other regulatory regions, including exon VII (Dittmer, 2003). The overall structure, and roles of each domain in regulating Ets-1 activity, is depicted in figure 4.

The secondary and tertiary structures of the ETS domain within Ets-1 as well as a related Ets protein (Fli-1) were found to be very similar to the winged helix turn helix (wHTH) motif of proteins within the wHTH structural family, containing a three helix bundle with an anti-parallel β-sheet against which the helices pack (the turn of the HTH motif) (Donaldson *et al.* 1996). NMR studies have demonstrated that the helix H3 of this protein subunit actually binds to target DNA sites, interacting with the GGAA element on the major DNA groove whereas the turn contributes to binding to the minor groove (Werner *et al.* 1997).

The pointed (PNT) domain is not common to all Ets proteins and is a characteristic of the subgroup of proteins in which Ets-1 has been placed (Laudet *et al.* 1999). This domain is made up of a number of α helices, five in the case of Ets-1 and appears to be pivotally involved in the interaction with other proteins involved in determining Ets-1 target specificity and in regulating Ets-1 activity (Mackereth *et al.* 2004). It is also of note that a key threonine which is a target for phosphorylation lies just to the N terminus of the Ets-1 PNT domain. This residue plays an important role in regulating Ets-1 activity (Mackereth *et al.* 2004). The transactivation domain of this factor is characterized by a high content of acidic residues and is essential for transcription activation activity (Gegonne *et al.* 1992). The significance of the exon VII and C terminus regulatory domains will be further discussed in the context of the regulation of Ets-1 activity.

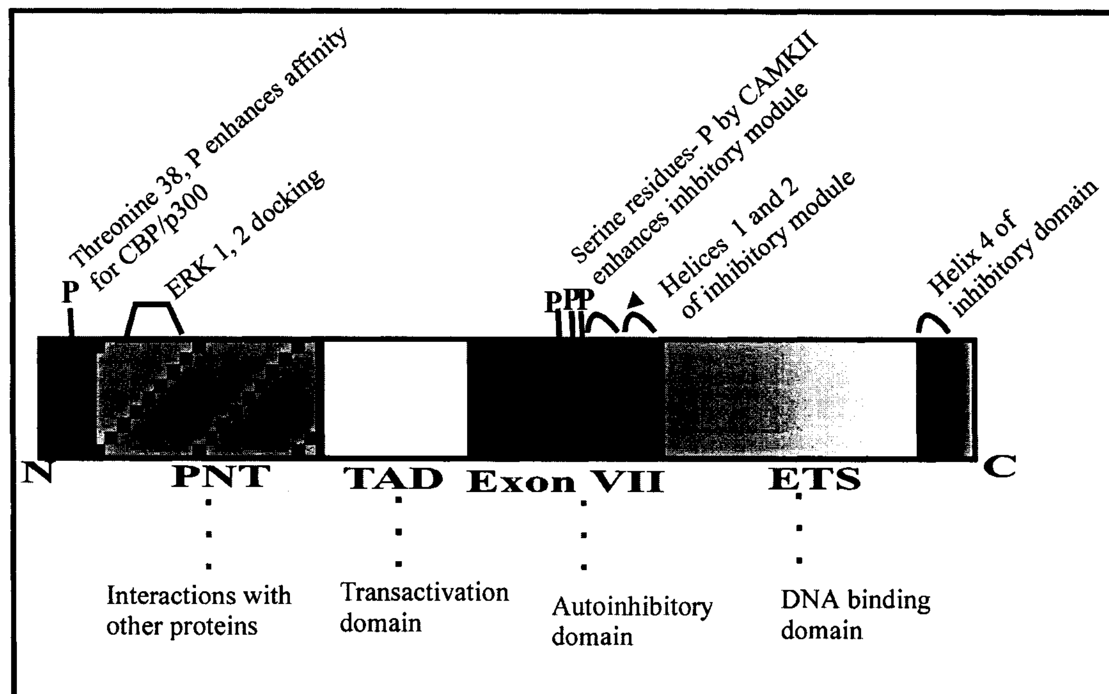


Figure 4: Schematic diagram of Ets-1.
The six domains of the Ets-1 protein are shown, as are key phosphorylation sites, an Erk 1, 2 docking site and approximate locations of helices which are involved in the formation of an autoinhibitory domain.

Though the major form of Ets-1 found within cells is the full length form, the p42 spliced variant is of interest as it appears to play distinct roles to the full length form (Lionneton *et al.* 2003; Li *et al.* 1999). The p42 variant in mouse cells was found to bind the same core DNA sequence as the full length and is capable of inducing transcription of identical target genes, however the sequences flanking the core element, which typically affect Ets-1 binding, did not affect p42 to the same extent. The capacity to bind non-optimal sequences was increased in the case of p42 (Lionneton *et al.* 2003). This may partially explain why p42 appeared to have the capability to induce apoptosis in human

colon carcinoma cells, potentially via caspase1, whereas full length Ets-1 could not (Li *et al.* 1999).

The normal expression of Ets-1 seems to be limited to adult lymphoid and vascular tissues, expressed in T, B and natural killer cells as well as endothelial and vascular smooth muscle cells (Ghysdael *et al.* 1986; Tanaka *et al.* 1998; Naito *et al.* 1998). The list of Ets-1 expressing tissues has since grown to encompass tumors, where expression appears to have a negative effect (Dittmer, 2003). Original observations of a relatively limited expression of this factor led investigators to examine the effects of knocking out this gene on the mouse lymphoid system (Bories *et al.* 1995). These studies involved introducing *ets-1* deficient embryonic stem cells into *recombination activating gene-2* (*RAG-2*) knockout blastocysts. Since *RAG-2* deficiency prevents the development of B and T cells, the only such cells found in resulting chimaeric mice originated from the *ets-1* deficient stem cells (Bories *et al.* 1995). Using this system, it was found that in *ets-1* deficient mice, the thymocyte population contained a much larger proportion of immature cells suggesting a defect in T cell development. Furthermore, mature T cells derived from *ets-1* deficient mice were much more prone to apoptosis. B cells, though similar in number in the *ets-1* knockout mice, displayed an increased proportion of mature plasma cells (antibody-secreting cells) (Bories *et al.* 1995). The defect in B cell development was confirmed in full *ets-1* knockout mice where it was seen that the B cell receptor did not respond efficiently to stimulation (Eyquem *et al.* 2004). It was also further shown that *ets-1* is required for natural killer cell development as well as differentiation and function of the Th1 subset of cells in adaptive immunity (Barton *et al.*

1998; Grenningloh *et al.* 2005). There is thus clearly a biological role for Ets-1 in the development of the lymphoid system. A role in the vascular system is also suggested by its expression profile.

1.4.2- Regulation of Ets-1 activity

All members of the ETS family bind a core GGAA/T element in target promoters, with some preferences shown for nucleotides surrounding this core. The expression of Ets proteins overlaps in many cell types (Graves & Petersen, 1998), thus, there are several levels of regulation required to direct specific Ets proteins to target promoters and to activate or repress these promoters under different cellular conditions. In the case of Ets-1, post-translational regulatory mechanisms controlling protein activity include autoinhibition, phosphorylation and the interaction with other transcription factors and co-activators (Dittmer, 2003).

Autoinhibition is a mechanism used to hold a protein's activity in check until an appropriate signal or co-activator is present to relieve such inhibition and is frequently observed among phosphatases, kinases and transcription factors (Cowley & Graves, 2000). In the case of Ets-1, DNA binding is regulated by an autoinhibitory module, the structure of which has been described (Lee *et al.* 2005). Sequences flanking the ETS domain (shown in figure 3), contain four helices, 2 N terminal and 2 C terminal, which pack together with helix 1 of the DNA binding domain to form a stable inhibitory module (Lee *et al.* 2005). The role of this inhibitory domain is demonstrated in the p42 splice variant of Ets-1 which does not contain the inhibitory helices 1 and 2 due to the lack of exon VII and displays a 10-20 fold enhanced affinity for DNA sites, emphasizing the role

of the inhibitory module in limiting the DNA binding of Ets-1 (Fisher *et al.* 1994). This module can, however, be disrupted upon DNA binding at which time helix 1 of the ETS domain is capable of a helix-coil transition. DNA binding therefore leads to a disruption of the autoinhibitory module, providing a regulatory mechanism for Ets-1 activity (Lee *et al.* 2005; Petersen *et al.* 1995).

Regulatory control of Ets-1 via the autoinhibitory domain has been revealed to be complex given that phosphorylation events can also influence this property (Cowley & Graves, 2000). It was observed that calcium dependent phosphorylation of Ets-1 via the calmodulin-dependent kinase II (CAMKII) occurs on four serine residues within exon VII, near inhibitory helices 1 and 2 and inhibits DNA binding (Rabault & Ghysdael, 1994). This led to speculation that this phosphorylation was encouraging the formation of the autoinhibitory module of the protein. CAMKII was confirmed to specifically phosphorylate serine residues within exon VII and following treatment with this kinase, DNA binding by Ets-1 was reduced; moreover, phosphatase treatment was capable of restoring DNA binding affinity (Cowley & Graves, 2000). When the inhibitory domain was permanently modulated by mutating key residues within the inhibitory helices, the effect of phosphorylation on DNA binding was lost- implying that the phosphorylation mediated inhibition was via a direct effect on the inhibitory module. Thus, the proposed model is that calcium-dependent phosphorylation of serines within exon VII inhibits DNA binding by favoring the folded, inhibitory state of the protein (Cowley & Graves, 2000).

Phosphorylation mediated by the MAPKs Erk 1 or 2 as well as by protein kinase C α (PKCα) also play regulatory roles in controlling Ets-1 activity. The MAPK-mediated phosphorylation of Ets-1 was proposed upon observation of a docking site for Erk within the PNT domain of Ets-1 and Ets-2 (Seidel & Graves, 2002). It had also been previously noted that phosphorylation mediated by the Ras/MAPK pathway was capable of superactivating genes which contained proximal Ets and AP-1 sites within their promoters (Yang *et al.* 1996). Ets-1 and AP-1 were found to be responsive to Ras signaling to activate such elements- termed Ras Responsive Elements (RREs). Mutation of threonine 38 within the Ets-1 PNT domain, close to the Erk docking site, abrogated this Ets, Ras cooperation. Thus, Ras mediated phosphorylation on threonine 38 increased the transactivation potential of Ets-1, yet it did not appear to be via enhanced DNA binding nor did it occur on every Ets target promoter (Yang *et al.* 1996). It was later clarified that Erk-mediated phosphorylation of Ets-1 actually increases its affinity for a key cellular co-activator, CREB binding protein (CBP) and the p300 homologue (Foulds *et al.* 2004). A previous report had demonstrated that Ets-1 co-immunoprecipitated CBP/p300 and that this association was necessary for transactivation of certain target genes, however, the phosphorylation status of Ets-1 was not addressed (Yang *et al.* 1998). The requirement of CBP/p300 in order to direct activation of target genes is likely due to the intrinsic histone acetylase activity of this co-activator, activating chromatin repressed promoters. Additionally, these co-activators may be providing a bridge between Ets-1 and the basic transcriptional machinery (Foulds *et al.* 2004). Regardless of the mechanism, a clear association was found between phosphorylation of Ets-1 on

threonine 38 by Erk and enhanced binding of this co-activator providing an explanation for the increased Ets-1 activity following MAPK-mediated phosphorylation (Foulds *et al.* 2004).

Effects of PKCα on Ets-1 activity are less well described. It was demonstrated that PKCα, a serine-threonine kinase activated by calcium and 1,2- diacylglycerol, is capable of phosphorylating exon VII of Ets-1 (the autoinhibitory domain) (Lindemann et *al.* 2003). It was later shown that decreasing levels of this kinase within cells led to a corresponding drop in Ets-1 levels as well as levels of Ets-1 target genes (Vetter *et al.* 2005). This loss in Ets-1 was not at the transcriptional level, but rather via loss of protein stability and new protein synthesis. Inhibition of CAMKII activity partially blocked this response indicating that perhaps Ets-1 phosphorylation by PKCα prevents CAMKII phosphorylation. Consequently, PKCα activity may protect Ets-1 from the effects of CAMKII phosphorylation, namely enhanced autoinhibitory protein formation and a reported destabilization of the protein (Vetter *et al.* 2005).

An ability to bind co-activator or co-repressor proteins on or near gene promoters is a key Ets-1 property that directs target gene specificity. One example is the RRE- RREs are found in the promoters of various genes including cytokines, matrix metalloproteinases (MMPS), glutathione *S*-transferase Ya (*GST Ya*) as well as viral genes (Li *et al.* 2000). There appears to be a physical association between the Ets-1 DNA binding domain and AP-1 in activated human T cells allowing for this cooperative transcriptional activation (Bassuk & Leiden, 1995). Other examples of protein partners for Ets-1 which enhance target gene expression and thus modulate Ets-1 activity include

NFκB, upstream transcription factor-1 (USF-1), pituitary-specific transcription factor (Pit-1), acute myeloid leukemia-1 (AML-1), CREB binding protein α2 (CBPα2), HIF-2α and paired box gene-5 (Pax-5) (Li *et al.* 2000; Elvert *et al.* 2003; Garvie *et al.* 2001). In the case of NFκB, a physical interaction between Ets-1 and NFκB was necessary for the transactivation of HIV-I and HIV-II genes, this association appeared to be between the ETS domain of Ets-1 and the p50 subunit of NFκB (Bassuk *et al.* 1997). USF-1 is a further example of a protein that directly interacts with the DNA binding domain of Ets-1 resulting in a cooperative transactivation of HIV-I (Li *et al.* 2000).

An alternative mechanism used by Ets-1 binding partners to enhance transactivation potential involves the modulation of the Ets-1 autoinhibitory domain. One example is the Pit-1- Ets-1 interaction (Bradford *et al.* 1997). Pit-1 is a factor expressed in pituitary cell types and is required for the expression of rat prolactin, growth hormone and thryotropin. In the case of prolactin, an Ets binding site is located adjacent to a low affinity Pit-1 binding site, a complex of the two factors is essential to induce a Ras mediated increase in promoter activity (Bradford *et al.* 1997). The mechanism by which Pit-1 increases Ets-1 activation is by means of binding the exon VII inhibitory domain and blocking stabilization of this module (Augustijn *et al.* 2002). Similarly, AML-1, CBPα2 and HIF-2α bind and disrupt the autoinhibitory module. In the case of CBPα2, a reciprocal effect of Ets-1 on CBPα2 occurs; Ets-1 increases the DNA binding ability of this protein partner demonstrating the cooperative effect of these two proteins on target genes (Goetz *et al.* 2000). Similarly, an interaction between AML-1 and Ets-1 is cooperative. AML-1 relieves Ets-1 from its inhibitory conformation, and Ets-1

disrupts a negative regulator domain of AML-1 (Kim *et al.* 1999). HIF-2α similarly can interfere with the Ets-1 inhibitory module through an interaction with the exon VII region (Elvert *et al.* 2003). A different mechanism allows for the cooperative effect of Ets-1 and Pax5 binding on target genes- Pax5 directly binds the ETS domain of Ets-1 and causes a structural alteration allowing Ets-1 to bind normally unfavorable sequences (Garvie *et al.* 2001).

There are also several examples of protein partners that repress Ets-1 activity- including MafB, the Ets-1 associated proteins I (EAPI/Daxx) and II (EAPII) as well as SP100. A yeast one hybrid system identified MafB as a factor which binds the ETS domain of Ets-1 via its own DNA binding domain (Sieweke *et al.* 1996). MafB is capable of repressing transactivation mediated by Ets-1, specifically inhibiting expression of transferrin receptor and as a result, the differentiation of erythroid cells. The proposed mechanism for MafB mediated repression of Ets-1 activity is by blocking the Ets-1 transactivation domain (Sieweke *et al.* 1996). Daxx and EAPII also directly interact with Ets-1 and cause repression of target genes such as *MMP1* and B-cell leukemia/lymphoma 2 associated gene (*Bcl2)* (Li *et al.* 2000). EAPII binds both the ETS and PNT domains of Ets-1, has no apparent effect on the DNA binding ability of Ets-1 but likely blocks any other protein interactions (Pei *et al.* 2003). Sp100 is an interesting example of a repressor of Ets-1 activity- it is not a transcription factor itself but a member of the nuclear body, a subnuclear organelle (Yordy *et al.* 2004). Sp100 is capable of both *in vitro* and *in vivo* interactions with Ets-1 which results in a down-regulation of Ets-1 targets such as *MMP1*

and urokinase plasminogen activator (*uPA*). Sp100 appears to mediate such effects by inhibiting Ets-1 DNA binding (Yordy *et al.* 2004).

1.4.3- Transcriptional control of the ets-1 gene

Clearly there are multiple levels of post-translational control of Ets-1 activity. The limited expression of Ets-1 in normal tissues and the aberrant expression within tumors, indicates that the *ets-1* gene is also under transcriptional control. To identify possible tumor derived factors that up-regulate *ets-1* expression, fibroblasts were cultured in conditioned media from tumor cells and were shown to display increased *ets-1* expression. Growth factors [PDGF, basic fibroblast growth factor (bFGF), EGF] as well as cytokines [tumor necrosis factor α (TNFα), IL-1α] were all shown to induce *ets-1* expression albeit with different timing and levels of up-regulation (Gilles *et al.* 1996). Further evidence for growth factor mediated up-regulation of Ets-1 includes the effects of EGF in breast cancer cell lines, bFGF in glioma cell lines, vascular endothelial growth factor (VEGF) in bovine retinal endothelial cells and PDGF-BB and endothelin-1 in vascular smooth muscle cells (Watabe *et al.* 1998; Kitange *et al.* 1999; Watanabe *et al.* 2004; Naito *et al.* 1998).

ROS is also a proposed regulator of *ets-1* up-regulation based on studies on angiogenesis using bovine aortic endothelial cells (Yasuda *et al.* 1999; Shimuzu *et al.* 2004). Angiogenesis is initiated by H_2O_2 and nitric oxide (NO) in this model. Both of these agents also led to increased *ets-1* mRNA expression and treatment with *ets-1* antisense oligonucleotides prevented the observed ROS mediated tube formation indicating that angiogenesis was activated by Ets-1 (Yasuda *et al.* 1999; Shimizu *et al.*

2004). There is also evidence that Ets-2, a closely related Ets family member to Ets-1, is transcriptionally up-regulated by H_2O_2 treatment in NIH 3T3 fibroblasts and that cells with inherent defects to their antioxidant defense systems display endogenously increased level of Ets-2 (Sanij *et al.* 2001).

In order to address how proposed mediators of a transcriptional up-regulation of *ets-1* are acting, the gene promoter must be analyzed. The *ets-1* gene promoter was independently cloned in two separate laboratories in 1991and 1992 by different methods (Oka *et al.* 1991; Majerus *et al.* 1992). The gene promoter region was found to contain several transcription initiation sites with no obvious TATA or CAT box (Oka *et al.* 1991; Majerus *et al.* 1992). Binding sites for various transcription factors were observed clustered around a region approximately 400-800 base pairs upstream from the proposed transcription initiation sites which became regarded as an enhancer region (Oka *et al.* 1991). Binding sites included those for AP-1, activating enhancer binding protein-2 (AP-2), Ets-1 itself as well as Sp-1. AP-2, AP-1 and Ets-1 are all capable of activating the *ets-1* promoter and promoter binding of AP-2 was confirmed by gel shift analysis (Oka *et al.* 1991; Majerus *et al.* 1992). Outside of the enhancer region, negative regulatory elements- with as of yet unidentified binding proteins- were observed within the promoter, as were an additional ETS binding site, preferential for the Ets protein polyoma enhancer activator 3 (PEA3) and binding sites for octamer binding transcription factors 1 and 2 (Oct 1 and Oct 2) (Chen & Wright, 1993). Analysis and comparison of the abilities of several putative activators indicated that the strongest activator of the promoter was PEA3, followed by AP-2, OCT 1 and 2 and finally Ets-1. Though a combination of these

factors increased transactivation it did not appear to be in a cooperative fashion, effects were simply additive (Chen & Wright, 1993).

Later *ets-1* promoter studies further characterized regions of the promoter that may explain the *ets-1* gene responsiveness to specific agents and conditions- namely retinoic acid and hypoxia (Raouf *et al.* 2000; Oikawa *et al.* 2001). Retinoic acid influences cell development and differentiation through its effects on retinoic receptors which interact directly with retinoic acid response elements (RARE) within promoters of target genes (Raouf *et al.* 2000). Two independent groups showed that retinoic acid was capable of inducing *ets-1* gene expression but analysis of the responsible promoter region was inconclusive. The two groups identified different putative RAREs, neither correlated exactly to the consensus accepted RARE sequence and it remains unclear whether either site identified is a true RARE (So & Crowe, 2000; Raouf *et al.* 2000). With regards to the hypoxic induction of *ets-1*, expression was induced in bladder cancer cells exposed to hypoxia. Promoter activity was shown to be directly induced under hypoxic conditions and a protein complex containing HIF-1α appeared to bind a putative hypoxia response element (HRE) within the promoter in a hypoxia inducible fashion (Oikawa *et al.* 2001). The idea that Ets-1 expression can be induced in hypoxic conditions is furthered by evidence in a rat adjuvant-induced arthritis model (Peters *et al.* 2004). In this model, the synovium becomes hypoxic as shown by a hypoxyprobe that can be taken up *in vivo* and binds tissues of low O_2 concentrations. The location of such regions is then evaluated *ex vivo* with a specific antibody. Joint inflammation was found to be associated with increased hypoxia as well as increased HIF-1α and Ets-1 protein expression, suggesting

that hypoxia and specifically HIF-1α mediate Ets-1 up-regulation in these particular tissue types (Peters *et al.* 2004).

Though several elements have been identified within the *ets-1* promoter, the tissue specific expression of this transcription factor remains unresolved. Transgenic mice generated with constructs containing *ets-1* promoter and gene segments adjacent to an *Escherichia Coli* (*E. Coli*) *β-D-galactosidase (lacZ)* reporter were used to analyze the elements necessary for tissue specificity (Jorcyk *et al.* 1997). None of the transgenic mice with *ets-1* 5' promoter element constructs displayed lacZ activity in tissues where *ets-1* would be expected to be expressed. Only one construct tested, correlating to intron 1 of the *ets-1* gene, appeared to direct appropriate tissue expression of Ets-1 in the developing mouse. The identity of this intronic element capable of directing appropriate Ets-1 expression has yet to be deduced. It should also be noted that none of the *ets-1* gene regions tested could direct expression of Ets-1 to the lymphoid tissue, indicating that tissue specificity requires further investigation (Jorcyk *et al.* 1997).

Among the known and putative regulators of *ets-1* transcription, H_2O_2 is an interesting candidate as a tumor derived factor that may cause elevated expression of *ets-1*. If indeed H_2O_2 is capable of transcriptionally up-regulating *ets-1*, candidate promoter elements that would mediate such a response include the AP-1, HRE and antioxidant response element (ARE). In the case of an AP-1 binding site, a functional site has already been identified within the *ets-1* enhancer region and could well be responsible for an H_2O_2 mediated *ets-1* promoter induction (Oka *et al.* 1991). The components of the AP-1 heterodimer, c-*jun* and c-*fos*, are MAPK-responsive genes and the MAPK pathway

is activated by H_2O_2. A specific increase in AP-1 binding following H_2O_2 treatment has been seen in various AP-1 target gene promoters including *Growth Arrest and DNA Damage inducible gene 153 (GADD153)* and *GST* (Guyton *et al.* 1996B; Duvoix *et al.* 2004). Furthermore, an assay designed to test the direct effect of oxidative stress on AP-1 activity indicated that pro-oxidant treatment led to direct activation of reporter constructs containing an AP-1 binding element (TGACTCA) (Zhou *et al.* 2001). This same study confirmed the activation of NFκB target genes by oxidative stress, however NFκB is an unlikely candidate for the mediator of oxidant induced expression of *ets-1* since no consensus NFκB site has been identified within the promoter (Zhou *et al.* 2001). With regards to the possibility that a proposed H_2O_2 induction of *ets-1* could act through an HRE, this comes from evidence that a functional HRE, capable of binding HIF-1α in a hypoxic inducible fashion, lies within the *ets-1* enhancer (Oikawa *et al.* 2001) and that HIF-1α is a proposed target of H_2O_2 via protein stabilization (Chandel *et al.* 1998).

The final proposed element involved in a H_2O_2 mediated induction of *ets-1* is the ARE. Though an ARE has not been identified within this promoter to date, it is considered a possible mediator as it is a well-known oxidative stress induced activator of target genes (Nguyen *et al.* 2003). This element was first identified in the rat subunit *GSTA2* gene as the sequence responsible for promoting gene induction by agents such as phenolic antioxidants, which are capable of undergoing transformations to free radicals and H_2O_2, as well as H_2O_2 itself. Deletion and mutational analysis of the promoter identified a core region required for induction of this gene by these agents: TGACnnnGC (Rushmore *et al.* 1991). Note that this element consists of a partial AP-1 binding element

(TGAC) separated by three nucleotides from what is termed the GC box (Rushmore *et al.* 1991). This element was later found to activate the expression of a large group of genes involved in controlling the cellular redox status, including those encoding enzymes associated with glutathione biosynthesis, proteins with sulfhydryl residues as well as drug metabolizing enzymes (Nguyen *et al.* 2003). Agents that are capable of activating ARE driven gene expression, as well as H_2O_2; include several compounds that react with sulfhydryl groups such as phenolic antioxidants which undergo metabolism to quinines as well as flavonoids and GSH depleting agents (Nguyen *et al.* 2003).

The fact that the consensus ARE sequence is very close to the accepted AP-1 binding site led to initial proposals that it was this transcription factor that regulated a coordinated response of free radical detoxifying proteins in response to ARE inducing agents (Nguyen *et al.* 1994). It has since been established, however, that the ARE is a distinct element with an independent signaling pathway controlling its regulation. This was demonstrated using 12-O-tetradecanoyl-phorbol-13-acetate (TPA), a well-known inducer of AP-1 target genes, which is also capable of causing a slight up-regulation in ARE driven gene expression (Nguyen *et al.* 1994). If the *GST2A* core ARE was altered to match the exact AP-1 sequence with a flanking GC box maintained, TPA induction was drastically increased- however a mutation to the GC box, while maintaining TPA responsiveness, no longer allowed a response to traditional ARE inducers such as *tert*-butylhydroquinone (tBHQ). Thus AP-1 and ARE elements appear to be regulated by distinct factors (Nguyen *et al.* 1994).

The main binding protein responsible for the regulation of ARE driven genes was later identified as the nuclear factor (erythroid-derived) factor 2 (NF-E2) related factor 2 (Nrf2) (Moi *et al.* 1994). NF-E2 is a nuclear-erythroid factor; expression is limited to hematopoietic cells where it was originally identified as a key regulator of globin gene expression (Nguyen *et al.* 2003). Nrf2 was isolated from a cDNA library using a probe corresponding to an NF-E2 recognized site and was found to be highly homologous to NF-E2 with a similar basic leucine zipper DNA binding domain and a functional transactivation domain (Moi *et al.* 1994). Unlike NF E2, Nrf2 was found to be expressed in virtually every tissue tested (Moi *et al.* 1994). The first clue that Nrf2 is the factor driving ARE activation came from transfection experiments where Nrf2, or the closely related Nrf1, were observed to increase ARE-CAT reporter activity, but were unable to drive activity of a mutated ARE reporter (Venugopal & Jaiswal, 1996). An Nrf2 knockout mouse later confirmed the key role of this factor in ARE activation as basal levels of enzymes which are clearly activated via an ARE within their promoters, *GSTA1* and *NAD(P)H: quinone reductase* (*NQO1*), were drastically reduced in knockout mice and incapable of being induced following treatment with typical ARE inducers (Itoh *et al.* 1997). Analysis of gene expression in knockout mice as compared to wildtype by gene arrays has also allowed the identification of several Nrf2 target genes. As well as the expected loss of expression of several genes encoding antioxidant defense enzymes, protein chaperones as well as proteasome subunits were also identified as Nrf2 targets indicating that there are several types of protective genes induced via the ARE (Kwak *et al.* 2003).

The ubiquitous expression of Nrf2 indicates that there must be post-translational regulation of the activity of this factor allowing for controlled, appropriate induction of target genes. It is well established that Nrf2, like other members of the NF E2 family, cannot bind DNA alone as a monomer, or as a homodimer- a second factor is required to aid in transactivation of target genes (Moi *et al.* 1994). In the case of NF E2, these binding partners are members of the small Maf protein family. Maf proteins are leucine zipper domain containing proteins, capable of binding DNA but incapable of transactivation (Dhakshinamoorthy & Jaiswal, 2000). Unlike NF E2, it appears that the binding of small Maf proteins with Nrf2 actually represses target gene expression, as seen in the *NQO1* and *GST Ya* genes (Dhakshinamoorthy & Jaiswal, 2000). There are reports of Jun proteins cooperatively binding with Nrf2 and activating target gene expression, but for the most part, identification of protein partners remains unresolved (Nguyen, 2003).

There are also several questions regarding the control of Nrf2 by way of various signaling pathways. Due to the common feature of most ARE inducing agents, the ability to react with sulfhydryl groups, a proposed regulatory mechanism for Nrf2 activity is via modification of such residues within the protein. Though no key cysteine residues required for Nrf2 activity itself have been identified, a binding partner for Nrf2 in the cytoplasm identified via yeast two-hybrid analysis appears to be regulated in this fashion (Itoh *et al.* 2004). This Nrf2 interacting protein, Kelch-like ECH associating protein 1 (Keap1), contains domains capable of binding Nrf2 as well as actin and appears to localize to the cytoskeleton (Itoh *et al.* 2004). It was found in co-transfection experiments that Keap1 is capable of sequestering Nrf2 in the cytoplasm and thereby

repressing its activity, however, treatment of cells with oxidative stress inducing agents releases Nrf2. Two cysteine residues within the Nrf2 binding domain of Keap1 are required for this redox-sensitive interaction (Itoh *et al.* 1995; Itoh *et al.* 1999; Zhang & Hannink, 2003). Characteristics of Keap knockout mice confirm the regulatory role of this protein on Nrf2 function, as Nrf2 protein was active in the nucleus of embryonic fibroblasts from these mice and ARE target genes were constitutively expressed without controlled up-regulation under typical ARE inducing conditions (Wakabayashi *et al.* 2003). More recent evidence indicates that the pivotal role of the Keap1-Nrf2 interaction is to promote ubiquitination and subsequent degradation of Nrf2. Therefore, the Keap1 regulatory role may be to promote degradation of Nrf2 rather than to sequester it in the cytoplasm, appropriate signals leading to a dissociation of the complex would protect Nrf2 from this degradation (Nguyen *et al.* 2005)

There are indications that the repressive effect of Keap1 is not the only regulatory mechanism determining Nrf2 nuclear localization and activation; phosphorylation events are reportedly also involved in the regulation of Nrf2 activity. One example is the phosphorylation mediated by PKC, which phosphorylates Nrf2 in response to inducers and positively affects nuclear localization (Huang *et al.* 2000). The MAPK pathway also appears to be involved in Nrf2 regulation, as agents such as tBHQ induce phosphorylation of Erk 2 leading to ARE target gene activation. Though the JNK pathway also appears to be a positive regulator of Nrf2 activity, p38 kinase phosphorylation actually seems to repress the activity of an ARE reporter under both basal and induced conditions (Nguyen, 2003). An indication that phosphorylation events

are critical in regulating ARE driven transcriptional activation comes from a study demonstrating that H_2O_2 activation of the ARE specifically involves the JNK and Erk pathways with no apparent involvement of thiol containing antioxidants, indicating that the MAPK pathway may be of more importance in H_2O_2 mediated ARE activation than a Keap1 modification (Go *et al.* 2004). There are evidently several regulatory mechanisms controlling the expression of genes regulated by an ARE, allowing for specific induction in conditions of oxidative stress. The ARE is therefore a plausible promoter element involved in a specific H_2O_2 mediated gene up-regulation. Thus, in the case of *ets-1*, AP-1, HRE and potentially ARE sequences within the promoter region may all be involved in a proposed H_2O_2 mediated regulation.

1.5- Effect of Ets-1 overexpression: correlation with cancer

1.5.1- Ets-1 expression and cancer

It has become increasingly evident that Ets-1 expression is linked to the progression of cancer in several tissues. *Ets-1* itself is thought to have oncogenic potential due to the ability to transform NIH3T3 cells and more recently has been shown to increase the transformed phenotype of a human epithelial tumor cell line (HeLa cells) (Seth & Papas, 1990; Hahne *et al.* 2005). In the latter case, HeLa cells with stable increased expression of Ets-1 displayed enhanced migration, invasion and anchorage independent growth- hallmarks of a transformed phenotype (Hahne *et al.* 2005). Ets-1 is highly expressed in several different cancer tissues and appears to correlate specifically with more advanced, invasive tumors. Well-described examples include breast, ovarian and colorectal carcinomas (Span *et al.* 2000; Davidson *et al.* 2001). In some cases,

expression is also linked to poor survival. Increasing evidence indicates that a correlation between Ets-1 expression and tumor progression is a general phenomenon as evidence for such a correlation has also been found in prostate, gastric, oral and thyroid cancers as well as melanoma and meningioma (summarized on table 2).

An early study looking at the expression of Ets-1 in breast cancer tissues showed that Ets-1 was expressed in over 60% of 123 primary breast cancer samples analyzed. The patients whose samples were used in this study were followed for clinical data and a significant correlation was identified between Ets-1 expression and reduced relapse free survival indicating that Ets-1 is an independent predictor of poor prognosis in breast cancer (Span *et al.* 2002). More recent studies on breast cancer specimens have provided further evidence for a association between Ets-1 expression and breast cancer- an examination of the expression of both mRNA and protein levels in benign fibroadenomas as well as primary carcinomas identified that while mRNA levels were similar in all tissues, Ets-1 protein expression was significantly higher in carcinoma tissues as well as within stromal cells surrounding the tumor (Buggy *et al.* 2004). When samples of differing invasive capabilities were tested for Ets-1 expression using immunohistochemistry, it was noted that neither normal breast epithelium nor non-invasive carcinomas expressed Ets-1 protein whereas samples taken from invasive ductal carcinomas displayed intense staining for Ets-1 protein (Katayama *et al.* 2005). Thus a significant correlation between Ets-1 expression and breast carcinoma, particularly invasive carcinoma is evident and Ets-1 appears to be a marker of poor prognosis in these cases.

Table 2: Association between Ets-1 expression and cancer

Type of cancer	Ets-1 expression findings	Association between Ets-1 expression and clinical outcomes	Reference
Breast	-Protein expression found in >60% primary cancer samples	-Significant correlation between expression and poor relapse free survival	-Span *et al.* 2002
	-mRNA expression high in benign and primary carcinomas, protein significantly higher in carcinoma and surrounding stroma	-No data reported	-Buggy *et al.* 2004
	-No protein detected in normal epithelia and non-invasive carcinoma, high levels in invasive ductal carcinoma	-No data reported	-Katayama *et al.* 2005
Ovarian	-mRNA detected in 42% carcinomas tested, also noted in surrounding stromal cells	-mRNA expression significantly associated with poor survival, particularly if detected in stromal cells	-Davidson *et al.* 2001
	-Protein and mRNA detected in carcinoma and endothelial cells, higher levels in metastatic lesions	-Lower 24 month survival rate if higher Ets-1 levels in metastatic lesion than in primary cancer	-Fujimoto *et al.* 2004
Colorectal	-Increased levels in stroma surrounding cells developing to invasive carcinoma; low levels detected in less aggressive forms (HNPCC)	-No data reported	-Behrens *et al.* 2003
	-48% of all specimens tested positive for Ets-1 (65% positive in endothelial cells, 28% in stromal cells)	-Survival rate of patients with Ets-1 detected in vascular tissue significantly lower	-Tokuhara *et al.* 2003
Melanoma	-Higher levels of Ets-1 in invasive melanoma as compared to non-invasive	-No data reported	-Keehn *et al.* 2003
Prostate	-Protein expression not detected in normal, benign hyperplasia; expressed in 77% of malignant cases tested; protein levels significantly higher in clinical versus latent carcinoma	-No data reported	-Alipov *et al.* 2005

Oral squamous cell carcinoma	-Normal tissue negative, 47% of potentially malignant samples positive, 58% malignant positive- particularly in invasive front, surrounding stroma and endothelial cells of well vascularized tumors	-Expression significantly correlated with lymph node involvement, clinically advanced stage tumors most frequently positive	-Pande *et al.* 1999
Esophageal squamous cell carcinoma	-Overexpression in 80% malignant samples tested	-Expression correlated significantly with poor disease free survival	-Mukherjee *et al.* 2003
Gastric	-No protein observed in normal epithelia, detected in 52% of primary gastric cancers tested	-Significant correlation between expression and poor survival rates	-Tsutsumi *et al.* 2005
Thyroid	-8 to 10 fold up-regulation of protein in papillary thyroid carcinomas as compared to normal samples, 3 to 4 fold up-regulation in follicular thyroid neoplasia	-No data reported	-Fuhrer *et al.* 2005
Meningioma (tumors of the CNS)	-38% of benign tissue positive, 100% of atypical and anaplastic cells positive for protein expression. Increasing levels of protein noted with higher tumor grade.	-87% of samples which showed infiltration to the brain positive for Ets-1 expression	-Kitange *et al.* 2000
Testicular germ cell tumors	-Expression significantly higher and more frequently observed in metastatic tumors as compared to non-metastatic	-No data reported	-Adam *et al.* 2003

The examination of ovarian carcinoma samples has yielded similar results (Davidson *et al.* 2001; Fujimoto *et al.* 2004). An attempt to correlate *ets-1* expression and survival data from advanced ovarian carcinoma patients was performed by analyzing sections from 42 patients classified as either short term or long term survivors using mRNA *in situ* hybridization (Davidson *et al.* 2001). *Ets-1* expression was noted in both tumor and surrounding stromal cells, as well as in endothelial cells within stroma in some cases. In samples where expression was noted in both tumor and stromal cells, a significant association with poor survival was found, with a particularly strong correlation with expression in stromal cells (Davidson *et al.* 2001). A separate study confirmed this correlation- Ets-1 protein and mRNA expression was examined in primary tumors as well as metastatic lesions from 30 patients with stage III ovarian cancer (Fujimoto *et al.* 2004). Investigators noted that Ets-1 was expressed in a high percentage of cancer cells and vascular endothelial cells tested, with significantly higher levels noted within metastatic lesions. Furthermore, the 24 month survival rate of patients with increased *ets-1* mRNA levels in the metastatic lesions as compared to the primary tumor was significantly lower than patients with unchanged levels, indicating a negative effect of increasing levels of *ets-1* in tumor progression (Fujimoto *et al.* 2004).

Various studies have also illustrated a role for Ets-1 in aggressive colorectal cancers. A comparison of Ets-1 levels in sporadic colorectal cancers and the less invasive and metastatic hereditary nonpolyposis colorectal carcinomas (HNPCC), was performed in order to evaluate whether levels of the protein are elevated in the more aggressive variant (Behrens *et al.* 2003). An increased expression of Ets-1 was noted in

the stroma surrounding cancer cells in the stage where sporadic adenomas develop to invasive carcinomas whereas HNPCC tumors displayed lower levels of Ets-1 expression, indicating that Ets-1 is specifically associated with the more invasive, metastatic variant of colorectal carcinoma (Behrens *et al.* 2003). A correlation with patient survival was noted in another colorectal study where expression of Ets-1 in vascular tissue feeding the cancerous specimens was associated with lower survival rates (Tokuhara *et al.* 2003). Further examples indicating that there is an association between Ets-1 expression and increased tumor progression or invasive capacity comes from melanocytic lesions where immunohistochemical expression of Ets was found to be highest in invasive melanomas as compared to benign and non invasive lesions (Keehn *et al.* 2003). Prostate carcinoma data also indicates that Ets-1 is overexpressed in malignant tissue as compared to benign with significantly higher levels noted in clinical versus latent samples implying a role for Ets-1 in progression (Alipov *et al.* 2005). All studies cited as well as further examples from oral, gastric, and thyroid carcinomas as well as meningioma, demonstrating an association between Ets-1 expression and tumor progression, are summarized in table 2.

1.5.2- Ets-1 target genes: possible explanation for link to cancer

The link between Ets-1 and cancer can potentially be explained by the list of known target genes regulated by this transcription factor. The normal expression of Ets-1 in lymphoid and vascular tissues as well as data obtained from a targeted *ets-1* knockout, suggest that target genes are generally grouped into roles in lymphoid cell development and angiogenesis (Bories *et al.* 1995; Naito *et al.* 1998). There certainly is a growing list of lymphoid tissue related gene products regulated by Ets-1 including T cell receptors,

cytokines and cytokine receptors (Sementchenko & Watson, 2000; Aringer *et al.* 2003). However, what has become apparent is that the products of several Ets-1 regulated genes are generally involved in the degradation of extracellular matrix as well as the promotion of migration and angiogenesis and thus may have key roles in mediating tumor progression (Sementchenko & Watson, 2000).

Degradation of the extracellular matrix (ECM), composed of collagen, proteoglycans and adhesive matrix proteins surrounding a tumor cell allows for a tumor cell to begin to spread to distant sites of metastasis. Mediators of proteolytic degradation of ECM are typically proteases such as MMPs and uPA (a serine protease that converts plasminogen to plasmin, plasmin is capable of directly degrading ECM as well as activating MMPs) (Mignatti & Rifkin, 1993; Murphy *et al.* 1992). Ets-1 has been found to regulate these proteases in several different cell types. The EGF mediated up-regulation of *uPA* in a breast cancer cell line was determined to be via AP-1 and ETS elements within the *uPA* gene promoter (Watabe *et al.* 1998). Though transfection of Ets-1 activated this promoter directly, the EGF stimulated phosphorylation of Ets proteins 1 and 2 led to a greatly enhanced activation of the promoter indicating that *uPA* activation by Ets in cancer cells is enhanced by phosphorylation events (Watabe *et al.* 1998). A similar positive association between Ets-1 and *uPA* expression was noted in astrocytic tumors (Nakada *et al.* 1999). In this case, *uPA* levels and the invasive activity of human gliomas were directly linked. The expression of a dominant negative Ets-1 protein, with no transactivation domain, decreased levels of *uPA* mRNA as well as

invasive capability in a 3D collagen gel, confirming the proposed role of Ets-1 in regulating uPA expression (Nakada *et al.* 1999).

Ets-1 has also been implicated as a regulator of MMP1 and MMP9 (Behrens *et al.* 2001). The expression of these MMPs in breast cancer lesions was particularly noted in invasive and ductal carcinomas in endothelial and surrounding fibroblastic cells; Ets-1 was co-expressed with these MMPs in the tissues studied displaying an *in vivo* correlation of protein expression (Behrens *et al.* 2001). A bronchial asthma study demonstrates a direct regulation of MMPs by Ets-1 (Nakamura *et al.* 2004). TNFα activated bronchial fibroblasts exhibited both increased Ets-1 and MMP9 expression; treatment with *ets-1* antisense oligonucleotides led to a MMP9 down-regulation (Nakamura *et al.* 2004). Further evidence for specific MMP regulation was demonstrated in glioma cells where a specific Ets-1 decoy oligodesoxynucleotide caused reduced MMP9 expression (Sahin *et al.* 2005).

The ability of a cancer cell to invade surrounding tissue relies on the degradation of surrounding matrix as well as the induced ability to migrate, a process that Ets-1 also appears to regulate in cancer cells (Kita *et al.* 2001; Oda *et al.* 1999). Glioma cells stably transfected with a dominant negative Ets-1 were observed to display poor adhesion and spreading on fibronectin coated dishes as compared to untransfected cells indicating impaired function of fibronectin receptors. It was confirmed that loss of Ets-1 activity led to a down-regulation of *Integrin α5*, a key component of the fibronectin receptor (Kita *et al.* 2001). *Integrin β3* was also identified as an Ets-1 target in endothelial cells (Oda *et al.* 1999).

The growth and spreading of tumors relies greatly on the ability to sprout new blood vessels from preexisting ones, a process termed angiogenesis (Folkman & Shing, 1992). The expression of Ets-1 in vascular cells in normal tissues and within endothelial cells surrounding tumors, as well as the documented regulation of this factor by VEGF indicate that Ets-1 may be a key regulator of angiogenesis (Dittmer, 2003). This putative role for Ets-1 was demonstrated when the expression of specific antisense oligonucleotides for *ets-1* in endothelial cells inhibited angiogenesis (Iwasaka *et al.* 1996). Possible Ets-1 targets underlying this capability include *MMPs 1*, *3* and *9* as well as *Integrin β3*, all shown to be up-regulated in murine endothelial cells stably over expressing *ets-1* and down-regulated in cells expressing antisense *ets-1* (Oda *et al.* 1999). Though these gene targets were described in the context of ECM degradation and migration, clearly these processes are also involved in the degradation of vascular basement membranes as well as migration of endothelial cells involved in angiogenesis, thus linking these Ets-1 targets to the angiogenic process.

Various studies have also identified a link between Ets-1 expression and angiogenesis *in vivo* (Nakano *et al.* 2000; Pourtier-Manzanedo *et al.* 2003; Hashiya *et al.* 2004). Murine endothelial cells stably expressing a dominant-negative Ets-1 protein displayed reduced cell migration and invasion. This was evaluated *in vivo* with the use of a matrigel plug assay, where an adenoviral dominant-negative Ets-1 vector was added to a matrigel plug implanted into the subcutaneous tissue of mice for five days (Nakano *et al.* 2000). Evaluation of the development of neovessels in the plug indicated that growth of new vessels into the plug was reduced when dominant-negative Ets-1 was present.

Investigators suggested that the dominant-negative Ets-1 was capable of altering gene expression in invading endothelial cells (Nakano *et al.* 2000). A mouse ear model which allows the evaluation of growth factor- and tumor-induced angiogenesis, indicated that dominant negative Ets-1expression inhibited FGF-induced angiogenesis (Pourtier-Manzanedo *et al.* 2003). Finally, an HVJ liposome method used to directly transfect rat hindlimbs, indicated that transfection with the human *ets-1* gene caused enhanced capillary density and blood flow in hindlimbs compared to control, emphasizing the role of Ets-1 in stimulating angiogenesis (Hashiya *et al.* 2004). As well as gene products involved in ECM degradation and migration, other Ets-1 targets such as *angiopoeitin-2*, an angiogenic factor involved in destabilizing blood vessels and *member of the myocyte enhancer factor 2* (*MEF2c*) involved in vascular endothelium development may also be components of the pro-angiogenic capability of this factor (Hegen *et al.* 2004; De Val *et al.* 2004).

The process of apoptosis, a term encompassing the events leading to programmed cell death in response to various cellular stresses and damage, is frequently associated with cancer in that cancerous cells use various mechanisms to avoid this process and continue to proliferate. There is no clear role for the involvement of Ets-1 targeted genes in apoptosis, despite the fact that early data from the *ets-1* knock out mouse indicated that *ets-1* played a role in protecting T cells from apoptosis (Bories *et al.* 1995). Contradicting evidence indicates that the p42 isoform of Ets-1 has pro-apoptotic effects in colon carcinoma cells via the activation of *caspase1* (Huang *et al.* 1997). There are also indications that Ets-1 has a pro-apoptotic capability in HUVEC cells, though this did

not appear to be as a result of the *ets-1* regulation of *caspase1* (Teruyama *et al.* 2001). A more recent study in vascular smooth muscle cells indicates that Ets-1 protects cells from undergoing apoptosis via the regulation of *p21WAF1/Cip1* (cyclin-dependent kinase inhibitor) (Zhang *et al.* 2003). Thus the role of Ets-1 in apoptosis remains unclear.

The list of Ets-1 target genes grows as research into the role of this factor continues. Though many genes regulated by Ets-1 have clear roles in mediating migration, invasion and angiogenesis and thus tumor progression, several other target genes may also have a less clearly defined role in carcinogenesis and or cancer progression. Examples include growth factors and growth factor receptors such as *PDGF A* and *c-met* (hepatocyte growth factor receptor), both potent mitogens (Jiang *et al.* 2001; Santiago & Khachigian. 2004). Parathyroid hormone related protein (*PTHrP*) has been found to be regulated by Ets-1 and Smad3 cooperatively, PTHrP may be an important factor in increasing the metastatic capability of cancer cells, especially to the bone as it has bone resorptive activity (Lindemann *et al.* 2001). Oligosaccharide branching on glioma associated glycoproteins has been associated with increased metastatic potential of these cells, particularly β-1,6 bearing glycans; it was noted that the enzyme responsible for synthesizing these branches is regulated by Ets-1 (Yamamoto *et al.* 2000). Finally, a modulator of NFκB activity (*IκKα* subunit of IκBα kinase) that has well described roles in increased proliferation and survival of cancer cells, has recently been identified as an *ets-1* responsive gene (Gu *et al.* 2004). The association between Ets-1 expression and cancer cells, particularly aggressive variants, can rationally be explained

by the various gene products up-regulated by Ets-1 activity which have multiple roles in promoting tumor progression.

1.6- Use of an ovarian carcinoma, *cis*-platinum (II)- diammine dichloride (cisplatin) resistance, model to study the effect of mitochondrial dysfunction on nuclear gene expression (retrograde regulation)

The human ovarian carcinoma model used in this study makes use of 2008 and C13* ovarian carcinoma cells. 2008 cells are a parental cell line, established from a patient with adenocarcinoma of the ovary and C13* cell variants were derived following 13 successive cisplatin treatments. 2008 cells are sensitive to cisplatin treatment whereas C13* are 8-15 fold more resistant (Andrews *et al.* 1988). It was observed that in acquiring resistance to cisplatin, the mitochondria of the C13* cells become altered (Andrews *et al.* 1992). Andrews *et al.* demonstrated by electron microscopy that the mitochondrial membranes of C13* cells are less electron dense and that the cristae within the mitochondria are irregular and often absent. Membrane potential analysis also indicated that the membrane potential of C13* cells is 2.3 times elevated over that of 2008 cells (Andrews *et al.* 1992).

Though it is the mitochondrial alterations in C13* cell variants that are of interest in this study, the fundamental characteristic of the 2008/C13* model is the development of cisplatin resistance, a feature that has been extensively studied. Platinum compounds such as cisplatin are DNA damaging molecules widely used as anti-cancer agents (Brabec & Kasparkova, 2002). They have proven to be effective in the treatment of testicular and ovarian cancer as well as in combination for various other carcinomas (Weiss &

Christian, 1993). Cisplatin acts as a DNA alkylator and crosslinker, forming crosslinks between guanine bases. These cisplatin-DNA adducts are presumed to initiate apoptosis in treated cells (Siddik, 2003). The major limitation of cisplatin therapy is the development of drug resistance within tumor cells (Brabec & Kasparkova, 2002). Cisplatin resistance is frequently attributed to reduced adduct formation, decreased accumulation or increased efflux of the drug, enhanced repair of DNA adducts as well as increased survival due to an inhibition of apoptosis (Wernyj & Morin, 2004). A limited ability to form adducts with DNA can partly be attributed to detoxification by thiol containing agents that are able to coordinate with the drug and form a less toxic conjugate. As a result, increased glutathione as well as elevated metallothionein levels have been associated with cell resistance to cisplatin (Zhang *et al.* 2001; Hagrman *et al.* 2003). In contrast, the binding of polyamines to DNA appears to enhance the ability of cisplatin to alkylate DNA (Marverti *et al.* 2005). Accordingly, a down-regulation of the rate-limiting enzyme in the polyamine catabolic pathway (spermidine/spermine N^1-acetyltransferse (SSAT)) leads to enhanced cisplatin resistance (Marverti *et al.* 2005).

The major repair mechanism linked to cisplatin resistance is nucleotide excision repair (NER), accordingly elevated levels of the rate-limiting enzyme in this pathway [excision repair cross-complementing 1 (ERCC1)] is associated with resistance (Rosell *et al.* 2002). Thymidylate synthetase, an enzyme involved in the *de novo* pyrimidine biosynthetic pathway and essential for DNA replication and repair, and breast cancer 1, early onset associated protein (BRCA1), are examples of other DNA repair enzymes with putative roles in mediating cisplatin resistance (Lenz *et al.* 1996; Husain *et al.* 1998).

Drug efflux, or limited drug accumulation, can often be attributed to the expression of proteins involved in the multi drug resistance (MDR) phenotype. Cisplatin resistance, however, is not typically a component of this phenotype and only one MDR related protein has been associated with cisplatin resistance (MRP2) (Wernyj &Morin, 2004). Proteins involved in copper transport, however, seem to play a role in the accumulation and efflux of platinum containing drugs. This role was proposed following the finding that cells resistant to the toxic effects of copper are often cross resistant to compounds such as cisplatin (Safaei & Howell, 2005). It was confirmed that the expression of proteins such as copper transporter receptor 1 (CTR1) and ATPase copper transporting alpha and beta polypeptides (ATP7A and ATP7B), all involved in regulating cellular copper homeostasis, are altered in various cisplatin resistance models and regulate the cellular uptake as well as efflux of the drug (Safaei & Howell, 2005).

Though the above-mentioned mechanisms are well described in many *in vitro* models of cisplatin resistance, seldom does one mechanism completely account for acquired resistance indicating that resistance is a multifactorial phenomenon. This is exemplified by the 2008/C13* human ovarian carcinoma cell model. Researchers have identified several determinants of cisplatin resistance within this model, including the reduced accumulation of cisplatin within C13* cells and the increased expression of DNA polymerase β in C13* variants (Bergoglio *et al.* 2001; Mann *et al.* 1990). The reduced accumulation of cisplatin is likely related to an increased expression of the copper transporter ATP7A, overexpression of this protein in 2008 cells is able to establish increased cisplatin resistance due to increased efflux of the drug as well as

sequestration of the drug in vesicular compartments (Samimi *et al.* 2004). DNA polymerase β enhances translesion synthesis of platinum lesions and therefore increases C13* cell survival following cisplatin treatment (Bergoglio *et al.* 2001).

Although it was originally reported that defective DNA repair does not account for cisplatin sensitivity in the 2008 cell line (Moorehead *et al.* 1996), recent studies have shown that the Fanconi Anaemia complement protein FANCF is not expressed in 2008 cells due to promoter methylation. In C13* cells on the other hand, the promoter is demethylated and the protein expressed. Restoration of the expression of this DNA damage inducible protein, which plays a role in DNA repair, partially induces cisplatin resistance in the 2008 cells but does not completely account for the resistance of C13* cells (Taniguchi *et al.* 2003). Genes which have been observed to be up-regulated in C13* variants include those coding for *dihydrodiol dehydrogenase*, *tropomyosin* isoforms, *apoliprotein*, *glucose-6-phosphate dehydrogenase* and heat shock proteins (Deng *et al.* 2002; Yamamoto *et al.* 2001). In some cases, overexpression of these proteins in 2008 cells induces partial cisplatin resistance, the mechanisms of which are not fully described (Deng *et al.* 2002; Yamamoto *et al.* 2001). Activity of the MAPK signaling pathways in these cell variants have also been studied; sensitivity to cisplatin appears to require a sustained activation of the JNK and p38 kinase enzymes which then lead to an up-regulation of Fas ligand and eventual cell death by apoptosis. The C13* cells do not display this prolonged activation (Mansouri *et al.* 2003). Transcription factors, c-Fos for example, have been implicated in cisplatin resistance due to the activation of several genes potentially mediating enhanced survival; the association

between c-Fos and cisplatin resistance was addressed in the 2008/C13* model (Moorehead & Singh, 2000). Although expression levels of this protein did not appear to differ significantly between the cells in question, c-*fos* antisense treatment in C13* cells led to increased cisplatin sensitivity indicating that this transcription factor does play a role in the 2008/C13* model (Moorehead & Singh, 2000). Finally, the observed alterations to the mitochondria of C13* cells may be involved in cisplatin resistance. Revertant cells selected on the basis of low membrane potential demonstrated increased sensitivity to the drug (Zinkewich-Peotti & Andrews, 1992). Thus, there are several lines of research describing resistance mechanisms in C13* cells but evidently there is no single mechanism.

Changes to nuclear gene expression in C13* cell variants as compared to parental 2008 cells were the stimulus for this study, in that changes which appeared to be related to altered mitochondrial properties were assumed to represent example targets of mitochondria to nucleus signaling. Since the mitochondrial alterations of C13* variants appear to play a role in inducing cisplatin resistance, it follows that changes to gene expression that result from these alterations may be at least in part responsible for mediating this enhanced resistance. Therefore, any findings were also considered in the context of cisplatin resistance

1.7- Rationale, hypothesis and objectives

Rationale:

In the 2008/C13* cell model of mitochondrial dysfunction, *ets-1* mRNA was observed to be up-regulated in the C13* variants (Kenji Sakata, personal

communication). The fact that Ets-1 is related to a transcription factor (NRF-2/GABP) that acts to regulate the expression of genes encoding mitochondrial proteins, indicates that Ets-1 may also have this ability under certain conditions (Scarpulla, 2002). Ets-1 was therefore postulated to be a mediator of mitochondria-nucleus crosstalk, up-regulated in situations of mitochondrial stress and capable of responding by mediating nuclear gene expression to compensate for such changes.

Hypothesis

Expression of the transcription factor Ets-1 is induced by increased levels of ROS produced by mitochondrial alterations, and, in turn, affects mitochondrial functions via the altered expression of appropriate nuclear genes. Specifically, in the ovarian cancer cell model under study, the up-regulation of Ets-1 leads to decreased electron transport chain function, resulting in greater reliance on glycolysis as well as enhanced cisplatin resistance.

Objectives

1. Evaluate the levels of intracellular ROS in the 2008 and C13* cell model and characterize the expression of Ets-1 in these cells under basal conditions as well as following exposure to H_2O_2, a reducing agent and mitochondrial inhibitors.
2. Evaluate whether the *ets-1* gene promoter is responsive to H_2O_2; if so, determine the approximate promoter region responsible for this responsiveness.
3. Determine the element or elements within the *ets-1* promoter responsible for transcriptional up-regulation under conditions of oxidative stress and evaluate whether a protein complex forms on this element following H_2O_2 induction.

4. Stably alter the expression of Ets-1 in C13* and 2008 cells and examine the effects on mitochondrial functions. Extend these studies into a second cell model.
5. Stably alter the expression of Ets-1 in C13* and 2008 cells and examine the effects on cisplatin resistance. Extend these studies into a second cell model.

2. MATERIALS AND METHODS

Cell Culture-

Human ovarian carcinoma cells (2008 and C13*) were provided by Dr. Paul Andrews, Georgetown University, Rockville MD (Andrews *et al.* 1988). COS-1 transformed monkey kidney fibroblasts were from ATCC (ATCC# CRL-1650) as were human HT29 colorectal adenocarcinoma cells (ATCC# HTB-38) and ES2 ovarian carcinoma cells (ATCC# CRL-1978). 2008 and C13* cells were maintained in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) solution, COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% P/S solution, HT29 cells were maintained in minimum essential medium supplemented with 10% FBS and 1% P/S and ES-2 cells were maintained in McCoy's 5A medium similarly supplemented (media from HyClone & McMaster Media prep; FBS from Cansera and HyClone; P/S from Invitrogen). Cells were passaged by trypsinization with 1X Trypsin/ ethylenediaminetetraaceticacid (EDTA) (Invitrogen) and maintained below passage 20 in 100 mm diameter tissue culture dishes (Falcon Becton Dickenson). All cells were kept at 37°C in a humidified atmosphere of 5% carbon dioxide (CO_2).

Cell treatments-

Oxidant/Antioxidant treatment: In order to manipulate the cellular redox status (control treatments for intracellular H_2O_2 assay), cells were treated with exogenous H_2O_2 directly (100 μM, BDH) or with *N*-acetyl-L-cysteine (NAC) (50 mM, Sigma). Treatments with these same reagents for Western blotting purposes were as follows: 0.1-100 μM H_2O_2 for 30 minutes followed by 0-6 hours of recovery time prior to lysate harvesting and 5 or 50 mM NAC for 4 hours continuous treatment. H_2O_2 was also used as an activator of promoter activity for luciferase and electrophoretic mobility shift assays (EMSA), in these cases a concentration of 100 μM was administered for 30 minutes followed by cell recovery for up to 4 hours. tBHQ (Sigma) was also used for luciferase assays- cells were treated for 6 hours continuously with a concentration of 200 μM.

Inhibition of transcription: Actinomycin D (5 μg/mL, Sigma) was added to either untreated cells or to cells following 30 minute treatment with 0.1 μM H_2O_2. Cell lysates were made after a 6 hour incubation time.

Mitochondrial inhibitors: The mitochondrial ETC was specifically inhibited using the complex I inhibitor rotenone (5 μM, Sigma) and the complex V inhibitor oligomycin B (0.5 μM, Sigma). Treatments were for 2 hours at which time cells were left to recover in normally supplemented media for 0-4 hours.

Treatments with glycolysis inhibitor and cisplatin: Reagents 2-deoxy-D-glucose (2-DG, Sigma), an inhibitor of glycolysis, and cisplatin (Sigma) were used in growth inhibition assays. 2-DG was administered at concentrations ranging from 0.5-7.5 mM for 96 hours

continuously, cisplatin was administered at concentrations of 5-50 µM for a 1 hour pulse, following which cells were grown for 96 hours.

Induction of gene expression: Tetracycline (tet) (Sigma) was used to induce gene expression from a tet-inducible promoter at a concentration of 0.2 µg/mL for 24 hours.

Hypoxia: Cells were placed in hypoxic conditions for 6 hours. Hypoxia was generated using a compact O_2 controller (Biospherix) connected to an O_2 controlling device (PRO-OX110, Biospherix), providing 1% O_2, 94.75% nitrogen (N_2), 4.25% CO_2 (Praxair).

Plasmids-

The following plasmids were used directly in appropriate experiments or as backbones in the construction of various plasmids to be described in the following sections. pcDNA6/TR is a vector encoding TetR, this protein was isolated from the Tn10 transposon in bacteria and confers resistance to tet in bacteria via the induction of various resistance genes. TetR has a high affinity for tet operator sequences within enhancer regions as well as for tet itself. When tet is not present, TetR binds tet operator sequences as a dimer and prevents transcription of genes driven by this operator sequence, when tet is added to cells it binds to TetR which then undergoes a conformational change and dissociates from the operator sequence (Meyer-Ficca *et al.* 2004). pcDNA6/TR encodes the gene for TetR protein under the control of a human cytomegalovirus immediate-early (CMV) promoter and also contains a blasticidin resistance gene. This plasmid, as well as the inducible *ets-1* plasmid, pcDNA4/TO/*ets-1*, made by ligating the full length *ets-1* cDNA into the multiple cloning site (MCS) of pcDNA4/TO (Invitrogen), were kindly provided by Dr. Hiro Yamamoto (Chicago

Institute for Neurosurgery and Neuroresearch, Ill). In the pcDNA4/TO/*ets-1* vector, the *ets-1* gene was placed under the control of a CMV promoter adjacent to a tet operator; this vector expresses a zeocin resistance gene. A pCMVSPORT6 plasmid containing the full length cDNA encoding *Nrf2* was obtained from Invitrogen (mammalian gene collection). The pGL3 basic vector, used to construct luciferase vectors as well as for control purposes, was from Promega. pGL3 contains the firefly luciferase gene but lacks eukaryotic promoter and enhancer sequences allowing the cloning of regulatory sequences into the MCS upstream from the luciferase gene. The pRLTK plasmid, also from Promega, is a vector encoding the *Renilla* luciferase gene under the control of a thymidine kinase promoter and acts as an internal control in luciferase experiments. Unmodified pcDNA3.0 from Invitrogen was also used for control experiments where appropriate. pDRIVE cloning vector, containing U overhang elements at both 3' ends and used to clone polymerase chain reaction (PCR) fragments was from Qiagen (within Qiagen PCR cloning kit).

Construction of pcDNA3-ets-1 expression plasmid-

Human *ets-1* cDNA was obtained by reverse transcription PCR (RT-PCR) on RNA extracted from C13* cells using a gene specific reverse primer. RNA was first extracted from C13* cells using an RNA extraction kit from Qiagen, prepared following the manufacturers protocol and was quantified by measuring the absorbance of 260nm wavelength ultraviolet (UV) light (A_{260}) using a Beckman DU spectrophotometer. 2 µg of this RNA was used in the RT-PCR reaction. RNA was combined with a gene specific reverse primer (shown on table 3, *ets-1* reverse primer), and MilliQ water up to a volume

of 10 μL, denatured at 65°C for 10 minutes and placed on ice for 10 minutes. Denatured RNA/primer was then mixed with 4 μL 5X 1st strand buffer, 2 μL 10 mM dNTP mix, 1 μL 0.1 mM dithiothreitol (DTT), 1μL RNAse out, 1 μL MilliQ water treated with diethyl pyrocarbonate (DEPC) and 1 μL of the reverse transcriptase enzyme (all reagents from Invitrogen). The mixture was left at room temperature for 10 minutes followed by a complementary DNA (cDNA) synthesis step at 50°C for 1 hour and finally a 10 minute termination reaction at 85° C. Following the termination reaction, RNAse H was added for 20 minutes at 37°C in order to denature the original RNA template.

The resulting cDNA was then used in a PCR amplification step using primers introducing flanking *Hind*III sites (shown on table 3, *ets-1* forward and reverse primers). PCR conditions were as follows: 2 μL of cDNA template from the RT reaction, with 5 μL of 10X PCR buffer, 1.5 μL 50 mM $MgCl_2$, 1 μL 10 mM dNTP mix, 1 μL of each primer in a 10 μM solution, 1 μL of 5 X diluted Taq polymerase and DEPC treated MilliQ water up to a 50 μl total volume (all reagents from Invitrogen). The PCR program, run on a Perkin Elmer GeneAmp 9600 PCR machine, consisted of a 35 second denaturation step at 94°C, 35 second annealing at 55°C and 55 seconds extension at 72°C. After 35 cycles, the PCR product was separated by electrophoresis on a 1% agarose gel, made with 1X TAE buffer (40mM Tris, 2 mM $Na_2EDTA \cdot 2H_2O$, 1 M glacial acetic acid and 0.5 μg/mL ethidium bromide). The appropriate sized product (1.4 kB) was visualized under UV light and extracted from the agarose gel into 30 μL of TE buffer (10 mM Tris Base, 1 mM EDTA, pH 8.0), using a Qiagen gel extraction kit following the manufacturers protocol.

10 μL of the extracted product was used in a restriction digestion reaction with 1 μL *Hind*III enzyme (10 U), 2 μL React 2 buffer and MilliQ water up to 20 μL, 1 μg of pcDNA3.0 was similarly restricted with *Hind*III- both reactions were left at 37° C for 1 hour (all restriction enzymes and digestion buffers were from Invitrogen). Following digestion, products were again run on a 1% agarose gel and the cut DNA fragments were extracted and used in a ligation reaction. In order to set up a ligation reaction, approximate amount of restricted plasmid and cDNA were estimated by running a small amount of each on a 1% agarose gel adjacent to a quantitative ladder which allowed a visual estimation of DNA concentration (i.e. samples of DNA of known concentration are on the ladder, intensity of sample DNA samples is compared to these known values to estimate a concentration of DNA in ng/μL). Based on this quantification, ligation reactions were set up according to the following calculation: 20 *f*m vector DNA and 60 *f*m insert (*ets-1* cDNA) were used, where *f*m= {(ng/μL DNA * 2)/MW(approximately 650 * # of base pairs)}*10^6. Ligation reactions were made up of the calculated amounts of each DNA, 1 μL T4 DNA ligase enzyme, 2 μL 10X ligation buffer and MilliQ water up to a volume of 20 μL (all reagents from Invitrogen). Reactions were left at room temperature overnight and used for transformations the following day. Transformation procedures, as well as the replication of resulting plasmids is described in the plasmid replication and purification section.

Table 3: Primers used in PCR reactions and plasmid construction

Primer description	**Primer sequence (5′ to 3′)**
ets-1 forward	CCCAAGCTTATGAAGGCGGCCGTCGATCTC
ets-1 reverse	CCCAAGCTTTCACTCGTCGGCATCTGGCTTTAC
ets-1 promoter forward	GGGGGTACCCCGAGCTCAGTGCCTGGTTCT
ets-1 promoter reverse	CCGCTCGAGACGTACGGGATGGTAGCAAGT
AP-1 mutation, forward	AGAAACACACGCCC**G**GACTCAAGAT
AP-1 mutation, reverse	GATCTTGAGTC**C**GGGCGTGT
HRE mutation, forward	CATCCACATGCCTCA**AA**TCCTGTGTGT
HRE mutation, reverse	CTGACACACAGGA**TT**TGAGG
ARE mutation 1, forward	G**G**GACCAAGCCCTCAAGAATGCGT
ARE mutation 1, reverse	GCATTCTTGAGGGCTTGGTC**C**CCCG
ARE mutation 2, forward	ACCAA**AT**CCTCAAGAATGCGTGGAG
ARE mutation 2, reverse	CTCCACGCATTCTTGAGG**AT**TTGGT

Construction of pcDNA3-Nrf2-

Human full length *Nrf2* cDNA, contained within a pCMVSPORT6 vector, was isolated by restriction digestion. 1 μg of the pCMVSPORT6-*Nrf2* vector was digested with *Kpn*I and *Xho*I, as was an approximately equal amount of the pcDNA3.0 expression vector. For the digestion reaction, DNA was combined with 1μL *Kpn*I and 2μL React1 buffer in a total volume of 20 μL, digestion took place for 1 hour at 37° C at which time the NaCl concentration of the buffer was brought up to 50 mM with the addition of 1 μL 1M NaCl and 1 μL *Xho*I was added- DNA was digested for a further hour. Resulting digested DNA, (cDNA insert and digested pcDNA3.0 vector), were isolated by agarose gel electrophoresis followed by gel extraction as described for the construction of pCDNA3-*ets-1*. Following the estimation of DNA concentration, *Nrf2*+pcDNA3

ligations were set up overnight (again, as described for the construction of pcDNA3-*ets-1*). The ligation reaction was then used in transformation, plasmid replication and purification steps (described in appropriate sections).

Construction of full length ets-1 promoter pGL3 vector, pGL3-ets-1-wildtype (wt)-

A fragment of the 5′ *ets-1* promoter was cloned into a pGL3 basic luciferase plasmid. The sequence of the *ets*-1 promoter has been published and the primers were thus chosen to amplify a 1.4 kB region (Oka *et al.* 1991). The promoter was isolated by PCR amplification from DNA isolated from C13* ovarian carcinoma cells. DNA was extracted as follows: subconfluent monolayers of C13*cells were removed from a single 150 x 20 mm tissue culture plate, washed with phosphate buffered saline (PBS), and centrifuged to obtain a cell pellet. After aspiration of PBS, cells were suspended in lysis buffer (100 mM Tris (pH 8.3), 5 mM EDTA, 0.2% sodium dodecyl sulfate (SDS), 200 mM NaCl, 2.4 mg/ml RNase A) and incubated for 2 min at room temperature. Proteinase K was added at a final concentration of 0.4 mg/mL and incubated overnight at 55°C. One lysate volume of chloroform and NaCl (420 mM) was added and vortexed for 30 s. The mixture was then centrifuged for 10 min at 10000 rpm. The supernatant was collected and added to two supernatant volumes of absolute ethanol. Precipitated DNA was spooled out and placed in a solution of 500 mM Tris (pH 8.3)/100 mM EDTA. Samples were incubated for 30 min at 65°C with the lid open and an additional 2 hr at 4°C.

PCR amplification was done using primers introducing a *Kpn*I site in the 5'end and a *Xho*I site in the 3'end. Primer sequences are shown on table 3 (*ets-1* promoter forward and reverse). The PCR reaction and conditions were as follows: reaction

consisted of 15 ng DNA, 1 μL each primer (10 μM solutions), 10 μL betaine (Sigma), 1 μL 10 mM dNTP mix, 1.5 μL 50 mM $MgCl_2$, 5 μL 10X PCR buffer, 1 μL 5X dilution Taq polymerase, up to 50 μL volume with MilliQ water. Reaction conditions were identical to those described for the construction of pcDNA3-*ets-1* except that the annealing temperature was set at 62° C. Once separated and extracted from an agarose gel as described in the previous section, this DNA fragment as well as the pGL3 basic vector were digested in a two step reaction with *Kpn*I and *Xho*I (as described in the construction of pcDNA3-*Nrf2* section). Following digestion, DNA and vector fragments were ligated as described for pcDNA3-*ets-1*. The overall cloning strategy for this plasmid, as well as for the truncations to be described in the following section, is summarized in figure 5.

Construction of ets-1 promoter truncation plasmids: pGL3-ets-1-1000 and pGL3-ets-1-570-

In order to create truncations of the *ets-1* promoter of approximate sizes of 1000 bp and 570 bp, two internal restriction sites (*Hind*III at approximate position 400 of the promoter region and *Sma*I at position 830) were chosen to use for appropriate digestions. The smaller truncation was made by digesting the pGL3-*ets-1*-wt plasmid with *Sma*I and *Xho*I in a 2 step reaction to extract the region of interest from the wildtype construct. The digestion reaction was done using 2 μg of plasmid DNA, 1 μL *Sma*I enzyme, 2 μL React 4 buffer and MilliQ water up to a volume of 20 μL, after 1 hour of 30°C incubation, the *Sma*I enzyme was heat inactivated at 65°C for 10 minutes. 1 μL *Xho*I was then added along with 2 μL React2 buffer, incubation continued for another hour at 37° C. The

resulting approximately 570 bp fragment was isolated and purified as described in prior plasmid preparation sections and ligated into a pGL3 vector which had also been digested with these two enzymes.

This type of strategy was not possible for the 1000bp truncation- a *Hind*III site is located downstream of the *Xho*I site in the pGL3 vector, thus cutting with *Hind*III and *Xho*I to create a ligation site in the vector would cause a *Hind*III-*Xho*I fragment of the promoter to be cloned in backwards. Thus, the *Hind*III to *Xho*I 1000 bp fragment of the promoter was cut out of the wildtype vector and ligated into a pDRIVE cloning vector as a first step. Extraction of the appropriate fragment involved a double digestion with 1 μL *Hind*III, 1 μL *Xho*I and 2 μL React2 buffer for 1 hour at 37° C. Digestion was followed by purification via extraction from an agarose gel as described in previous sections. Ligation into the pDRIVE vector involved a 30 minute reaction with the ligation master mix provided (Qiagen) as well as appropriate amounts of DNA, estimated as described for the ligation reaction for the construction of pcDNA3-*ets-1*. Once the pDRIVE-*ets-1*-1000 plasmid was purified, the promoter region of interest was cut out using *Kpn*I and *Xho*I, as these sites are present outside the promoter region in the correct orientation. The pGL3 vector was similarly digested with *Kpn*I and *Xho*I, (digestion conditions as previously described), and the resulting DNA fragments were ligated. The cloning strategy for these promoter truncations is depicted in figure 5.

Figure 5: Cloning strategy for *ets-1* promoter & *ets-1* promoter truncation constructs. A) The *ets-1* promoter was cloned according to the following steps: 1- The promoter region of interest was amplified by PCR from a DNA template using primers introducing *Kpn*I and *Xho*I sites. 2- The amplified DNA fragment was digested with *Kpn*I and *Xho*I restriction enzymes, as was the pGL3 basic vector. 3- The digested DNA fragment and vector were ligated to create the pGL3-*ets-1*-wt construct. B) The *ets-1* promoter 570 bp truncation was cloned as follows: 1- The pGL3-*ets-1*-wt vector was digested with *Sma*I and *Xho*I, as was the pGL3 basic vector. 2- The digested DNA fragment and vector were ligated to create the pGL3-*ets-1*-570 construct. C) The *ets-1* promoter 1000 bp truncation was cloned as follows: 1- The pGL3-*ets-1*-wt vector was digested with *Hind*III and *Xho*I. 2- The digested DNA fragment was ligated into a pDRIVE vector to produce pDRIVE-*ets-1*-1000. 3- The pDRIVE-*ets-1*-1000 vector was digested with *Kpn*I and *Xho*I, as was the pGL3 basic vector. 4- The digested DNA fragment and vector were ligated to create the pGL3-*ets-1*-1000 construct.

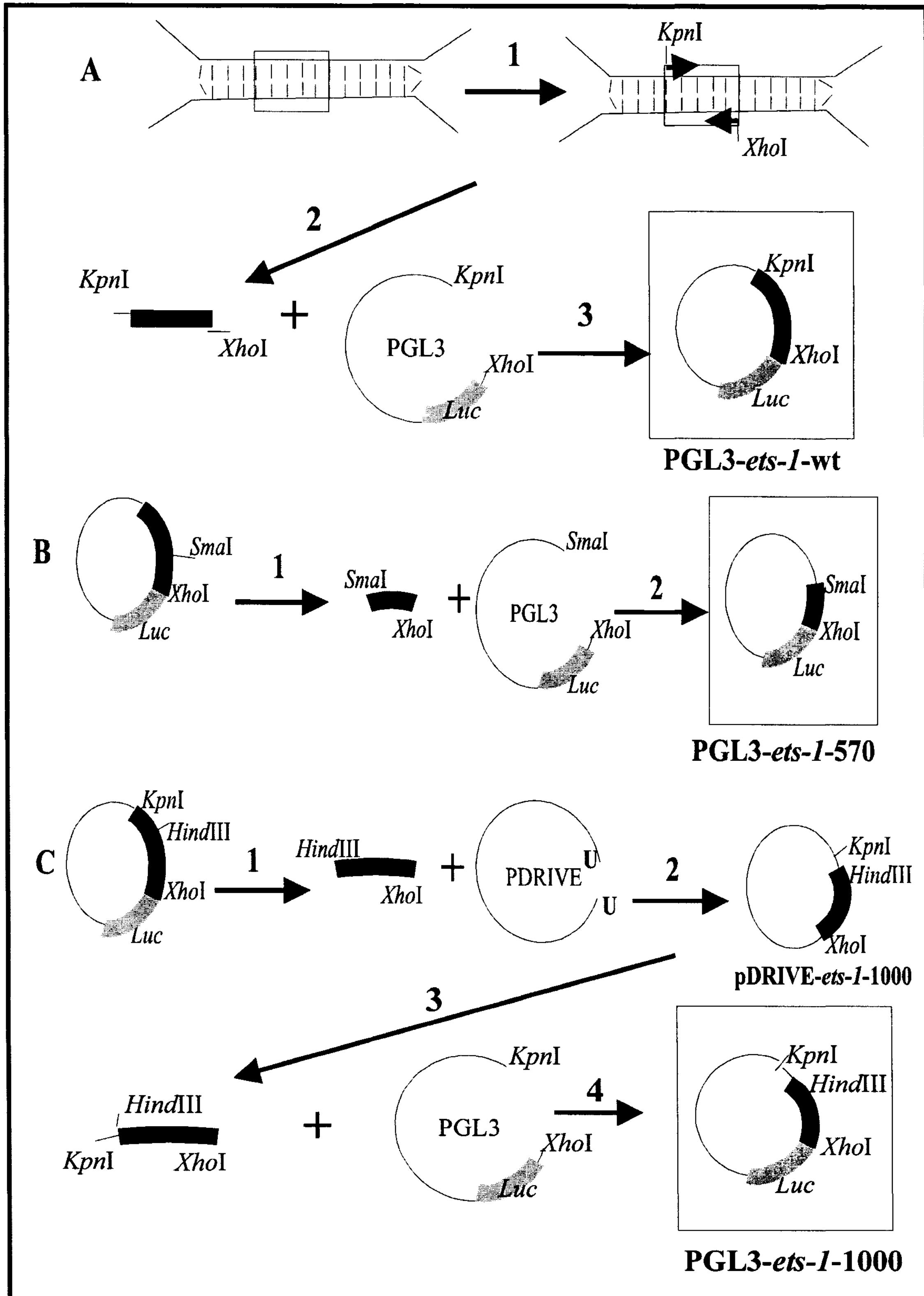


Figure 5: Cloning strategy for *ets-1* promoter & *ets-1* promoter truncation constructs

Construction of mutant promoter plasmids: mutAP-1, mutHRE, mutARE-

The creation of point mutations at specific sites within the *ets-1* promoter was achieved using overlap extension mutagenesis. The principle of this technique is depicted in figure 6, it can be seen that primers used introducing the mutation of interest are designed in both the forward and reverse direction. In conjunction with primers for the entire region (in this case the forward and reverse primers for the wild type *ets-1* promoter), two fragments are initially amplified containing the point mutation. A second PCR step then uses both fragments as the DNA template and amplifies the entire promoter region with the mutation of interest hopefully in place. In the case of the desired AP-1 mutation, a T to G change was attempted in the following region: **T**GACTCA to **G**GACTCA. PCR reaction number 1 made use of the *ets-1* promoter forward primer (table 3) and a reverse primer introducing the desired mutation (AP-1 mutation, reverse primer on table 3) in one reaction and the *ets-1* promoter reverse primer with a forward primer introducing the same mutation (AP-1 mutation, forward primer, table 3) in another. In both cases, reactions contained 1 μL of each primer, 1 μg of pGL3-*ets-1*-wt plasmid DNA, 1 μL dNTP mix (10 mM), 5 μL 10X PCR buffer, 1 μl 5X dilution of Taq polymerase, 1.5 μL $MgCl_2$ (50mM), 10 μL Betaine, made up to 50 μL with MilliQ water. Reaction conditions were as described for pcDNA3-*ets-1*, with the annealing temperature set at 55°C. The products of these PCR reactions were run on a 1% agarose gel, expected size bands at 880 bp (from AP-1 mutation forward primer) and 535 bp (from AP-1 mutation reverse primer) were observed and extracted into 30 μL MilliQ water as previously described. The second PCR reaction made use of 1 μL of

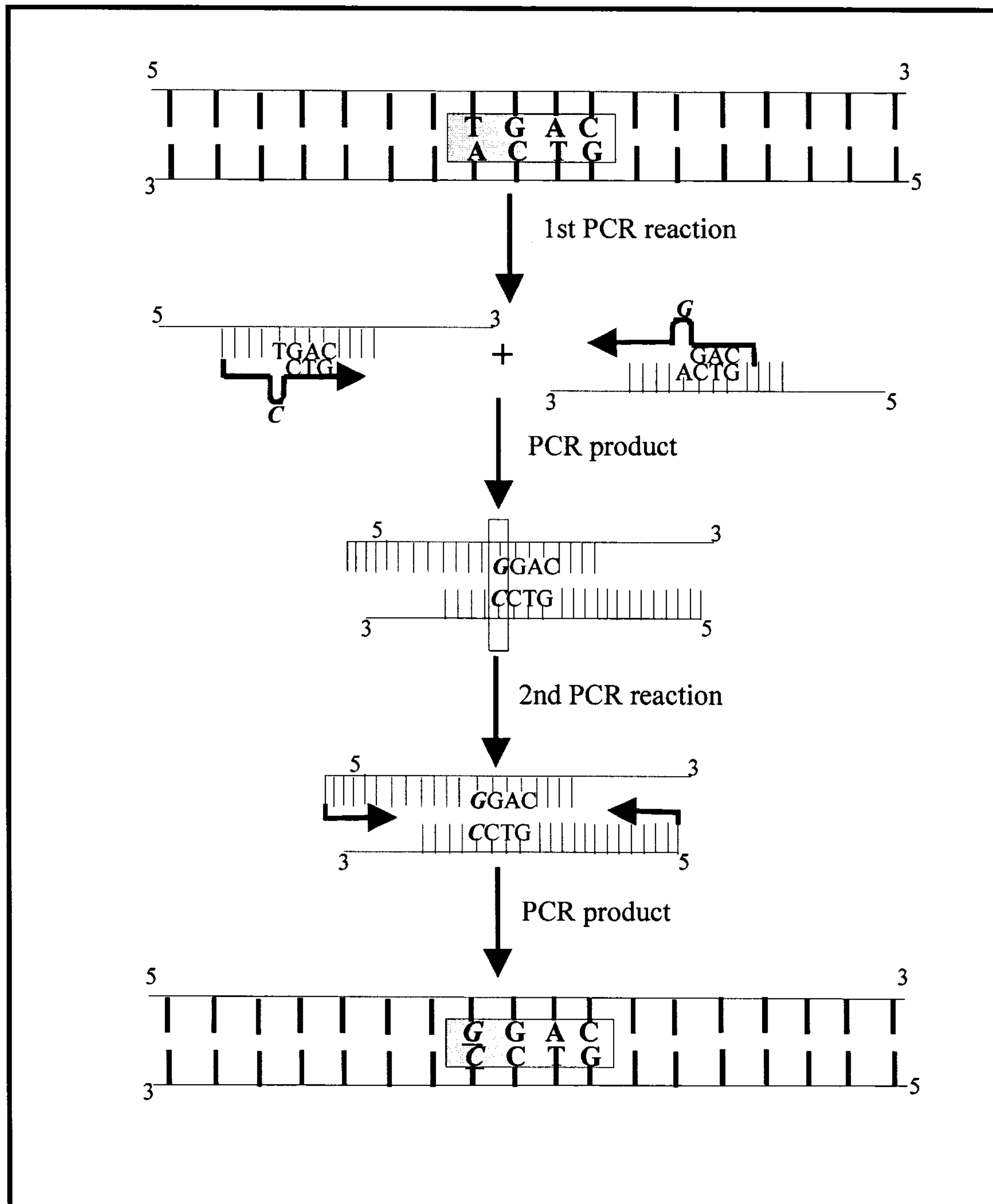


Figure 6: Use of overlap extension mutagenesis to create site specfic mutations in the *ets-1* promoter. The DNA template is PCR amplified in 2 steps, the first is actually two separate reactions, introducing the desired mutation in both directions. The mutation shown here is a T to G change. The resulting PCR products from both reactions are combined and used as the template for a 2nd PCR reaction, using primers for the entire region of interest. The resulting fragment harbors the desired mutation.

each extracted product as the DNA template, 1 μL of each *ets-1* promoter full length primer and all other reagents and conditions as in the first reaction. The resulting amplified fragment ran at the expected 1.4 kB size, was extracted, digested with *Kpn*I and *Xho*I and ligated into a *Kpn*I, *Xho*I digested pGL3 vector (digestion and ligation steps as described in previous sections) to create the plasmid pGL3-mutAP-1.

The HRE mutation desired, a CG to AT change in the sequence TCA**CG**TCC to TCA**AA**TCC was similarly generated by two PCR reactions, the first using the full length *ets-1* promoter forward primer with the mutation generating reverse primer (HRE mutation reverse primer, table 3) in one reaction and the second with the full length *ets-1* reverse primer and a mutation generating forward primer (HRE mutation forward primer, table 3). These amplified fragments of sizes 830 (mutation forward primer) and 585 (mutation reverse primer) bp were extracted and used as the template for a second PCR reaction. Reaction conditions and subsequent cloning steps were as described for thegeneration of the mutAP-1 plasmid, resulting plasmid with this mutation was designated pGL3-mutHRE.

In the case of the desired ARE mutant, two mutations were necessary within a certain promoter region. These two mutations were thus created in succession. The first involved a T to G change in the following sequence, **T**GACCAAGC to **G**GACCAAGC and made use of the ARE mutation 1 forward and reverse primers, shown on table 3, used in separate reactions with the corresponding *ets-1* promoter primers. Resulting fragment sizes of 695 (ARE mutation 1 forward primer) and 720 (ARE mutation 2 reverse primer) bp were extracted and used for a second PCR reaction as the DNA template. The 1.4 kB

PCR product of this second reaction was then cloned into a pGL3 vector. The resulting plasmid was used as a template to create a second mutation in the sequence, a GC to AT change as follows: GGACCAA**GC** to GGACCAA**AT**. The first PCR reaction introducing this mutation made use of the ARE mutation 2 forward and reverse primers, shown on table 3, again used in separate reactions with full length *ets-1* promoter primers. The resulting amplified fragments of 689 (ARE mutation 2 forward primer) and 726 (ARE mutation 2 reverse primer) bp sizes were extracted and used in the second PCR reaction as described for the pGL3-mutAP-1 plasmid. The resulting plasmid constructed from this amplified sequence was designated pGL3-mutARE. Sequences of all plasmids constructed were confirmed by sequencing analysis at Mobix facility (McMaster University).

Plasmid replication and purification-

Transformation-

Competent *E.Coli* DH5α cells (Invitrogen) were transformed with the plasmids constructed (as described above) following a 24 hour ligation period or with expression plasmids provided. 2 or 10 μL of a ligation mixture or a volume equivalent to 1 μg of provided plasmids was added to 50 μL of competent cells which were incubated on ice for 30 minutes followed by a 1 minute heat shock step at 42° C. Cells recovered on ice for 1 minute and were then added to LB solution (1% w/v bactotryptone, 0.5% w/v bactoyeast extract, 170 mM NaCl, 5 mM NaOH) up to a volume of 1 mL and were shaken for 45 minutes at 37° C. 100 μL of this solution, or 100 μL of a 10X dilution,

was then plated onto LB agar plates [plates made with LB supplemented with 1.5% w/v bactoagar and 100 μg/mL ampicillin (Sigma)]. Plates were incubated overnight at 37° C.

Plasmid miniprep-

Successful transformation resulted in the appearance of distinct colonies following 24 hours which were picked and shaken at 37°C, 250 rpm in 5 mL LB with 100 μg/mL ampicillin for 8-12 hours. 1 mL of this solution was aliquoted into 1.5 mL tubes and cells were pelleted (13000 rpm, 30 seconds). The pellet was re-suspended in 100 μL of solution 1 (50 mM D-glucose, 25 mM Tris-HCL pH 8.0, 10 mM EDTA) and vortexed. 200 μL of solution 2 was then added (1 % SDS, 0.2 M NaOH), re-vortexed and finally 150 μL of ice cold solution 3 (5 M potassium acetate) was added; cells were mixed and left on ice for 10 minutes. Tubes were then centrifuged at 13000 rpm, at 4° C, for 10 minutes and the supernatant was collected. An equal volume of 1:1 phenol (Invitrogen) chloroform (Caledon) was added to the solution, vortexed on low for 5 seconds and centrifuged for 5 minutes at 13000 rpm. The top layer of the resulting separated solution was collected and 0.1 volume of 3 M sodium acetate (pH 5.5) was added. 2 volumes of ice cold absolute ethanol (commercial alcohols inc.) was added and following vortexing, the solution was centrifuged at 13000 rpm, at 4° C for 20 minutes. The supernatant was discarded and the pellet was washed with 500 μL of 70% ethanol and recentrifuged at 13000 RPM for 5 minutes. Ethanol was then aspirated off and the pellet was dried and resuspended in 30 μL TE buffer (10 mM Tris Base, 1 mM EDTA pH 8). The isolation of the correct plasmid was verified by restriction digest analysis on 10

μL of the miniprepped solution, using appropriate enzymes depending on the specific plasmid. Resulting cut plasmids were electrophoresed on a 1% agarose gel containing ethidium bromide and visualized under UV light to visualize band sizes (digestion and agarose gel details as described in construction of plasmid sections).

Plasmid maxiprep-

The remaining 4 mL of LB in which cultures had been grown up, and had been confirmed to contain the correct plasmids by miniprep analysis, was added to 250 mL of LB with 100 μg/mL ampicillin and shaken overnight at 37° C and 250 rpm. Cells were then pelleted at 5000g for 10 minutes and plasmid DNA was extracted from the resulting pellet using either a Qiagen plasma maxi kit or a Sigma GenElute HP plasmid maxiprep kit according to manufacturers protocols. The resulting pelleted DNA was resuspended in 200 μL TE buffer and DNA concentration was evaluated by measuring the absorbance of 260 nm wavelength UV light (A_{260}) using a Beckman DU spectrophotometer.

Stable transfection of inducible ets-1 gene into 2008 cells and constitutively expressed ets-1 into HT29 and ES-2 cells-

2008 cells were plated in a 6 well tissue culture plate at 250 000 cells per well and incubated for 24 hrs. Cells were then transfected with 2.0 μg pcDNA6/TR by liposome-mediated transfer using Lipofectamine™ (Invitrogen/life technologies). Following 3 weeks of selection in 10 μg/mL Blasticidin (Invitrogen/life technologies), surviving clones were isolated. Tetracycline repressor (TetR) protein expression was evaluated. The surviving clone with highest expression of TetR was then similarly transfected with

the pcDNA4/TO/*ets-1* vector. Following 3 weeks of selection in 0.1 mg/mL Zeocin (Invitrogen/Life technologies), surviving clones were isolated and tested for inducible *ets-1* expression (Ets-1 levels were evaluated following 24 hrs of treatment with 2 μg/mL tet). HT29 cells and ES-2 were similarly transfected with pcDNA3-*ets-1* (2 μg) and treated for three weeks with 1mg/mL (HT29) or 0.4 mg/mL (ES-2) Geneticin® (Invitrogen/ Life Sciences) at which time surviving clones were isolated and evaluated for Ets-1 expression.

Transient transfections and luciferase assays-

Cells were seeded in 6 well plates at a density of 2.5 x 10^5 cells/well and grown overnight. The following day, cells were transfected using Lipofectamine reagent (Invitrogen). The transfection complex containing 2 μg plasmid DNA and 0.2 μg of pRLTK (Promega) and the transfection reagent was added to each well in the absence of FBS. 5 hours after transfection, media was replaced with supplemented medium containing 5% FBS. Cells were incubated for a further 20 hours at which time they were incubated with inducers or left untreated for specified amounts of time. Following treatments, cells were lysed using the lysis buffer provided by the manufacturer of the Dual luciferase reporter assay kit (Promega). Lysis buffer was diluted 5X in MilliQ water, cells were washed with ice cold PBS and then scraped using a rubber policeman into 200 μL of diluted lysis buffer. Lysates were transferred to 1.5 mL tubes and underwent one freeze thaw cycle (frozen at –80 C). *Renilla* and firefly luciferase were then measured in these lysates using the dual luciferase assay kit (Promega) with a microplate luminometer (Tropix, Bedford, MA). Briefly, the substrate for firefly

luciferase, containing beetle luciferin, was prepared in 10 mL of supplied buffer and aliquoted into the luminometers first injector. The *Renilla* substrate, stop and glo, which quenches firefly luciferase activity and simultaneously provides the *Renilla* substrate coelenterazine was diluted in provided buffer from a 50X stock solution and aliqoted into the second injector of the luminometer. Injectors were primed with each solution prior to assay and 20 μL of lysates was added to a 96 well, opaque plate (Tropix). The luminometer was programmed to deliver 100 μL of substrate 1 to each well in use followed by a 2 second pre-measurement delay and 10 second measurement time period, 100 μL of the second substrate was then delivered with an identical program. Luciferase activities were normalized to *Renilla* activities, and those reported are from 3- 5 different transfections.

Cell proliferation assay-

Cellular growth rate and the inhibition of growth following the administration of various agents were evaluated by assessing total cell number. 2000 cells were plated onto 96 well tissue culture plates (Falcon Becton Dickinson) and were allowed to adhere- treatment of various agents occurred 24 hours later. Cells were then cultured for a further 96 hrs at 37°C and 5% CO_2 at which time a Hoechst DNA content assay was performed. The DNA binding bisbenzimidazo Hoechst 33258 (H 33258) (Calbiochem- Novabiochem) is a fluorochrome used for the quantitation of cell number. Following treatment, cells were washed and lysed in MilliQ water and 2 μg/mL Hoechst 33258 stain diluted in TNE buffer (10 mM Tris, 1 mM EDTA, 2 M NaCl, pH 7.4) was added. Fluorescence, an indication of total cellular DNA content, was then evaluated using a

Cytofluor series 4000 multiwell plate reader (excitation 350 nm, emission 460 nm) (PerSeptive Biosystems, Framingham, MA). Cell number was standardized to fluorescence for each cell type by comparison with a standard curve generated from seeding known cell numbers.

For growth in glucose free media, glucose free RPMI (Invitrogen) supplemented with 110mg /L pyruvate (Invitrogen) was added to cells following overnight adherence. Plates of cells were frozen every 24 hrs up to and including the 96 hr time point and Hoechst assays were done on each time point. All growth assays were repeated at least three times; representative growth curves are presented.

Measurement of intracellular ROS-

Intracellular ROS production was assessed using 2'7'- dichlorodihydrofluorescin diacetate (DCFH-DA) (Molecular probes). DCFH-DA is a fluorescent probe capable of permeating cell membranes due to the lipid soluble acetate group which is subsequently cleaved by intracellular esterases. The resulting cleaved form, DCFH, does not easily leak out of cells and can be oxidized by intracellular H_2O_2 to the fluorescent product DCF (Oyama *et al.* 1994). 500 000 subconfluent cells were suspended in serum-free media containing 10 μM DCFH-DA for 15 minutes, they were then washed and resuspended in serum free media for a further 15 minutes. During this 15 minute recovery time, samples were treated with antioxidant or oxidizing agent. Cell fluorescence was measured using a flow cytometer equipped with an argon laser lamp (Beckman Coulter). Cells were passed through the argon laser beam at an excitation wavelength of 488 nm, light emission was measured in the FL1 filter region, detecting wavelengths less than 550 nm-

the number of cells positive for the fluorescence emitting DCF were counted. Dead cells and debris were excluded from analysis by gating of forward and side scatter measurements.

Preparation of whole cell lysates-

100 mm plates of cells were washed 2X with ice cold PBS. Cells were scraped into 2 mL of ice cold PBS using a rubber policeman, were pelleted at 1000 RPM for 5 minutes and re-suspended in 100 μL of NP40 lysis buffer [1% NP40 (or igepal, Sigma), 0.05 M Tris pH 7.4, 0.005 M EDTA pH 8.0, 0.4 M NaCl, protease inhibitors added just before use in the form of a protease cocktail pill (Roche)]. The suspension was left on ice for 30 minutes, rocked on a rocking platform in a 4° C coldroom for 30 minutes,spun at 13000 rpm at 4° C and the supernatant was collected.

Protein quantitation -

Total protein was measured in cells lysates using the Bio-Rad protein assay (Bio-Rad) which is based on the Bradford assay involving a protein binding dye which is colourimetric (Bradford, 1976). The Coomassie brilliant blue g-250 dye involved binds to basic and aromatic amino acid side chains resulting in a colour change shift to red. Bovine serum albumin (Sigma) standard solutions ranging from 0 to 0.5 mg/mL BSA in MilliQ water were prepared and used to generate a standard curve, with the colour change measured as absorbance at a wavelengh of 570 nm using a BioTek instruments EL 340 microplate biokinetics reader. 10 μL of each standard dilution as well as 10-20X dilutions of cell lysates were aliquoted into wells of a 96 well plate at which time 200 μL

of a 5X dilution of the Bio-Rad protein quantitation reagent was added, absorbance at 570 nm was read after 5 minutes.

Treatment of cell lysates with Calf intestine alkaline phosphatase (CIAP)-

30-50 μg of cell lysate, prepared in lysis buffer without protease inhibitors, was aliquoted into 1.5 mL tubes. 3 μL of 10X dephosphorylation buffer, 1 mM $MgCl_2$ and 3 μL (30 U) CIAP were added and the solution was brought to a volume of 30 μL. This solution was then incubated at 30°C for 2 hours at which time sample loading buffer, containing SDS, was added to terminate the reaction. Samples were then prepared for Western blot analysis.

Western blot analysis -

30-50 μg of whole cell lysate protein, (determined using the Bradford protein assay), was denatured in SDS containing buffer by boiling for 5 minutes, samples were then separated by electrophoresis in SDS-10% polyacrylamide gel. Once separated, protein was transferred onto a nitrocellulose membrane (Amersham Biosciences) in transfer buffer (0.25 M Tris, 2 M glycine, 20% methanol) at 60 volts for 90 minutes. The membrane was then blocked with 5% skim milk in Tris-buffered saline plus Tween-20 (TBST) (10mM Tris-HCl, 150mM NaCl, 0.05% Tween-20) for 1 hour. Membranes were then incubated overnight with either a monoclonal mouse anti-Ets-1 antibody (BD Biosciences), a monoclonal mouse anti-β-Actin antibody (MP Biomedicals), a monoclonal mouse anti-HIF-1α antibody (Transduction laboratories), a mix of mouse monoclonal anti-TetR antibodies (Molecular Biologische Technologie), a polyclonal rabbit anti-Ets-2 or an anti-Nrf2 antibody (Santa Cruz). Following washing and 1 hour

incubation in horseradish peroxidase-linked anti mouse or rabbit IgG secondary (Santa Cruz), membranes were washed again in TBST. Proteins were detected by chemiluminescence of the secondary antibody induced using ECL (Amersham biosciences) and detected by development on Kodak X-Omat AR film.

Nuclear lysate preparation-

2 100 mm plates of untreated cells or cells treated with H_2O_2 following various recovery times were washed with PBS and cells were scraped into 2 mL PBS (cells from 2 plates were combined for each condition). Cells were pelleted by centrifugation at 1000 rpm for 5 minutes and the supernatant was removed. Pellets were re-suspended in 200 µL ice cold buffer 1 (Cer I from Pierce NE-PER nuclear and cytoplasmic extraction kit), transferred to 1.5 mL tubes and left on ice for 10 minutes. 11 µL of buffer 2 (Cer II) as well as 1X protease inhibitor cocktail in MilliQ water (Roche) was added, solutions were vortexed and left on ice for a further minute. Tubes were then centrifuged at maximum speed for 5 minutes and the supernatant was collected (cytoplasmic fraction). The remaining pellet was resuspended in 100 µL buffer 3 (NER) and left on ice for 40 minutes, and was vortexed every 10 minutes during this time period. Tubes were spun at maximum speed for 10 minutes and the resulting supernatant, the nuclear fraction, was kept and stored at -80°C.

Electrophoretic mobility shift assay-

The *ets-1* gene ARE (in a 25mer oligonucleotide: sense: GAGAGCGGG**TGACCAAGC**CCTCAAG, antisense: CTTGAGG**GCTTGGTCA**CCCGCTCTC) was annealed in annealing buffer (1.75 µg of

each strand, 0.4 M Tris-HCl, pH 8.0, 0.1 M $MgCl_2$) at 100° C for 5 minutes and left at room temperature overnight. The annealed oligonucleotide was then endlabeled with γ-P^{32}-ATP (Perkin Elmer) and T4 polynucleotide kinase (New England Biolabs) as was an annealed, mutated version of this oligonucleotide (corresponding to sequence of mutARE plasmid, sense: GAGAGCGGG**GGACCAAAT**CCTCAAG, antisense: CTTGAGG**ATTTGGTCC**CCCGCTCTC) and a control annealed oligonucleotide, containing the NQO1 gene ARE (sense: CAGTACAG**TGACTCAGC**AGAATCT antisense: GATTCT**GCTGAGTCA**CTGTGACTG) (Dhakshinamoorthy & Jaiswal, 2000). 10 μg of nuclear extracts were pre-incubated with binding buffer (50 mM TrisCl pH 7.5, 20% glycerol, 5 mM $MgCl_2$, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM DTT, 0.25 mg/mL poly dIdC) for 10 minutes, binding reactions were then labeled with oligonucleotide probes for 30 minutes at room temperature. Specific binding was confirmed by using 100 fold excess unlabeled *ets-1*, mut or NQO1 ARE oligonucleotides as competitors. Unlabeled probes were added to lysates in binding buffer and kept on ice for 15 minutes prior to the addition of the labeled probe. For supershift analysis, nuclear extracts were incubated with 1-2 μl Nrf2 antibody overnight at 4°C prior to the binding reaction. Protein-DNA complexes were separated by gel electrophoresis on a 5% polyacrylamide gel, prepared with Tris-Borate (0.25 TBE) and glycerol (1.25%) which had been pre-run for 90 minutes at 180 volts. The gel was then transferred onto a Whatman filter paper, covered with plastic wrap and dried under vacuum at 80°C for 60 minutes. The dried filter was autoradiographed overnight and phosphorescence was

quantified using a STORM 860 phosphoimager and ImageQuant 5.0 software (Amersham Pharmacia Biotech).

Oligonucleotide array analysis-

Total RNA was purified using the RNeasy mini kit (Qiagen, Valencia, CA) from 2008 (tet-induced for 24 hrs), 2008-*ets-1*-5B and 2008-*ets-1*-5B (tet-induced for 24 hrs) cells. The relative abundance of specific RNA species was evaluated through the use of the Human Genome Focus array (Affymetrix, Santa Clara, CA) that contains probes (small DNA fragments chemically synthesized on a coated quartz surface) for the detection of approximately 8500 human gene sequences. Details for the preparation of biotinylated cRNA (generated from the RNA samples) and its use in probing the array is available from the array manufacturer (Affymetrix) and was performed at the Affymetrix location at the Hospital for Sick Children, Toronto, Ont. Briefly, double stranded cDNA was prepared from sample mRNA and made into biotinylated cRNA. This cRNA was then fragmented to fragments of 50-100 nucleotides that were hybridized with appropriate controls to the Affymetrix human genome focus array. Each gene on the array was represented by 11-20 25mer oligonucletides, each in pairs of perfect match and mismatch oligonucleotides, (mismatch differs at position #13). The hybridization signal generated for 1 gene incorporates data from all 11- 20 pairs.

Data analysis using Affymetrix software-

Output files were analyzed using the Affymetrix microarray suite 5.0 software. Fluorescence intensity measured for each sample's chip was normalized to the average

fluorescence for the entire group of samples (values were scaled to 150 so that chips could be directly compared). To identify differentially expressed transcripts, 2008 with tet treatment was considered the baseline sample and 2008-*ets-1*-5B and 2008-*ets-1*-5B + tet were separately considered experimental samples. Only genes with a significant detection in all 3 samples (considered significant based on a P detection value of < 0.05, calculated based on differences between perfect match and mismatch oligo hybridization) were considered in the identification of differentially expressed transcripts. Out of these transcripts, pair-wise comparison analysis was carried out (comparing the difference in values of each probe pair in the baseline array to the matching probe pair on the experimental array). A P change value was generated and significant changes were denoted as increase or decrease in gene expression. The fold change in signal intensity of genes where a change was noted was calculated and only those with a 2 or greater fold increase or decrease in the highest Ets-1 expressor (2008-*ets-1*-5B + tet) over the baseline (2008 + tet) were considered as examples of increased or decreased gene expression.

O_2 *consumption assay-*

150 mm plates of cells were trypsinized and pelleted by centrifugation at 1000 rpm for 5 minutes. The cell pellets were resuspended in 500 uL PBS and counted, a cell solution was then made up to 1 x10^6 cells/100 uL. 300 uL was used for analysis and added to the OROBOROS oxygraph chambers once the oxygraph was calibrated. The respirometer used allows for a homogenously stirred closed chamber, constant temperature and minimum O_2 diffusion. O_2 consumption is calculated by polarographic

O_2 sensors which measure dissolved O_2 (Gnaiger *et al.* 2001). Each chamber was filled up to 1.8 mL with potassium media (80 mM KCl, 10 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EDTA, 5 mM K_2HPO_4, pH 7.4) and cells were added. The O_2 consumption of cells was evaluated at 37° C, OROBOROS software was used for data acquisition and analysis. O_2 concentration and flux were recorded at 1 second intervals. Once the O_2 consumption of cells stabilized, cells were permeabilized with low levels of digitonin at which time complex I substrates (glutamate, malate) were added followed by ADP to evaluate complex I activity, inhibition by rotenone confirmed an intact, functional ETC complex. Similarly, complex II/III substrate and inhibitors (succinate and antimycin A respectively) were added to cells as were COX substrates and inhibitors [ascorbate, N,N,N',N'-tetramethyl-p-phenylene diamine (TMPD), KCN]. O_2 consumption of cells was expressed as pmoles O_2/sec/$1x10^6$ cells.

Statsitical analysis and median dose calculation-

Where appropriate, data was expressed as mean +/- standard deviation based on at least three separate experiments. To determine whether significant differences between parameters in sample groups existed, a students paired or un-paired two-tailed, t test was performed using Excel software. A P value of <0.05 was considered significant. Calculations of median dose, or IC_{50}, for cisplatin and 2-DG were done according to the Chou median effect equation (Chou, 1984), by first plotting the log of dose versus log [(fa)/(1-fa)] where fa= fraction affected (1-relative cell number). From this plot, the median dose (Dm) was calculated using the formula y intercept= -slopelog(Dm).

3. Results

3.1- Evaluation of the levels of intracellular ROS in the 2008 and C13* cell model as well as the expression of Ets-1 in these cells under basal conditions and following exposure to H_2O_2, a reducing agent and mitochondrial inhibitors (Objective 1).

3.1.1- Evaluation of intracellular ROS levels in 2008 and C13 cells*

To test whether C13* cells, displaying altered mitochondria relative to their parental cell line (Andrews *et al.* 1992), also display an elevated production of ROS, 2008 and C13* cells were probed using DCFH-DA. The product of DCFH oxidation, DCF, fluoresces and can be quantified in a population of cells using flow cytometry (Keston & Brandt, 1965). Using this technique, 2008 and C13* cells were tested for intracellular levels of ROS. The C13* cells were found to have approximately 4 fold higher levels of intracellular ROS than the parental 2008 cells (figure 7A). Treatment of C13* cells with an antioxidant, NAC, led to a decrease in fluorescence and treatment with H_2O_2 led to an increase. Combination treatment caused no change in fluorescence confirming that DCF fluorescence reflects changes to levels of ROS within cells (figure 7B).

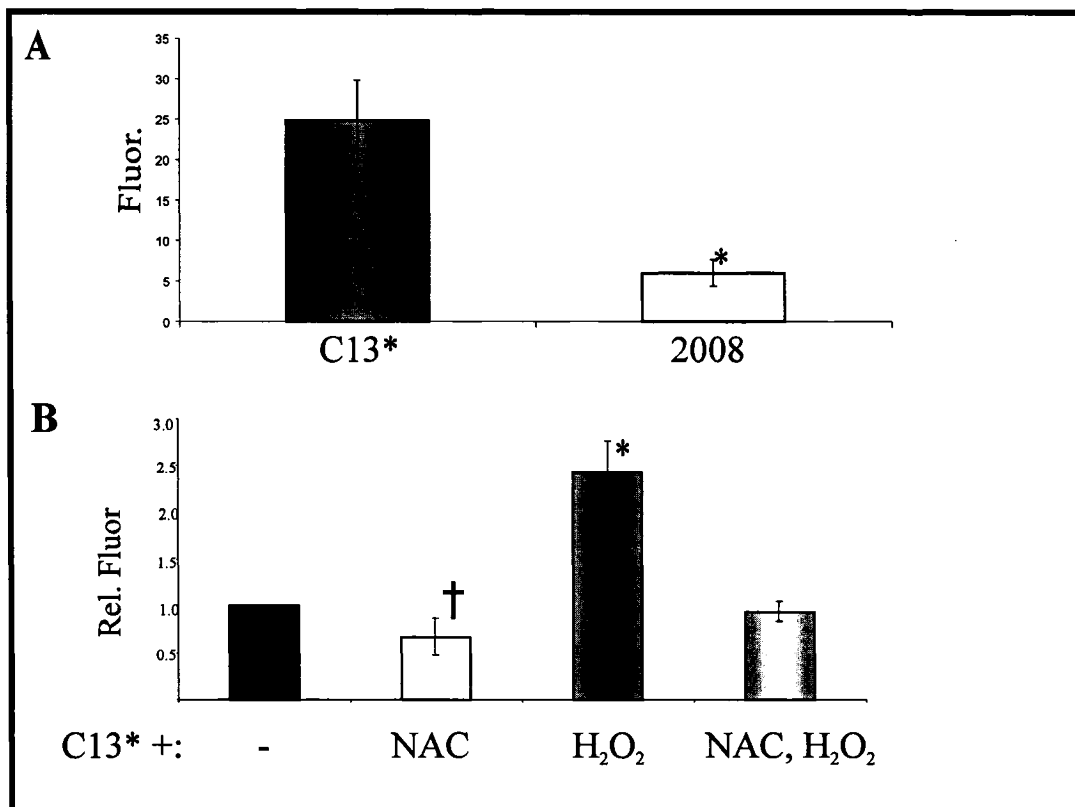


Figure 7: Levels of intracellular ROS in 2008 and C13* cells. A) The fluorescent probe DCFH-DA was used to detect intracellular levels of reactive oxygen species in the cell lines indicated. 500 000 cells were incubated in serum free media containing 10 μM DCFH-DA for 15 minutes and recovered in DCFH-DA free media for a further 15 minutes. DCF fluorescence was detected using flow cytometry. Data shown represents the average fluorescence and standard deviation detected in each cell line following five independent trials. Fluorescence detected within 2008 cells was approximately 4 times less than that in C13* cells. * value is significantly lower than C13* average fluorescence ($P<0.05$). B) C13* cells recovered in media containing 50 mM NAC, 1 mM H_2O_2 or both, following incubation with DCFH-DA to evaluate the effect of these reagents on DCF fluorescence. Data shown represents the mean fluorescence, relative to the untreated control, and standard deviation detected in each cell line following five independent trials. Control reagents used demonstrated an expected drop in DCF fluorescence (NAC) or increase in fluorescence (H_2O_2). * Average fluorescence value is significantly higher than C13* average fluorescence ($P<0.05$), † Average fluorescence value is significantly lower than C13* average fluorescence ($P<0.05$).

3.1.2- Basal levels of Ets-1 protein in C13 and 2008 cells.*

Prior to this study, an observation made in our lab indicated that *ets-1* mRNA levels are elevated in C13* cells relative to 2008 cells (Kenji Sakata, personal communication). Ets-1 protein expression was therefore examined in 2008 and C13* cells by Western blot analysis in order to verify whether differences in expression were also observed at the protein level. Ets-1 protein is expressed at a higher level in the C13* variants as compared to the parental 2008 cells (figure 8A). Simultaneously, examination of levels of other Ets proteins in the lysates showed expression of a closely related Ets family member, Ets-2 and of a distantly related member, PEA3, were similar in both 2008 and C13* cells (figure 8B,C).

3.1.3- Regulation of Ets-1 protein levels by H_2O_2

Though the DCFH assay cannot specify the form of ROS present within cells, previous investigations indicate that the dye may preferentially react with H_2O_2 (LeBel *et al.* 1992). To test the hypothesis that it is elevated levels of H_2O_2 within C13* cells that has led to an elevation in Ets-1 levels, the effect of exogenous H_2O_2 treatment on Ets-1 protein levels was examined. Western blot analysis indicates that Ets-1 protein expression is elevated following H_2O_2 treatment at the lowest doses tested. The increase peaks at 6 hours following treatment (figure 9A). The specificity of this up-regulation was tested by evaluating Ets-2 levels in cell lysates following similar H_2O_2 treatment. Lysates were collected at 2-6 hours recovery time following low and high dose H_2O_2 treatments. Ets-2 protein levels were stable, unaffected by treatment.

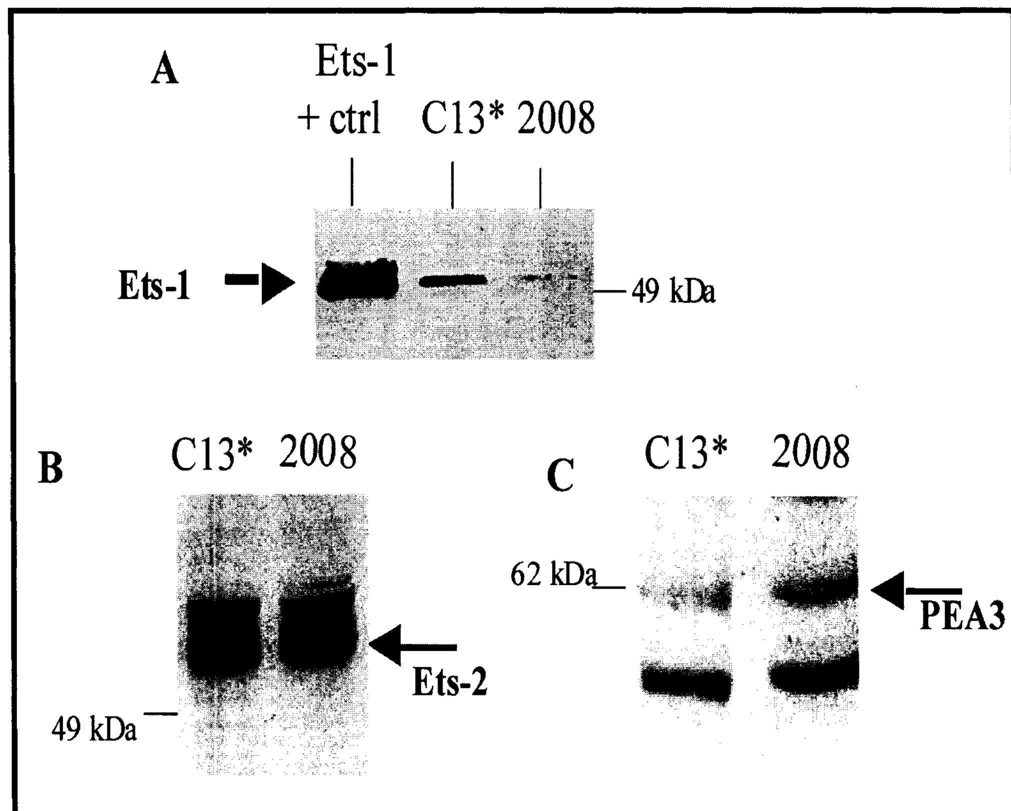


Figure 8: Levels of Ets proteins in 2008 and C13* cells. Western blot analysis for the detection of Ets-1, Ets-2 and PEA3 protein levels in cell lines indicated. 50 μg of whole cell lysates from 2008 and C13* cells were analyzed using a monoclonal antibody against Ets-1 and PEA3 and a polyclonal antibody against Ets-2. The positive control for Ets-1 (Ets-1 +ctrl) used is 5μg protein lysate from COS-1 cells transiently transfected with pcDNA3-*ets-1*. Ets-1 is indicated at 51 kDa (A), Ets-2 at 55 kDa (B) and PEA3 at 62 kDa (C). Results indicate that Ets-1 is specifically up-regulated in C13* cells as compared to 2008 cells.

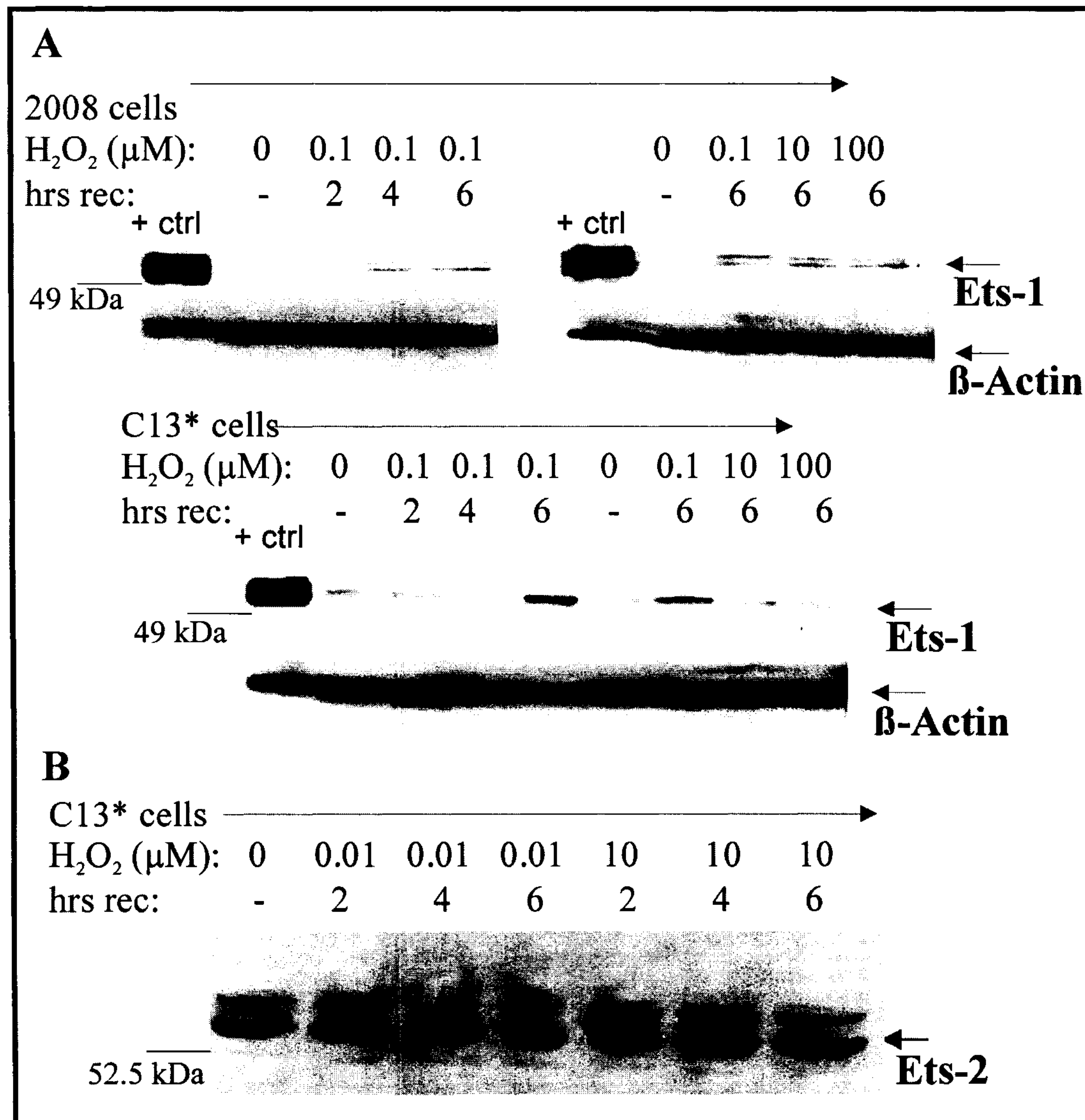


Figure 9. Specific up-regulation of Ets-1 by H_2O_2 in 2008 and C13* cells.
A) 2008 and C13* cells were treated with a range of H_2O_2 concentrations as indicated for 30 minutes and recovered for the time periods indicated. Ets-1 levels were analyzed by Western blotting and are indicated at 51 kDa. The lowest dose of H_2O_2 caused an up-regulation of Ets-1 in both cell lines tested, with optimal increases in protein levels observed at 6 hrs recovery time. Positive control used is as described in figure 8. β- Actin levels in lysates were also analyzed by Western blotting in order to control for loading accuracy.
B) C13* cells were treated with the indicated concentrations of H_2O_2 for 30 minutes at which time cells recovered in media for the time periods indicated. Ets-2 levels in the resulting lysates were analyzed by Western blotting. Ets-2 is indicated at 55 kDa, levels were not affected by H_2O_2 treatment.

3.1.4- Evaluation of the nature of the H_2O_2 mediated up-regulation of ets-1

The recovery time required to observe an increase in Ets-1 protein levels following H_2O_2 treatment suggests a transcriptional up-regulation. In order to test this hypothesis, a specific inhibitor of transcription (actinomycin D), was used. Actinomycin D is a widely used inhibitor of transcription which actually intercalates into DNA causing the RNA polymerase to stall (Kimura *et al.* 2002). Western blot analysis showed increased Ets-1 levels following 6 hours recovery in cells treated only with H_2O_2, however, there was no increase in Ets-1 in cells treated with actinomycin D. In fact expression of Ets-1 is decreased in cells treated with actinomycin D alone as well as in lysates from H_2O_2 treated cells (figure 10). The loss of Ets-1 expression with actinomycin D treatment indicates that the H_2O_2-mediated increase is at the level of gene transcription.

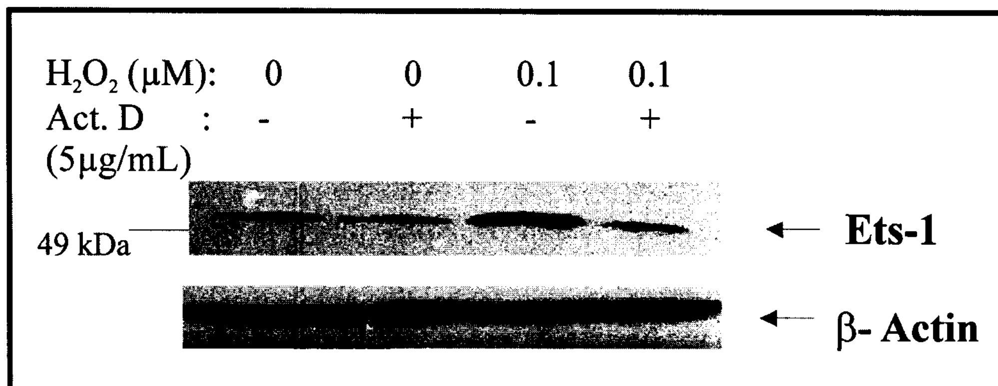


Figure 10: Transcriptional up-regulation of Ets-1 by H_2O_2. C13* cells were treated with the indicated concentrations of H_2O_2 for 30 minutes at which time cells recovered in media with or without 5 μg/mL actinomycin D. Ets-1 levels in the resulting lysates were analyzed by Western blotting as were β-Actin levels to indicate equal loading. Results indicate that the H_2O_2 mediated up-regulation of Ets-1 does not occur in the presence of actinomycin D.

3.1.5- Effect of a reducing agent on Ets-1 expression

To test the effect of a reducing agent on Ets-1 expression levels, 2008 cells were treated with NAC. NAC functions as a direct reducing agent by elevating the availability of intracellular cysteine and thereby detoxifying ROS (De Vries *et al.* 1993; Sen, 1998). . At the lower concentration of NAC used, no change in Ets-1 expression was observed, however, a decrease in Ets-1 protein expression was observed at a concentration of 50 mM NAC (figure 11). These observations suggest that Ets-1 is specifically up-regulated by oxidative stress.

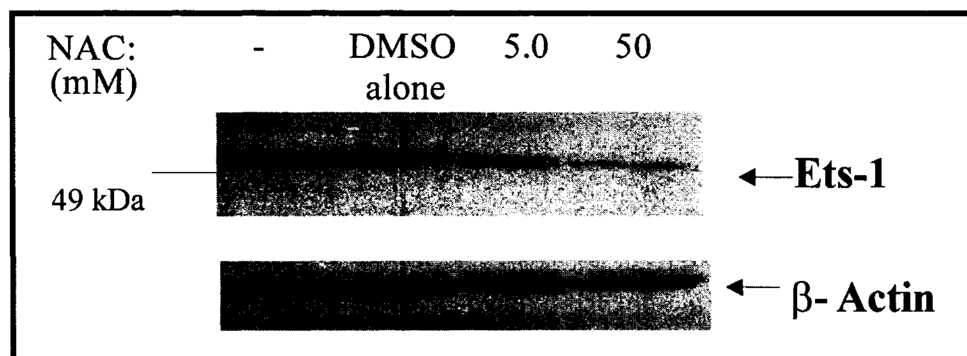


Figure 11: Ets-1 is down-regulated by a reducing agent
2008 cells were treated with the NAC concentrations indicated, or the solvent DMSO, for 4 hours at which time whole cell lysates were made and Ets-1 and β-Actin levels were detected by Western blot analysis. Results indicate that treatment with 50 mM NAC causes a decrease in Ets-1 protein levels.

3.1.6- Effect of mitochondrial inhibitors on Ets-1 expression

The transcriptional up-regulation of Ets-1 by oxidative stress, specifically H_2O_2, indicates that mitochondrial dysfunction may directly alter levels of this transcription factor. The induction of mitochondrial stress by specific electron transfer chain inhibitors, rotenone and oligomycin B, which inhibit complex I of the electron transport chain and F_0F_1-ATP synthase respectively, was performed and the effect on Ets-1 protein levels was evaluated. Western blot analysis of these lysates indicates that Ets-1 protein expression is up-regulated by both drugs with an optimal increase observed at 6 hours recovery time (figure 12A,B).

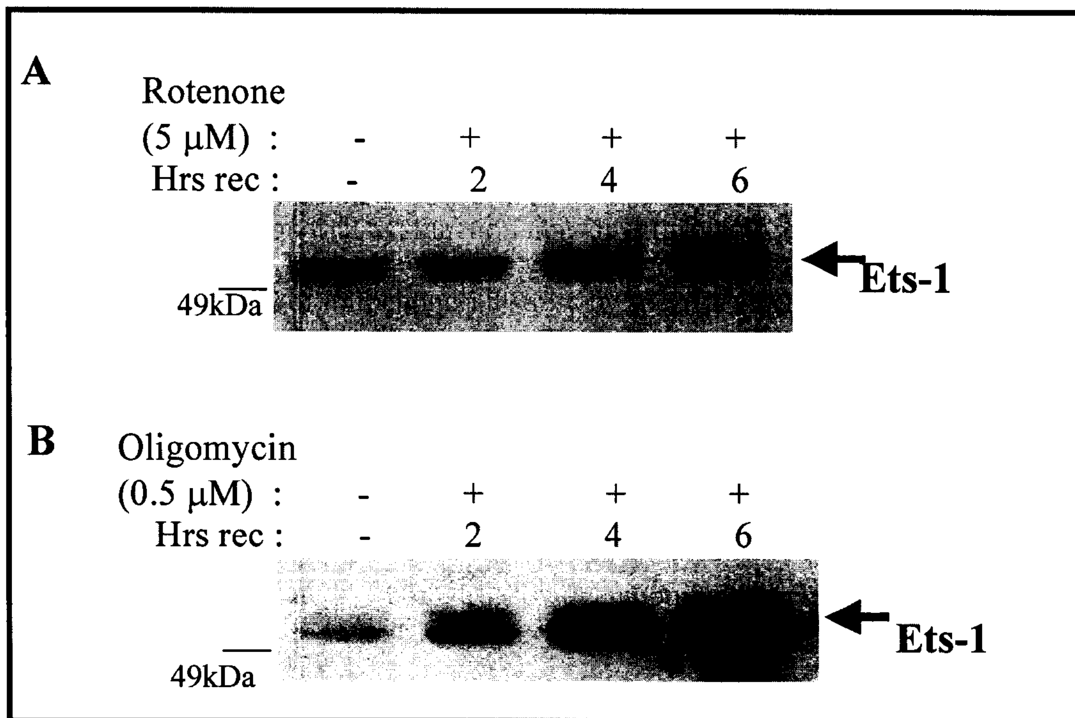


Figure 12: Up-regulation of Ets-1 by mitochondrial stress in C13* cells. C13* cells were treated with 5 μM rotenone (A) or 0.5 μM oligomycin B (B) for 2 hrs at which time cells were allowed to recover for 2-6 hrs (Hrs rec= hours of recovery). Ets-1 levels were analyzed by Western blotting and are seen to be up-regulated following treatment with optimal increases observed at 6 hrs recovery time.

3.2- Evaluation of the response of the *ets-1* gene promoter to H_2O_2 and determination of the responsive region within this promoter (objective 2)

3.2.1- Basal activity of the ets-1 promoter

In order to address the mechanism of the H_2O_2 mediated transcriptional up-regulation of *ets-1*, the direct effect of H_2O_2 on the *ets-1* gene promoter region was evaluated. Promoter activity of *ets-1* was measured by using a luciferase reporter system- the *ets-1* promoter region was cloned into a basic luciferase vector in which the transcription of firefly luciferase became under the control of the *ets-1* promoter. Treatments of cells transfected with this vector were then performed in order to test whether H_2O_2 enhances transcriptional activation of *ets-1* in 2008 cells. A 1.4 kB region of the *ets-1* promoter, containing the elements required for activation of transcription, has previously been described (Oka *et al.* 1991) and was thus isolated by PCR amplification from genomic DNA isolated from C13* cells. The sequence of the isolated promoter matched that previously published (Oka *et al.* 1991). A full length promoter luciferase vector was constructed, pGL3-*ets-1*-wt (wild type) as were two truncated promoter vectors. Truncated promoters were created by deleting sections of the full length promoter construct, the resulting vectors are designated pGL3-*ets-1*-1000 and pGL3-*ets-1*-570- numbers indicate the length of promoter sequence remaining in the vector in base pairs. Vectors constructed are depicted in figure 13A.

Ets-1 promoter constructs were transfected into 2008 cells along with a control *Renilla* luciferase vector. The *Renilla* vector was co-transfected as an internal control, to normalize the activity of the experimental vector to a baseline activity value and thus

minimize experimental variability. 24 hours following transfection, cell lysates were prepared and the activities of luciferase and *Renilla* were independently measured using a multiplate luminometer. Luciferase assay results indicate that truncated construct pGL3-*ets-1*-1000 exhibits similar basal levels of transcriptional activity as the full length promoter construct whereas basal activity of the truncation pGL3-*ets-1*-570, which includes a deletion of the putative enhancer region, is significantly reduced (figure 13B). These data indicate that the *ets-1* promoter is capable of driving transcription and that this capability is largely located within the region between 570-1000 base pairs upstream from the transcriptional start site.

3.2.2- Evaluation of the transcriptional activity of the ets-1 promoter and certain promoter truncations following H_2O_2 treatment.

In order to evaluate whether promoter constructs are responsive to H_2O_2, 24 hours following transfection cells were treated with H_2O_2 for 30 minutes and left to recover for 4 hours at which time lysates were prepared for luciferase and *Renilla* measurements. Following H_2O_2 treatment, the wild type *ets-1* promoter construct consistently displayed an approximate 2.5 fold increase in luciferase activity as did truncated promoter construct pGL3-*ets-1*-1000 (Figure 13C). Construct pGL3-*ets-1*-570 however, showed no significant increase in promoter activity following treatment, indicating that the element or elements involved in H_2O_2 inducibility lie within the putative enhancer region located between 570-1000 bps upstream of the transcriptional start site (Figure 13C). Similar treatments were done following transfection with the basic pGL3 vector; no increase in luciferase activity was noted following H_2O_2 treatment.

Figure 13: Basal and H_2O_2 inducible activity of ets-1 promoter. A) A 1.4 kB fragment of the *ets-1* promoter was isolated by PCR amplification and cloned into a pGL3 basic vector (pGL3-*ets-1*-wt). Similar methods were used to clone 1000bp and 570bp promoter truncations into luciferase vectors (pGL3-*ets-1*-1000 and pGL3-*ets-1*-570). Approximate location of promoter elements of interest, (AP-1, ARE, HRE) are shown within the promoter. B) The basal activities of promoter fragments were tested by transfecting 2008 cells with the appropriate luciferase vector along with a *Renilla* control vector. 24 hours following transfection, luciferase and *Renilla* activities were measured in cell lysates. The relative light units (RLU) shown represent the ratio of luciferase to *Renilla* activity (activity of the full length promoter was considered a value of 1, activity of truncations are shown relative to this value). Data shown represent mean and standard deviation following at least three independent trials. The pGL3-*ets-1*-570 construct displays reduced transcriptional activity as compared to pGL3-*ets-1*-wt whereas pGL3-*ets-1*-1000 displays similar activity. * luciferase/*Renilla* ratio is significantly lower than that of wildtype promoter. C) Similar luciferase/*Renilla* measurements were taken following H_2O_2 treatment (30 minute treatment with 100 μM H_2O_2, followed by 4 hours recovery time at which point lysates were made and luciferase/*Renilla* activity measured). The activity of both pGL3-*ets-1*-wt and pGL3-*ets-1*-1000 was significantly induced following H_2O_2 treatment, whereas there was no significant effect on the activity of the pGL3-*ets-1*-570 construct. † luciferase/*Renilla* ratio is significantly higher than that of construct prior to H_2O_2 induction.

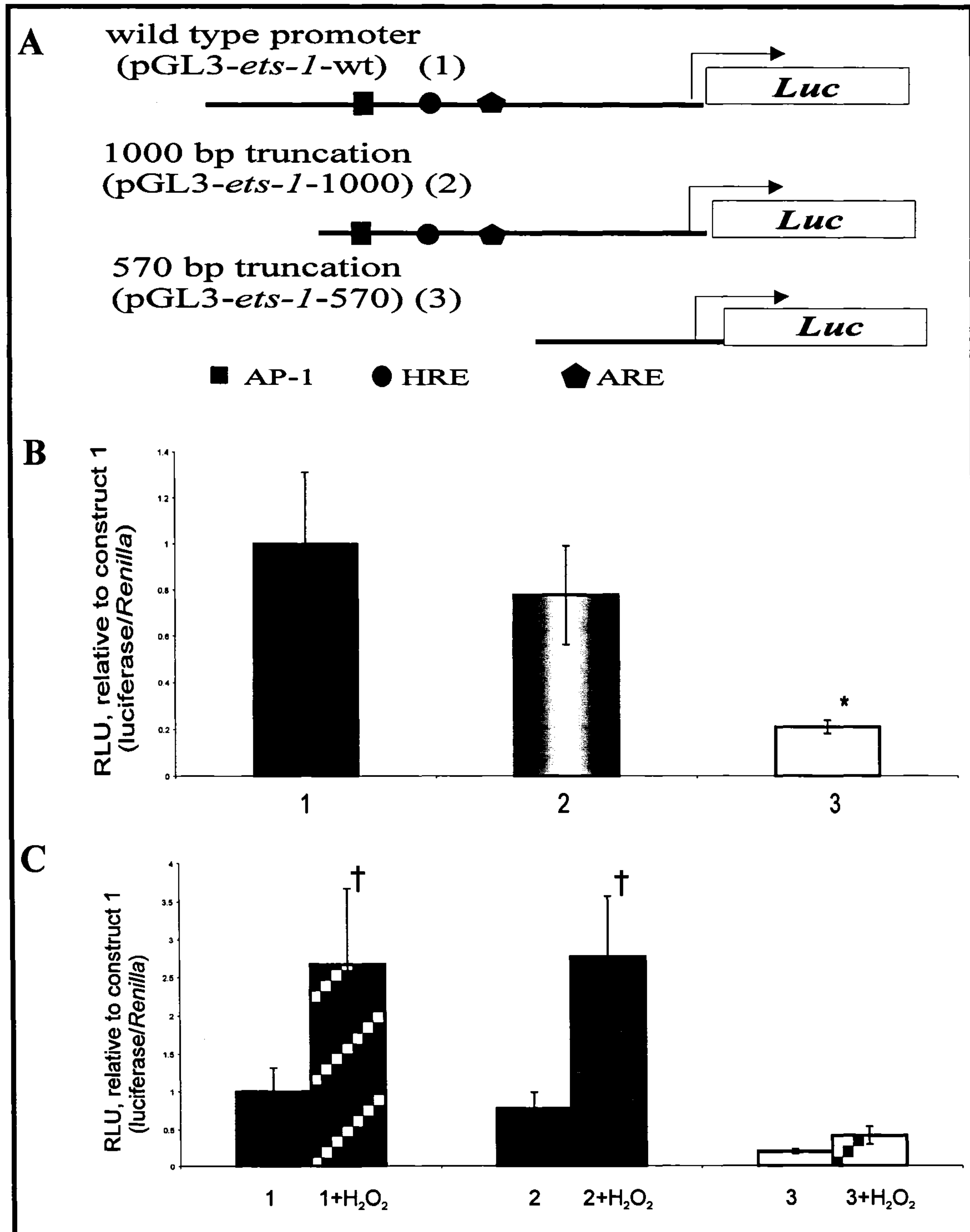


Figure 13: Basal and H_2O_2 inducible activity of *ets-1* promoter.

3.3- Determination of the elements within the *ets-1* promoter responsible for the H_2O_2 mediated gene up-regulation and characterization of the protein complex which forms on these elements in a H_2O_2 dependent manner (objective 3).

3.3.1- ets-1 promoter mutants created to delineate H_2O_2 responsive element

Within the proposed enhancer region of the *ets-1* promoter several transcription binding sites are found including previously identified AP-1 and HIF-1α sites as well as a putative ARE, identified in this study, all of which may be involved in mediating an activation response via H_2O_2 (Oka *et al.* 1991; Oikawa *et al.* 2001). To evaluate the individual contribution of these sites, site directed mutagenesis was employed to disrupt each site within the pGL3-*ets-1*-wt construct, the consequences of which were examined using luciferase assays. All mutations made were chosen according to published studies indicating that the specific base changes lead to disruptions of transcription factor binding to the elements in question (Guyton *et al.* 1996; Coulet *et al.* 2003; Rushmore *et al.* 1991). Resulting plasmids pGL3-*ets-1*-mutAP-1 (mutAP-1), pGL3-*ets-1*-mutHRE (mutHRE) and pGL3-*ets-1*-mutARE (mutARE) are depicted in figure 14A.

3.3.2- Evaluation of basal and H_2O_2 inducible activity of ets-1 promoter mutants

The basal transcriptional activity of all three mutated promoters as well as activity following induction by H_2O_2 was compared to that of pGL3-*ets-1*-wt. MutAP-1 and mutHRE displayed similar basal transcriptional abilities to that of the full length promoter whereas the basal activity of mutARE was significantly lower (figure 14B). Activation of mutAP-1 and mutHRE by H_2O_2 reached similar levels as the full length promoter, with a 2.69 fold activation of mutAP-1 and a 2.15 fold activation of mutHRE

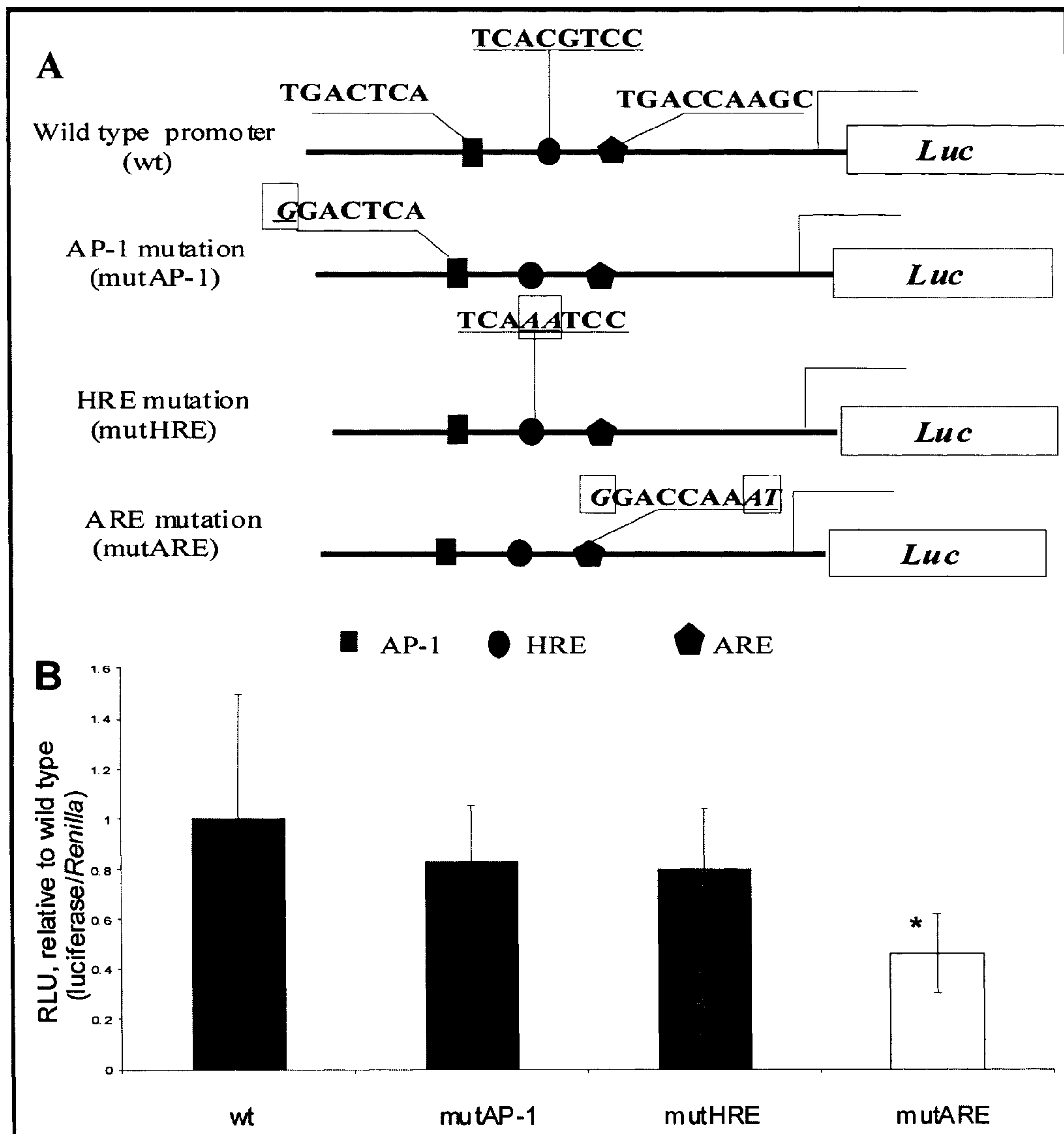


Figure 14: Construction and evaluation of *ets-1* promoter mutants A) Mutations were made to elements within the enhancer region of the *ets-1* promoter by site directed mutagenesis and mutated promoters were cloned into the pGL3 basic vector.
B) The basal luciferase/*Renilla* activity of all mutated promoters was measured as described in figure 13. RLU shown represents the ratio of luciferase to *Renilla* activity relative to the activity of the wt promoter (considered a value of 1). Data shown represents the average and standard deviation following at least three independent trials. MutAP-1 and mutHRE constructs displayed similar basal transcriptional activiteis as the wt construct whereas the activity of mutARE was reduced. *Luciferase/*Renilla* ratio is significantly lower than that of the wt promoter.

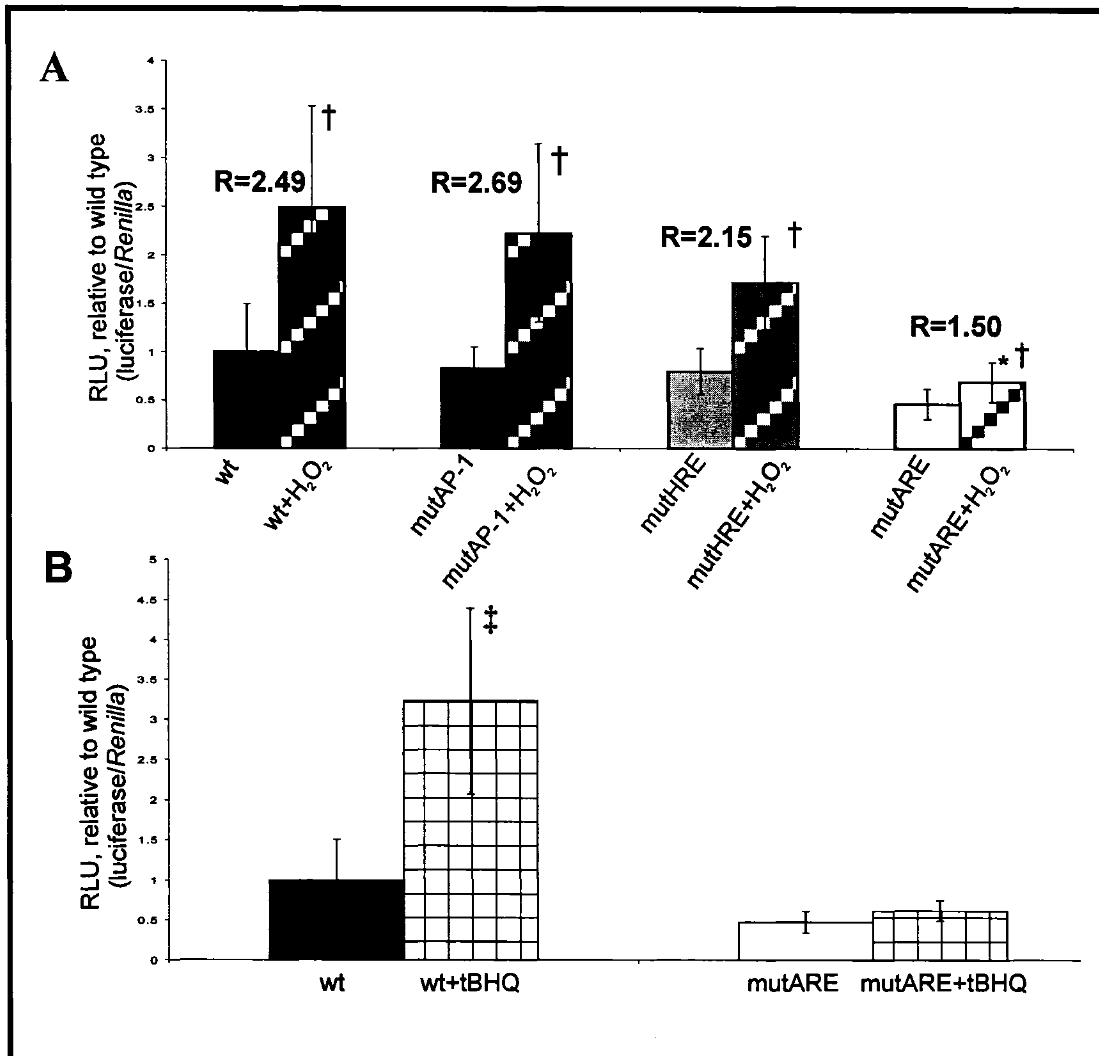


Figure 15: ARE mediates the inducibility of the *ets-1* promoter.
A) Luciferase/*Renilla* activities of mutated promoters were measured before and after H_2O_2 induction (as described in figure 13). Data shown represents average and standard deviation from at least three independent trials, R values represent the ratios of activities of induced constructs to un-induced. MutAP-1 and mutHRE promoter activity was increased following H_2O_2 induction, to a level similar to that of the wt promoter, mutARE activity showed significantly less induction † luciferase/*Renilla* ratio is significantly higher than that of construct prior to H_2O_2 induction. *luciferase/*Renilla* ratio is significantly lower than that of the wt promoter following H_2O_2 induction. B) 2008 cells were transfected with either the wt promoter or the mutARE construct. 24 hours later, cells were treated with 200μM tBHQ for 6 hours and lysates were made. Resulting luciferase/*Renilla* activities were plotted alongside activities of untreated cells (with untreated wt promoter activity considered 1, other values plotted relative to this value). Data shown represents average and standard deviation from at least three independent trials. tBHQ treatment significantly increased the activity of the wt promoter but had no effect on the mutARE promoter activity. ‡ luciferase/*Renilla* is significantly higher than that of the construct prior to tBHQ treatment.

observed (figure 15A). The mutant ARE construct demonstrated very little overall luciferase activity with only a 1.5 fold activation noted following H_2O_2 treatment. Though this induction was significant, the overall luciferase activity was well below that of other constructs and the fold induction was the lowest observed (figure 15A). Taken together, these data indicate that the ARE is pivotal for both basal and inducible activity of the *ets-1* promoter.

In order to evaluate whether the proposed ARE within the *ets*-1 promoter will respond to a traditional ARE inducer, 2008 cells were first transfected with either the *ets*-1 full length promoter construct or the mutARE construct as well as a control *Renilla* vector and subjected to 6 hours of treatment with 200µM tBHQ. tBHQ is a phenolic antioxidant used as a model inducing agent for ARE driven transcription (Jaiswal, 2004). Lysates were made following tBHQ treatment and evaluated for luciferase activity. A significant, 3 fold increase in luciferase activity was noted in lysates from cells transfected with the full length promoter, whereas transcription driven by the mutARE promoter did not change following treatment indicating the increase in activity in the full length promoter acted through the ARE (figure 15B).

3.3.3- Characterization of the protein containing complex that binds to the ets-1 ARE in a H_2O_2 inducible fashion

To identify whether protein complexes increasingly bind to the *ets-1* ARE under induced conditions, nuclear extracts from untreated 2008 cells and cells treated with H_2O_2 (at varying recovery times) were incubated with a radiolabeled *ets-1* ARE probe and gel shift assays were performed. Following treatment with H_2O_2, an increase in

binding of nuclear proteins to the *ets-1* ARE was evident with an optimal increase at 3hrs recovery time (figure 16). In order to attempt to identify whether Nrf2, a transcription factor typically involved in ARE mediated gene regulation (Nguyen *et al.* 2003), is a component of the protein complex, nuclear lysates extracted from H_2O_2 treated cells were pre-incubated with an anti-Nrf2 antibody prior to the binding reaction with the labeled oligonucleotide. Previous studies have shown that such a pre-incubation may prevent formation of a DNA-protein complex (Martin *et al.*, 2004).

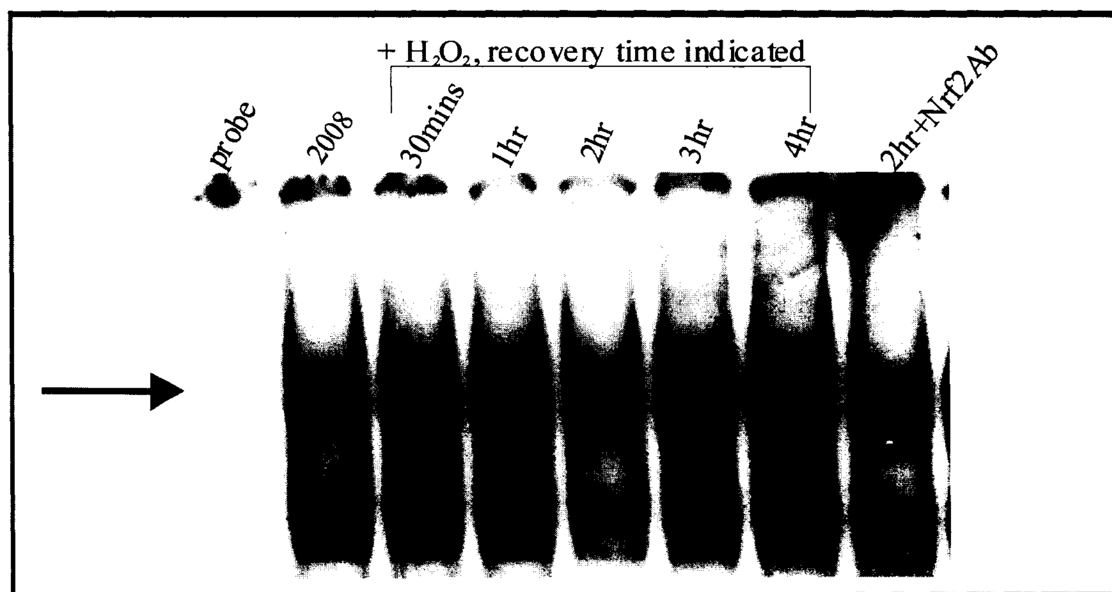


Figure 16: Gel shift analysis of protein-*ets-1* ARE complex. A 25 bp *ets-1* ARE oligonucleotide was end labeled with γ-P^{32}-ATP and incubated with 10 μg nuclear extract from untreated 2008 cells or cells treated with H_2O_2 (100uM, 30 minutes) with varying recovery times, as indicated, and gel shift analysis was performed. A specific ARE-protein complex increasingly formed following H_2O_2 treatment and is indicated with an arrow. Nuclear lysate from treated cells (at 2 hrs recovery time) was pre-incubated overnight at 4°C with Nrf2 antibody and run in the final lane of the gel. The loss of the protein complex following this treatment indicates that the complex contains Nrf2. Lane marked probe indicates that a labeled probe alone was run in this lane.

The intensity of the complex formed did drop following pre-incubation indicating that Nrf2 is a component of the protein complex bound to the *ets-1* ARE (figure 16, lane 8).

DNA binding of the Nrf2 containing complex was also tested using an *ets-1* mutARE as well as a *NQO1* ARE. NQO1 is a flavoprotein involved in the detoxification of quinones and is a typical example of an ARE regulated gene. The ARE within the *NQO1* promoter has been extensively studied and is most notably regulated by Nrf2, thus the *NQO1* ARE sequence was used as a positive control to demonstrate the binding of this transcription factor (Dhakshinamoorthy and Jaiswal, 2000)). Similar to the *ets-1* ARE, substantial binding of presumably the same complex (as it ran at the same position on the gel) was noted on the *NQO1* ARE, with enhanced binding following H_2O_2 treatment of cells (figure 17A, lanes 8,9). No binding of such a complex was noted following incubation of extracts with the mutARE oligonucleotide indicating that mutations to this element prevent the formation of an Nrf2 containing complex (figure 17A, lanes 6,7). Competition assays were performed in order to definitively establish whether the protein complex formed on the *ets-1* ARE is identical to that formed on the *NQO1* ARE. Nuclear lysates were pre-incubated with cold *ets-1* ARE, *NQO1* ARE and mutARE probes prior to the binding reaction with labeled *ets-1* ARE probe. Pre-incubation with either *ets-1* or *NQO1* probes completely prevented the protein complex from forming on the labeled probe, indicating that the two sites bind the same complex (Figure 17A, lanes 3,4). The mutARE, however, was not able to compete for the protein complex furthering evidence that mutations to the *ets-1* ARE prevent protein complex binding (Figure 17A, lane 5). The reverse reaction was also performed, where lysates

were pre-incubated with a cold *ets-1* or *NQO1* ARE prior to incubation with a labeled *NQO1* ARE probe. In this case, cold competition with a *NQO1* probe completely prevented complex formation on the labeled probe whereas competition with the *ets-1* ARE probe only partially prevented complex formation- indicating that the *NQO1* ARE displays greater affinity for the protein complex (figure 17B, lanes 5,6).

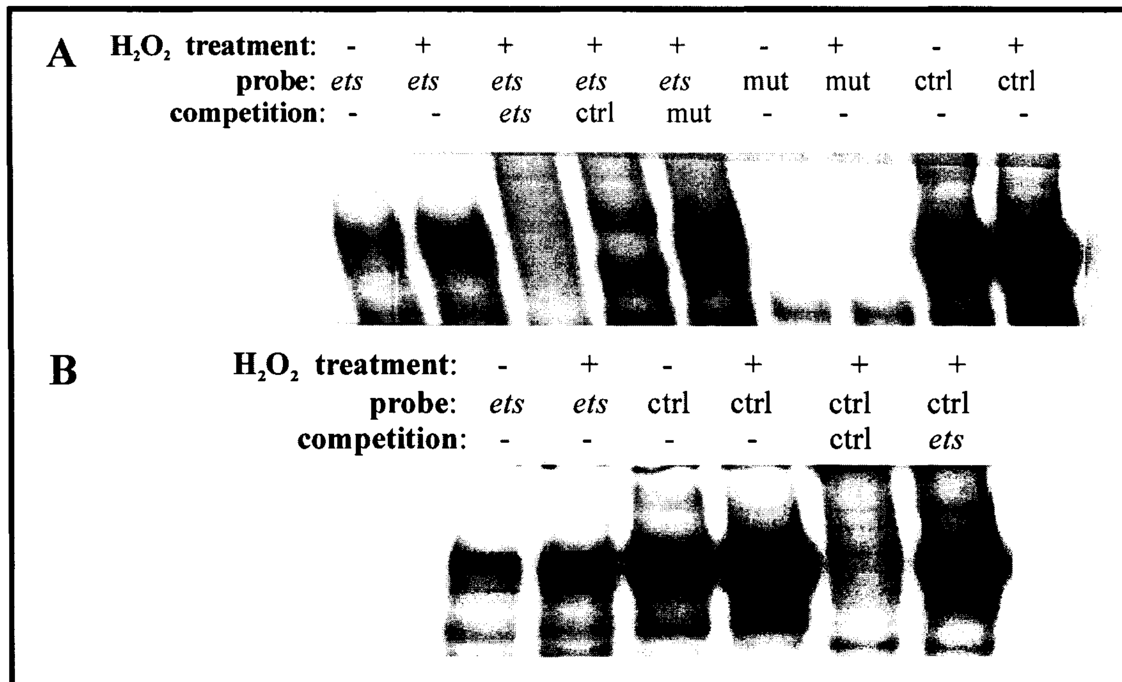


Figure 17: Gel shift analysis of *ets-1, NQO1* and mut ARE complexes (competition assays). A) *ets-1* ARE, mutARE (mut) and *NQO1* ARE (ctrl) 25 bp oligonucleotides were end labeled and incubated with nuclear extract from untreated and H_2O_2 treated 2008 cells (3 hrs recovery time) and gel shift assays were performed. For competition assays, nuclear extract from treated cells was incubated with excess unlabeled *ets-1*, ctrl or mut probes for 15 minutes on ice prior to the binding reaction with the *ets-1* oligonucleotide. Competition with both *ets* and ctrl probes prevented formation of a protein complex on the *ets-1* ARE. B) Repeat of gel shift analysis using *ets-1* and ctrl probes with extracts from H_2O_2 treated and untreated 2008 cells. Competition assays performed in this case involved the pre-incubation of extracts from treated cells with excess unlabeled *ets-1* and ctrl probes prior to the binding reaction with a labeled ctrl oligonucleotide. Competition with the ctrl probe completely prevented complex formation and competition with the *ets-1* probe partially prevented complex formation.

3.3.4- Expression of Nrf2 in 2008 cells

Both luciferase assays and gel shift analysis have indicated that an ARE in the *ets-1* promoter is pivotal in regulating basal and H_2O_2-induced promoter activity. Gel shift analysis further indicated that Nrf2 is a part of a protein complex binding to this site. In order to confirm that this protein is expressed in the cell line under study, cytoplasmic and nuclear lysates were collected from 2008 cells before and after 30 minute, 100 μM H_2O_2 treatment at increasing recovery time points and Western blot analysis, for the detection of Nrf2, was performed. The use of a positive control (COS-1 cells transiently transfected with pcDNA3-*Nrf2*) demonstrated that Nrf2 runs at approximately 100 kDa in a doublet. Only one form of the protein was detected in cytoplasmic fractions, whereas the two forms appeared to be present in the nucleus with the lower molecular weight form accumulating following H_2O_2 treatment (at approximately 1 hour recovery time). Both forms of the protein appeared to display higher levels of expression in the nucleus

(figure 18).

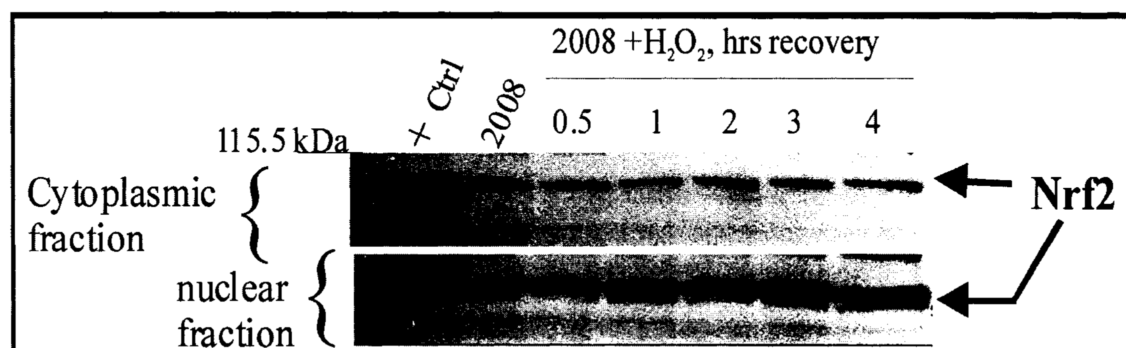


Figure 18: Nrf2 expression in 2008 cytoplasmic and nuclear cell fractions. Nrf2 expression was evaluated in cytoplasmic and nuclear fractions of untreated and H_2O_2 treated 2008 cells. Positive control used is 30 μg of whole cell lysates from COS-1 cells transiently transfected with pcDNA3-*Nrf2*. Nrf2 appears as a doublet at 100 kDa, one form is expressed at all timepoints in both the cytoplasmic and nuclear fractions, a lower molecular weight form is only seen in the nuclear fraction and appears at the 1 hr recovery timepoint.

3.3.5- Expression of HIF-1α in 2008 cells

Luciferase assays have indicated that the HRE within the *ets-1* promoter plays a small role in the H_2O_2 inducibiilty of the promoter. In order to investigate whether HIF-1α, the protein responsible for the activation of HRE sites, is stabilized following H_2O_2 induction in 2008 cells, Western blot analysis was performed on treated lysates. The basal levels of HIF-1α were first evaluated in 2008 and C13* cells, and compared to levels observed in cells following 6 hour incubation in a hypoxic chamber (1% O_2). It was demonstrated that C13* cells display a higher basal level of HIF-1α than 2008 cells, but that the protein is stabilized or induced in both cell lines following exposure to hypoxia (figure 19A). HIF-1α levels were then evaluated in 2008 cells following treatment with a low dose of H_2O_2 for 30 minutes and increasing recovery times. Western blot analysis of the resulting lysates indicated that HIF-1α levels were

transiently up-regulated following H_2O_2 treatment at 2 hours recovery time (figure 19B). Finally, to evaluate whether a hypoxic or H_2O_2 mediated up-regulation of HIF-1α necessarily leads to an up-regulation of Ets-1 expression, lysates from cells incubated in a hypoxic chamber, which displayed increased levels of HIF-1α, were screened for Ets-1 protein levels and compared to normal cell lysates. No change in Ets-1 protein levels was observed (Figure 19C).

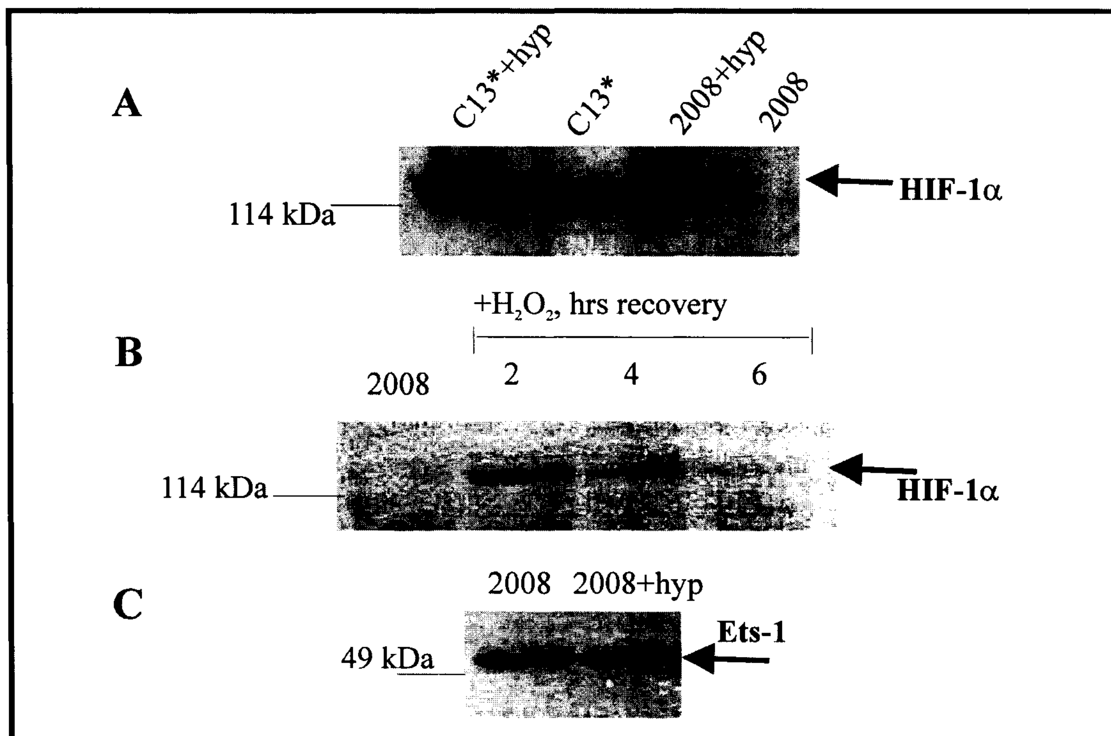


Figure 19: HIF-1α expression in 2008 and C13* cells. A) Expression of HIF-1α in untreated 2008 and C13* cells as well as in cells incubated in a hypoxic chamber for 6 hours. HIF-1α is indicated at 120 kDa and is expressed at a higher level in C13* cells than in 2008 cells; HIF-1α accumulates in both cell lines following hypoxic treatment. B) HIF-1α expression in lysates from untreated 2008 cells as well as cells treated with 0.05 μM H_2O_2 for 2- 6 hours of recovery time. HIF-1α is up-regulated at 2 hrs recovery time. C) Ets-1 protein expression in untreated 2008 cells and cells incubated in a hypoxic chamber for 6 hours. Ets-1 is indicated at 51 kDa, levels appear unchanged following exposure to hypoxia.

3.4- Evaluation of the effects of Ets-1 overexpression on mitochondrial function (objective 4)

3.4.1- Creation of stable cell lines expressing increased levels of Ets-1

In order to evaluate the effect of Ets-1 expression on mitochondrial properties as well as cisplatin resistance of cells, the expression of the protein was stably altered in 2008 cells and various properties of these stable cell lines were evaluated. In previous experiments, 2008 stable cell lines with high, constitutive expression of Ets-1 were not obtained. The approach thus chosen to stably alter expression is by making use of an inducible system. The vectors pcDNA4/TO/*ets-1* and pcDNA6/TR were sequentially transfected into 2008 cells using the lipofectamine liposome transfection system. . Cells were first transfected with pcDNA6/TR and stable clones were selected by Blasticidin treatment (the TR vector also contains a Blasticidin resistance gene). Clones were screened for TetR expression by Western blotting using a mix of monoclonal antibodies against the TetR protein. One TetR positive 2008 clone (2008-TetR-2) was identified (Figure 20A). These cells, with high stable expression of tet repressor, were then transfected a second time, with the pcDNA6/TO/*ets-1* vector. If successfully incorporated, the *ets*-1 gene should be silent in cells expressing TetR until induced by tet. Following selection in Zeocin, (resistance marker in pcDNA4/TO/*ets-1*), clones were screened for inducible Ets-1 expression. Cells were treated for 24 hours with 0.2 μg/mL tet and Ets-1 protein levels evaluated by Western blotting. Clone #'s 2008-TR2-*ets-1*/TO-4 (2008-*ets-1*-4) and 2008-TR2-*ets-1*/TO-5B(2008-*ets-1*-5B) were found to have high, inducible expression of Ets-1 (figure 20B). Cells were cultured for up to 20

passages to observe the stability of inducible Ets-1 expression. Although it was still possible to observe induced expression of Ets-1 following numerous passages, increased Ets-1 expression was also observed in the 2008-*ets-1*-5B clone without tet treatment, indicating that there is "leakiness" in the system. The TetR repressor protein doesn't completely inhibit transcription of *ets-1* (figure 20C). Ets-1 overexpressing cells were also noted to typically display a doublet band for Ets-1 in Western blots. In order to identify the two bands, cell lysates were incubated with CIAP for 2 hours prior to Western blot analysis. This treatment caused a decreased expression of the higher molecular weight band in the Ets-1 doublet, indicating that it is a phosphorylated form of the protein (Figure 20D)

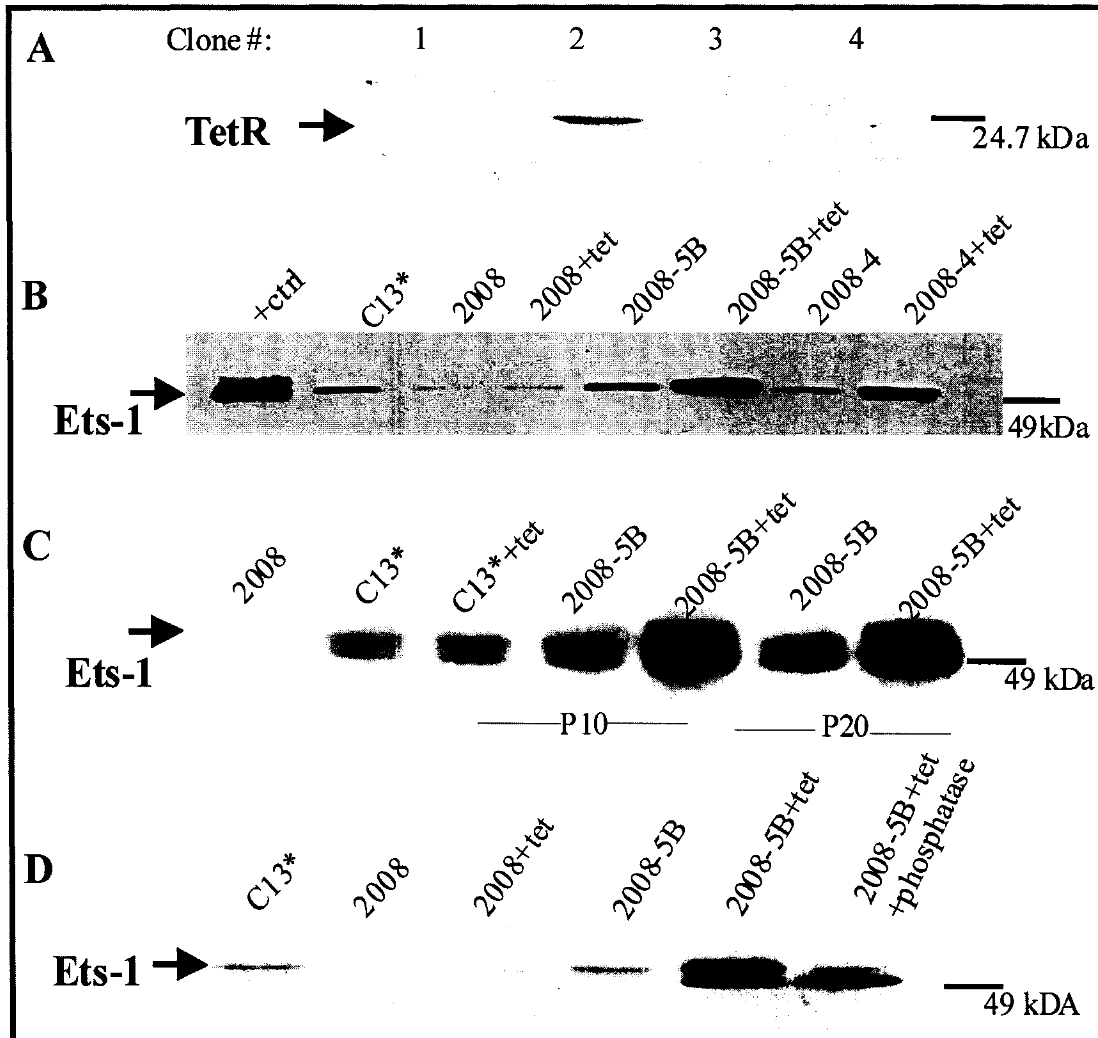


Figure 20: Creation of Ets-1 inducible stable cell lines and evaluation of inducibility/stability. A) 2008 cells were stably transfected with pcDNA6/TR in order to express the tet repressor (Tet R) protein. Clones were screened for stable expression of Tet R by Western blot analysis. 2008 clone 2 was used in further experiments as these cells displayed high expression of Tet R. Tet R is indicated at 24 kDa. B) 2008-TetR-2 cells were stably transfected with pcDNA4/TO/*ets-1* in order to incorporate a tetracycline inducible *ets-1* gene. Western blot analysis for the detection of Ets-1 in lysates from different clones before and after induction is shown. Clones 5B (2008-*ets-1*-5B, or 2008-5B) and 4 (2008-*ets-1*-4/2008-4) were used in further experiments. C) Stability and inducibility of Ets-1 expression was evaluated in the 2008-5B clone following numerous cell culture passages (P10= 10 passages, P20=20 passages). Ets-1 expression was evaluated with or without tet treatment by western blot analysis and was found to be up-regulated in clone 5B even in an un-induced state. D) Lysate of tet induced 2008-5B was treated with CIAP in order to characterize the upper band of the doublet often seen in Ets-1 Western blots. CIAP treatment caused a decrease in the expression of the higher molecular weight form of Ets-1.

3.4.2- Microarray analysis of stable cells overexpressing Ets-1

Ets-1 is a transcription factor and it is expected that altering this proteins expression will have an effect on the expression of a wide variety of genes. Specifically, we hypothesize that the expression of genes encoding mitochondrial proteins or proteins controlling the expression of mitochondrial genes may be affected by Ets-1 expression. In order to evaluate changes to gene expression in stable cell lines with high Ets-1 expression as compared to basal expression, RNA was extracted from 2008 (tet treated), 2008 clone 5B and 2008-5B treated with tet. These conditions represent a range of Ets-1 expression, low in 2008, medium in 2008-5B and high expression in induced clone 5B. This RNA was then used to probe an Affymetrix human genome focus array. This technology allows the simultaneous monitoring of the activity of thousands of genes.

The array chosen represents over 8500 verified human sequences and is annotated in such a way to easily view changes to gene expression in the cells in question. Extracted RNA was biotinylated and used to probe the array. Increasing levels of *ets-1* expression were verified in the microarray data- with a 3.6 fold increase in the 2008-*ets-1*-5B sample over basal (2008 +tet) and a 27.3 fold increase in 2008-*ets-1*-5B with tet treatment over basal. A total of 50 up-regulated genes, with significant detection in all samples tested, were identified with at least a 2 fold increase in expression in the highest Ets-1 expression cell line, (2008-*ets-1*-5B +tet) over the basal *ets-1* expression sample and a total of 94 down-regulated genes, again with significant detection in all samples, showing at least a two fold decrease in expression were similarly identified (complete array results are appended in appendix 1). Within these data, certain trends were observed: increased

expression of genes involved in antioxidant defense and DNA repair and decreased expression of genes encoding mitochondrial proteins involved in oxidative phosphorylation as well as metabolic enzymes potentially involved in generating substrates for the citric acid cycle and thus oxidative phosphorylation. Up-regulated genes involved in antioxidant defense included metallothioneins, thioredoxin and enzymes leading to glutathione synthesis. Those involved in DNA repair were thymidylate synthase and mutY homolog. In each case, gene expression in all three samples tested correlated to the levels of Ets-1 within the cell lines (2008-*ets*-1-5B+tet > 2008-*ets*-1-5B > 2008+tet). Genes encoding mitochondrial proteins observed to be down-regulated included components of the ATP synthase, succinate dehydrogenase and NADH dehydrogenase complexes, as well as cytochrome c1. Several metabolic enzymes involved in the metabolism of fatty acids and the processing of substrates for the citric acid cycle, such as pyruvate dehydrogenase, succinate coA ligase and fatty-acyl-coA ligases were down-regulated- again, gene expression levels correlated to the level of Ets-1 expression within cells (table 4).

3.4.3- Evaluation of glycolytic capability of stable ets-1 overexpressing cell lines

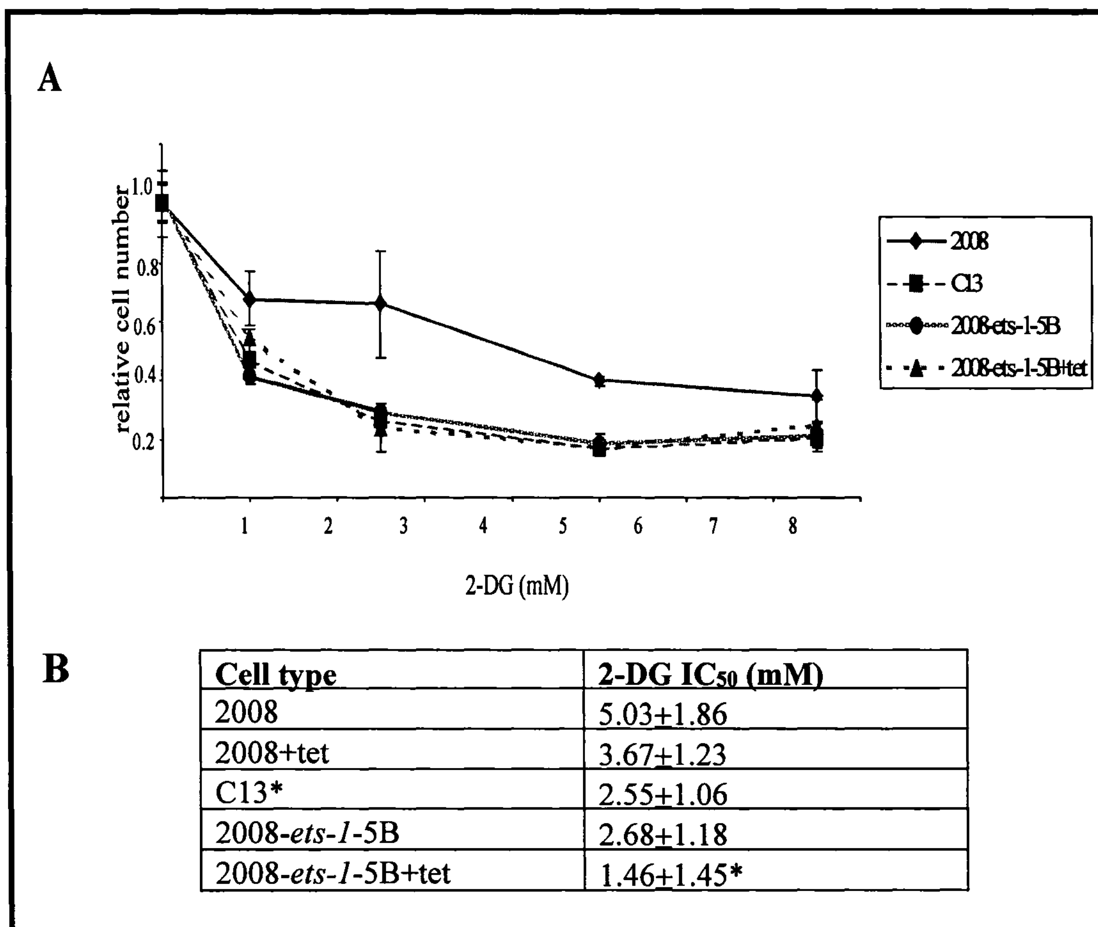
In order to evaluate whether cells with stable overexpression of Ets-1 favor glycolysis over oxidative phosphorylation as an ATP source, as would be predicted by the microarray data, cells were grown in media containing a glycolytic inhibitor and in

Table 4. Genes differentially regulated in 2008 cells stably transfeted with *ets-1*. Gene expression in 2008+tet, 2008-*ets-1*-5B (2008-5B) and 2008-*ets-1*-5B+tet (2008-5B+tet) was evaluated via hybridization of biotinylated cRNA from respective cell lines to an Affymetrix human genome focus array. Genes differentially expressed in the given cell lines were identified based on a significant P detection value in all 3 samples, as well as a significant P change value when samples were directly compared. Sample genes picked up in array analysis are grouped by function. Ratios shown are hybridization signals in stables, induced or uninduced, over basal signals (2008-5B+tet/2008 and 2008-5B/2008 respectively).

Up-regulated genes	**20085B+tet/ 2008**	**20085B/ 2008**
Ets-1	***27.30***	***3.59***
Antioxidant defense		
Metallothionein 2A	3.93	2.76
Glutathione peroxidase 2	3.40	3.28
Metallothionein 1X	2.79	1.72
Thioredoxin	2.08	1.84
DNA repair		
Thymidylate synthase (TYMS)	3.02	2.82
mutY (E Coli) homolog (MUTYH)	2.51	2.42
Down-regulated genes		
Mitochondrial proteins		
Succinate dehydrogenase subunit B	0.28	0.53
NADH dehydrogenase 1, αβ subcomplex	0.40	0.46
ATP synthase,H+ transporting mitochondrial F1 complex β	0.45	0.50
Ferredoxin 1	0.46	0.50
Mitofilin (inner mitochondrial membrane)	0.46	0.65
NADH dehydrogenase 1, β subcomplex	0.47	0.55
ATP synthase, H+ transporting mitochondrial FI complex α	0.48	0.66
Cytochrome- c1	0.48	0.81
Metabolic enzymes		
Asparagine synthetase	0.31	0.44
Arginosuccinate synthetase	0.35	0.44
Ornithine decarboxylase 1	0.40	0.46
Fatty-acid-Coenzyme A ligase long chain 4	0.40	0.55
Glutamine synthase	0.43	0.45
Succinate-coA ligase α subunit	0.46	0.50
Fatty-acid-Coenzyme A ligase long chain 3	0.46	0.68
Oxidized low-density lipoprotein receptor	0.47	0.53
Pyruvate dehydrogenase α1	0.49	0.52

Table 4: Genes differentially regulated in 2008 cells stably transfected with *ets-1*.

glucose-free media. The growth of cells in media containing 2-DG, an analog of glucose and inhibitor of glycolysis, was inhibited to a greater extent in cells with a higher expression of Ets-1. Cells were grown in the presence of varying amounts of 2-DG, representative growth curves for each cell line are shown in figure 21A. The 2-DG IC_{50} doses, or doses where 50% of the cells have stopped proliferating, were calculated from 4 independent trials. Results indicate that 2008-*ets-1*-5B+tet cells are the most sensitive to 2-DG, with an IC_{50} of 1.46 mM (significantly lower than that of 2008 cells), next are C13 cells with an IC_{50} of 2.55 mM and finally 2008-*ets-1*-5B with an IC_{50} of 2.68 mM. 2008 cells were far more resistant than all other cells tested, with an IC_{50} of 5.03 mM. Tet treatment of 2008 cells alone had no significant effect on the growth inhibition by 2-DG (figure 21B). Growth of cells in normal or glucose-free media (supplemented with pyruvate) was compared over a 96 hr time period and it was observed that growth of cells with increased expression of Ets-1 was significantly inhibited as compared to the parental 2008 cells. Figure 22 indicates that the growth rate of all cells in glucose free media is decreased as compared to normally supplemented media, however at 48 hrs there is a distinct divergence in cell growth. Glucose availability is obviously a critical parameter for the growth of cells with higher expression of Ets-1 at this point, as their numbers drastically decrease. 2008 cells do display slowed growth at this timepoint, however, cell number did not decrease as was the case for the other cell types tested. These data indicate that Ets-1 expression leads cells to rely more heavily on glycolysis as an energy source. Again, tet treatment of 2008 cells had no effect on growth in glucose-free media.



Cell type	2-DG IC_{50} (mM)
2008	5.03±1.86
2008+tet	3.67±1.23
C13*	2.55±1.06
2008-*ets-1*-5B	2.68±1.18
2008-*ets-1*-5B+tet	1.46±1.45*

Figure 21: Effect of 2-DG on cell growth. A) Cells were grown in the presence of varying concentrations of 2-DG for 4 days at which time cell number was determined using the Hoechst DNA quantitation assay. Characteristic growth curves are shown for each cell line tested. Each point represents the mean and standard deviation of triplicate samples from one representative experiment. It is demonstrated that cells with a higher expression of Ets-1 than 2008 cells display reduced survival in 2-DG containing media as compared to 2008 cells. B) Median doses of 2-DG calculated for all cell lines tested. Values shown are the average and standard deviation from 4 independent trials (except for 2008+tet, 2 trials only). +tet indicates cells were treated with 0.2 μg/mL tet for 24 hours prior to incubation in 2-DG containing media. All cell lines tested have lower 2-DG IC_{50} values than 2008 cells. * IC_{50} is significantly lower than 2008 IC_{50} value, $P<0.05$.

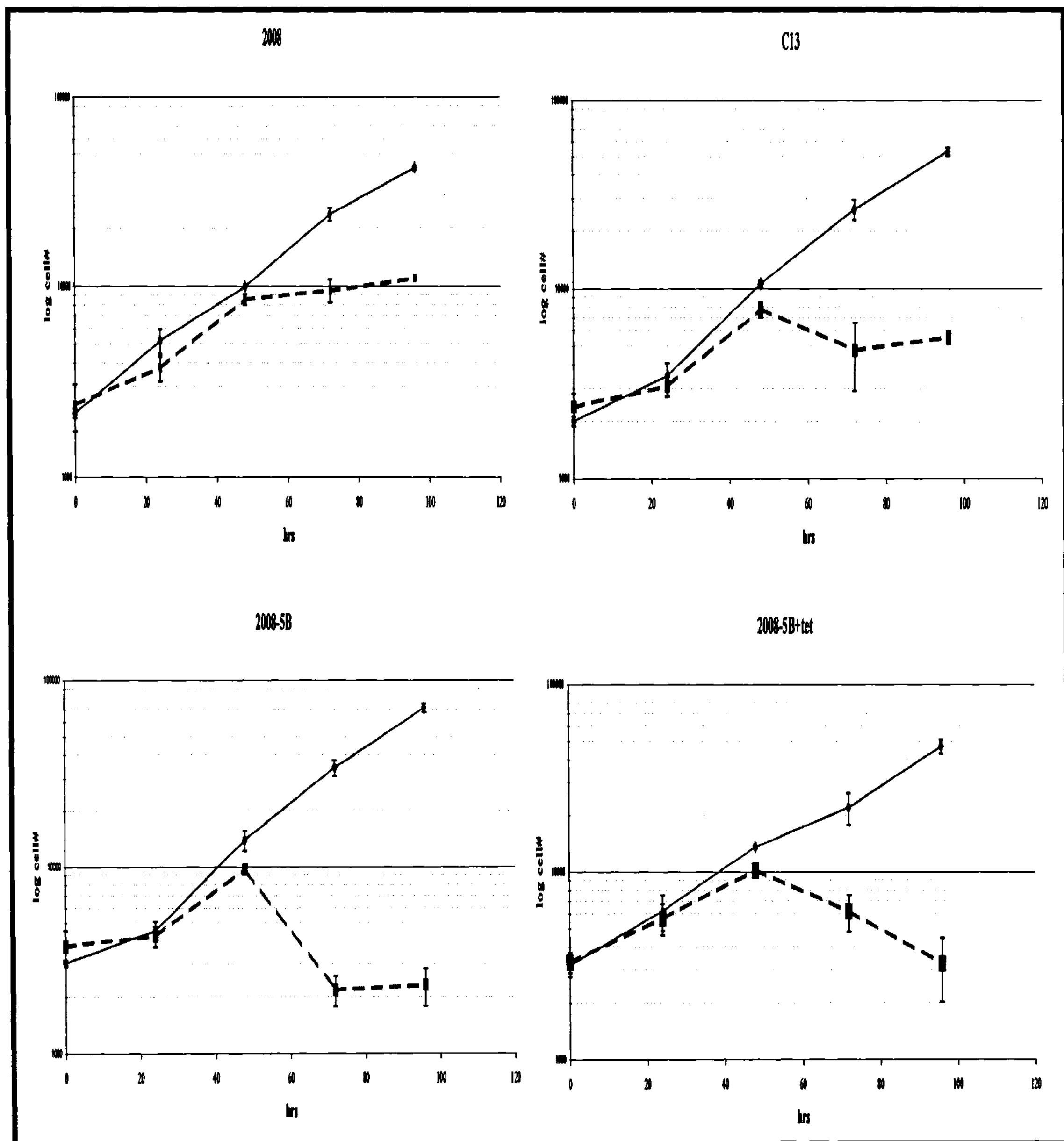


Figure 22: Growth of cells in glucose-free media. Cells were grown in glucose-free media supplemented with pyruvate for 4 days. Every 24 hrs, cell number for each cell line was determined using a Hoechst DNA quantitation assay. The log of cell number at each time point for all 4 cell types tested is shown. Cell number represents the average and standard deviation of 3 samples from one representative experiment. Solid line is growth curve in normal media and dashed line is growth in glucose free media supplemented with pyruvate. There is a distinct divergence in the growth characteristics of cells at 48 hours, 2008 cells display slowed growth whereas all other cell types begin to decrease in number at this timepoitnt.

3.4.4- Evaluation of the O_2 consumption by Ets-1 over-expressing cells

The overall trend of down-regulation of genes encoding mitochondrial proteins, including components of the electron transport chain within Ets-1 overexpressing cells, would indicate that these cell lines would display decreased O_2 consumption. This was tested using high-resolution respirometry. Cells were added to chambers of an oxygraph, in a concentration of 1.5 million cells per mL in KCl media. Figure 23A shows representative measurements of the respiratory rates of 2008 and 2008-*ets-1*-5B cells. Following addition of detergent, to allow free access of substrates to the mitochondria, the activity of each complex was tested with the addition of specific substrates and inhibitors. Results indicated that complexes functioned normally following permeabilization. The basal respiration rates of cells, prior to permeabilization, from three separate trials were analyzed; figure 23B represents the mean and standard deviation of these rates (where respiration rate indicates pmol O_2 consumed per second per million cells). Results indicate that 2008 cells overexpressing Ets-1 display significantly reduced O_2 consumption as compared to parental cells.

3.4.5- Effect of Ets-1 overexpression on metabolic properties of ES-2 cells

To evaluate whether observations made in 2008 *ets-1* overexpressing cell lines are cell-specific, the gene was overexpressed in another ovarian carcinoma cell line, ES-2. Cells were transfected with pcDNA3-*ets-1* to produce stable cell lines constitutively

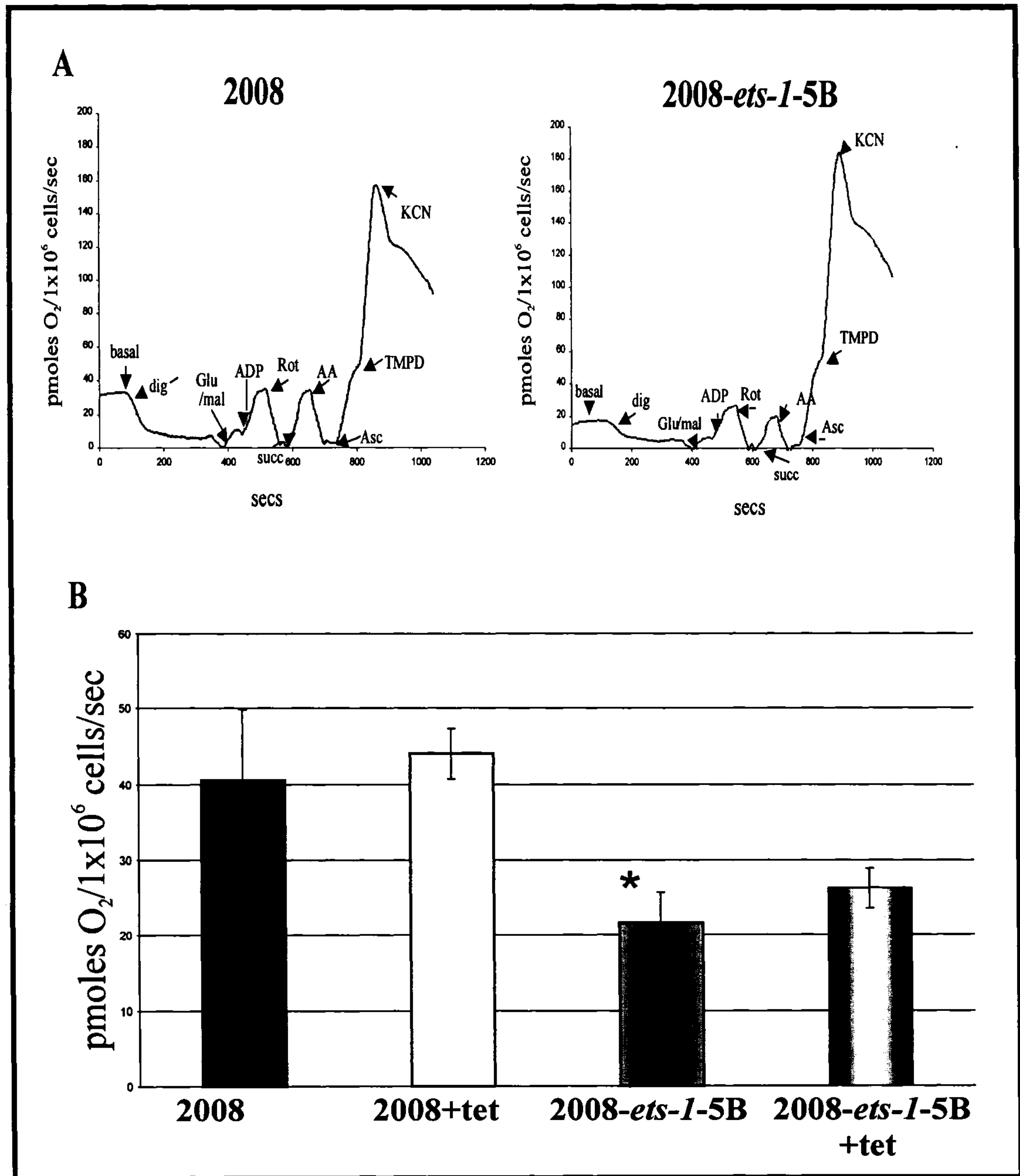


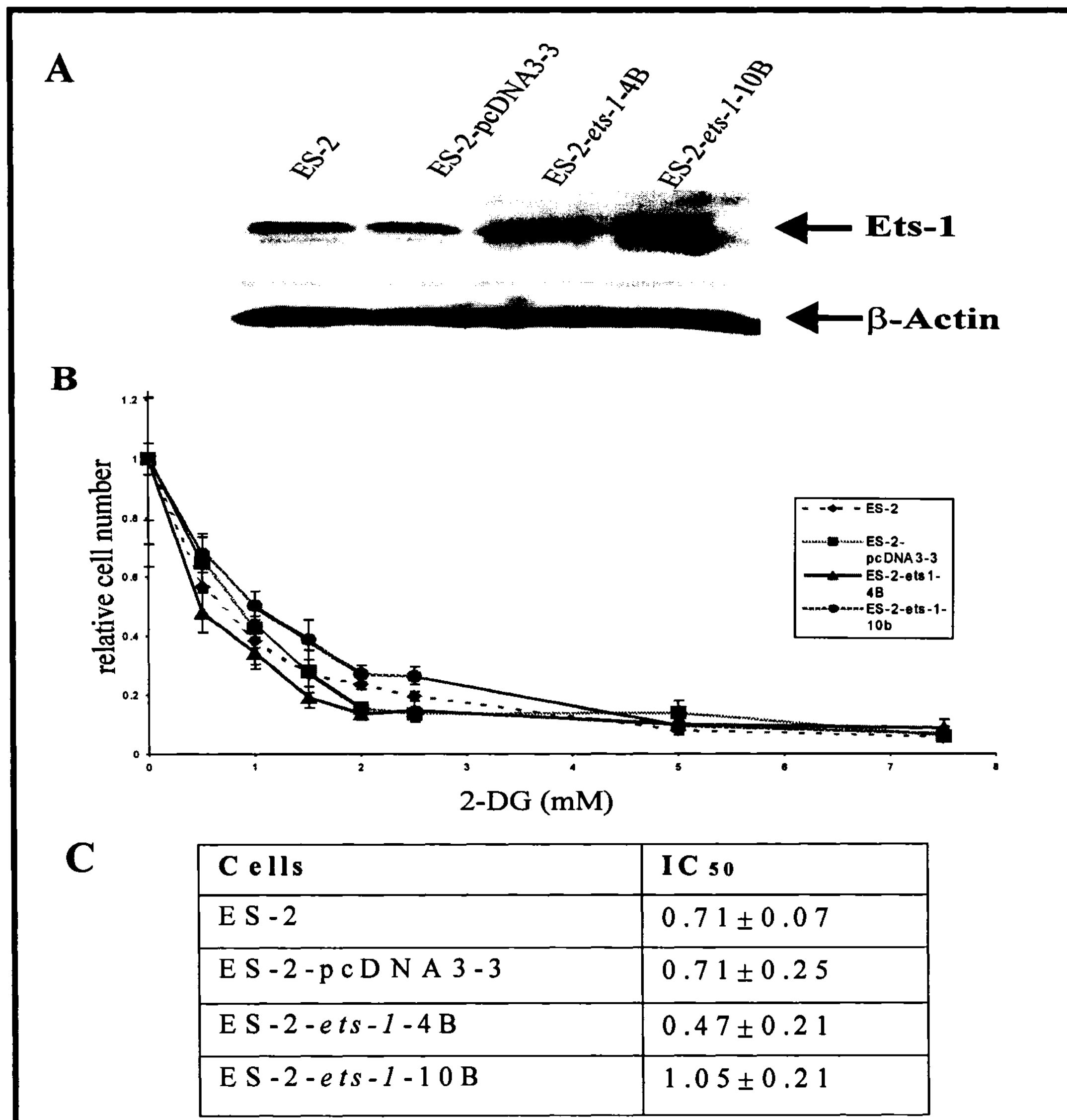
Figure 23: Oxygen consumption by parental and Ets-1 overexpressing cells.
A) Oxygen consumption by digitonin permeabilized 2008 and 2008-*ets-1*-5B cells. Oxygen consumption was measured using 1.5 million cells/mL KCl buffer. Subsequent addition of digitonin to permeabilize cells (dig), substrates for electron transport chain complexes glutamate/malate (glu/mal), ADP, succinate (succ), ascorbate (asc) and TMPD are indicated as are additions of inhibitors rotenone (rot), antimycin a (aa) and potassium cyanide (KCN). B) Basal respiration rates. Initial respiration rates, prior to permeabilization, of cells in question were tabulated from three independent experiments. Results represent mean and standard deviation, it is evident that 2008-*ets-1*-5B, tet induced and uninduced cells, consume less oxygen than 2008 cells.
* respiration rate is significantly lower than that of 2008 cells.

overexpressing *ets-1*. Following transfection and selection in G418, several clones were selected and screened for Ets-1 protein expression. Two clones (ES-2-*ets-1*-4B and ES-2-*ets-1*-10B) displayed increased Ets-1 protein expression as compared to untransfected and cells transfected with an empty vector (ES-2-pcDNA3-3) (figure 24A). The response of these cells to 2-DG treatment was then evaluated. Growth of all cells in 2-DG containing media was severely inhibited indicating that these cells, including untransfected ES-2 cells, are highly glycolytic. Sample growth curves are shown in figure 24B. IC_{50} values calculated from three independent assays indicate that all cell lines are highly sensitive and no significant differences were noted between parental and transfected cell lines (figure 24C). As for the O_2 consumption of the cells, basal respiration rates were quite low prior to permeabilization- again perhaps reflecting a glycolotic phenotype. Results showed a trend, though not significant, towards lower basal respiration rates of the *ets-1* transfected cell lines. However, even the empty vector transfected cell line displayed a lower rate of respiration than the parental cells.

3.5- Evaluation of the effect of increased Ets-1 expression on cisplatin resistance (objective 5).

3.5.1- Ets-1 is a cisplatin responsive gene

The fundamental difference between 2008 and C13* cell lines is resistance to cisplatin treatment. C13* cells are variants of 2008 cells displaying an approximate 8 fold resistance to cisplatin treatment, believed to be related to the observed mitochondrial differences within this cell line (Andrews *et al.* 1992). The up-regulation of Ets-1 in



Cells	IC 50
ES-2	0.71±0.07
ES-2-pcDNA3-3	0.71±0.25
ES-2-*ets-1*-4B	0.47±0.21
ES-2-*ets-1*-10B	1.05±0.21

Figure 24: Effect of 2-DG on cell growth of ES-2, Ets-1 overexpressing cells. A) ES-2 cells were stably transfected with pcDNA3 empty vector or pcDNA3-*ets-1*. Ets-1 levels in lysates of selected clones were evaluated by Western blotting. Clones 4B and 10B were used in further experiments based on the observed increased expression of Ets-1 in these cell lines. B) Cells were grown in the presence of varying concentrations of 2-DG for 4 days at which time cell number was determined using the Hoechst DNA quantitation assay. Characteristic growth curves are shown for each cell line tested. Each point represents the mean and standard deviation of triplicate samples from one representative experiment. Curves for all cell lines tested indicate that all cells display similar survival characteristics in 2-DG. C) IC_{50} doses of 2-DG calculated for all cell lines tested. Values shown are the mean and standard deviation from 4 independent trials. No significant differences between IC_{50} values were noted.

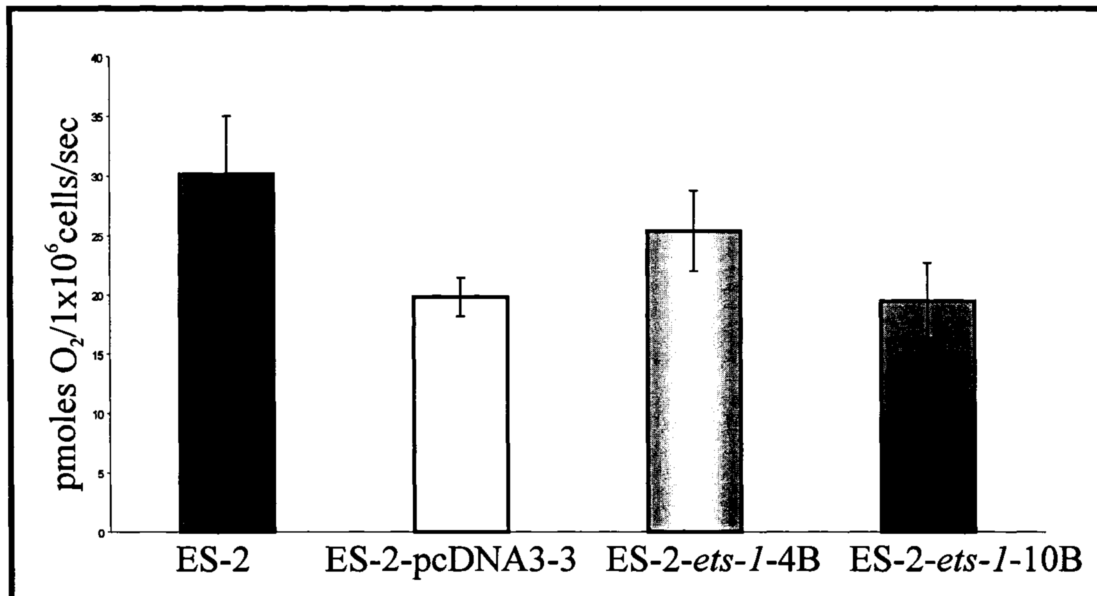


Figure 25: Oxygen consumption by ES-2, Ets-1 overexpressing cells. Basal respiration rates of all cell lines is indicated. Initial respiration rates, prior to permeabilization, of cells in question were tabulated. Results represent mean and standard deviation from three separate experiments. No significant changes in respiration rates were observed in *ets-1* transfected cells as compared to untransfected and empty vector transfected cells.

C13* cells appears to be as a result of altered mitochondria, thus the transcription factor may itself play a part in cisplatin resistance. To probe the role of Ets-1 in the cellular response to cisplatin, changes in Ets-1 expression following cisplatin treatment were evaluated. The western blot shown demonstrates the increase in Ets-1 protein levels in 2008 cells following cisplatin treatment with a range of concentrations at 4 and 6 hours recovery time (figure 26).

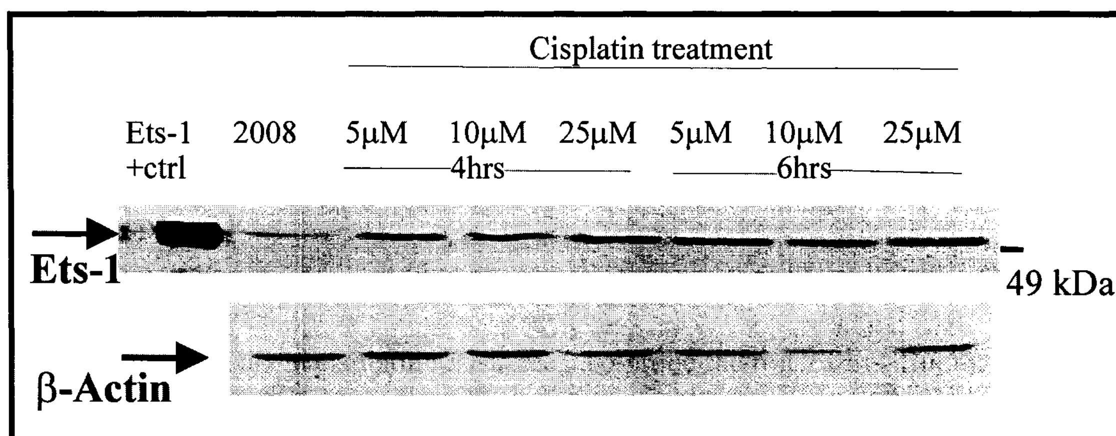


Figure 26. Up-regulation of Ets-1 by cisplatin treatment. 2008 cells were treated with 5-25 μM cisplatin for 1 hour, at which time media was replaced and cells recovered for 4-6 hours. Western blot analysis of Ets-1 protein levels indicates that cisplatin treatment causes an increase in Ets-1 expression at all concentrations and time-points tested. β-Actin levels in lysates were also analyzed and indicate equal gel loading.

3.5.2- Effect of increased Ets-1expression on the cisplatin resistance of 2008 cells

To test whether Ets-1 plays a role in mediating cisplatin resistance, inducible stables were screened for their response to cisplatin. Cisplatin cytotoxicity profiles of 2008, C13*, and Ets-1 overexpressing clones were determined using a DNA binding agent (Hoechst) to assess cell number. Cells were treated with a 1-hour pulse of various concentrations of cisplatin and cell number was determined 4 days later. The cytotoxic

responses to cisplatin of 2008 and C13* cells was first plotted and confirms the resistance of C13* cells relative to 2008 cells (figure 27). Resistance of cells stably overexpressing Ets-1 was then evaluated by the same method, representative survival curves of all cell lines tested (2008-*ets-1*-4 and 2008-*ets-1*-5B, induced and un-induced) are plotted alongside the 2008 curve for comparison purposes (figures 28 A,B).

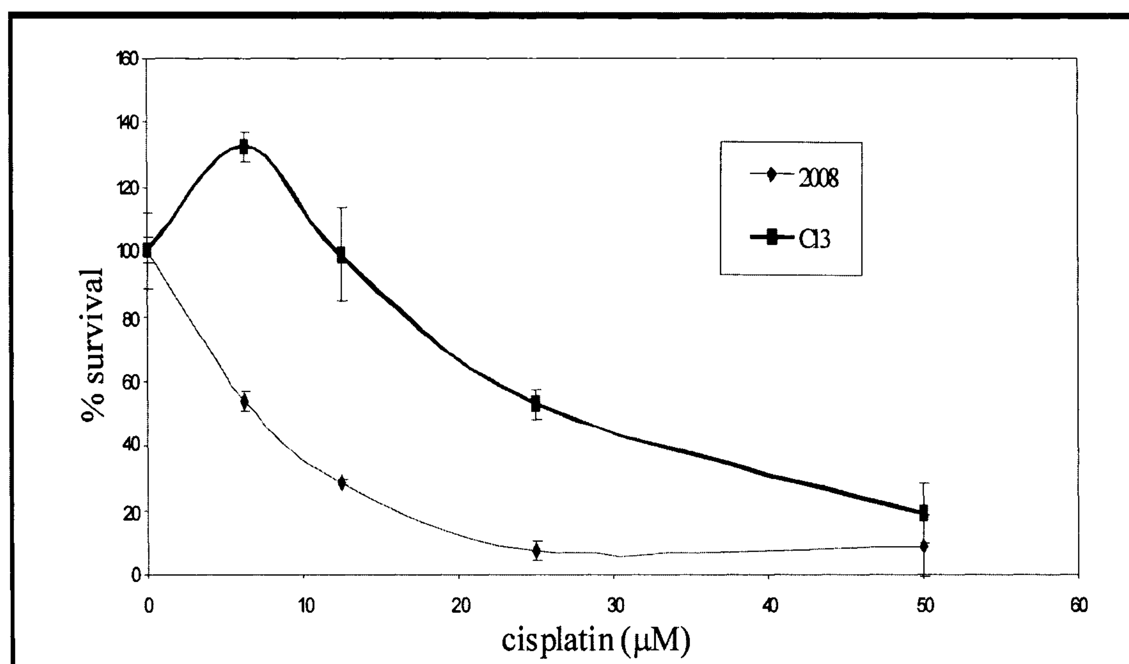


Figure 27: Response of 2008 and C13* cells to cisplatin. Representative cisplatin cytotoxicity curves for 2008 and C13* cells. Cells were continuously treated with concentrations of cisplatin shown for 4 days at which time cell number was determined using the Hoechst assay to quantitate total DNA content. Cell number relative to cells grown in cisplatin-free media for 4 days was calculated at each concentration tested and percent survival is shown. Values represent the mean and standard deviation of 3 samples in one representative experiment. Enhanced survival of C13* cells following cisplatin treatment is demonstrated. Experiments were repeated three times yielding similar results.

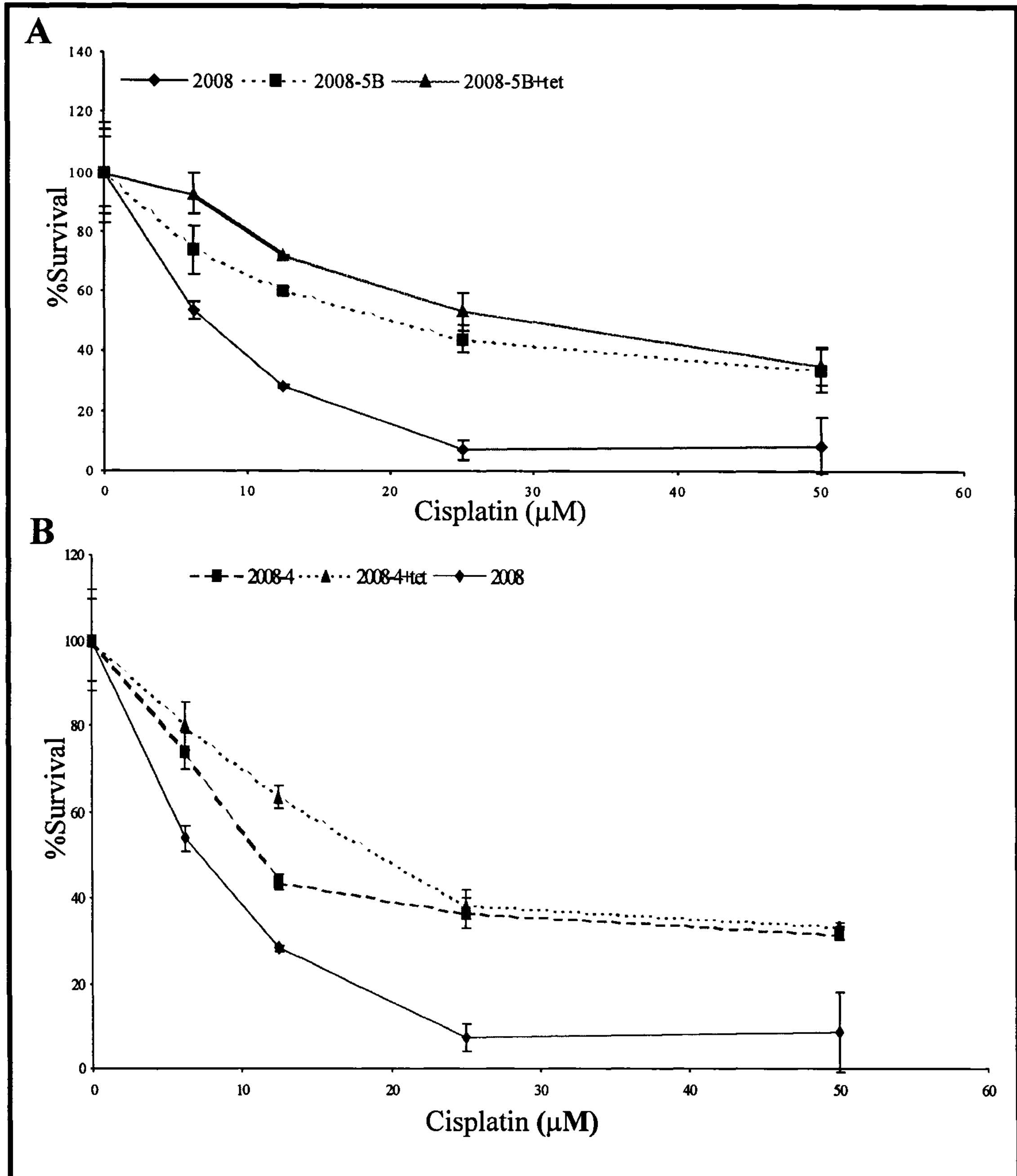


Figure 28: Effect of overexpression of Ets-1 on cisplatin resistance of 2008 cells. Representative cisplatin cytotoxicity curves for 2008 Ets-1 overexpressing clones 2008-*ets-1*-5B and 2008-*ets-1*-5B +tet, as compared to a cisplatin cytotoxicity curve for 2008 cells (A) and for 2008-*ets-1*-4 and 2008-*ets-1*-4 +tet, as compared to a cisplatin cytotoxicity curve for 2008 cells (B). Cell treatment and calculation of % survival at each cisplatin concentration was performed as described in figure 27. In both cases, Ets-1 overexpressing cells appear more resistant to cisplatin than parental 2008 cells.

The cisplatin IC_{50} values are shown on table 5 and indicate that C13* as well as Ets-1 overexpressing clones display significantly higher cisplatin IC_{50} values than that of 2008 cells. Tet treatment enhanced the cisplatin resistance characteristics of *ets-1* tet-inducible clones, whereas tet treatment alone had no significant effect on the response of 2008 cells to cisplatin.

3.5.3- Effect of overexpression of Ets-1 on the cisplatin resistance of a colorectal carcinoma cell line.

In order to determine whether the increased cisplatin resistance observed in 2008 cells following stable transfection with an *ets-1* vector is a cell-specific phenomenon, an unrelated colon carcinoma cell line was similarly transfected with an *ets-1* vector. In this case, transfection with a constitutively expressed *ets-1* vector was performed. Increased expression of Ets-1 protein was confirmed in clones 2 and 3 as compared to cells transfected with an empty vector (pcDNA3) (figure 29A). Cisplatin cytotoxicity assays were performed on HT29 cells stably transfected with empty vector and *ets-1*. Survival curves indicate that HT29-*ets-1*-2 and HT29-*ets-1*-3 have greater survival rates at higher concentrations of cisplatin (figure 29B). IC_{50} values confirm the statistically significant increase in the cisplatin resistance of *ets-1* overexpressing clones as indicated in table 5.

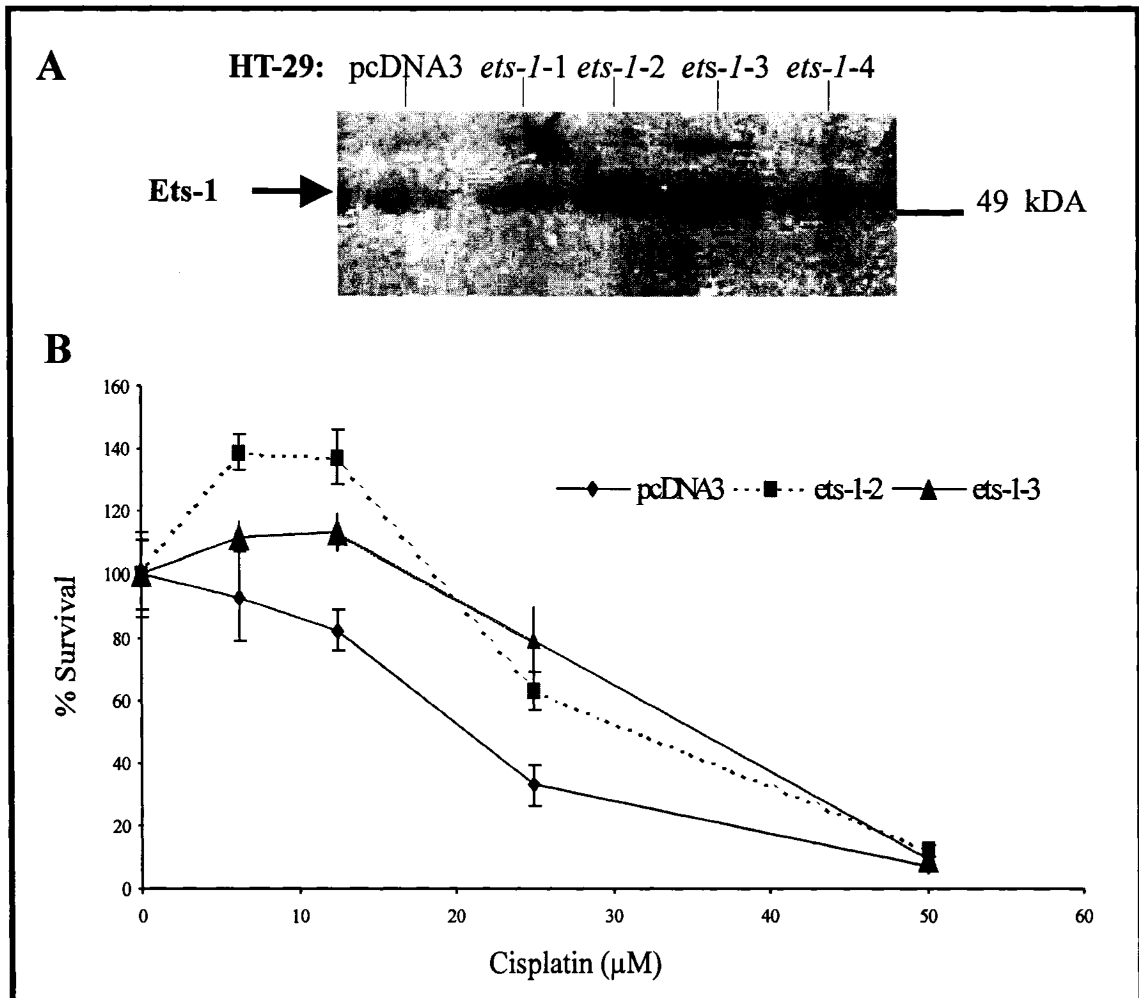


Figure 29: Effect of overexpression of Ets-1 on cisplatin resistance of HT-29 cells. A) HT-29 cells were stably transfected with pcDNA3-*ets-1* or pcDNA3 alone. Western blot analysis for the detection of Ets-1 protein levels in lysates from samples is shown. HT-29 *ets-1*-2 and *ets-1*-3 were used for further experiments based on the observed increased levels of Ets-1 in cell lysates. B) Representative cisplatin cytotoxicity curves for HT-29 stable transfectants. Cells were continuously treated with concentrations of cisplatin shown for 4 days at which time cell number was determined using the Hoechst assay to quantitate total DNA content. Cell number relative to cells grown in cisplatin-free media for 4 days was calculated at each concentration tested and percent survival is shown. The enhanced survival, and thus increased cisplatin resistance, of *ets-1*-2 and *ets-1*-3 clones as conpared to an empty vector control is evident Values represent the mean and standard deviation of 3 samples in one representative experiment. Experiments were repeated three times yielding similar results.

Cells	IC_{50} (µM)
2008	4.87 ± 1.12
2008 + tet	7.79 ± 5.02
C13*	30.07 ± 8.43*
2008-*ets-1*-5B	19.23 ± 1.95*
2008-*ets-1*-5B + tet	37.84 ± 6.33*‡
2008-*ets-1*-4	21.11 ± 7.30*
2008-*ets-1*-4 + tet	21.70 ± 3.03*
HT29-pcDNA3	23.56 ± 3.29
HT29-*ets-1*-2	39.23 ± 6.92†
HT29-*ets-1*-3	37.41 ± 4.44†

Table 5: Cisplatin IC_{50} values. IC_{50} doses of cisplatin calculated for all cell lines tested. Values shown are the mean and standard deviation from at least 3 independent trials. +tet indicates cells were treated with 0.2 µg/mL tet for 24 hours prior to the delivered cisplatin pulse. In both cell models, an increased expression of Ets-1 led to increased cisplatin resistance. *IC_{50} is significantly higher than 2008 IC_{50} value. ‡ IC_{50} value is significantly higher than 2008-*ets-1*-5B IC50 value. † IC_{50} value is significantly higher than HT29-pcDNA3 IC_{50} value.

4. Discussion

4.1- H_2O_2 mediated up-regulation of *ets-1*

Expression of the transcription factor Ets-1 in tumor cells as well as in surrounding stromal and endothelial cells has been repeatedly associated with increasing aggressiveness of tumors as well as overall poor patient prognosis (see table 2). The normal expression of Ets-1 is limited to lymphoid tissues as well as to areas undergoing active angiogenesis (Ghysdael *et al.* 1986; Naito *et al.* 1998). The aberrant expression in cancerous tissues and mechanisms leading to this increased expression is thus an important area of research.

This study has focused on H_2O_2, generated by dysfunctional mitochondria of cancer cells, as a possible mediator of increased Ets-1 expression. The observations that *ets-1* mRNA and protein expression is specifically elevated in C13* cells as compared to 2008 cells (Kenji Sakata, personal communication, figure 8) led to the overall hypothesis that H_2O_2 causes an up-regulation of Ets-1. C13* cells are cisplatin-resistant variants of 2008 cells with observable mitochondrial differences when compared to the parental cells (Andrews *et al.* 1992). These mitochondrial differences were hypothesized to elicit a resulting increase in H_2O_2 production by C13* cells, a proposal that was confirmed via the administration of an oxidant sensitive dye, DCFH-DA, to 2008 and C13* cells (figure 7). Though this dye is commonly used to detect intracellular levels of H_2O_2, the increased levels of fluorescent DCF, (the product of DCFH oxidation), observed in C13* cells cannot necessarily be concluded to be representative of higher intracellular levels of H_2O_2. There are indications that the oxidation rate of DCFH by H_2O_2 is in fact slow, and

that the dye will also be oxidized by secondary reactions and thus only indirectly by H_2O_2 (Royall & Ischiropoulos, 1993). A thorough analysis of the effect of different reactive species on DCF fluorescence identified mediators of DCFH oxidation as H_2O_2 as well as hydroxide ion and peroxynitrite (Myhre *et al.* 2003). Thus though H_2O_2 may certainly be the ROS responsible for the observed differences in DCF fluorescence in the cells tested, particularly since direct treatment of cells with H_2O_2 using this protocol led to increased dye fluorescence, the possibility that other species have led to DCFH oxidation cannot be ignored.

The increased production of H_2O_2 by C13* cells coupled to enhanced expression of Ets-1 led to further investigations into whether this transcription factor can be specifically up-regulated by H_2O_2. The hypothesis that genes are directly up-regulated by H_2O_2 is not unfounded- several genes have been identified to be H_2O_2 responsive. Examples include the gene encoding the antiapoptotic protein *Bfl-1* (*Bcl-2-related protein A1*), the up-regulation of which is mediated by an H_2O_2 activation of NFκB, as well as *GADD153*, *p21* and *monocyte chemoattractant protein 1*, all regulated by the H_2O_2 responsive transcription factor complex AP-1 (Kim *et al.* 2005; Chung *et al.* 2002; Guyton *et al.* 1996; Wung *et al.* 1997). Metallothionein-1 is an example of a protein encoded by a gene that demonstrates H_2O_2 inducibility by way of an ARE within the gene promoter (Dalton *et al.* 1994). In many of these cases, however, non physiological levels of H_2O_2 were administered to cells prior to the observed gene or protein up-regulation. Physiological levels have been estimated to be in the range of 0.01 to 0.1 μM (Chance *et al.* 1979); thus, studies where the levels were not in this range may not be

providing relevant results regarding the effects of H_2O_2 on gene expression as high levels may cause severe oxidative modifications within the cell (Antunes & Cadenas, 2001). In this study, a range of H_2O_2 levels, (0.01-100 μM) were administered to cells and it was found that the lower dose of 0.1 μM, similar to the estimated physiological level, consistently led to increased Ets-1 protein levels indicating this H_2O_2 mediated up-regulation may occur *in vivo* (figure 9).

The time course of Ets-1 up-regulation by H_2O_2, (4-6 hours to observe a clear protein up-regulation), suggests that up-regulation is at the level of gene transcription. In order to test this hypothesis, actinomycin D was administered to cells following H_2O_2 addition. Actinomycin D is an agent that intercalates into DNA and appears to stall the RNA polymerase enzyme, thereby inhibiting further transcription (Kimura *et al.* 2002). Administration of this drug prevented the H_2O_2 mediated up-regulation of Ets-1, indicating that this increased expression is in fact by way of a transcriptional mechanism (figure 10). It was also noted that administration of the drug alone led to a down-regulation of Ets-1. This is not surprising as the half life of Ets-1 has been estimated to be in the 4 hour range (Gilles *et al.* 1996) and thus during the 6 hour incubation time with actinomycin D, production of new *ets-1* transcripts following the degradation of endogenous levels was prevented.

Further evidence that Ets-1 levels are up-regulated by oxidative stress came from results of NAC administration to cells. NAC acts as a reducing agent by specifically elevating cell GSH levels via the increased availability of intracellular cysteine-intracellular thiols thus accumulate (Sen, 1998). In such a reduced environment, Ets-1

levels were down-regulated (figure 11). NAC, however, has low lipid solubility and higher levels of the agent, 50 mM, were necessary to attain a down-regulation of Ets-1 expression (Grinberg *et al.* 2005).

The up-regulation of Ets-1 by oxidative stress and the observation that levels of this protein are increased in a cell variant with disrupted mitochondria and a resulting elevated production of ROS, indicate that the induction of mitochondrial stress would lead to an up-regulation of Ets-1. To address this possibility, specific electron transport chain inhibitors were administered to cells and the resulting effect on Ets-1 levels was examined by Western blotting. Two hour treatment with rotenone, an inhibitor of complex I which acts to inhibit NADH reductase activity possibly by binding to the ND1 or ND4 site (Degli, 1998), led to an increase in Ets-1 protein levels; particularly evident at 4-6 hours recovery time. Treatment with oligomycin B, an inhibitor of the F_0 subunit of ATP synthase (Devenish *et al.* 2000), similarly led to increased Ets-1 protein levels at 4-6 hours recovery time (figure 12). The time course of induction again indicated that this regulation was likely transcriptional. Since it has been shown that increased H_2O_2 will lead to an up-regulation of Ets-1, a probable mechanism for the increase in Ets-1 levels mediated by ETC inhibitors is via the increased production of H_2O_2, produced as a byproduct of inefficient electron transport. This proposal was tested by evaluating changes in DCF fluorescence in cells treated with the drugs in question, however, no changes were noted at the various doses and time points tested (data not shown). It is possible that changes in ROS levels using this method were not picked up due to the timing of administration- it is unclear whether DCFH-DA should have been administered

to cells immediately following treatment or at some point during cell recovery. The observation of increased Ets-1 protein levels 4-6 hours following drug administration is similar to the time course of protein induction observed following H_2O_2 administration- indicating that the drugs may be resulting in H_2O_2 increases almost instantaneously. This was not picked up using the DCF assay.

Though any conclusions regarding the direct effect of these treatments on H_2O_2 levels could not be made in this study, there have been several studies published indicating that ETC inhibitors will cause increased ROS production. Complex I is considered a major site of electron leakage along the ETC and thus inhibition of this site with rotenone would be expected to elicit an increase in H_2O_2 production (Liu *et al.* 2002). This appears to be the case since several independent studies have confirmed that rotenone administration causes increased H_2O_2 production intracellularly or extracellularly using fluorescent dyes such as DCFH-DA and amplex red (used to evaluate extracellular H_2O_2 levels) (Liu *et al.* 2002; Sipos *et al.* 2003; Vanden Hoek *et al.* 1997). These studies examined the effects of rotenone on cardiomyocytes and synaptosomes, as well as in rat mitochondria and used doses of rotenone in the range of 50 nM-50 uM, thus the dose of 5 μM used in this study likely led to a similar increased production of H_2O_2 (Liu *et al.* 2002; Sipos *et al.* 2003; Vanden Hoek *et al.* 1997).

The effects of oligomycin B on H_2O_2 production are less well described. This drug is typically used to lower cellular ATP levels (Gong & Agani, 2005); however, this effect is not necessarily achieved following drug administration to cancer cells, given that these cells display an enhanced glycolytic phenotype. In this case, transient inhibition of

ATP synthase will not necessarily lead to an inhibition of ATP production (Gong & Agani, 2005). There are reports indicating that inhibition of ATP synthase will lead to an increase in the production of ROS; contradictory studies however claim that oligomycin will in fact reduce the production of ROS (Gong & Agani, 2005; Carriere *et al.* 2003; Sipos *et al.* 2003). A reduction in ROS production following oligomycin administration may be expected since a drop in ATP synthesis will lead to a decreased electron flow along the ETC and a resulting decrease in electron leakage and ROS production; however the opposite may also occur. In the *Ant1* knockout mouse, the ATP synthase enzyme is inhibited as a result of the lack of ATP and ADP exchange resulting in an increased production of ROS. It was argued in this study that stalling electron flow following inhibition of the ATP synthase enzyme causes electron accumulation at complex I, where they are available to be donated to O_2, thereby generating ROS (Esposito *et al.* 1999). Therefore, it is not clear whether both ETC inhibitors elicited increases in Ets-1 via increased ROS production; however, the conclusion that can be made is that mitochondrial stress, in the form of a block in electron flow along the ETC, or in the production of ATP by the ETC, will lead to increased Ets-1 protein levels. Ets-1 is then responsive, at a transcriptional level, to changes in intracellular H_2O_2 levels as well as to a general malfunctioning of the mitochondria.

4.2- ARE within *ets-1* promoter is H_2O_2 responsive element

In order to clarify whether the up-regulation of Ets-1 by H_2O_2 is in fact via an activation of gene transcription, the promoter of the *ets-1* gene was tested for H_2O_2 responsiveness. The 5' region of *ets-1*, necessary and sufficient to promote transcription

of the gene, has previously been characterized; the promoter region used in this study was accordingly isolated (Oka *et al.* 1991). In order to test the activity of the promoter, as well as various promoter truncations and mutations, constructs were made in which the promoter region of interest was cloned adjacent to a firefly luciferase gene whose activation is easily evaluated. The drawback of this type of system is that lysates from differently transfected populations of cells are directly compared in order to evaluate differences in transcriptional activation due to cell treatments, or as a result of different promoter regions and mutations tested. Experimental variability always occurs when transfecting due to transfection efficiency, amount of cell death, pipetting errors etc; therefore it was necessary to include an internal control in all of these experiments. The *Renilla* luciferase vector provides a suitable control as it is transfected along with the reporter vector under every condition and provides a background level of *Renilla* luciferase activity (the *Renilla* luciferase enzyme is distinct from the firefly, the two enzymes have unique substrate requirements and thus can be independently evaluated in a cell lysate). One possible limitation of the *Renilla* control is that this gene is under the control of a thymidine kinase promoter, which could itself respond to different treatments. This issue was noted in a study evaluating the effects of different hormone treatments on promoter activity: the thymidine kinase promoter was activated by dihydrotestosterone and repressed by dexamethasone treatment (Ibrahim *et al.* 2000). Clearly, when using this assay method, the internal control plasmid response to various treatments must also be monitored. In the case of H_2O_2 treatments used in this study, the *Renilla* luciferase activity remained fairly constant throughout and thus provided a

suitable internal control. All reporter luciferase levels were expressed as a ratio to *Renilla* luciferase values.

Initial luciferase assays, testing the activity of the *ets-1* promoter region indicated that the 1.4 kB region evaluated consistently displayed transcriptional activity. Truncations were then made to assess the activity of a 1000 bp region, which according to published accounts contains an enhancer region with several transcription factor binding sites, and of a 570 bp region, where this enhancer region was deleted (Oka *et al.* 1991; Majerus *et al.* 1992). Luciferase activity driven by these promoter truncations confirmed that the 1000 bp region retained transcriptional activation capacity with luciferase activity levels comparable to the 1.4 kB region tested, whereas the 570 bp region lost a significant amount of this ability- luciferase activity was significantly reduced as compared to the full length promoter tested (figure 13B). These results indicate that the enhancer region within the region 570-1000 base pairs upstream of the *ets-1* transcriptional start site is essential for full basal activity of the *ets-1* promoter, confirming previous studies which drew similar conclusions (Oka *et al.* 1991; Majerus *et al.* 1992). The effect of H_2O_2 on these constructs was then tested and a response by both the full length and 1000 bp truncation promoters was detected, displaying a 2-3 fold increase in luciferase activity following treatment as compared to untreated. There was no significant increase in promoter activity of the 570 bp region following H_2O_2 treatment, however, indicating that the element or elements responsible for mediating an H_2O_2 effect on the promoter lie within the enhancer region (figure 13C).

Within the enhancer region, several transcription factor binding sites have been characterized which were then evaluated for their possible roles in causing an H_2O_2 mediated increase in promoter activity. A plausible element which has been fairly well studied in the context of the *ets-1* promoter is a perfect consensus AP-1 binding site located approximately 870 base pairs upstream of the transcriptional start site and therefore within the enhancer region (Oka *et al.* 1991; Majerus *et al.* 1992). AP-1 is a known transcription factor capable of responding to oxidative stress in activating genes such as *GADD153* (Guyton *et al.* 1996B). The study describing a *GADD153* up-regulation by H_2O_2 via enhanced AP-1 binding to the promoter identified the key nucleotides required for AP-1 binding. The AP-1 binding site- TGACTCA was mutated to *G*GACTCA resulting in a loss of protein binding to this site following H_2O_2 induction (Guyton *et al.* 1996B). This same mutation was consequently made in the full length *ets-1* promoter construct for this study, in order to evaluate the contribution of the AP-1 site within the *ets-1* enhancer region to the overall H_2O_2 responsiveness of the promoter. The basal level of luciferase activity generated from the AP-1 mutant promoter was similar to that of the full length promoter (figure 14B). Following H_2O_2 induction, the AP-1 mutant promoter was activated 2.7 fold, an increase in activity that is not significantly different from the observed 2.5 fold activation of the full length promoter (figure 15A). The AP-1 site does not therefore appear to contribute to the overall luciferase activity of the *ets-1* promoter, as disrupting this site has no effect on basal or induced activity.

The AP-1 site within the *ets-1* promoter is assumed to be a functional site, as studies in which both B lymphoid Daudi and HeLa cells were transfected with *ets-1*

promoter constructs along with c-Jun and c-Fos, the protein components of AP-1, an activation of the promoter was noted (Oka *et al.* 1991; Majerus *et al.* 1992). In the case of Daudi cells, it was noted that c-Jun significantly activated the *ets-1* promoter; however, a simultaneous transfection of c-Fos did not synergistically increase activation of the promoter (Oka *et al.* 1991). A combination of c-Jun and c-Fos did enhance activation of the *ets-1* promoter in HeLa cells, indicating that the site is a representative AP-1 site. It is possible that in certain cell lines a different Fos protein family member may be involved in site binding (Majerus *et al.* 1992). This may be the case in the 2008 cells tested in this study, in which the AP-1 site did not appear to be involved in promoter basal and induced activity. An inability to activate an authentic AP-1 site has been observed in hepatic stellate cells isolated from rat liver where an intact AP-1 site was not responsive to TPA, a well characterized inducer of AP-1 activity (Reichard & Petersen, 2004). In this particular case, investigators identified Fra1 and Fra2 as the major Fos proteins bound to the AP-1 site of a test promoter, proteins which are known inhibitors of AP-1 mediated activation (Reichard & Petersen, 2004). It is therefore a possibility that though the AP-1 site located within the *ets-1* promoter is an authentic site, protein components of AP-1 needed to activate this site are not present in 2008 cells.

A second site investigated in the *ets-1* promoter enhancer region was an HRE located approximately 770 base pairs upstream from the transcriptional start site, again within the enhancer region of interest. This site was originally identified in a study indicating that the *ets-1* promoter responds to hypoxia via the enhanced binding of HIF-1α at the region corresponding to a putative HRE (Oikawa *et al.* 2001). The HRE has

been identified in promoters of several genes known for their ability to respond, and up-regulate encoded proteins in response to hypoxia. Examples include VEGF, EPO and enzymes in the glycolytic pathway (Forsythe *et al.* 1996; Semenza *et al.* 1996). In the analysis of the minimal promoter region required to elicit a hypoxic response in all of these genes, a consensus HRE was identified: G/C/T- ACGTGC-G/T in which the CGTG core appears to be particularly required (Forsythe *et al.* 1996; Semenza *et al.* 1996). The element considered to be an HRE in the *ets-1* promoter does not perfectly match the consensus HRE, but differs in a central base (CACGT**C**CT). This element was evaluated as a possible contributor to the H_2O_2 inducible activity of the *ets-1* promoter since HIF-1α has been shown to be stabilized by ROS and hypoxic conditions themselves are associated with increased levels of ROS (Chandel *et al.* 2000; Schroedl *et al.* 2002). H_2O_2 treatment may simulate a hypoxic environment, lead to a stabilization of HIF-1α and a subsequent binding of this factor to responsive elements and target gene activation. In order to investigate the role of this element in the 2008 cell model, the *ets-1* full length promoter was mutated from the wildtype CACGTCCT to CA*AA*TCCT, a substitution that had been noted to abolish the hypoxia mediated induction of the endothelial nitric oxide synthase gene (Coulet *et al.* 2003). Basal activity of this promoter construct was not significantly lower than that of the wildtype promoter, indicating that this element is not of importance in regulating *ets-1* activation under normal conditions (figure 14B). The H_2O_2-induced activity of the HRE mutant luciferase construct was significantly elevated as compared to basal activity levels, with a 2.15 fold activation; however the overall luciferase activity following H_2O_2 induction did decrease as compared to the induced

activity of the wild type promoter indicating that this element does contribute to the H_2O_2-induced activation of the promoter (figure 15A). Nevertheless- the mutant HRE promoter retained a significant ability to respond to H_2O_2, indicating that this is not the sole, and likely not the most important element involved in the inducible activity of the promoter.

In order to further investigate the possibility that HIF-1α may make some contribution to the H_2O_2 responsiveness of the *ets-1* promoter, HIF-1α protein levels under normal, hypoxic and H_2O_2 treated conditions were evaluated. HIF-1α was noted to be expressed at a higher basal level in the cell line with elevated endogenous ROS levels (C13*), and was inducible in both 2008 and C13* cell variants following incubation in a hypoxic chamber. H_2O_2 treatment also up-regulated HIF-1α levels in 2008 cells (figure 19 A,B). The induction of HIF-1α stabilization by H_2O_2 appeared to be transient, with increased protein levels observed at 2 hours recovery time following treatment and a loss of detectable protein by 4 hours recovery time. This time course may indicate that at the 2 hour time point, HIF-1α is capable of binding target promoters such as the *ets-1* promoter and activating transcription. If indeed HIF-1α is responsible for regulating the H_2O_2 mediated up-regulation of *ets-1*, it would be expected that any conditions capable of up-regulating or stabilizing HIF-1α levels would consequentially cause an up-regulation of *ets-1*. This was not the case in 2008 cells, where hypoxic incubation did not lead to an increased level of Ets-1 protein despite an observed increase in HIF-1α levels (figure 19C). Given that a hypoxic stabilization of HIF-1α did not up-regulate Ets-1, it is

possible that in this cell line HIF-1α does not bind the *ets*-1 promoter or may lack required binding partners, preventing promoter transactivation.

These results, implicating only a minimal role for the *ets-1* HRE in H_2O_2 mediated up-regulation of the gene, despite observed H_2O_2 mediated increases in HIF-1α protein, are in contrast to those observed using a bladder cancer cell line (Oikawa *et al.* 2001). In the latter case, hypoxia led to a direct transcriptional up-regulation of *ets-1* via the element described and tested in this study. Furthermore, EMSA analysis confirmed that both HIF-1α and β bound to the region of interest under induced conditions (Oikawa *et al.* 2001). Differences may be due to the fact that different promoter regions were tested- in the bladder cancer study the HRE containing promoter fragment tested was only 479 base pairs, whereas the promoter studied here was approximately 1.4 kB (Oikawa *et al.* 2001). It is possible that in the larger promoter fragment, binding sites for HIF-1α lie further downstream in the promoter which may then sequester HIF-1α, or possibly repressive elements that would inhibit the activity of HIF-1α are present in the larger promoter fragment only. It is also of note that HIF-1α requires the co-activator CBP/p300, a limiting factor in this complex, for full transactivation ability (Ruas *et al.* 2005). CBP/p300 is a co-activator used by several other transcription factors, thus in the context of the full length *ets-1* promoter tested, several other factors may be bound to CBP/p300, limiting co-factor availability for HIF-1α- a phenomenon which may not exist in the smaller promoter fragment tested.

The results of this study indicate that the AP-1 binding element and HRE do not substantially contribute to an H_2O_2 mediated induction of the *ets-1* promoter; therefore,

additional investigations were directed towards a further characterization of the enhancer region of the promoter, with particular attention paid to the possible identification of a consensus ARE within this region. The ARE is a well described element involved in the inducibility of several promoters to agents such as pro-oxidant xenobiotics as well as H_2O_2 itself (Nguyen *et al.* 2003). A search for the ARE consensus TGACNNNGC was performed on the *ets-1* promoter and one such element was identified 690 base pairs upstream from the transcriptional start site, within the enhancer region. A thorough analysis of each nucleotide in the consensus ARE has identified the T and the GC box at the 3' end as critical in ARE inducibility, thus these nucleotides were replaced as follows to create a mutant ARE *ets-1* promoter construct: G̲GACNNNA̲T̲ (Rushmore *et al.* 1991). The luciferase activity driven by this mutant promoter was significantly lower than that of the wildtype as was the H_2O_2-induced activity (figures 14B, 15A). Though the ARE mutant promoter was still significantly induced by H_2O_2, the ratio of induced to un-induced was only 1.5, the lowest observed of all promoter mutants tested (figure 15B). The ARE contributes to the H_2O_2 inducibility of the *ets-1* promoter to the greatest extent of all elements tested, at both the basal and induced levels.

In order to evaluate whether the ARE identified to be a pivotal element in the regulation of *ets-1*, is an authentic ARE, an ARE inducing agent frequently used to demonstrate the inducibility of promoters with ARE elements, tBHQ, was used. tBHQ causes the up-regulation of several genes associated with resistance against oxidative stress including detoxifying enzymes NQO1 and subunits of GST and has a direct activating effect on promoter constructs containing consensus AREs (Hara *et al.* 2003).

Treatment of 2008 cells with this agent following transfection with either the *ets-1* wild type promoter construct or the mutant ARE construct led to a 3 fold increase in activity in the case of the wild type promoter, and no significant increase in the mutant promoter activity (figure 15B). These data indicate that the ARE within the *ets-1* promoter responds to typical ARE inducers, that the mutations created in key residues abolish this inducibility, and reinforces the role of the ARE in the transactivation of *ets-1*.

4.3- *ets-1* promoter ARE is bound by a Nrf2 containing complex

The next step in the evaluation of the ARE activity of the *ets-1* promoter involved EMSA analysis. This assay was used to determine whether proteins present in nuclear lysates from untreated or H_2O_2 treated cells are capable of binding the *ets-1* ARE. Nuclear proteins were observed to bind the *ets-1* ARE in an inducible fashion, with increased protein complex binding observed at approximately 3 hours of recovery time following H_2O_2 treatment (figure 16). In order to address whether Nrf2, the central transcription factor involved in binding and activating the ARE (Nguyen *et al.* 2003), is part of the protein complex bound to *ets-1,* supershift and competition assays were performed. Supershift assays involve the pre- or post-incubation of the radioactively labeled oligonucleotide/nuclear lysate protein complex with antibodies directed against proteins that may be components of the complex of interest. In this case, an anti-Nrf2 antibody was added to the *ets-1* ARE oligonucleotide following the binding reaction with lysate proteins or, alternatively, was incubated with nuclear lysate overnight prior to the binding reaction. Pre-incubation of nuclear lysates with anti-Nrf2 antibody led to decreased complex formation on the *ets-1* ARE probe, suggesting that incubation of

lysates with the anti-Nrf2 antibody depleted available Nrf2 from the complex and that Nrf2 is a part of the complex which typically forms on this site (figure 16). Incubation with the antibody following the binding reaction, under ideal conditions, should cause antibody binding to Nrf2 that is bound to the labeled probes and a resulting visible shift of the position of the probe following gel shift analysis. Several attempts to visualize this shift were not successful (data not shown). Thus, despite the data obtained following pre-incubation with the Nrf2 antibody, which suggests that Nrf2 is part of the protein complex bound to the *ets-1* ARE, this was not confirmed using the supershift technique. This may indicate that Nrf2 was not, in fact, part of the complex and that pre-incubation with the antibody was causing a depletion of non specific proteins; however this technique has been used by different investigators and appears to be specific for the depletion of Nrf2 (Martin *et al.* 2004; Suh *et al.* 2004). It may be possible that the complex was not bound by an anti-Nrf2 antibody because the complex is formed in such a way that the Nrf2 epitope is masked and unable to bind the antibody. Alternatively, the assay conditions used may not have been optimal to allow antibody-complex binding.

Supershift data from EMSAs performed using the *ets-1* ARE did not definitively determine whether Nrf2 binds this specific element and therefore, competition assays were attempted to further address this issue. In these assays, lysates were first incubated with excess unlabelled probes corresponding to the *ets-1*, mutant or control (*NQO1*) ARE for 15 minutes followed by incubation with the labeled *ets-1* or control ARE. If the unlabeled probe binds a similar complex to that of the labeled probe, it will deplete the lysate sample of the component proteins and subsequent binding to the labeled probe will

not occur. Using this technique, it was observed that unlabelled *ets-1* ARE competed for the complex typically formed on the labeled *ets-1* ARE, confirming the validity of this technique. An unlabeled mutant ARE probe did not complete for the complex, confirming that the mutation created in the *ets-1* ARE prevented complex binding, and finally the *NQO1* Control ARE did compete for the *ets-1* ARE bound complex (figure 17A). In the final case, it was interesting to note that though the complex consistently observed on the *ets-1* ARE was competed out with the *NQO1* ARE, it appeared that 2 unique complexes formed in the absence of the standard complex (figure 17A). Thus, it appears that alternate factors will bind the *ets-1* ARE if the characteristic binding complex is not able to form- perhaps indicative of an existent competition mechanism within cells. Competition assays using the *NQO1* labeled probe, competing with the *ets-1* ARE indicated that the *ets-1* ARE will also compete for the factors that bind the *NQO1* ARE, but this competition was not able to completely prevent complex formation on the *NQO1* ARE indicating that the *NQO1* ARE has much greater affinity for the complex (figure 17B).

The overall results from EMSA data analysis suggest that an Nrf2 containing complex binds the *ets-1* ARE and that this same complex binds the *NQO1* ARE. A probe corresponding to the *NQO1* ARE was chosen as a control in these experiments since the *NQO1* gene is a well described ARE regulated gene, up-regulated by factors such as tBHQ, and is bound and activated by Nrf2. This control probe then presumably provided a positive control for Nrf2 binding (Dhakshinamoorthy & Jaiswal, 2000). It is important to note, however, that the *NQO1* ARE has also been found to bind

several other proteins, typically binding partners for Nrf2, such as small Maf proteins as well as Maf G, Maf K, Jun Fos and Fra proteins, and in one case appeared to preferentially bind Nrfl rather than Nrf2 under induced conditions (Dhakshinamoorthy & Jaiswal, 2002; Vasiliou *et al.* 2003). It is therefore possible that proteins other than Nrf2 are uniquely involved in the regulation of the *ets-1* ARE, a possibility that requires further studies. There is precedence for the apparent regulation of an ARE by different factors than those that regulate *NQO1* and other control ARE genes- this was observed in the ARE-mediated regulation of the transcription factor c-*jun* (Radjendirane & Jaiswal, 1999). The near consensus ARE identified within the gene promoter was activated by inducers such as tBHQ but not to the same level of activity of the *NQO1* ARE. EMSA analysis showed two bands indicative of protein binding to the c-*jun* ARE following induction, but only one was competed out by the *NQO1* ARE, implying that there is a unique complex bound to this element (Radjendirane & Jaiswal, 1999). It is interesting to note that this example describes the regulation of a transcription factor, an atypical ARE targeted gene much like *ets-1*.

The expression of Nrf2 was also evaluated in 2008 cells under basal and H_2O_2-induced conditions. If this factor was found to be expressed in the nucleus it would lend support to the possibility that it is involved in the regulation of the *ets-1* ARE. Nrf2 expression was confirmed in nuclear lysates under all conditions, with an apparent accumulation of two forms following H_2O_2 treatment- visible at approximately 1 hour of recovery time following treatment. Only one form was noted in the cytoplasmic fractions from the same cells and protein levels also appeared to be lower in the cytoplasm overall

(figure 18). Recent evidence, using a newly generated anti-Nrf2 antibody, has indicated that Nrf2 is actually localized to the nucleus under normal and stressed conditions (Nguyen *et al.* 2005). This antibody was used for both Western blotting and immunohistochemistry and these results call into question the Santa Cruz antibody used in this study and many others, which has indicated that Nrf2 is expressed in the cytoplasm (Nguyen *et al.* 2005). The novel mechanism proposed for controlling the inducibility of Nrf2 activity involves transient shuttling of the Nrf2 interactor Keap1 to the nucleus where interaction promotes ubiquitination of Nrf2 and subsequent degradation. Disruption of this process by ARE inducers is proposed to be via an interference with the interaction of Nrf2 and Keap1 (Nguyen *et al.* 2005). The form of Nrf2 which accumulates in the H_2O_2-induced nucleus of this study may then represent a form which does not interact with Keap1 and is able to transactivate target promoters.

It is of importance to fully characterize the regulatory mechanisms controlling activity of the *ets-1* ARE, to define whether this gene is regulated in a similar fashion to other ARE regulated genes and to possibly identify therapeutic targets for the prevention of *ets-1* up-regulation in tissues. Genes regulated by the ARE are thought be involved in cancer prevention, by preventing carcinogens from modifying DNA, detoxifying harmful agents and increasing the cells antioxidant capability (Hayes & McMahon, 2001). Enzymes involved in these processes, whose encoding genes have been identified as targets for ARE regulation, include NQO1, heme oxygenase 1, GST, aldehyde dehydrogenase and many others (Hayes & McMahon, 2001). It has thus been proposed as an anticarcinogenic strategy to target the up-regulation of ARE regulated genes with

various agents such as chemicals with active thiol moieties as well as inhibitors of Keap1 activity (Zhang & Gordon, 2004; Devling *et al.* 2005). However, the results of this study indicate that such agents could produce a simultaneous up-regulation of *ets-1*, a gene with clear deleterious effects in tumors. A better understanding of the factors involved in regulating the *ets-1* ARE is therefore required.

It would also be of interest to evaluate whether other members of the ETS family are similarly regulated via an ARE within gene promoters. Ets-2 is the closest family member to Ets-1 and thus expression of this factor was evaluated following H_2O_2 treatment; however, no change in expression was noted (figure 9B), which is contrary to a report indicating Ets-2 was induced by H_2O_2 in a mouse fibroblast model (Sanij *et al.* 2001). The mouse fibroblast study used high doses of H_2O_2, which are likely less physiologically relevant than those used in the present study, possibly explaining the different findings (Sanij *et al.* 2001). An analysis of the *ets-2* promoter did, however, reveal that a consensus ARE lies at approximately position 722 from the 5' end. Promoter activity studies on the *ets-2* promoter have only been conducted from position 890 on however, and have revealed that regions beyond position 1460 are essential for promoter activity (Mavrothalassitis *et al.* 1990). Further studies would then be required to evaluate whether the ARE identified plays a part in regulating Ets-2 expression, a factor which has relatively unrestricted expression.

4.4- Ets-1 as a mediator of mitochondria to nucleus crosstalk

A likely source of H_2O_2 within cells is the mitochondria, and factors such as NRF-1 and NRF-2 which are able to regulate the expression of mitochondrial proteins and

respond to levels of H_2O_2 indicate that this ROS is involved in mitochondria- nucleus signaling (Suliman *et al.* 2003; Martin *et al.* 1996). Data indicating that expression of the transcription factor Ets-1 is up-regulated by H_2O_2 has led to the proposal that this factor is similarly involved in mediating mitochondria-nucleus crosstalk. In order to test this hypothesis, 2008 cells were stably transfected to over-express Ets-1 and the consequences of this overexpression were evaluated.

A tet-inducible system was used to generate stable Ets-1 over-expressing cell lines. The expected result was the generation of cells which, under normal conditions express close to endogenous levels of Ets-1, and only following tet induction express the protein at a higher level. However, the system was found to be leaky, in that un-induced cells consistently displayed higher levels of Ets-1 than untransfected cells, particularly following several passages (figures 20 B,C). Tet induction certainly led to increased expression, but rather than having a cell line that only displayed increased Ets-1 following induction, cells used in assays exhibited a range of Ets-1 levels: low in 2008 cells, higher in 2008-*ets-1*-5B (clone chosen for high expression of Ets-1) and highest in 2008-*ets-1*-5B+tet (figure 20). The leakiness problem with this system has been documented elsewhere- an evaluation of various inducible expression systems indicated that a tet inducible promoter linked to a reporter gene displayed considerable background levels of the reporter gene prior to tet induction, but with induction was driven to express higher levels of the reporter gene as compared to other systems tested- demonstrating the advantage and disadvantage of using this system (Meyer-Ficca *et al.* 2004). A suggested

explanation for the leakiness is the possible presence of trace amounts of tet in cell media supplemented with FBS (Meyer-Ficca *et al.* 2004).

Another interesting note regarding cell lines stably overexpressing Ets-1 was that two forms of the protein were consistently observed by western blotting (figure 20C). These two forms were also often seen in C13* cells but infrequently observed in untransfected 2008 cells. CIAP, a general serine/threonine phosphatase, was used to dephosphorylate proteins in cell lysates following harvesting and led to decreased expression of the higher molecular weight band in high Ets-1 expressors indicating that this is a phosphorylated form. Therefore, Ets-1 over-expression involves over-expression of both an un-phosphorylated and a phosphorylated form (figure 20D). Though it cannot be deduced which phosphorylated form of the protein is present, more specific phosphatases would be necessary for further evaluation, it is possibly the ERK phoshorylated form which displays enhanced activity, or alternatively, the CAMKII phosphorylated form which exhibits less transactivation function. Preliminary results using an Ets-1 responsive promoter reporter construct have indicated that over-expressed forms of Ets-1 in 2008 cells are able to increase target gene transactivation (data not shown). These assays were performed using a *uPA* promoter luciferase construct, (provided by Mike Ostrowski, Ohio State University, Columbus, Ohio). The *uPA* reporter has been shown to respond to Ets proteins such as Ets-1, Ets-2 and PEA3, and is therefore not specific for Ets-1 (Stacey *et al.* 1995). Initial luciferase assays demonstrating an activation of the uPA reporter by Ets-1 did not include co-transfections with the internal control *Renilla* vector and are thus only indicative of a possible

increased Ets-1 activity in 2008-*ets-1*-5B and 2008-*ets-1*-5B +tet lysates as compared to untransfected 2008 cell lysates; however, the results do encourage the conclusion that the over-expressed protein is in fact active and not present mainly in an in-active form.

In order to evaluate what changes in gene expression may arise as a result of Ets-1 over-expression in 2008 cells, RNA samples from 2008 (treated with tet to control for effects of tet alone on gene expression), 2008-*ets-1*-5B and 2008-*ets-1*-5B+tet were hybridized to an Affymetrix human genome focus array. This array technology allows the simultaneous monitoring of 8500 genes in the samples tested and thus, generates a large amount of data. The main advantages of the Affymetrix array technology is that each gene on the array is represented by 11 probe sets, each set containing a perfect match probe and a mismatch probe (mismatch probe includes an alternate base at the 13th position of the oligonucleotide). Data generated for one gene therefore includes the hybridization signals of sample RNA to all 11 probes, with each hybridization signal incorporating differences in affinity to the perfect match and the mismatch probes. In the analysis of data produced using this method, genes can be chosen that are significantly detected in all three samples tested and that display a significant increase or decrease in the highest Ets-1 expressor (2008-*ets-1*-5B+tet) as compared to 2008 cells. For this study, the P value of detection was set at 0.05 and genes were only considered as examples of increased or decreased expression if there was at least a 2 fold change in hybridization signal when comparing 2008 and 2008-*ets-1*-5B+tet values. Results are listed in table 4. The increased expression of *ets-1* in the samples tested was confirmed on the array and several trends were noted in the gene expression changes. Genes involved

in antioxidant defense and DNA repair were up-regulated and several genes that encode mitochondrial proteins were down-regulated, as were metabolic enzymes.

The down-regulation of several genes was an un-expected result. Though there are several examples of members of the Ets family acting as transcriptional repressors, there is little evidence that Ets-1 has this capability (Mavrothalassitis & Ghysdael, 2000). Factors which act as repressors typically do so by displacing activating factors, binding to and masking activation domains of activators or directly inhibiting transcription by affecting chromatin structure and DNA accessibility (Mavrothalassitis & Ghysdael, 2000). The Ets proteins TEL, Ets-2 repressor factor (ERF), Elk1, PU.1 and NET have all demonstrated repressor activity. ERF is a unique Ets protein in that, though it contains an ETS DNA binding domain, it has no other homologous regions to other Ets proteins and contains a distinct carboxy terminus domain which recruits a histone deacetylase that then prevents transcription of target promoters (Wright *et al.* 2005). Histone deacetylase enzymes function by deacetylating histones, increasing the net positive charge and resulting DNA binding affinity of these proteins. The increased binding of histones to DNA prevents transcription of underlying genes (Thiel *et al.* 2004). The TEL protein forms oligomers which are capable of recruiting co-repressors, also linked to histone deacetylases in order to repress target gene expression. Net and PU.1 have also been shown to act via interactions with co-repressors with histone deacetylase activity (Mavrothalassitis & Ghysdael, 2000; Hwang *et al.* 2004). Elk-1 is first modified by small ubiquitin- like modifier (SUMO) which allows recruitment of the histone deacetylase HDAC-2 and repression of target gene expression (Yang & Sharrocks, 2004).

Ets-1 has not been described as a repressor and does not appear to have the ability to recruit histone deacetylase enzymes to promoters; however repression of specific promoters has been demonstrated via the interaction of Ets-1 with other factors such as EAPII and MafB (Sieweke *et al.* 1996; Li *et al.* 2000). The observed down-regulation of various genes in this cell model is thus not likely to be via direct repression of promoters by Ets-1, but rather via the ability of the over-expressed protein to bind EBS not typically occupied by Ets-1, thereby displacing Ets factors that would be capable of activating these promoters, causing an overall down-regulation of the genes in question. This is an interesting possibility when it is considered that many of the genes identified by array analysis are in fact targets of the Ets protein GABP/NRF-2 (such as *succinate dehydrogenase subunit B* and *ATP synthase β*). GABP has also been noted to display reduced DNA binding capability under conditions of oxidative stress, thus it can be hypothesized that in conditions where a cells mitochondria are inefficient and producing ROS as a byproduct, Ets-1 levels are up-regulated and GABP displays reduced binding to sites which are then taken up by Ets-1 (Martin *et al.* 1996). Ets-1 may be incapable of activating transcription of these genes, likely due to the inability to bind co-activators at these sites, and as a result, genes encoding mitochondrial proteins involved in oxidative phosphorylation would be down-regulated, and cells would require alternate methods of ATP production. Alternatively, since GABP and Ets-1 share the requirement for the co-activator CBP/p300 for enhanced transcriptional activation, overexpression of Ets-1 may tie up cellular sources of this co-factor and leave GABP lacking co-activator activity

(Bannert *et al.* 1999). These alternative hypotheses for transcriptional repression mediated by Ets-1 are summarized in figure 30.

The limitation of the results observed using this array technology is that they were generated from only one trial. It would have been preferable to test 2-3 different RNA samples to confirm consistent results. It is also advisable to verify any array changes in gene expression observed by Northern blot or real time PCR analysis. In this study, rather than confirming individual changes in gene expression picked up by the array, functional changes that would be predicted from trends in gene changes were instead evaluated. Given that components of every complex of the ETC appeared to be down-regulated, and that enzymes which generate substrates for the citric acid cycle, and ultimately the reducing equivalents needed for electron transport, were similarly down-regulated, it appeared that Ets-1 over-expressing cells had less capacity to generate ATP via oxidative phosphorylation. The capacity for oxidative phosphorylation of all cells in this model was therefore evaluated by various methods.

Cancer cells with reduced oxidative phosphorylation switch to a more glycolytic phenotype (Warburg, 1956). This switch was hypothesized for C13*, 2008-*ets-1*-5B and 2008-*ets-1*-5B+tet, all with increased Ets-1 expression and a resulting down-regulation of genes involved in oxidative phosphorylation as compared to 2008 cells. One method used to examine glycolytic capability of cells, and therefore indirectly evaluate oxidative phosphorylation capacity, is to treat cells with an inhibitor of glycolysis, 2-DG. 2-DG is a glucose analogue, in which a hydrogen ion substitutes for a hydroxyl group on the 2nd

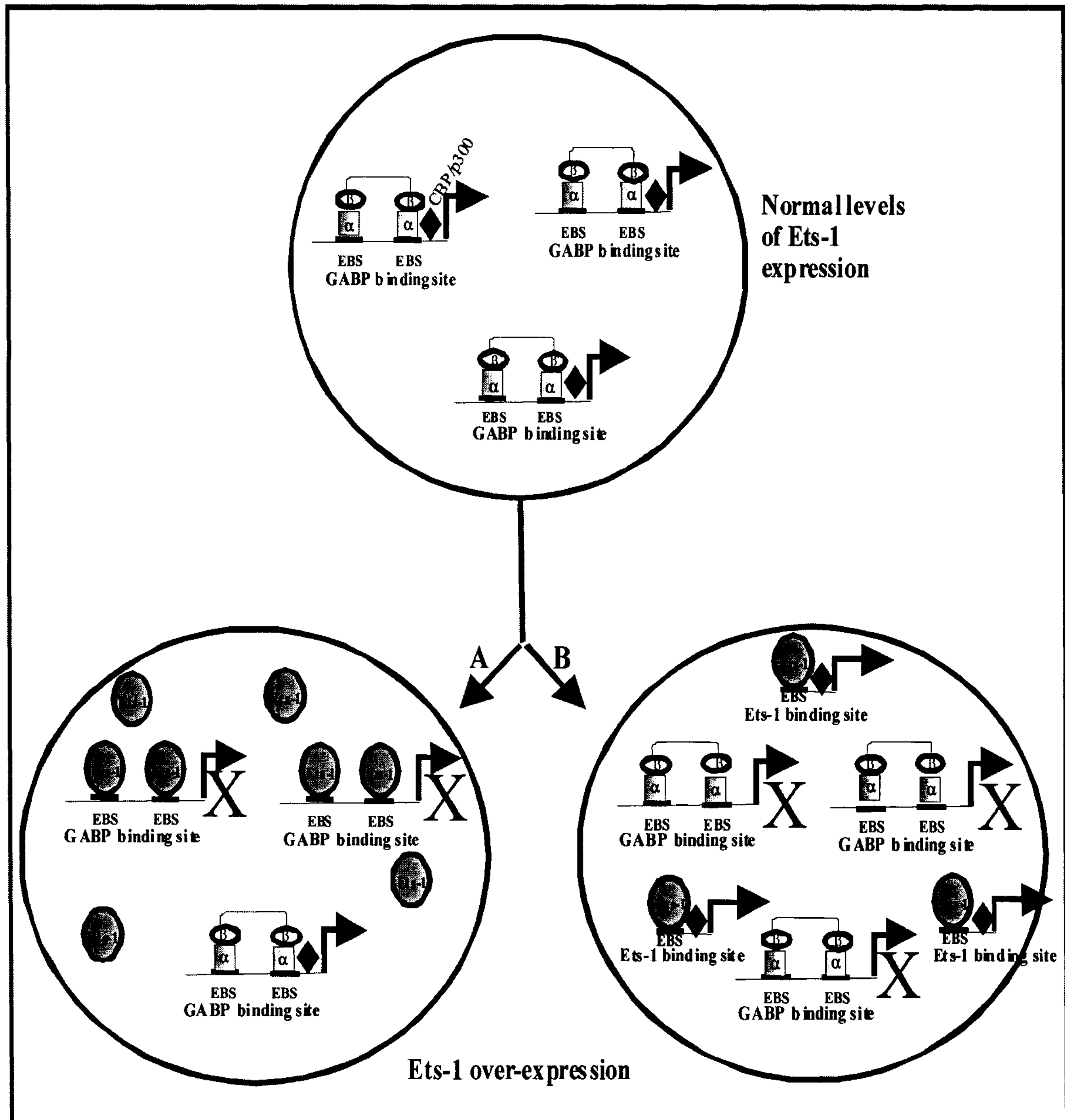


Figure 30: Possible mechanisms for the down-regulation of GABP target genes by Ets-1. Under normal conditions, GABP target genes are appropriately regulated by GABP (subunits α and β shown). However, under conditions of Ets-1 over-expression, two possible scenarios are suggested accounting for an Ets-1 mediated down-regulation of GABP target genes. A) Excess Ets-1 protein binds to GABP target sites, preventing GABP binding and the activation of these promoters. B) Excess Ets-1 binds to its own target sites and in conjunction with CBP/p300 activates transcription. This co-activator is then not available for use by GABP to enhance transcription of its target genes.

carbon (Aft *et al.* 2002). When 2-DG enters cells through glucose transporters, it is phosphorylated to 2-DG-P by hexokinase; however, this substrate cannot be used further by the enzyme glucose-6-P-dehydrogenase, and thus accumulates and inhibits key glycolytic enzymes (Aft *et al.* 2002). Due to the unusual glycolytic phenotype of most cancer cells, 2-DG and other glucose analogs have been tested as cytotoxic agents in different cancer cell models (Zhu *et al.* 2005; Aft *et al.* 2002). 2-DG was shown to reduce the incidence of mammary carcinomas in a Sprague-Dawley rat model as well as cell growth of human breast cancer MCF-7 cells (Zhu *et al.* 2005). Four other breast cancer cell lines similarly displayed sensitivity to 2-DG, with cell growth inhibition observed following administration of 4-8 mM of the drug. Analysis of the possible mechanisms of growth inhibition by 2-DG indicated that the drug actually caused cell death via apoptosis in these particular cell lines (Aft *et al.* 2002). Treatment of cells with 2-DG in the present study also led to growth inhibition, particularly evident in cells which are characterized by a higher expression of Ets-1 (figure 21). IC_{50} values calculated from three independent trials indicate that the cells with highest 2-DG sensitivity are 2008-*ets-1*-5B+tet, with an IC_{50} value of 1.46 mM [significantly lower than the observed IC_{50} for 2008 cells (5.03 mM)], 2-DG IC_{50} values for 2008-*ets-1*-5B and C13* cells were calculated to be 2.68 and 2.55 mM respectively. Therefore, cells with higher Ets-1 expression and reduced expression of genes encoding proteins involved in oxidative phosphorylation are more sensitive to 2-DG treatment indicating that these cells are more reliant on glycolysis for ATP production.

Cell growth of all cell variants was also monitored in glucose free media supplemented with pyruvate. Glucose deprivation caused all cells tested, except 2008 cells, to die following 48 hours of incubation, emphasizing the glucose requirement by the Ets-1 overexpressing cells (figure 22). In both 2-DG treatment and glucose deprivation assays, the mechanisms of inhibited cell growth or cell death were not evaluated, though based on observations made in studies mentioned above, it is likely via apoptosis (Zhu *et al.* 2005; Aft *et al.* 2002).

A more direct method to evaluate the oxidative phosphorylation capacities of the different cell variants is by means the evaluation of O_2 consumption by these cells. O_2 consumption by a population of cells reflects the rate that electrons are passing along the ETC and reducing O_2 to water. The polarographic system used to measure O_2 consumption includes sensors that yield a current proportional to the partial pressure of O_2 in cell containing media by consuming O_2 in a cathode half reaction, this signal responds exponentially to changes in O_2 pressure within the sample (Gnaiger, 2001). In the absence of specific complex substrates and ADP, which stimulates respiration, the basal rate of O_2 consumption was measured. Cells were then permeabilized to allow substrates access to the mitochondria directly. The addition of complex specific substrates and inhibitors, along with ADP, allows the dissection of any specific defects in particular mitochondrial ETC complexes. Responses to the oxidizable substrates glutamate and malate and inhibitor rotenone are indicative of complex I activity, succinate and antimycin A of complex II and III and ascorbate/TMPD as well as KCN of complex IV (Latini *et al.* 2005; Peterside *et al.* 2003). This method was used to evaluate

the O_2 consumption by 2008, 2008+tet, 2008-*ets-1*-5B and 2008-*ets-1*-5B +tet cells on three separate occasions. Typical results, corresponding to the initial rate of O_2 consumption and that following the addition of various substrates and inhibitors, for 2008 and 2008-*ets-1*-5B cells are shown in figure 23A. All complexes of the ETC appeared to respond as expected to their respective substrates and inhibitors, thus no obvious defects in mitochondrial function were picked up through this analysis. It can be seen that prior to permeabilization with digitonin, 2008 cells typically consume more O_2 than the Ets-1 overexpressing 2008-*ets-1*-5B cells. This was a consistent finding, average O_2 consumption values for all cells are tabulated in figure 23C. A significant decrease in O_2 consumption by 2008-*ets-1*-5B cells as compared to 2008 cells was confirmed following three independent experiments. Tet induction however, did not further enhance this drop in O_2 consumption, as would be expected from microarray data- instead the difference between 2008 and tet induced 2008-*ets-1*-5B cells lost significance. This is likely due to effects of tet alone on O_2 consumption, as tet induced 2008 cells exhibited a trend towards increased O_2 consumption. This effect of tet may be due to mitochondrial toxicity induced by the drug; though the doses of tet used for gene induction are much lower than those that should affect the mitochondria (Van Den *et al.* 1989).

The problems with the tet inducible system encouraged the establishment of a second cell model which could be used to evaluate the effects of Ets-1 overexpression on glycolysis and oxidative phosphorylation. O_2 consumption data using the tet-inducible system appeared to indicate that the tet treatment alone introduced extraneous effects. Furthermore, the leakiness of the system meant that in all

experiments, there is no true empty vector control- all cells compared to parental have greatly increased levels of Ets-1 and thus a transfected cell line, lacking increased expression of the gene of interest, is not available. For these reasons, and also to attempt to verify whether observations made using the 2008 cells can be extended to other cells and are therefore not cell specific, ES-2 ovarian clear cell carcinoma cells were stably transfected with either empty pcDNA3 vector or pcDNA3-*ets-1*. Resulting clones were evaluated for Ets-1 expression and two clones (ES-2-*ets-1*-4B and ES-2-*ets-1*-10B) were selected for further study based on the observed increased expression of Ets-1 as compared to the empty vector control (ES-2-pcDNA3-3) (figure 24A). Analysis of the growth of these cell lines following 2-DG treatment indicated that all cells tested, including parental, were highly susceptible to the cytotoxicity of 2-DG. IC_{50} values calculated for these cells ranged from 0.47 to 1.05 mM, approximately 5 to 10 fold lower than the IC_{50} value calculated for 2008 cells (figure 24C). These data indicate that these cells are extremely glycolytic and rely on glucose for survival, and therefore increases in Ets-1 expression have no obvious effect on this characteristic. An apparently glycolytic phenotype was further confirmed following analysis of O_2 consumption by these cells. Again, all displayed low basal rates, approximately 2 fold lower than those observed for 2008 cells, indicative of a minor role for oxidative phosphorylation as a source of ATP for these cells (figure 25). Overexpression of Ets-1 did appear to cause a trend towards decreasing the basal rate of O_2 consumption by ES-2 cells, though it was not significant, and it is probable that these differences are solely due to effects of transfection and selection since the empty vector transfected ES-2 cells displayed a similar trend. Thus,

observations made using the 2008 cell model were not confirmed using an ES-2 cell model. It is probable that the high intrinsic glycolytic rate of these cells prevents any further changes to metabolic properties from being observed following stable transfection of Ets-1. A highly glycolytic cell may not be able to be induced to further enhance their dependence on glycolysis for energy requirements.

The observations made in this section of this study require further verification, possibly using a third Ets-1 overexpression model in cells which display a high O_2 capacity normally, such as human breast carcinoma MCF-7 cells (data not shown). A down-regulation of Ets-1 in C13* cells would also be useful in order to evaluate whether down-regulation of this gene would then lead to a reduced dependence on glycolysis and increased O_2 consumption by cells. This was attempted using *ets-1* specific antisense vectors, as well as dominant negative Ets-1 protein lacking a transactivation domain. Following attempts to stably transfect C13* cells with either antisense or dominant negative Ets-1 vectors, no surviving clones remained (data not shown). This indicates that knocking out this gene is not viable for C13* cells, it is likely that this is due to non-specific effects of these vectors leading to the knockout of several other similar Ets proteins. It is thus necessary to optimize a more specific method to knock out Ets-1 in target cells, possibly by RNAi technology, in order to attempt these experiments.

4.5- Ets-1 as a mediator of cisplatin resistance

The 2008/C13* cell model used in this study is fundamentally one of cisplatin resistance. C13* cell variants were established following 13 consecutive rounds of cisplatin treatment (Andrews *et al.* 1988). One of the many mechanisms suggested to

account for the increased cisplatin resistance of C13* cells were the mitochondrial alterations observed in C13* cells (Andrews *et al.* 1992). The contribution of mitochondrial alterations to drug resistance was concluded following the generation of revertant cells from the C13* variants with restored low mitochondrial membrane potential and a resulting increased sensitivity to cisplatin (Zinkewich-Peotti & Andrews, 1992). The mechanism by which mitochondrial dysfunction would lead to enhanced cisplatin resistance is not clear, though one plausible mechanism is the increased production of ROS which then behave as signaling molecules. In this scenario the up-regulation of Ets-1 may be contributing to the mitochondria-mediated increase in cisplatin resistance. To test this theory, two different Ets-1 overexpression cell models were tested for their response to cisplatin treatment.

Data presented in this thesis indicates that increased expression of Ets-1 in a cisplatin sensitive ovarian carcinoma cell line (2008 cells) induces up to 7 fold cisplatin resistance (figure 28, table 5). This surpasses the fold resistance of C13* cells we have noted in this study (figure 27), but it should be pointed out that expression levels of Ets-1 in the most resistant clone (2008-*ets-1*-5B +tet) were higher than those of C13* cells. Furthermore, HT-29 colon carcinoma cells stably transfected with an Ets-1 expression vector displayed altered cisplatin cytotoxicity profiles and increased IC_{50} values. However, this increase was not as dramatic as observed in the 2008 cells, with a 1.7 fold increase in IC_{50} (figure 29, table 5). This may be due to the fact that expression levels of Ets-1 achieved in stable clones were not as high as those in 2008 transfectants and that the inherent cisplatin sensitivity of these cells is higher (increased expression of Ets-1

may only lead to increased resistance up to a certain threshold). This threshold effect may be due to auto-inhibition by the transcription factor itself or perhaps by the transactivation of genes that counteract the effects of up-regulated gene products with roles in cisplatin resistance. This possibility may explain why in one 2008 *ets-1* overexpressing clone (2008-*ets-1*-4), enhanced expression of Ets-1 in the un-induced cells did increase cisplatin resistance by approximately 4 fold, whereas further increases in protein following tet induction had no significant effect on resistance.

A microarray was performed on RNA extracted from 2008 cells and 2008 clones displaying increased expression of Ets-1. This microarray, previously described, was performed, not only to identify putative targets of Ets-1 which may implicate Ets-1 as a mediator of mitochondria to nucleus crosstalk but also to identify targets that may account for cisplatin resistance. The array identified many transcriptional alterations, most with no obvious connection to cisplatin resistance; however enzymes involved in antioxidant defense and DNA repair were recognized (table 4). Specifically, expression of two metallothionein genes correlated with Ets-1 expression. Although there is no published evidence for the involvement of an Ets protein in the transcriptional control of metallothionein 2A, it has been observed that this gene is a target for c-Fos, and thus may represent an example of a promoter responsive to the synergistic activation by c-Fos and Ets-1 (Scanlon *et al.* 1991). The up-regulation of a DNA repair enzyme, thymidylate synthetase, was also noted in Ets-1 overexpressing cells. Thymidylate synthetase is of importance as the rate limiting step in the *de novo* pyrimidine synthesis pathway. It has been reported that cisplatin inhibits the salvage pyrimidine biosynthesis pathway in lung

cancer cells; thus, an up-regulation in this enzyme may compensate for such an effect (Futami *et al.* 1989). It is also of high interest that promoter studies on thymidylate synthetase regulatory elements have shown that a 20 nucleotide region containing an ETS binding element is sufficient and necessary for transcription and Ets-1 is presumably involved in the regulation of this gene (Dong *et al.* 2000). There is also evidence for the involvement of c-Fos in regulating the thymidylate synthetase gene and thus this provides another possible target for the combined action of c-Fos and Ets-1 (Scanlon *et al.* 1991). To further investigate other possible mechanisms of cisplatin resistance, such as decreased drug accumulation, enhanced NER or base excision repair (BER) as well as decreased induction of apoptosis, the expression of genes whose products are implicated in these processes was examined on the array. Though these genes were not picked up in the initial analysis as being significantly up-or down-regulated in the *ets-1* overexpressing clones, this second analysis did identify a trend for higher expression of other DNA repair enzymes in the *ets-1* overexpressing clones [such as *BRCA1*, *BRCA1 associated ring domain 1(BARD1)*, *ERCC1* and *xeroderma pigmentosum complementation group a* (*XPA*)]. The expression of genes involved in cisplatin accumulation and apoptosis appeared to be unaltered. Expression of dihydrodiol dehydrogenase was also notably higher in the stable cell lines, a gene previously implicated in the C13* resistance model (Deng *et al.* 2000). Finally, expression of SSAT, which has been demonstrated to restore cisplatin sensitivity to C13* cells, was noted to be decreased in 2008, Ets-1 over-expressing cells (Marverti *et al.* 2005) (summarized in table 6). The overall suggestion from array results is that there is an

increase in thiol containing molecules as well as up-regulated DNA repair enzymes in the Ets-1 overexpressing cell lines which may mediate the cisplatin resistance of these cell lines.

It is apparent that the transcription factor Ets-1 is a marker of poor prognosis in a range of tumor types (Span *et al.* 2002; Davidson *et al.* 2001). The finding that expression of this factor increases cisplatin resistance reveals a possible mechanism that may underlie its role as a prognostic marker. Importantly, this finding may be of therapeutic significance as therapy aimed at inhibiting the actions of this transcription factor, such as the use of *ets-1* targeted antisense or RNAi molecules, may be beneficial. This type of approach has been attempted with components of the AP-1 transcription factor as targets. c-*fos* antisense treatment increased cisplatin sensitivity in C13* cells and c-*jun* antisense was used in the A2780 human ovarian carcinoma cell line with derived cisplatin resistance (Moorehead & Singh, 2000; Pan *et al.* 2002). In the A2780 model, investigators noted a 5 fold decrease in the cisplatin IC_{50} of their resistant cells following antisense treatment (Pan *et al.* 2002). This has not, however, been attempted *in vivo* and in the case of Ets-1 there could be several limitations to this type of therapy. These include the likely inhibition of many other Ets-1 target genes as well as potentially inhibiting the action of other Ets proteins that share sequence homology to *ets-1*.

Table 6: Further analysis of *ets-1* overexpression array (genes potentially involved in cisplatin resistance)

Mechanism of increased cisplatin resistance	Gene	(A) 2008	(B) 2008-*ets-1*-5B	(C) 2008-*ets-1*-5B + tet	Expression ratios *B/A*	*C/A*
Reduced cisplatin accumulation	ATP7A	90.7	39	Absent	0.43	-
	ATP7B	91.4	82	77.8	0.90	0.85
	MDR1	Absent	Absent	Absent	-	-
DNA repair	Topoisomerase2	965	662.3	779.9	0.68	0.81
	BRCA I	**299.7**	**413**	**469.7**	**1.38**	**1.57**
	BRCA II	Absent	11.5	45.2	-	-
	BARD I	Absent	**128.5**	**151.5**	-	-
	ERCC I	**157.5**	**286**	**206.3**	**1.82**	**1.31**
	XPA	**76.6**	**131**	**149**	**1.71**	**1.95**
	hMSH2	167.3	193.2	140.7	1.15	0.84
	hMLH1	462.5	398.8	426	0.86	0.92
	DNA-Pol-β	356	233.7	Absent	0.66	-
Apoptosis	Fas	Absent	Absent	Absent	-	-
	Fas Ligand	Absent	Absent	Absent	-	-
	Caspase 3	107.8	119.4	88.4	1.11	0.82
	Caspase 8	101.3	135.3	195.8	1.33	1.93
	Caspase 9	88.2	116	96.9	1.31	1.10
	Bcl-2	Absent	Absent	Absent	-	-
	Bax	Absent	Absent	Absent	-	-
Previously identified in 2008/C13* model	FANCF	Absent	Absent	Absent	-	-
	Tropomyosin-(skeletal)	Absent	Absent	Absent	-	-
	Dihydrodiol-dehydrogenase	**1067.1**	**2301.5**	**1855**	**2.16**	**1.74**
	SSAT	**1202.1**	**260.7**	**295.4**	**0.22**	**0.26**

4.6- Overall conclusions and significance

The transcriptional up-regulation of *ets-1* by H_2O_2 described in this thesis is a novel mechanism by which this transcription factor may be regulated in the tumor environment. Previous studies describing such an up-regulation of *ets-1* involved endothelial cells and failed to address mechanisms of up-regulation (Yasuda *et al.* 1999). In the present work, it has been confirmed that *ets-1* is transcriptionally up-regulated by H_2O_2 in tumor cells. Increased levels of H_2O_2 are frequently observed in cancer cells therefore this may be a critical mechanism leading to the up-regulation of *ets-1* in tumor tissues (Szratowski & Nathan, 1991). The increased expression of Ets-1 in tumors has deleterious effects, such as increased metastatic capability (see table 2). It was thus of importance to elucidate the mechanism of *ets-1* regulation by H_2O_2, as prevention of this up-regulation in cancer cells may provide a therapeutic target.

Analysis of the *ets-1* promoter led to the identification of an ARE which proved to be pivotal in regulating the expression of *ets-1* under both basal and H_2O_2-induced conditions. To our knowledge, there are few studies reporting the regulation of a transcription factor by such a promoter element. The transcription factor c-Jun has been noted to be under such regulation. However, the ARE described in this case did not match the perfect consensus and the element appeared to bind unique factors under induced conditions as compared to a control ARE (Radjendirane & Jaiswal, 1999). A second example is Nrf2, the key factor involved in regulating the ARE which is autoregulated given that an ARE within the Nrf2 gene promoter is critical in regulating gene expression (Kwak *et al.* 2002). This second example is logical; a feedback loop to

enhance the expression of signaling proteins required to maintain a response in the face of oxidative stress may be required. The regulation of *ets-1* via an ARE is, however, curious. Array analysis of *ets-1* overexpressing cells indicates that genes involved in antioxidant defense were up-regulated by Ets-1, which may then provide a protective effect when Ets-1 levels are increased by oxidative stress. However, several genes involved in extracellular matrix degradation, migration and angiogenesis have been well described as Ets-1 target genes and thus in response to ARE inducing stress would likely also be up-regulated (Watabe *et al.* 1998; Nakada *et al.* 1999; Behrens *et al.* 2001). Products of most ARE target genes are considered to be protective against carcinogens and targeting the ARE as a cancer preventive therapy has been proposed. Clearly, an up-regulation of Ets-1 would not be desired in such a scenario, the data provided in this work then must be considered carefully before instigating such therapies (Hayes & McMahon, 2001).

A novel role for Ets-1 was elucidated in this work following array analysis and functional assays performed to confirm an interesting trend observed in cells overexpressing Ets-1. It was unexpected to note a number of down-regulated genes in Ets-1 overexpressing cells, including many that encode mitochondrial proteins involved in oxidative phosphorylation as well as metabolic enzymes, potential generators of ETC required substrates. Functional assays confirmed that Ets-1 overexpressing cells display reduced oxidative phosphorylation capabilities as well as enhanced glycolysis. This type of a response is understandable in light of the fact that H_2O_2, possibly generated by dysfunctional mitochondria, up-regulates Ets-1. An overall scenario can be proposed

where damage to the mitochondria results in increased production of H_2O_2, a resulting up-regulation of Ets-1 then acts to reduce mitochondrial protein expression and reliance on this organelle for ATP requirements. Ets-1 can then be grouped with other transcription factors considered to be mediators of mitochondria-nucleus crosstalk. Typically, such factors have been observed to up-regulate the expression of mitochondrial proteins (NRFs) or genes involved in glycolysis (HIF-1α) in response to specific stresses; thus Ets-1 represents a novel factor with a role in down-regulating genes in response to mitochondrial stress (Scarpulla, 2002; Semenza, 2003).

The final observation regarding the effect of Ets-1 overexpression also represents a novel finding regarding roles for this protein. Ets-1 expression was clearly associated with increased cisplatin resistance in this study. Observations that Ets-1 expression is associated with poor prognosis in cancer patients may then be as a result of therapy resistance as well as the apparent effect on enhancing metastatic characteristics of tumor cells (Span *et al.* 2002; Davidson *et al.* 2001). An Ets-1 expression profile of tumor samples may then provide an idea of the type of therapy which should be attempted as well as a marker of prognosis.

A model of the findings from this work is represented in figure 31, summarizing the mechanism of *ets-1* up-regulation revealed in this thesis as well as novel findings regarding consequences of this up-regulation.

Figure 31: Summary of the role of Ets-1 as a mediator of mitochondria-nucleus crosstalk. The findings of this study and proposed significance are summarized as follows: 1) Damage to the mitochondria, possibly provoking mtDNA mutagenesis, as well as hypoxic conditions promote the formation of ROS. 2) ROS behave as signaling molecules by stabilizing the expression of HIF-1α and thereby up-regulating the expression of HIF-1α target genes such as *ets-1*. 3) *ets-1* is also directly up-regulated by ROS, via the activation of an ARE within the *ets-1* promoter. 4) Up-regulation of Ets-1 leads to downstream effects on several Ets-1 targets such as: A) Up-regulation of *MMP*s, *uPA* and *Integrins* ultimately leading to enhanced ECM degradation, migration and angiogenesis, culminating in increased tumorigenesis. B) Up-regulation of Antioxidant defense genes such as *metallothionein* and *thioredoxin*, resulting in a feedback protective effect countering the increased levels of ROS. These genes, along with those involved in DNA repair, may also mediate the increased cisplatin resistance in Ets-1 over-expressing cells. C) Down-regulation of genes encoding mitochondrial proteins, such as ETC components, as well as metabolic enzymes involved in producing substrates for the ETC, leads to an overall inhibition of oxidative phosphorylation and an increased reliance on glycolysis.

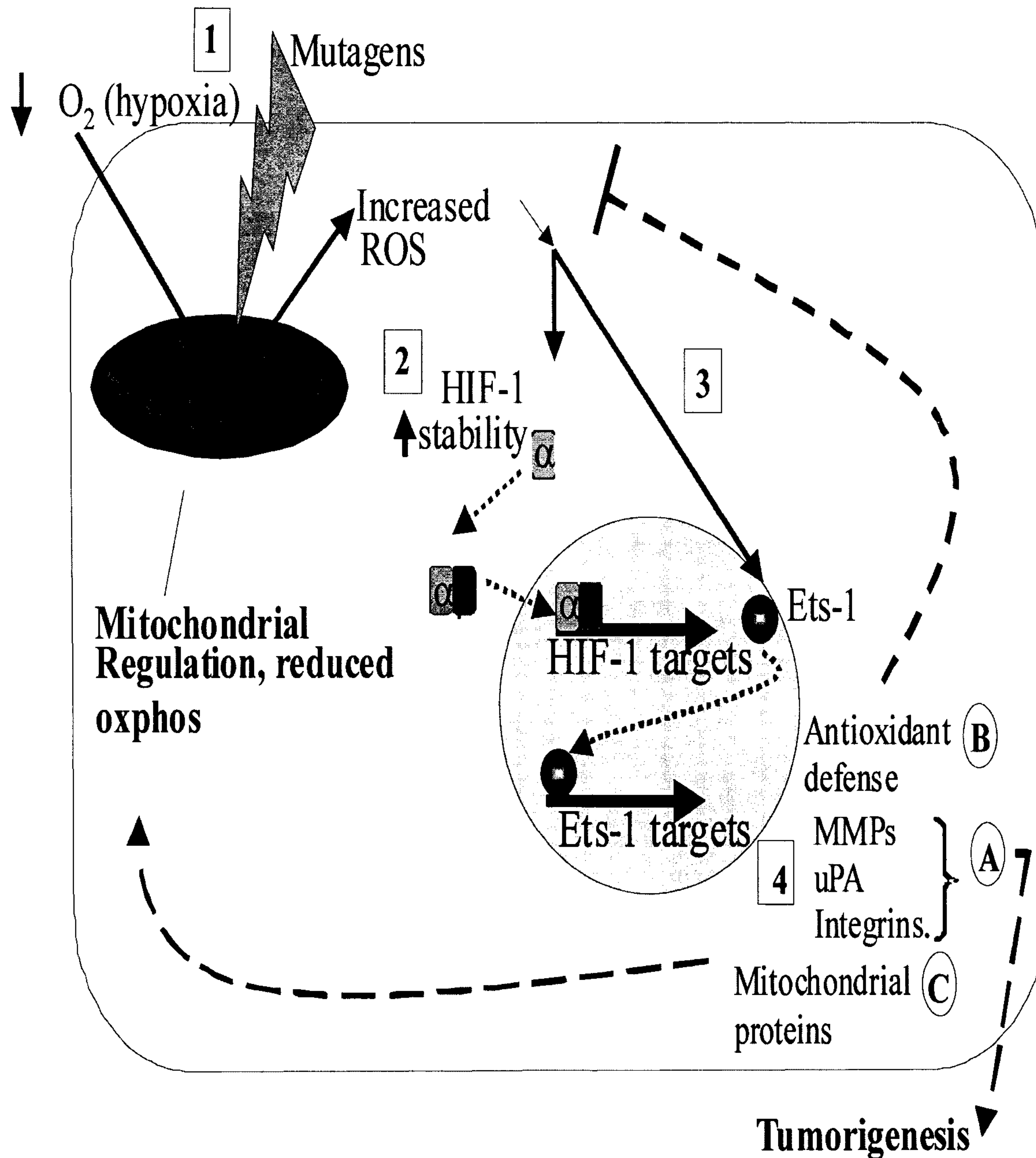


Figure 31: Summary of the role of Ets-1 as a mediator of mitochondria-nucleus crosstalk

4.7- Future directions

One of the key areas of future research stemming from this study involves further elucidation of the signaling pathway involved in regulating the ARE within the *ets-1* promoter. This study identified this element as critical in regulating the *ets-1* gene, and EMSA analysis appeared to indicate that Nrf2, a well known factor involved in ARE regulation is involved in this process. Evidence for Nrf2 involvement was not definitive, however, and further evidence is required to determine whether this factor is in fact required for *ets-1* promoter activation, and also to determine what other binding partners for Nrf2 are involved in the *ets-1* ARE regulation. Proposed experiments to address these issues include chromatin immunoprecipitation (ChIP) analysis, where plausible factors binding to the *ets-1* ARE can be directly evaluated via immunoprecipitation followed by PCR analysis. Reporter assays using the *ets-1* promoter luciferase construct, co-transfected with Nrf2 should also be attempted to evaluate whether overexpression of Nrf2 will inevitably cause an up-regulation in *ets-1* promoter activity. Finally, further supershift assays should be attempted using antibodies directed against suspected proteins that may bind the *ets-1* ARE. The importance of fully describing the regulation of the *ets-1* ARE is two-fold, it may allow the clarification of a signaling pathway that can be targeted therapeutically in order to prevent the inappropriate up-regulation of *ets-1* in cancer cells, and also should be characterized in order to determine whether typical ARE signaling pathways will necessarily cause an up-regulation of *ets-1*, or if in fact the factors involved are unique.

Array data obtained in this study require further verification, and the overall functional changes in 2008 Ets-1 overexpressing cells which corresponded to trends picked up in the array need to be evaluated in other cell models. This was attempted in ES-2 ovarian carcinoma cells, however, these cells are naturally glycolytic and it is proposed that increasing the expression of Ets-1 in these cells was incapable of further affecting cell properties related to metabolism. It would be desirable to overexpress Ets-1 in a cell line which relies on oxidative phosphorylation as an ATP source and is therefore less glycolytic. In this scenario, according to the hypothesis put forward in this study, these cells should display enhanced glycolytic capabilities and less O_2 consumption. It would also be desirable to specifically down-regulate *ets-1* in C13* cells or a similar cell line with high endogenous levels of Ets-1, and observe whether this down-regulation would reverse the effects seen with Ets-1 overexpression. RNAi methods appear to be the most suitable for these types of experiments.

A model where *ets-1* has been down-regulated would also be of use to evaluate whether cisplatin sensitivity can be increased with decreased expression of Ets-1. It would also be interesting to evaluate the cisplatin sensitivity of *ets-1* expressing tumors *in vivo*, thus the creation of an animal model with tumors expressing this factor should be attempted. This type of model could also be used to evaluate the effects of drugs targeted towards the inhibition of glycolysis, such as 2-DG, on Ets-1 expressing tumors. If this type of therapy proved useful against Ets-1 expressing tumors, it would be of importance as several different tumor types are consistently characterized as Ets-1 positive and therapeutic options for these types of tumors could be re-evaluated.

5. References

Abate C, Patel L, Rauscher FJ, III, Curran T (1990), Redox regulation of fos and jun DNA-binding activity in vitro, *Science* 249: 1157-1161

Abu-Amero KK, Alzahrani AS, Zou M, Shi Y (2005), High frequency of somatic mitochondrial DNA mutations in human thyroid carcinomas and complex I respiratory defect in thyroid cancer cell lines, *Oncogene* 24: 1455-1460

Adam M, Schmidt D, Wardelmann E, Wernert N, Albers P (2003), Angiogenetic protooncogene ets-1 induced neovascularization is involved in the metastatic process of testicular germ cell tumors, *Eur.Urol.* 44: 329-336

Adler V, Yin Z, Tew KD, Ronai Z (1999), Role of redox potential and reactive oxygen species in stress signaling, *Oncogene* 18: 6104-6111

Aft RL, Zhang FW, Gius D (2002), Evaluation of 2-deoxy-D-glucose as a chemotherapeutic agent: mechanism of cell death, *Br.J.Cancer* 87: 805-812

Agani FH, Pichiule P, Chavez JC, LaManna JC (2000), The role of mitochondria in the regulation of hypoxia-inducible factor 1 expression during hypoxia, *J.Biol.Chem.* 275: 35863-35867

Akimoto M, Niikura M, Ichikawa M, Yonekawa H, Nakada K, Honma Y, Hayashi J (2005), Nuclear DNA but not mtDNA controls tumor phenotypes in mouse cells, *Biochem.Biophys.Res.Commun.* 327: 1028-1035

Alipov G, Nakayama T, Ito M, Kawai K, Naito S, Nakashima M, Niino D, Sekine I (2005), Overexpression of Ets-1 proto-oncogene in latent and clinical prostatic carcinomas, *Histopathology* 46: 202-208

Amuthan G, Biswas G, Zhang SY, Klein-Szanto A, Vijayasarathy C, Avadhani NG (2001), Mitochondria-to-nucleus stress signaling induces phenotypic changes, tumor progression and cell invasion, *EMBO J.* 20: 1910-1920

Amuthan G, Biswas G, Ananadatheerthavarada HK, Vijayasarathy C, Shephard HM, Avadhani NG (2002), Mitochondrial stress-induced calcium signaling, phenotypic changes and invasive behavior in human lung carcinoma A549 cells, *Oncogene* 21: 7839-7849

Andrews PA, Velury S, Mann SC, Howell SB (1988), cis-Diamminedichloroplatinum(II) accumulation in sensitive and resistant human ovarian carcinoma cells, *Cancer Res.* 48: 68-73

Andrews PA, Albright KD (1992), Mitochondrial defects in cis-diamminedichloroplatinum(II)-resistant human ovarian carcinoma cells, *Cancer Res.* 52: 1895-1901

Andreyev AY, Kushnareva YE, Starkov AA (2005), Mitochondrial metabolism of reactive oxygen species, *Biochemistry (Mosc.)* 70: 200-214

Antunes F, Cadenas E (2001), Cellular titration of apoptosis with steady state concentrations of H(2)O(2): submicromolar levels of H(2)O(2) induce apoptosis through Fenton chemistry independent of the cellular thiol state, *Free Radic.Biol.Med.* 30: 1008-1018

Aringer M, Hofmann SR, Frucht DM, Chen M, Centola M, Morinobu A, Visconti R, Kastner DL, Smolen JS, O'Shea JJ (2003), Characterization and analysis of the proximal Janus kinase 3 promoter, *J.Immunol.* 170: 6057-6064

Augustijn KD, Duval DL, Wechselberger R, Kaptein R, Gutierrez-Hartmann A, van der Vliet PC (2002), Structural characterization of the PIT-1/ETS-1 interaction: PIT-1 phosphorylation regulates PIT-1/ETS-1 binding, *Proc.Natl.Acad.Sci.U.S.A* 99: 12657-12662

Bannert N, Avots A, Baier M, Serfling E, Kurth R (1999), GA-binding protein factors, in concert with the coactivator CREB binding protein/p300, control the induction of the interleukin 16 promoter in T lymphocytes, *Proc.Natl.Acad.Sci.U.S.A* 96: 1541-1546

Barber DA, Harris SR (1994), Oxygen free radicals and antioxidants: a review, *Am.Pharm.* NS34: 26-35

Barton K, Muthusamy N, Fischer C, Ting CN, Walunas TL, Lanier LL, Leiden JM (1998), The Ets-1 transcription factor is required for the development of natural killer cells in mice, *Immunity.* 9: 555-563

Bassuk AG, Leiden JM (1995), A direct physical association between ETS and AP-1 transcription factors in normal human T cells, *Immunity.* 3: 223-237

Bassuk AG, Anandappa RT, Leiden JM (1997), Physical interactions between Ets and NF-kappaB/NFAT proteins play an important role in their cooperative activation of the human immunodeficiency virus enhancer in T cells, *J.Virol.* 71: 3563-3573

Behrens P, Rothe M, Wellmann A, Krischler J, Wernert N (2001), The Ets-1 transcription factor is up-regulated together with MMP 1 and MMP 9 in the stroma of pre-invasive breast cancer, *J.Pathol.* 194: 43-50

Behrens P, Mathiak M, Mangold E, Kirdorf S, Wellmann A, Fogt F, Rothe M, Florin A, Wernert N (2003), Stromal expression of invasion-promoting, matrix-degrading proteases

MMP-1 and -9 and the Ets 1 transcription factor in HNPCC carcinomas and sporadic colorectal cancers, *Int.J.Cancer* 107: 183-188

Bergoglio V, Canitrot Y, Hogarth L, Minto L, Howell SB, Cazaux C, Hoffmann JS (2001), Enhanced expression and activity of DNA polymerase beta in human ovarian tumor cells: impact on sensitivity towards antitumor agents, *Oncogene* 20: 6181-6187

Betteridge DJ (2000), What is oxidative stress?, *Metabolism* 49: 3-8

Biswas G, Adebanjo OA, Freedman BD, Anandatheerthavarada HK, Vijayasarathy C, Zaidi M, Kotlikoff M, Avadhani NG (1999), Retrograde Ca2+ signaling in C2C12 skeletal myocytes in response to mitochondrial genetic and metabolic stress: a novel mode of inter-organelle crosstalk, *EMBO J.* 18: 522-533

Bories JC, Willerford DM, Grevin D, Davidson L, Camus A, Martin P, Stehelin D, Alt FW (1995), Increased T-cell apoptosis and terminal B-cell differentiation induced by inactivation of the Ets-1 proto-oncogene, *Nature* 377: 635-638

Brabec V, Kasparkova J (2002), Molecular aspects of resistance to antitumor platinum drugs, *Drug Resist.Updat.* 5: 147-161

Bradford AP, Wasylyk C, Wasylyk B, Gutierrez-Hartmann A (1997), Interaction of Ets-1 and the POU-homeodomain protein GHF-1/Pit-1 reconstitutes pituitary-specific gene expression, *Mol.Cell Biol.* 17: 1065-1074

Bradford MM (1976), A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal.Biochem.* 72: 248-254

Brambilla L, Cairo G, Sestili P, O'Donnel V, Azzi A, Cantoni O (1997), Mitochondrial respiratory chain deficiency leads to overexpression of antioxidant enzymes, *FEBS Lett.* 418: 247-250

Buggy Y, Maguire TM, McGreal G, McDermott E, Hill AD, O'Higgins N, Duffy MJ (2004), Overexpression of the Ets-1 transcription factor in human breast cancer, *Br.J.Cancer* 91: 1308-1315

Burdon RH (1995), Superoxide and hydrogen peroxide in relation to mammalian cell proliferation, *Free Radic.Biol.Med.* 18: 775-794

Butow RA, Avadhani NG (2004), Mitochondrial signaling: the retrograde response, *Mol.Cell* 14: 1-15

Cadenas E, Boveris A, Ragan CI, Stoppani AO (1977), Production of superoxide radicals and hydrogen peroxide by NADH-ubiquinone reductase and ubiquinol-cytochrome c reductase from beef-heart mitochondria, *Arch.Biochem.Biophys.* 180: 248-257

Carew JS, Huang P (2002), Mitochondrial defects in cancer, *Mol.Cancer* 1: 9

Carriere A, Fernandez Y, Rigoulet M, Penicaud L, Casteilla L (2003), Inhibition of preadipocyte proliferation by mitochondrial reactive oxygen species, *FEBS Lett.* 550: 163-167

Chance B, Sies H, Boveris A (1979), Hydroperoxide metabolism in mammalian organs, *Physiol Rev.* 59: 527-605

Chandel NS, Maltepe E, Goldwasser E, Mathieu CE, Simon MC, Schumacker PT (1998), Mitochondrial reactive oxygen species trigger hypoxia-induced transcription, *Proc.Natl.Acad.Sci.U.S.A* 95: 11715-11720

Chandel NS, McClintock DS, Feliciano CE, Wood TM, Melendez JA, Rodriguez AM, Schumacker PT (2000), Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1alpha during hypoxia: a mechanism of O2 sensing, *J.Biol.Chem.* 275: 25130-25138

Chen F, Castranova V, Shi X (2001), New insights into the role of nuclear factor-kappaB in cell growth regulation, *Am.J.Pathol.* 159: 387-397

Chen JH, Wright CD (1993), PEA3, Oct 1 and Oct 2 positively regulate the human ETS1 promoter, *Oncogene* 8: 3375-3383

Chen K, Albano A, Ho A, Keaney JF, Jr. (2003A), Activation of p53 by oxidative stress involves platelet-derived growth factor-beta receptor-mediated ataxia telangiectasia mutated (ATM) kinase activation, *J.Biol.Chem.* 278: 39527-39533

Chen K, Thomas SR, Albano A, Murphy MP, Keaney JF, Jr. (2004), Mitochondrial function is required for hydrogen peroxide-induced growth factor receptor transactivation and downstream signaling, *J.Biol.Chem.* 279: 35079-35086

Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ (2003B), Production of reactive oxygen species by mitochondria: central role of complex III, *J.Biol.Chem.* 278: 36027-36031

Chinenov Y, Henzl M, Martin ME (2000), The alpha and beta subunits of the GA-binding protein form a stable heterodimer in solution. Revised model of heterotetrameric complex assembly, *J.Biol.Chem.* 275: 7749-7756

Chinnery PF, Turnbull DM (1999), Mitochondrial DNA and disease, *Lancet* 354 Suppl 1: SI17-SI21

Choi MH, Lee IK, Kim GW, Kim BU, Han YH, Yu DY, Park HS, Kim KY, Lee JS, Choi C, Bae YS, Lee BI, Rhee SG, Kang SW (2005), Regulation of PDGF signalling and vascular remodelling by peroxiredoxin II, *Nature* 435: 347-353

Choi YS, Lee KU, Pak YK (2004), Regulation of mitochondrial transcription factor A expression by high glucose, *Ann.N.Y.Acad.Sci.* 1011: 69-77

Chou TC, Talalay P (1984), Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors, *Adv.Enzyme Regul.* 22: 27-55

Chung-man HJ, Zheng S, Comhair SA, Farver C, Erzurum SC (2001), Differential expression of manganese superoxide dismutase and catalase in lung cancer, *Cancer Res.* 61: 8578-8585

Chung YW, Jeong DW, Won JY, Choi EJ, Choi YH, Kim IY (2002), H(2)O(2)-induced AP-1 activation and its effect on p21(WAF1/CIP1)-mediated G2/M arrest in a p53-deficient human lung cancer cell, *Biochem.Biophys.Res.Commun.* 293: 1248-1253

Colman MS, Afshari CA, Barrett JC (2000), Regulation of p53 stability and activity in response to genotoxic stress, *Mutat.Res.* 462: 179-188

Connor KM, Subbaram S, Regan KJ, Nelson KK, Mazurkiewicz JE, Bartholomew PJ, Aplin AE, Tai YT, Aguirre-Ghiso J, Flores SC, Melendez JA (2005), Mitochondrial H2O2 regulates the angiogenic phenotype via PTEN oxidation, *J.Biol.Chem.* 280: 16916-16924

Coulet F, Nadaud S, Agrapart M, Soubrier F (2003), Identification of hypoxia-response element in the human endothelial nitric-oxide synthase gene promoter, *J.Biol.Chem.* 278: 46230-46240

Cowley DO, Graves BJ (2000), Phosphorylation represses Ets-1 DNA binding by reinforcing autoinhibition, *Genes Dev.* 14: 366-376

Cuezva JM, Krajewska M, de Heredia ML, Krajewski S, Santamaria G, Kim H, Zapata JM, Marusawa H, Chamorro M, Reed JC (2002), The bioenergetic signature of cancer: a marker of tumor progression, *Cancer Res.* 62: 6674-6681

Dalton T, Palmiter RD, Andrews GK (1994), Transcriptional induction of the mouse metallothionein-I gene in hydrogen peroxide-treated Hepa cells involves a composite major late transcription factor/antioxidant response element and metal response promoter elements, *Nucleic Acids Res.* 22: 5016-5023

Dang CV, Semenza GL (1999), Oncogenic alterations of metabolism, *Trends Biochem.Sci.* 24: 68-72

Davidson B, Reich R, Goldberg I, Gotlieb WH, Kopolovic J, Berner A, Ben Baruch G, Bryne M, Nesland JM (2001), Ets-1 messenger RNA expression is a novel marker of poor survival in ovarian carcinoma, *Clin.Cancer Res.* 7: 551-557

De Val S, Anderson JP, Heidt AB, Khiem D, Xu SM, Black BL (2004), Mef2c is activated directly by Ets transcription factors through an evolutionarily conserved endothelial cell-specific enhancer, *Dev.Biol.* 275: 424-434

De Vries N, De Flora S (1993), N-acetyl-l-cysteine, *J.Cell Biochem.Suppl* 17F: 270-277

Degli EM (1998), Inhibitors of NADH-ubiquinone reductase: an overview, *Biochim.Biophys.Acta* 1364: 222-235

Delsite R, Kachhap S, Anbazhagan R, Gabrielson E, Singh KK (2002), Nuclear genes involved in mitochondria-to-nucleus communication in breast cancer cells, *Mol.Cancer* 1: 6

Deng HB, Parekh HK, Chow KC, Simpkins H (2002), Increased expression of dihydrodiol dehydrogenase induces resistance to cisplatin in human ovarian carcinoma cells, *J.Biol.Chem.* 277: 15035-15043

Devenish RJ, Prescott M, Boyle GM, Nagley P (2000), The Oligomycin Axis of Mitochondrial ATP Synthase: OSCP and the Proton Channel, *J.Bioenerg.Biomembr.* 32: 507-515

Devling TW, Lindsay CD, McLellan LI, McMahon M, Hayes JD (2005), Utility of siRNA against Keap1 as a strategy to stimulate a cancer chemopreventive phenotype, *Proc.Natl.Acad.Sci.U.S.A* 102: 7280-7285A

Dhakshinamoorthy S, Jaiswal AK (2000), Small maf (MafG and MafK) proteins negatively regulate antioxidant response element-mediated expression and antioxidant induction of the NAD(P)H:Quinone oxidoreductase1 gene, *J.Biol.Chem.* 275: 40134-40141

Dhakshinamoorthy S, Jaiswal AK (2002), c-Maf negatively regulates ARE-mediated detoxifying enzyme genes expression and anti-oxidant induction, *Oncogene* 21: 5301-5312

Dittmer J (2003), The biology of the Ets1 proto-oncogene, *Mol.Cancer* 2: 29

Donaldson LW, Petersen JM, Graves BJ, McIntosh LP (1996), Solution structure of the ETS domain from murine Ets-1: a winged helix-turn-helix DNA binding motif, *EMBO J.* 15: 125-134

Dong S, Lester L, Johnson LF (2000), Transcriptional control elements and complex initiation pattern of the TATA-less bidirectional human thymidylate synthase promoter, *J.Cell Biochem.* 77: 50-64

Duvoix A, Schnekenburger M, Delhalle S, Blasius R, Borde-Chiche P, Morceau F, Dicato M, Diederich M (2004), Expression of glutathione S-transferase P1-1 in leukemic cells is regulated by inducible AP-1 binding, *Cancer Lett.* 216: 207-219

Elvert G, Kappel A, Heidenreich R, Englmeier U, Lanz S, Acker T, Rauter M, Plate K, Sieweke M, Breier G, Flamme I (2003), Cooperative interaction of hypoxia-inducible factor-2alpha (HIF-2alpha) and Ets-1 in the transcriptional activation of vascular endothelial growth factor receptor-2 (Flk-1), *J.Biol.Chem.* 278: 7520-7530

Esposito LA, Melov S, Panov A, Cottrell BA, Wallace DC (1999), Mitochondrial disease in mouse results in increased oxidative stress, *Proc.Natl.Acad.Sci.U.S.A* 96: 4820-4825

Evans MJ, Scarpulla RC (1989), Interaction of nuclear factors with multiple sites in the somatic cytochrome c promoter. Characterization of upstream NRF-1, ATF, and intron Sp1 recognition sequences, *J.Biol.Chem.* 264: 14361-14368

Eyquem S, Chemin K, Fasseu M, Chopin M, Sigaux F, Cumano A, Bories JC (2004), The development of early and mature B cells is impaired in mice deficient for the Ets-1 transcription factor, *Eur.J.Immunol.* 34: 3187-3196

Felty Q, Xiong WC, Sun D, Sarkar S, Singh KP, Parkash J, Roy D (2005), Estrogen-induced mitochondrial reactive oxygen species as signal-transducing messengers, *Biochemistry* 44: 6900-6909

Fisher RJ, Fivash M, Casas-Finet J, Erickson JW, Kondoh A, Bladen SV, Fisher C, Watson DK, Papas T (1994), Real-time DNA binding measurements of the ETS1 recombinant oncoproteins reveal significant kinetic differences between the p42 and p51 isoforms, *Protein Sci.* 3: 257-266

Foley TD, Armstrong JJ, Kupchak BR (2004), Identification and H2O2 sensitivity of the major constitutive MAPK phosphatase from rat brain, *Biochem.Biophys.Res.Commun.* 315: 568-574

Folkman J, Shing Y (1992), Angiogenesis, *J.Biol.Chem.* 267: 10931-10934

Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL (1996), Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1, *Mol.Cell Biol.* 16: 4604-4613

Foulds CE, Nelson ML, Blaszczak AG, Graves BJ (2004), Ras/mitogen-activated protein kinase signaling activates Ets-1 and Ets-2 by CBP/p300 recruitment, *Mol.Cell Biol.* 24: 10954-10964

Fuhrer D, Eszlinger M, Karger S, Krause K, Engelhardt C, Hasenclever D, Dralle H, Paschke R (2005), Evaluation of insulin-like growth factor II, cyclooxygenase-2, ets-1 and thyroid-specific thyroglobulin mRNA expression in benign and malignant thyroid tumours, *Eur.J.Endocrinol.* 152: 785-790

Fujimoto J, Aoki I, Toyoki H, Khatun S, Sato E, Sakaguchi H, Tamaya T (2004), Clinical implications of expression of ETS-1 related to angiogenesis in metastatic lesions of ovarian cancers, *Oncology* 66: 420-428

Futami H, Shiotani T, Yamaji Y, Yamauchi N, Irino S (1989), Effects of the combination of acivicin and cis-diamminedichloroplatinum(II) on thymidylate synthesis of A549 lung cancer cells, *Biochem.Biophys.Res.Commun.* 161: 31-37

Garvie CW, Hagman J, Wolberger C (2001), Structural studies of Ets-1/Pax5 complex formation on DNA, *Mol.Cell* 8: 1267-1276

Gegonne A, Punyammalee B, Rabault B, Bosselut R, Seneca S, Crabeel M, Ghysdael J (1992), Analysis of the DNA binding and transcriptional activation properties of the Ets1 oncoprotein, *New Biol.* 4: 512-519

Ghysdael J, Gegonne A, Pognonec P, Dernis D, Leprince D, Stehelin D (1986), Identification and preferential expression in thymic and bursal lymphocytes of a c-ets oncogene-encoded Mr 54,000 cytoplasmic protein, *Proc.Natl.Acad.Sci.U.S.A* 83: 1714-1718

Gilles F, Raes MB, Stehelin D, Vandenbunder B, Fafeur V (1996), The c-ets-1 proto-oncogene is a new early-response gene differentially regulated by cytokines and growth factors in human fibroblasts, *Exp.Cell Res.* 222: 370-378

Gnaiger E (2001), Bioenergetics at low oxygen: dependence of respiration and phosphorylation on oxygen and adenosine diphosphate supply, *Respir.Physiol* 128: 277-297

Go YM, Gipp JJ, Mulcahy RT, Jones DP (2004), H2O2-dependent activation of GCLC-ARE4 reporter occurs by mitogen-activated protein kinase pathways without oxidation of cellular glutathione or thioredoxin-1, *J.Biol.Chem.* 279: 5837-5845

Goetz TL, Gu TL, Speck NA, Graves BJ (2000), Auto-inhibition of Ets-1 is counteracted by DNA binding cooperativity with core-binding factor alpha2, *Mol.Cell Biol.* 20: 81-90

Gong Y, Agani FH (2005), Oligomycin inhibits HIF-1alpha expression in hypoxic tumor cells, *Am.J.Physiol Cell Physiol* 288: C1023-C1029

Graves BJ, Petersen JM (1998), Specificity within the ets family of transcription factors, *Adv.Cancer Res.* 75: 1-55

Grenningloh R, Kang BY, Ho IC (2005), Ets-1, a functional cofactor of T-bet, is essential for Th1 inflammatory responses, *J.Exp.Med.* 201: 615-626

Grinberg L, Fibach E, Amer J, Atlas D (2005), N-acetylcysteine amide, a novel cell-permeating thiol, restores cellular glutathione and protects human red blood cells from oxidative stress, *Free Radic.Biol.Med.* 38: 136-145

Grist SA, Lu XJ, Morley AA (2004), Mitochondrial mutations in acute leukaemia, *Leukemia* 18: 1313-1316

Gu L, Zhu N, Findley HW, Woods WG, Zhou M (2004), Identification and characterization of the IKKalpha promoter: positive and negative regulation by ETS-1 and p53, respectively, *J.Biol.Chem.* 279: 52141-52149

Gugneja S, Virbasius JV, Scarpulla RC (1995), Four structurally distinct, non-DNA-binding subunits of human nuclear respiratory factor 2 share a conserved transcriptional activation domain, *Mol.Cell Biol.* 15: 102-111

Gugneja S, Scarpulla RC (1997), Serine phosphorylation within a concise amino-terminal domain in nuclear respiratory factor 1 enhances DNA binding, *J.Biol.Chem.* 272: 18732-18739

Guleng G, Lovig T, Meling GI, Andersen SN, Rognum TO (2005), Mitochondrial microsatellite instability in colorectal carcinomas--frequency and association with nuclear microsatellite instability, *Cancer Lett.* 219: 97-103

Guyton KZ, Liu Y, Gorospe M, Xu Q, Holbrook NJ (1996A), Activation of mitogen-activated protein kinase by H2O2. Role in cell survival following oxidant injury, *J.Biol.Chem.* 271: 4138-4142

Guyton KZ, Xu Q, Holbrook NJ (1996B), Induction of the mammalian stress response gene GADD153 by oxidative stress: role of AP-1 element, *Biochem.J.* 314 (Pt 2): 547-554

Ha PK, Tong BC, Westra WH, Sanchez-Cespedes M, Parrella P, Zahurak M, Sidransky D, Califano JA (2002), Mitochondrial C-tract alteration in premalignant lesions of the

head and neck: a marker for progression and clonal proliferation, *Clin.Cancer Res.* 8: 2260-2265

Habano W, Sugai T, Yoshida T, Nakamura S (1999), Mitochondrial gene mutation, but not large-scale deletion, is a feature of colorectal carcinomas with mitochondrial microsatellite instability, *Int.J.Cancer* 83: 625-629

Hagrman D, Goodisman J, Dabrowiak JC, Souid AK (2003), Kinetic study on the reaction of cisplatin with metallothionein, *Drug Metab Dispos.* 31: 916-923

Hahne JC, Okuducu AF, Kaminski A, Florin A, Soncin F, Wernert N (2005), Ets-1 expression promotes epithelial cell transformation by inducing migration, invasion and anchorage-independent growth, *Oncogene* 24: 5384-5388

Hara H, Ohta M, Ohta K, Kuno S, Adachi T (2003), Increase of antioxidative potential by tert-butylhydroquinone protects against cell death associated with 6-hydroxydopamine-induced oxidative stress in neuroblastoma SH-SY5Y cells, *Brain Res.Mol.Brain Res.* 119: 125-131

Hashiya N, Jo N, Aoki M, Matsumoto K, Nakamura T, Sato Y, Ogata N, Ogihara T, Kaneda Y, Morishita R (2004), In vivo evidence of angiogenesis induced by transcription factor Ets-1: Ets-1 is located upstream of angiogenesis cascade, *Circulation* 109: 3035-3041

Hayes JD, McMahon M (2001), Molecular basis for the contribution of the antioxidant responsive element to cancer chemoprevention, *Cancer Lett.* 174: 103-113

Hegen A, Koidl S, Weindel K, Marme D, Augustin HG, Fiedler U (2004), Expression of angiopoietin-2 in endothelial cells is controlled by positive and negative regulatory promoter elements, *Arterioscler.Thromb.Vasc.Biol.* 24: 1803-1809

Hibi K, Nakayama H, Yamazaki T, Takase T, Taguchi M, Kasai Y, Ito K, Akiyama S, Nakao A (2001), Mitochondrial DNA alteration in esophageal cancer, *Int.J.Cancer* 92: 319-321

Huang CC, Papas TS, Bhat NK (1997), A variant form of ETS1 induces apoptosis in human colon cancer cells, *Oncogene* 15: 851-856

Huang HC, Nguyen T, Pickett CB (2000), Regulation of the antioxidant response element by protein kinase C-mediated phosphorylation of NF-E2-related factor 2, *Proc.Natl.Acad.Sci.U.S.A* 97: 12475-12480

Huang LE, Gu J, Schau M, Bunn HF (1998), Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway, *Proc.Natl.Acad.Sci.U.S.A* 95: 7987-7992

Huo L, Scarpulla RC (2001), Mitochondrial DNA instability and peri-implantation lethality associated with targeted disruption of nuclear respiratory factor 1 in mice, *Mol.Cell Biol.* 21: 644-654

Husain A, He G, Venkatraman ES, Spriggs DR (1998), BRCA1 up-regulation is associated with repair-mediated resistance to cis-diamminedichloroplatinum(II), *Cancer Res.* 58: 1120-1123

Hwang CK, Kim CS, Choi HS, McKercher SR, Loh HH (2004), Transcriptional regulation of mouse mu opioid receptor gene by PU.1, *J.Biol.Chem.* 279: 19764-19774

Ibrahim NM, Marinovic AC, Price SR, Young LG, Frohlich O (2000), Pitfall of an internal control plasmid: response of Renilla luciferase (pRL-TK) plasmid to dihydrotestosterone and dexamethasone, *Biotechniques* 29: 782-784

Isidoro A, Martinez M, Fernandez PL, Ortega AD, Santamaria G, Chamorro M, Reed JC, Cuezva JM (2004), Alteration of the bioenergetic phenotype of mitochondria is a hallmark of breast, gastric, lung and oesophageal cancer, *Biochem.J.* 378: 17-20

Itoh K, Igarashi K, Hayashi N, Nishizawa M, Yamamoto M (1995), Cloning and characterization of a novel erythroid cell-derived CNC family transcription factor heterodimerizing with the small Maf family proteins, *Mol.Cell Biol.* 15: 4184-4193

Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yamamoto M, Nabeshima Y (1997), An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements, *Biochem.Biophys.Res.Commun.* 236: 313-322

Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, Yamamoto M (1999), Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain, *Genes Dev.* 13: 76-86

Itoh K, Tong KI, Yamamoto M (2004), Molecular mechanism activating Nrf2-Keap1 pathway in regulation of adaptive response to electrophiles, *Free Radic.Biol.Med.* 36: 1208-1213

Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin WG, Jr. (2001), HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing, *Science* 292: 464-468

Ivanova R, Lepage V, Loste MN, Schachter F, Wijnen E, Busson M, Cayuela JM, Sigaux F, Charron D (1998), Mitochondrial DNA sequence variation in human leukemic cells, *Int.J.Cancer* 76: 495-498

Iwasaka C, Tanaka K, Abe M, Sato Y (1996), Ets-1 regulates angiogenesis by inducing the expression of urokinase-type plasminogen activator and matrix metalloproteinase-1 and the migration of vascular endothelial cells, *J.Cell Physiol* 169: 522-531

Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ (2001), Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation, *Science* 292: 468-472

Jaiswal AK (2004), Nrf2 signaling in coordinated activation of antioxidant gene expression, *Free Radic.Biol.Med.* 36: 1199-1207

Jeronimo C, Nomoto S, Caballero OL, Usadel H, Henrique R, Varzim G, Oliveira J, Lopes C, Fliss MS, Sidransky D (2001), Mitochondrial mutations in early stage prostate cancer and bodily fluids, *Oncogene* 20: 5195-5198

Jiang Y, Xu W, Lu J, He F, Yang X (2001), Invasiveness of hepatocellular carcinoma cell lines: contribution of hepatocyte growth factor, c-met, and transcription factor Ets-1, *Biochem.Biophys.Res.Commun.* 286: 1123-1130

Jones JB, Song JJ, Hempen PM, Parmigiani G, Hruban RH, Kern SE (2001), Detection of mitochondrial DNA mutations in pancreatic cancer offers a "mass"-ive advantage over detection of nuclear DNA mutations, *Cancer Res.* 61: 1299-1304

Jorcyk CL, Garrett LJ, Maroulakou IG, Watson DK, Green JE (1997), Multiple regulatory regions control the expression of Ets-1 in the developing mouse: vascular expression conferred by intron I, *Cell Mol.Biol.(Noisy.-le-grand)* 43: 211-225

Kamata H, Hirata H (1999), Redox regulation of cellular signalling, *Cell Signal.* 11: 1-14

Katayama S, Nakayama T, Ito M, Naito S, Sekine I (2005), Expression of the ets-1 proto-oncogene in human breast carcinoma: differential expression with histological grading and growth pattern, *Histol.Histopathol.* 20: 119-126

Keehn CA, Smoller BR, Morgan MB (2003), Expression of the ets-1 proto-oncogene in melanocytic lesions, *Mod.Pathol.* 16: 772-777

KESTON AS, BRANDT R (1965), THE FLUOROMETRIC ANALYSIS OF ULTRAMICRO QUANTITIES OF HYDROGEN PEROXIDE, *Anal.Biochem.* 11: 1-5

Kim H, Kim YN, Kim H, Kim CW (2005), Oxidative stress attenuates Fas-mediated apoptosis in Jurkat T cell line through Bfl-1 induction, *Oncogene* 24: 1252-1261

Kim WY, Sieweke M, Ogawa E, Wee HJ, Englmeier U, Graf T, Ito Y (1999), Mutual activation of Ets-1 and AML1 DNA binding by direct interaction of their autoinhibitory domains, *EMBO J.* 18: 1609-1620

Kimura H, Sugaya K, Cook PR (2002), The transcription cycle of RNA polymerase II in living cells, *J.Cell Biol.* 159: 777-782

Kita D, Takino T, Nakada M, Takahashi T, Yamashita J, Sato H (2001), Expression of dominant-negative form of Ets-1 suppresses fibronectin-stimulated cell adhesion and migration through down-regulation of integrin alpha5 expression in U251 glioma cell line, *Cancer Res.* 61: 7985-7991

Kitange G, Shibata S, Tokunaga Y, Yagi N, Yasunaga A, Kishikawa M, Naito S (1999), Ets-1 transcription factor-mediated urokinase-type plasminogen activator expression and invasion in glioma cells stimulated by serum and basic fibroblast growth factors, *Lab Invest* 79: 407-416

Kitange G, Tsunoda K, Anda T, Nakamura S, Yasunaga A, Naito S, Shibata S (2000), Immunohistochemical expression of Ets-1 transcription factor and the urokinase-type plasminogen activator is correlated with the malignant and invasive potential in meningiomas, *Cancer* 89: 2292-2300

Klaunig JE, Kamendulis LM (2004), The role of oxidative stress in carcinogenesis, *Annu.Rev.Pharmacol.Toxicol.* 44: 239-267

Konstantinov AA, Peskin AV, Popova EY, Khomutov GB, Ruuge EK (1987), Superoxide generation by the respiratory chain of tumor mitochondria, *Biochim.Biophys.Acta* 894: 1-10

Kumimoto H, Yamane Y, Nishimoto Y, Fukami H, Shinoda M, Hatooka S, Ishizaki K (2004), Frequent somatic mutations of mitochondrial DNA in esophageal squamous cell carcinoma, *Int.J.Cancer* 108: 228-231

Kwak MK, Itoh K, Yamamoto M, Kensler TW (2002), Enhanced expression of the transcription factor Nrf2 by cancer chemopreventive agents: role of antioxidant response element-like sequences in the nrf2 promoter, *Mol.Cell Biol.* 22: 2883-2892

Kwak MK, Wakabayashi N, Itoh K, Motohashi H, Yamamoto M, Kensler TW (2003), Modulation of gene expression by cancer chemopreventive dithiolethiones through the Keap1-Nrf2 pathway. Identification of novel gene clusters for cell survival, *J.Biol.Chem.* 278: 8135-8145

Kwon J, Lee SR, Yang KS, Ahn Y, Kim YJ, Stadtman ER, Rhee SG (2004), Reversible oxidation and inactivation of the tumor suppressor PTEN in cells stimulated with peptide growth factors, *Proc.Natl.Acad.Sci.U.S.A* 101: 16419-16424

Lander HM (1997), An essential role for free radicals and derived species in signal transduction, *FASEB J.* 11: 118-124

Latini A, da Silva CG, Ferreira GC, Schuck PF, Scussiato K, Sarkis JJ, Dutra Filho CS, Wyse AT, Wannmacher CM, Wajner M (2005), Mitochondrial energy metabolism is markedly impaired by d-2-hydroxyglutaric acid in rat tissues, *Mol.Genet.Metab*

Laudet V, Hanni C, Stehelin D, Duterque-Coquillaud M (1999), Molecular phylogeny of the ETS gene family, *Oncogene* 18: 1351-1359

LeBel CP, Ischiropoulos H, Bondy SC (1992), Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress, *Chem.Res.Toxicol.* 5: 227-231

Lee GM, Donaldson LW, Pufall MA, Kang HS, Pot I, Graves BJ, McIntosh LP (2005), The structural and dynamic basis of Ets-1 DNA binding autoinhibition, *J.Biol.Chem.* 280: 7088-7099

Lee HC, Li SH, Lin JC, Wu CC, Yeh DC, Wei YH (2004), Somatic mutations in the D-loop and decrease in the copy number of mitochondrial DNA in human hepatocellular carcinoma, *Mutat.Res.* 547: 71-78

Lee K, Esselman WJ (2002), Inhibition of PTPs by H(2)O(2) regulates the activation of distinct MAPK pathways, *Free Radic.Biol.Med.* 33: 1121-1132

Lee MG, Pedersen PL (2003), Glucose metabolism in cancer: importance of transcription factor-DNA interactions within a short segment of the proximal region og the type II hexokinase promoter, *J.Biol.Chem.* 278: 41047-41058

Lenz HJ, Leichman CG, Danenberg KD, Danenberg PV, Groshen S, Cohen H, Laine L, Crookes P, Silberman H, Baranda J, Garcia Y, Li J, Leichman L (1996), Thymidylate synthase mRNA level in adenocarcinoma of the stomach: a predictor for primary tumor response and overall survival, *J.Clin.Oncol.* 14: 176-182

Li R, Pei H, Papas T (1999), The p42 variant of ETS1 protein rescues defective Fas-induced apoptosis in colon carcinoma cells, *Proc.Natl.Acad.Sci.U.S.A* 96: 3876-3881

Li R, Pei H, Watson DK (2000), Regulation of Ets function by protein - protein interactions, *Oncogene* 19: 6514-6523

Lievre A, Chapusot C, Bouvier AM, Zinzindohoue F, Piard F, Roignot P, Arnould L, Beaune P, Faivre J, Laurent-Puig P (2005), Clinical value of mitochondrial mutations in colorectal cancer, *J.Clin.Oncol.* 23: 3517-3525

Lindemann RK, Ballschmieter P, Nordheim A, Dittmer J (2001), Transforming growth factor beta regulates parathyroid hormone-related protein expression in MDA-MB-231 breast cancer cells through a novel Smad/Ets synergism, *J.Biol.Chem.* 276: 46661-46670

Lindemann RK, Braig M, Ballschmieter P, Guise TA, Nordheim A, Dittmer J (2003), Protein kinase Calpha regulates Ets1 transcriptional activity in invasive breast cancer cells, *Int.J.Oncol.* 22: 799-805

Lionneton F, Lelievre E, Baillat D, Stehelin D, Soncin F (2003), Characterization and functional analysis of the p42Ets-1 variant of the mouse Ets-1 transcription factor, *Oncogene* 22: 9156-9164

Liu VW, Shi HH, Cheung AN, Chiu PM, Leung TW, Nagley P, Wong LC, Ngan HY (2001), High incidence of somatic mitochondrial DNA mutations in human ovarian carcinomas, *Cancer Res.* 61: 5998-6001

Liu VW, Yang HJ, Wang Y, Tsang PC, Cheung AN, Chiu PM, Ng TY, Wong LC, Nagley P, Ngan HY (2003), High frequency of mitochondrial genome instability in human endometrial carcinomas, *Br.J.Cancer* 89: 697-701

Liu Y, Fiskum G, Schubert D (2002), Generation of reactive oxygen species by the mitochondrial electron transport chain, *J.Neurochem.* 80: 780-787

Mackereth CD, Scharpf M, Gentile LN, MacIntosh SE, Slupsky CM, McIntosh LP (2004), Diversity in structure and function of the Ets family PNT domains, *J.Mol.Biol.* 342: 1249-1264

Majerus MA, Bibollet-Ruche F, Telliez JB, Wasylyk B, Bailleul B (1992), Serum, AP-1 and Ets-1 stimulate the human ets-1 promoter, *Nucleic Acids Res.* 20: 2699-2703

Mambo E, Gao X, Cohen Y, Guo Z, Talalay P, Sidransky D (2003), Electrophile and oxidant damage of mitochondrial DNA leading to rapid evolution of homoplasmic mutations, *Proc.Natl.Acad.Sci.U.S.A* 100: 1838-1843

Mann SC, Andrews PA, Howell SB (1990), Short-term cis-diamminedichloroplatinum(II) accumulation in sensitive and resistant human ovarian carcinoma cells, *Cancer Chemother.Pharmacol.* 25: 236-240

Mansouri A, Ridgway LD, Korapati AL, Zhang Q, Tian L, Wang Y, Siddik ZH, Mills GB, Claret FX (2003), Sustained activation of JNK/p38 MAPK pathways in response to cisplatin leads to Fas ligand induction and cell death in ovarian carcinoma cells, *J.Biol.Chem.* 278: 19245-19256

Martin D, Rojo AI, Salinas M, Diaz R, Gallardo G, Alam J, De Galarreta CM, Cuadrado A (2004), Regulation of heme oxygenase-1 expression through the phosphatidylinositol

3-kinase/Akt pathway and the Nrf2 transcription factor in response to the antioxidant phytochemical carnosol, *J.Biol.Chem.* 279: 8919-8929

Martin ME, Chinenov Y, Yu M, Schmidt TK, Yang XY (1996), Redox regulation of GA-binding protein-alpha DNA binding activity, *J.Biol.Chem.* 271: 25617-25623

Martindale JL, Holbrook NJ (2002), Cellular response to oxidative stress: signaling for suicide and survival, *J.Cell Physiol* 192: 1-15

Marverti G, Monti MG, Pegg AE, McCloskey DE, Bettuzzi S, Ligabue A, Caporali A, D'Arca D, Moruzzi MS (2005), Spermidine/spermine N1-acetyltransferase transient over-expression restores sensitivity of resistant human ovarian cancer cells to N1,N12-bis(ethyl)spermine and to cisplatin, *Carcinogenesis*

Matsuyama W, Nakagawa M, Wakimoto J, Hirotsu Y, Kawabata M, Osame M (2003), Mitochondrial DNA mutation correlates with stage progression and prognosis in non-small cell lung cancer, *Hum.Mutat.* 21: 441-443

Matthews JR, Kaszubska W, Turcatti G, Wells TN, Hay RT (1993), Role of cysteine62 in DNA recognition by the P50 subunit of NF-kappa B, *Nucleic Acids Res.* 21: 1727-1734

Mavrothalassitis G, Ghysdael J (2000), Proteins of the ETS family with transcriptional repressor activity, *Oncogene* 19: 6524-6532

Mavrothalassitis GJ, Watson DK, Papas TS (1990), Molecular and functional characterization of the promoter of ETS2, the human c-ets-2 gene, *Proc.Natl.Acad.Sci.U.S.A* 87: 1047-1051

Maximo V, Soares P, Seruca R, Rocha AS, Castro P, Sobrinho-Simoes M (2001), Microsatellite instability, mitochondrial DNA large deletions, and mitochondrial DNA mutations in gastric carcinoma, *Genes Chromosomes.Cancer* 32: 136-143

Meyer-Ficca ML, Meyer RG, Kaiser H, Brack AR, Kandolf R, Kupper JH (2004), Comparative analysis of inducible expression systems in transient transfection studies, *Anal.Biochem.* 334: 9-19

Miceli MV, Jazwinski SM (2005), Common and cell type-specific responses of human cells to mitochondrial dysfunction, *Exp.Cell Res.* 302: 270-280

Michaels GS, Hauswirth WW, Laipis PJ (1982), Mitochondrial DNA copy number in bovine oocytes and somatic cells, *Dev.Biol.* 94: 246-251

Mignatti P, Rifkin DB (1993), Biology and biochemistry of proteinases in tumor invasion, *Physiol Rev.* 73: 161-195

Miyazono F, Schneider PM, Metzger R, Warnecke-Eberz U, Baldus SE, Dienes HP, Aikou T, Hoelscher AH (2002), Mutations in the mitochondrial DNA D-Loop region occur frequently in adenocarcinoma in Barrett's esophagus, *Oncogene* 21: 3780-3783

Moi P, Chan K, Asunis I, Cao A, Kan YW (1994), Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region, *Proc.Natl.Acad.Sci.U.S.A* 91: 9926-9930

Moorehead RA, Armstrong SG, Rainbow AJ, Singh G (1996), Nucleotide excision repair in the human ovarian carcinoma cell line (2008) and its cisplatin-resistant variant (C13*), *Cancer Chemother.Pharmacol.* 38: 245-253

Moorehead RA, Singh G (2000), Influence of the proto-oncogene c-fos on cisplatin sensitivity, *Biochem.Pharmacol.* 59: 337-345

Mukherjee T, Kumar A, Mathur M, Chattopadhyay TK, Ralhan R (2003), Ets-1 and VEGF expression correlates with tumor angiogenesis, lymph node metastasis, and patient survival in esophageal squamous cell carcinoma, *J.Cancer Res.Clin.Oncol.* 129: 430-436

Murphy G, Ward R, Gavrilovic J, Atkinson S (1992), Physiological mechanisms for metalloproteinase activation, *Matrix Suppl* 1: 224-230

Myhre O, Andersen JM, Aarnes H, Fonnum F (2003), Evaluation of the probes 2',7'-dichlorofluorescin diacetate, luminol, and lucigenin as indicators of reactive species formation, *Biochem.Pharmacol.* 65: 1575-1582

Nagy A, Wilhelm M, Kovacs G (2003), Mutations of mtDNA in renal cell tumours arising in end-stage renal disease, *J.Pathol.* 199: 237-242

Naito S, Shimizu S, Maeda S, Wang J, Paul R, Fagin JA (1998), Ets-1 is an early response gene activated by ET-1 and PDGF-BB in vascular smooth muscle cells, *Am.J.Physiol* 274: C472-C480

Nakada M, Yamashita J, Okada Y, Sato H (1999), Ets-1 positively regulates expression of urokinase-type plasminogen activator (uPA) and invasiveness of astrocytic tumors, *J.Neuropathol.Exp.Neurol.* 58: 329-334

Nakamura Y, Esnault S, Maeda T, Kelly EA, Malter JS, Jarjour NN (2004), Ets-1 regulates TNF-alpha-induced matrix metalloproteinase-9 and tenascin expression in primary bronchial fibroblasts, *J.Immunol.* 172: 1945-1952

Nakano T, Abe M, Tanaka K, Shineha R, Satomi S, Sato Y (2000), Angiogenesis inhibition by transdominant mutant Ets-1, *J.Cell Physiol* 184: 255-262

Nguyen T, Rushmore TH, Pickett CB (1994), Transcriptional regulation of a rat liver glutathione S-transferase Ya subunit gene. Analysis of the antioxidant response element and its activation by the phorbol ester 12-O-tetradecanoylphorbol-13-acetate, *J.Biol.Chem.* 269: 13656-13662

Nguyen T, Sherratt PJ, Pickett CB (2003), Regulatory mechanisms controlling gene expression mediated by the antioxidant response element, *Annu.Rev.Pharmacol.Toxicol.* 43: 233-260

Nguyen T, Sherratt PJ, Nioi P, Yang CS, Pickett CB (2005), NRF2 controls constitutive and inducible expression of are-driven genes through a dynamic pathway involving nucleocytoplasmic shuttling by keap1, *J.Biol.Chem.*

Nomura M, Ma W, Chen N, Bode AM, Dong Z (2000), Inhibition of 12-O-tetradecanoylphorbol-13-acetate-induced NF-kappaB activation by tea polyphenols, (-)-epigallocatechin gallate and theaflavins, *Carcinogenesis* 21: 1885-1890

Nunn MF, Seeburg PH, Moscovici C, Duesberg PH (1983), Tripartite structure of the avian erythroblastosis virus E26 transforming gene, *Nature* 306: 391-395

Oberley TD, Oberley LW (1997), Antioxidant enzyme levels in cancer, *Histol.Histopathol.* 12: 525-535

Oda N, Abe M, Sato Y (1999), ETS-1 converts endothelial cells to the angiogenic phenotype by inducing the expression of matrix metalloproteinases and integrin beta3, *J.Cell Physiol* 178: 121-132

Oikawa M, Abe M, Kurosawa H, Hida W, Shirato K, Sato Y (2001), Hypoxia induces transcription factor ETS-1 via the activity of hypoxia-inducible factor-1, *Biochem.Biophys.Res.Commun.* 289: 39-43

Oka T, Rairkar A, Chen JH (1991), Structural and functional analysis of the regulatory sequences of the ets-1 gene, *Oncogene* 6: 2077-2083

Oyama Y, Hayashi A, Ueha T, Maekawa K (1994), Characterization of 2',7'-dichlorofluorescin fluorescence in dissociated mammalian brain neurons: estimation on intracellular content of hydrogen peroxide, *Brain Res.* 635: 113-117

Pahl HL (1999), Activators and target genes of Rel/NF-kappaB transcription factors, *Oncogene* 18: 6853-6866

Pan B, Yao KS, Monia BP, Dean NM, McKay RA, Hamilton TC, O'Dwyer PJ (2002), Reversal of cisplatin resistance in human ovarian cancer cell lines by a c-jun antisense oligodeoxynucleotide (ISIS 10582): evidence for the role of transcription factor overexpression in determining resistant phenotype, *Biochem.Pharmacol.* 63: 1699-1707

Pande P, Mathur M, Shukla NK, Ralhan R (1999), Ets-1: a plausible marker of invasive potential and lymph node metastasis in human oral squamous cell carcinomas, *J.Pathol.* 189: 40-45

Parrella P, Xiao Y, Fliss M, Sanchez-Cespedes M, Mazzarelli P, Rinaldi M, Nicol T, Gabrielson E, Cuomo C, Cohen D, Pandit S, Spencer M, Rabitti C, Fazio VM, Sidransky D (2001), Detection of mitochondrial DNA mutations in primary breast cancer and fine-needle aspirates, *Cancer Res.* 61: 7623-7626

Parrella P, Seripa D, Matera MG, Rabitti C, Rinaldi M, Mazzarelli P, Gravina C, Gallucci M, Altomare V, Flammia G, Casalino B, Benedetti-Panici PL, Fazio VM (2003), Mutations of the D310 mitochondrial mononucleotide repeat in primary tumors and cytological specimens, *Cancer Lett.* 190: 73-77

Pastorino JG, Shulga N, Hoek JB (2002), Mitochondrial binding of hexokinase II inhibits Bax-induced cytochrome c release and apoptosis, *J.Biol.Chem.* 277: 7610-7618

Pedersen PL, Mathupala S, Rempel A, Geschwind JF, Ko YH (2002), Mitochondrial bound type II hexokinase: a key player in the growth and survival of many cancers and an ideal prospect for therapeutic intervention, *Biochim.Biophys.Acta* 1555: 14-20

Pei H, Yordy JS, Leng Q, Zhao Q, Watson DK, Li R (2003), EAPII interacts with ETS1 and modulates its transcriptional function, *Oncogene* 22: 2699-2709

Pelicano H, Carney D, Huang P (2004), ROS stress in cancer cells and therapeutic implications, *Drug Resist.Updat.* 7: 97-110

Penta JS, Johnson FM, Wachsman JT, Copeland WC (2001), Mitochondrial DNA in human malignancy, *Mutat.Res.* 488: 119-133

Peters CL, Morris CJ, Mapp PI, Blake DR, Lewis CE, Winrow VR (2004), The transcription factors hypoxia-inducible factor 1alpha and Ets-1 colocalize in the hypoxic synovium of inflamed joints in adjuvant-induced arthritis, *Arthritis Rheum.* 50: 291-296

Petersen JM, Skalicky JJ, Donaldson LW, McIntosh LP, Alber T, Graves BJ (1995), Modulation of transcription factor Ets-1 DNA binding: DNA-induced unfolding of an alpha helix, *Science* 269: 1866-1869

Peterside IE, Selak MA, Simmons RA (2003), Impaired oxidative phosphorylation in hepatic mitochondria in growth-retarded rats, *Am.J.Physiol Endocrinol.Metab* 285: E1258-E1266

Petros JA, Baumann AK, Ruiz-Pesini E, Amin MB, Sun CQ, Hall J, Lim S, Issa MM, Flanders WD, Hosseini SH, Marshall FF, Wallace DC (2005), mtDNA mutations increase tumorigenicity in prostate cancer, *Proc.Natl.Acad.Sci.U.S.A* 102: 719-724

Picardo M, Grammatico P, Roccella F, Roccella M, Grandinetti M, Del Porto G, Passi S (1996), Imbalance in the antioxidant pool in melanoma cells and normal melanocytes from patients with melanoma, *J.Invest Dermatol.* 107: 322-326

Pillar TM, Seitz HJ (1997), Thyroid hormone and gene expression in the regulation of mitochondrial respiratory function, *Eur.J.Endocrinol.* 136: 231-239

Policastro L, Molinari B, Larcher F, Blanco P, Podhajcer OL, Costa CS, Rojas P, Duran H (2004), Imbalance of antioxidant enzymes in tumor cells and inhibition of proliferation and malignant features by scavenging hydrogen peroxide, *Mol.Carcinog.* 39: 103-113

Polyak K, Li Y, Zhu H, Lengauer C, Willson JK, Markowitz SD, Trush MA, Kinzler KW, Vogelstein B (1998), Somatic mutations of the mitochondrial genome in human colorectal tumours, *Nat.Genet.* 20: 291-293

Pourtier-Manzanedo A, Vercamer C, Van Belle E, Mattot V, Mouquet F, Vandenbunder B (2003), Expression of an Ets-1 dominant-negative mutant perturbs normal and tumor angiogenesis in a mouse ear model, *Oncogene* 22: 1795-1806

Preston TJ, Muller WJ, Singh G (2001), Scavenging of extracellular H2O2 by catalase inhibits the proliferation of HER-2/Neu-transformed rat-1 fibroblasts through the induction of a stress response, *J.Biol.Chem.* 276: 9558-9564

Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM (1998), A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis, *Cell* 92: 829-839

Rabault B, Ghysdael J (1994), Calcium-induced phosphorylation of ETS1 inhibits its specific DNA binding activity, *J.Biol.Chem.* 269: 28143-28151

Radjendirane V, Jaiswal AK (1999), Coordinated induction of the c-jun gene with genes encoding quinone oxidoreductases in response to xenobiotics and antioxidants, *Biochem.Pharmacol.* 58: 597-603

Raouf A, Li V, Kola I, Watson DK, Seth A (2000), The Ets1 proto-oncogene is upregulated by retinoic acid: characterization of a functional retinoic acid response element in the Ets1 promoter, *Oncogene* 19: 1969-1974

Reichard JF, Petersen DR (2004), Hepatic stellate cells lack AP-1 responsiveness to electrophiles and phorbol 12-myristate-13-acetate, *Biochem.Biophys.Res.Commun.* 322: 842-853

Ricquier D, Bouillaud F (2000), Mitochondrial uncoupling proteins: from mitochondria to the regulation of energy balance, *J.Physiol* 529 Pt 1: 3-10

Ristevski S, O'Leary DA, Thornell AP, Owen MJ, Kola I, Hertzog PJ (2004), The ETS transcription factor GABPalpha is essential for early embryogenesis, *Mol.Cell Biol.* 24: 5844-5849

Rodriguez-Pena A, Escriva H, Handler AC, Vallejo CG (2002), Thyroid hormone increases transcription of GA-binding protein/nuclear respiratory factor-2 alpha-subunit in rat liver, *FEBS Lett.* 514: 309-314

Rosell R, Lord RV, Taron M, Reguart N (2002), DNA repair and cisplatin resistance in non-small-cell lung cancer, *Lung Cancer* 38: 217-227

Rosmarin AG, Resendes KK, Yang Z, McMillan JN, Fleming SL (2004), GA-binding protein transcription factor: a review of GABP as an integrator of intracellular signaling and protein-protein interactions, *Blood Cells Mol.Dis.* 32: 143-154

Rosson D, Keshgegian AA (2004), Frequent mutations in the mitochondrial control region DNA in breast tissue, *Cancer Lett.* 215: 89-94

Royall JA, Ischiropoulos H (1993), Evaluation of 2',7'-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular H2O2 in cultured endothelial cells, *Arch.Biochem.Biophys.* 302: 348-355

Ruas JL, Poellinger L, Pereira T (2005), Role of CBP in regulating HIF-1-mediated activation of transcription, *J.Cell Sci.* 118: 301-311

Rushmore TH, Morton MR, Pickett CB (1991), The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity, *J.Biol.Chem.* 266: 11632-11639

Safaei R, Howell SB (2005), Copper transporters regulate the cellular pharmacology and sensitivity to Pt drugs, *Crit Rev.Oncol.Hematol.* 53: 13-23

Sahin A, Velten M, Pietsch T, Knuefermann P, Okuducu AF, Hahne JC, Wernert N (2005), Inactivation of Ets 1 transcription factor by a specific decoy strategy reduces rat C6 glioma cell proliferation and mmp-9 expression, *Int.J.Mol.Med.* 15: 771-776

Samimi G, Safaei R, Katano K, Holzer AK, Rochdi M, Tomioka M, Goodman M, Howell SB (2004), Increased expression of the copper efflux transporter ATP7A mediates resistance to cisplatin, carboplatin, and oxaliplatin in ovarian cancer cells, *Clin.Cancer Res.* 10: 4661-4669

Sanij E, Hatzistavrou T, Hertzog P, Kola I, Wolvetang EJ (2001), Ets-2 is induced by oxidative stress and sensitizes cells to H(2)O(2)-induced apoptosis: implications for Down's syndrome, *Biochem.Biophys.Res.Commun.* 287: 1003-1008

Santiago FS, Khachigian LM (2004), Ets-1 stimulates platelet-derived growth factor A-chain gene transcription and vascular smooth muscle cell growth via cooperative interactions with Sp1, *Circ.Res.* 95: 479-487

Sawyer DE, Van Houten B (1999), Repair of DNA damage in mitochondria, *Mutat.Res.* 434: 161-176

Scanlon KJ, Jiao L, Funato T, Wang W, Tone T, Rossi JJ, Kashani-Sabet M (1991), Ribozyme-mediated cleavage of c-fos mRNA reduces gene expression of DNA synthesis enzymes and metallothionein, *Proc.Natl.Acad.Sci.U.S.A* 88: 10591-10595

Scarpulla RC (2002), Nuclear activators and coactivators in mammalian mitochondrial biogenesis, *Biochim.Biophys.Acta* 1576: 1-14

Schatz G (1995), Mitochondria: beyond oxidative phosphorylation, *Biochim.Biophys.Acta* 1271: 123-126

Schroedl C, McClintock DS, Budinger GR, Chandel NS (2002), Hypoxic but not anoxic stabilization of HIF-1alpha requires mitochondrial reactive oxygen species, *Am.J.Physiol Lung Cell Mol.Physiol* 283: L922-L931

Seidel-Rogol BL, Shadel GS (2002), Modulation of mitochondrial transcription in response to mtDNA depletion and repletion in HeLa cells, *Nucleic Acids Res.* 30: 1929-1934

Seidel JJ, Graves BJ (2002), An ERK2 docking site in the Pointed domain distinguishes a subset of ETS transcription factors, *Genes Dev.* 16: 127-137

Sementchenko VI, Watson DK (2000), Ets target genes: past, present and future, *Oncogene* 19: 6533-6548

Semenza GL, Wang GL (1992), A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation, *Mol.Cell Biol.* 12: 5447-5454

Semenza GL, Jiang BH, Leung SW, Passantino R, Concordet JP, Maire P, Giallongo A (1996), Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1, *J.Biol.Chem.* 271: 32529-32537

Semenza GL (2003), Targeting HIF-1 for cancer therapy, *Nat.Rev.Cancer* 3: 721-732

Sen CK (1998), Redox signaling and the emerging therapeutic potential of thiol antioxidants, *Biochem.Pharmacol.* 55: 1747-1758

Seth A, Papas TS (1990), The c-ets-1 proto-oncogene has oncogenic activity and is positively autoregulated, *Oncogene* 5: 1761-1767

Shannon AM, Bouchier-Hayes DJ, Condron CM, Toomey D (2003), Tumour hypoxia, chemotherapeutic resistance and hypoxia-related therapies, *Cancer Treat.Rev.* 29: 297-307

Sharrocks AD (2001), The ETS-domain transcription factor family, *Nat.Rev.Mol.Cell Biol.* 2: 827-837

Shaulian E, Karin M (2001), AP-1 in cell proliferation and survival, *Oncogene* 20: 2390-2400

Shidara Y, Yamagata K, Kanamori T, Nakano K, Kwong JQ, Manfredi G, Oda H, Ohta S (2005), Positive contribution of pathogenic mutations in the mitochondrial genome to the promotion of cancer by prevention from apoptosis, *Cancer Res.* 65: 1655-1663

Shimizu S, Kageyama M, Yasuda M, Sasaki D, Naito S, Yamamoto T, Kiuchi Y (2004), Stimulation of in vitro angiogenesis by nitric oxide through the induction of transcription factor ETS-1, *Int.J.Biochem.Cell Biol.* 36: 114-122

Siddik ZH (2003), Cisplatin: mode of cytotoxic action and molecular basis of resistance, *Oncogene* 22: 7265-7279

Sieweke MH, Tekotte H, Frampton J, Graf T (1996), MafB is an interaction partner and repressor of Ets-1 that inhibits erythroid differentiation, *Cell* 85: 49-60

Sipos I, Tretter L, Adam-Vizi V (2003), Quantitative relationship between inhibition of respiratory complexes and formation of reactive oxygen species in isolated nerve terminals, *J.Neurochem.* 84: 112-118

So EN, Crowe DL (2000), Characterization of a retinoic acid responsive element in the human ets-1 promoter, *IUBMB.Life* 50: 365-370

Span PN, Manders P, Heuvel JJ, Thomas CM, Bosch RR, Beex LV, Sweep CG (2002), Expression of the transcription factor Ets-1 is an independent prognostic marker for relapse-free survival in breast cancer, *Oncogene* 21: 8506-8509

Srinivas V, Leshchinsky I, Sang N, King MP, Minchenko A, Caro J (2001), Oxygen sensing and HIF-1 activation does not require an active mitochondrial respiratory chain electron-transfer pathway, *J.Biol.Chem.* 276: 21995-21998

Stacey KJ, Fowles LF, Colman MS, Ostrowski MC, Hume DA (1995), Regulation of urokinase-type plasminogen activator gene transcription by macrophage colony-stimulating factor, *Mol.Cell Biol.* 15: 3430-3441

Suh JH, Shenvi SV, Dixon BM, Liu H, Jaiswal AK, Liu RM, Hagen TM (2004), Decline in transcriptional activity of Nrf2 causes age-related loss of glutathione synthesis, which is reversible with lipoic acid, *Proc.Natl.Acad.Sci.U.S.A* 101: 3381-3386

Suliman HB, Carraway MS, Welty-Wolf KE, Whorton AR, Piantadosi CA (2003), Lipopolysaccharide stimulates mitochondrial biogenesis via activation of nuclear respiratory factor-1, *J.Biol.Chem.* 278: 41510-41518

Sun XZ, Vinci C, Makmura L, Han S, Tran D, Nguyen J, Hamann M, Grazziani S, Sheppard S, Gutova M, Zhou F, Thomas J, Momand J (2003), Formation of disulfide bond in p53 correlates with inhibition of DNA binding and tetramerization, *Antioxid.Redox.Signal.* 5: 655-665

Sundaresan M, Yu ZX, Ferrans VJ, Irani K, Finkel T (1995), Requirement for generation of H2O2 for platelet-derived growth factor signal transduction, *Science* 270: 296-299

Suzuki H, Kumagai T, Goto A, Sugiura T (1998), Increase in intracellular hydrogen peroxide and upregulation of a nuclear respiratory gene evoked by impairment of mitochondrial electron transfer in human cells, *Biochem.Biophys.Res.Commun.* 249: 542-545

Szatrowski TP, Nathan CF (1991), Production of large amounts of hydrogen peroxide by human tumor cells, *Cancer Res.* 51: 794-798

Takada Y, Mukhopadhyay A, Kundu GC, Mahabeleshwar GH, Singh S, Aggarwal BB (2003), Hydrogen peroxide activates NF-kappa B through tyrosine phosphorylation of I kappa B alpha and serine phosphorylation of p65: evidence for the involvement of I kappa B alpha kinase and Syk protein-tyrosine kinase, *J.Biol.Chem.* 278: 24233-24241

Takeuchi H, Fujimoto A, Hoon DS (2004), Detection of mitochondrial DNA alterations in plasma of malignant melanoma patients, *Ann.N.Y.Acad.Sci.* 1022: 50-54

Tan DJ, Bai RK, Wong LJ (2002), Comprehensive scanning of somatic mitochondrial DNA mutations in breast cancer, *Cancer Res.* 62: 972-976

Tanaka K, Oda N, Iwasaka C, Abe M, Sato Y (1998), Induction of Ets-1 in endothelial cells during reendothelialization after denuding injury, *J.Cell Physiol* 176: 235-244

Tang M, Baez S, Pruyas M, Diaz A, Calvo A, Riquelme E, Wistuba II (2004), Mitochondrial DNA mutation at the D310 (displacement loop) mononucleotide sequence in the pathogenesis of gallbladder carcinoma, *Clin.Cancer Res.* 10: 1041-1046

Taniguchi T, Tischkowitz M, Ameziane N, Hodgson SV, Mathew CG, Joenje H, Mok SC, D'Andrea AD (2003), Disruption of the Fanconi anemia-BRCA pathway in cisplatin-sensitive ovarian tumors, *Nat.Med.* 9: 568-574

Teruyama K, Abe M, Nakano T, Iwasaka-Yagi C, Takahashi S, Yamada S, Sato Y (2001), Role of transcription factor Ets-1 in the apoptosis of human vascular endothelial cells, *J.Cell Physiol* 188: 243-252

Thiel G, Lietz M, Hohl M (2004), How mammalian transcriptional repressors work, *Eur.J.Biochem.* 271: 2855-2862

Tokuhara K, Ogata Y, Nakagawa M, Shirouzu K (2003), Ets-1 expression in vascular endothelial cells as an angiogenic and prognostic factor in colorectal carcinoma, *Int.Surg.* 88: 25-33

Tong BC, Ha PK, Dhir K, Xing M, Westra WH, Sidransky D, Califano JA (2003), Mitochondrial DNA alterations in thyroid cancer, *J.Surg.Oncol.* 82: 170-173

Tsutsumi S, Kuwano H, Nagashima N, Shimura T, Mochiki E, Asao T (2005), Ets-1 expression in gastric cancer, *Hepatogastroenterology* 52: 654-656

Ubezio P, Civoli F (1994), Flow cytometric detection of hydrogen peroxide production induced by doxorubicin in cancer cells, *Free Radic.Biol.Med.* 16: 509-516

Unwin RD, Craven RA, Harnden P, Hanrahan S, Totty N, Knowles M, Eardley I, Selby PJ, Banks RE (2003), Proteomic changes in renal cancer and co-ordinate demonstration of both the glycolytic and mitochondrial aspects of the Warburg effect, *Proteomics.* 3: 1620-1632

Van den BC, Melis TE, Kroon AM (1989), Mitochondrial biogenesis during the activation of lymphocytes by mitogens: the immunosuppressive action of tetracyclines, *J.Leukoc.Biol.* 46: 128-133

Vanden Hoek TL, Shao Z, Li C, Schumacker PT, Becker LB (1997), Mitochondrial electron transport can become a significant source of oxidative injury in cardiomyocytes, *J.Mol.Cell Cardiol.* 29: 2441-2450

Vasiliou V, Qamar L, Pappa A, Sophos NA, Petersen DR (2003), Involvement of the electrophile responsive element and p53 in the activation of hepatic stellate cells as a response to electrophile menadione, *Arch.Biochem.Biophys.* 413: 164-171

Vaux EC, Metzen E, Yeates KM, Ratcliffe PJ (2001), Regulation of hypoxia-inducible factor is preserved in the absence of a functioning mitochondrial respiratory chain, *Blood* 98: 296-302

Vega A, Salas A, Gamborino E, Sobrido MJ, Macaulay V, Carracedo A (2004), mtDNA mutations in tumors of the central nervous system reflect the neutral evolution of mtDNA in populations, *Oncogene* 23: 1314-1320

Venugopal R, Jaiswal AK (1996), Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene, *Proc.Natl.Acad.Sci.U.S.A* 93: 14960-14965

Vetter M, Blumenthal SG, Lindemann RK, Manns J, Wesselborg S, Thomssen C, Dittmer J (2005), Ets1 is an effector of protein kinase Calpha in cancer cells, *Oncogene* 24: 4319

Virbasius CA, Virbasius JV, Scarpulla RC (1993), NRF-1, an activator involved in nuclear-mitochondrial interactions, utilizes a new DNA-binding domain conserved in a family of developmental regulators, *Genes Dev.* 7: 2431-2445

Virbasius JV, Scarpulla RC (1991), Transcriptional activation through ETS domain binding sites in the cytochrome c oxidase subunit IV gene, *Mol.Cell Biol.* 11: 5631-5638

Wakabayashi N, Itoh K, Wakabayashi J, Motohashi H, Noda S, Takahashi S, Imakado S, Kotsuji T, Otsuka F, Roop DR, Harada T, Engel JD, Yamamoto M (2003), Keap1-null mutation leads to postnatal lethality due to constitutive Nrf2 activation, *Nat.Genet.* 35: 238-245

Wang H, Morais R (1997), Up-regulation of nuclear genes in response to inhibition of mitochondrial DNA expression in chicken cells, *Biochim.Biophys.Acta* 1352: 325-334

Wang H, Meury L, Morais R (1997), Cloning and characterization of cDNAs encoding chicken mitogen-activated protein kinase kinase type 2, MEK2: downregulation of MEK2 in response to inhibition of mitochondrial DNA expression, *Biochemistry* 36: 15371-15380

Wang X (2001), The expanding role of mitochondria in apoptosis, *Genes Dev.* 15: 2922-2933

WARBURG O (1956), On the origin of cancer cells, *Science* 123: 309-314

Watabe T, Yoshida K, Shindoh M, Kaya M, Fujikawa K, Sato H, Seiki M, Ishii S, Fujinaga K (1998), The Ets-1 and Ets-2 transcription factors activate the promoters for invasion-associated urokinase and collagenase genes in response to epidermal growth factor, *Int.J.Cancer* 77: 128-137

Watanabe D, Takagi H, Suzuma K, Suzuma I, Oh H, Ohashi H, Kemmochi S, Uemura A, Ojima T, Suganami E, Miyamoto N, Sato Y, Honda Y (2004), Transcription factor Ets-1 mediates ischemia- and vascular endothelial growth factor-dependent retinal neovascularization, *Am.J.Pathol.* 164: 1827-1835

Watson DK, McWilliams-Smith MJ, Nunn MF, Duesberg PH, O'Brien SJ, Papas TS (1985), The ets sequence from the transforming gene of avian erythroblastosis virus, E26,

has unique domains on human chromosomes 11 and 21: both loci are transcriptionally active, *Proc.Natl.Acad.Sci.U.S.A* 82: 7294-7298

Weiss RB, Christian MC (1993), New cisplatin analogues in development. A review, *Drugs* 46: 360-377

Weitzel JM, Radtke C, Seitz HJ (2001), Two thyroid hormone-mediated gene expression patterns in vivo identified by cDNA expression arrays in rat, *Nucleic Acids Res.* 29: 5148-5155

Werner MH, Clore GM, Fisher CL, Fisher RJ, Trinh L, Shiloach J, Gronenborn AM (1997), Correction of the NMR structure of the ETS1/DNA complex, *J.Biomol.NMR* 10: 317-328

Wernyj RP, Morin PJ (2004), Molecular mechanisms of platinum resistance: still searching for the Achilles' heel, *Drug Resist.Updat.* 7: 227-232

Williams RS, Garcia-Moll M, Mellor J, Salmons S, Harlan W (1987), Adaptation of skeletal muscle to increased contractile activity. Expression nuclear genes encoding mitochondrial proteins, *J.Biol.Chem.* 262: 2764-2767

Wong LJ, Lueth M, Li XN, Lau CC, Vogel H (2003), Detection of mitochondrial DNA mutations in the tumor and cerebrospinal fluid of medulloblastoma patients, *Cancer Res.* 63: 3866-3871

Wong LJ, Tan DJ, Bai RK, Yeh KT, Chang J (2004), Molecular alterations in mitochondrial DNA of hepatocellular carcinomas: is there a correlation with clinicopathological profile?, *J.Med.Genet.* 41: e65

Wright E, Bain M, Teague L, Murphy J, Sinclair J (2005), Ets-2 repressor factor recruits histone deacetylase to silence human cytomegalovirus immediate-early gene expression in non-permissive cells, *J.Gen.Virol.* 86: 535-544

Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC, Spiegelman BM (1999), Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1, *Cell* 98: 115-124

Wung BS, Cheng JJ, Hsieh HJ, Shyy YJ, Wang DL (1997), Cyclic strain-induced monocyte chemotactic protein-1 gene expression in endothelial cells involves reactive oxygen species activation of activator protein 1, *Circ.Res.* 81: 1-7

Yakes FM, Van Houten B (1997), Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress, *Proc.Natl.Acad.Sci.U.S.A* 94: 514-519

Yamamoto H, Swoger J, Greene S, Saito T, Hurh J, Sweeley C, Leestma J, Mkrdichian E, Cerullo L, Nishikawa A, Ihara Y, Taniguchi N, Moskal JR (2000), Beta1,6-N-acetylglucosamine-bearing N-glycans in human gliomas: implications for a role in regulating invasivity, *Cancer Res.* 60: 134-142

Yamamoto K, Okamoto A, Isonishi S, Ochiai K, Ohtake Y (2001), Heat shock protein 27 was up-regulated in cisplatin resistant human ovarian tumor cell line and associated with the cisplatin resistance, *Cancer Lett.* 168: 173-181

Yang BS, Hauser CA, Henkel G, Colman MS, Van Beveren C, Stacey KJ, Hume DA, Maki RA, Ostrowski MC (1996), Ras-mediated phosphorylation of a conserved threonine residue enhances the transactivation activities of c-Ets1 and c-Ets2, *Mol.Cell Biol.* 16: 538-547

Yang C, Shapiro LH, Rivera M, Kumar A, Brindle PK (1998), A role for CREB binding protein and p300 transcriptional coactivators in Ets-1 transactivation functions, *Mol.Cell Biol.* 18: 2218-2229

Yang SH, Sharrocks AD (2004), SUMO promotes HDAC-mediated transcriptional repression, *Mol.Cell* 13: 611-617

Yasuda M, Ohzeki Y, Shimizu S, Naito S, Ohtsuru A, Yamamoto T, Kuroiwa Y (1999), Stimulation of in vitro angiogenesis by hydrogen peroxide and the relation with ETS-1 in endothelial cells, *Life Sci.* 64: 249-258

Yeh JJ, Lunetta KL, van Orsouw NJ, Moore FD, Jr., Mutter GL, Vijg J, Dahia PL, Eng C (2000), Somatic mitochondrial DNA (mtDNA) mutations in papillary thyroid carcinomas and differential mtDNA sequence variants in cases with thyroid tumours, *Oncogene* 19: 2060-2066

Yeh TS, Ho JD, Yang VW, Tzeng CR, Hsieh RH (2005), Calcium Stimulates Mitochondrial Biogenesis in Human Granulosa Cells, *Ann.N.Y.Acad.Sci.* 1042: 157-162

Yoon JC, Puigserver P, Chen G, Donovan J, Wu Z, Rhee J, Adelmant G, Stafford J, Kahn CR, Granner DK, Newgard CB, Spiegelman BM (2001), Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1, *Nature* 413: 131-138

Yordy JS, Li R, Sementchenko VI, Pei H, Muise-Helmericks RC, Watson DK (2004), SP100 expression modulates ETS1 transcriptional activity and inhibits cell invasion, *Oncogene* 23: 6654-6665

Zhang C, Wong-Riley MT (2000), Depolarizing stimulation upregulates GA-binding protein in neurons: a transcription factor involved in the bigenomic expression of cytochrome oxidase subunits, *Eur.J.Neurosci.* 12: 1013-1023

Zhang C, Kavurma MM, Lai A, Khachigian LM (2003), Ets-1 protects vascular smooth muscle cells from undergoing apoptosis by activating p21WAF1/Cip1: ETS-1 regulates basal and and inducible p21WAF1/Cip: ETS-1 regulates basal and inducible p21WAF1/Cip1 transcription via distinct cis-acting elements in the p21WAF/Cip1 promoter, *J.Biol.Chem.* 278: 27903-27909

Zhang DD, Hannink M (2003), Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress, *Mol.Cell Biol.* 23: 8137-8151

Zhang K, Chew M, Yang EB, Wong KP, Mack P (2001), Modulation of cisplatin cytotoxicity and cisplatin-induced DNA cross-links in HepG2 cells by regulation of glutathione-related mechanisms, *Mol.Pharmacol.* 59: 837-843

Zhang Y, Xiong Y (2001), A p53 amino-terminal nuclear export signal inhibited by DNA damage-induced phosphorylation, *Science* 292: 1910-1915

Zhang Y, Gordon GB (2004), A strategy for cancer prevention: stimulation of the Nrf2-ARE signaling pathway, *Mol.Cancer Ther.* 3: 885-893

Zhou LZ, Johnson AP, Rando TA (2001), NF kappa B and AP-1 mediate transcriptional responses to oxidative stress in skeletal muscle cells, *Free Radic.Biol.Med.* 31: 1405-1416

Zhu Z, Jiang W, McGinley JN, Thompson HJ (2005), 2-Deoxyglucose as an energy restriction mimetic agent: effects on mammary carcinogenesis and on mammary tumor cell growth in vitro, *Cancer Res.* 65: 7023-7030

Zinkewich-Peotti K, Andrews PA (1992), Loss of cis-diamminedichloroplatinum(II) resistance in human ovarian carcinoma cells selected for rhodamine 123 resistance, *Cancer Res.* 52: 1902-1906

6. Appendix

Complete table of results from microarray performed.

Genes up-regulated in cells overexpressing Ets-1	Hybridization signal			Expression ratios	
	A: 2008	B: 2-5B	C: 2-5B+tet	B/A	C/A
FOS-like antigen-1	191.4	1853.4	2259.7	9.683386	11.80617
serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2 (SERPINB2)	78.1	547.5	791.3	7.010243	10.13188
diaphorase (NADHNADPH) (cytochrome b-5 reductase) (DIA4)	445.8	2015.8	2660.5	4.521759	5.967923
Tara mRNA	142.5	242.1	698.5	1.698947	4.901754
FXYD domain-containing ion transport regulator 5 (FXYD5)	208.1	830.4	1014.4	3.990389	4.87458
UDP-N-acetylglucosamine-2-epimeraseN-acetylmannosamine kinase (GNE)	37.6	116.6	152.5	3.101064	4.055851
fatty acid desaturase 1	164	562.5	663.4	3.429878	4.045122
metallothionein 2A (MT2A)	1081.8	2982.7	4246.9	2.757164	3.925772
retinal short-chain dehydrogenasereductase retSDR2	54.3	132.7	204.6	2.443831	3.767956
macrophage myristoylated alanine-rich C kinase substrate (MACMARCKS)	112.5	322.1	396.1	2.863111	3.520889
glutathione peroxidase 2 (gastrointestinal) (GPX2)	206.1	677	700.1	3.284813	3.396895
Similar to adaptor-related protein complex 1, sigma 1 subunit	119.8	224.9	406.8	1.877295	3.395659
thymidylate synthetase (TYMS)	1952.8	5507.6	5900.8	2.820361	3.021712
ribosomal protein L31	58.9	170.1	175	2.887946	2.971138
craniofacial development protein 1 (CFDP1)	295.5	789.9	851.5	2.673096	2.881557
cathepsin L (CTSL)	326.2	707.9	931.9	2.170141	2.856836
metallothionein 1X (MT1X)	634.3	1094.1	1767	1.724894	2.785748
katanin p80 (WD40-containing) subunit B 1 (KATNB1)	174	354	480.6	2.034483	2.762069
glutamate-cysteine ligase, modifier subunit (GCLM)	352.6	960.9	968.1	2.725184	2.745604
PTPL1-associated RhoGAP 1 (PARG1)	267.7	680.7	715.6	2.542772	2.673142
1,2-cyclic-inositol-phosphate phosphodiesterase (ANX3)	184.2	471.1	490	2.557546	2.660152
Rho GDP dissociation inhibitor (GDI) beta (ARHGDIB),	467.2	727.8	1229.4	1.557791	2.631421
Similar to transforming growth factor beta 1 induced transcript 1	87.9	215.5	229.1	2.45165	2.606371
Decay accelerating factor for complement (CD55, Cromer blood group system) (DAF)	64.8	115.7	168.5	1.785494	2.600309
vaccinia related kinase 1 (VRK1)	335.6	779.2	863.5	2.321812	2.573004
Leman coiled-coil protein (LCCP	178.7	444.8	458.5	2.489088	2.565753
fasciculation and elongation protein zeta 2 (zygin II)	692.7	1164.8	1767.4	1.681536	2.551465
mutY (E. coli) homolog (MUTYH)	78.9	191.1	198.3	2.422053	2.513308
Fas (TNFRSF6)-associated via death domain (FADD)	109.4	189.5	273.6	1.732176	2.500914
sperm autoantigenic protein 17 (SPA17)	106.4	205.1	262.2	1.927632	2.464286
hxCT mRNA for cystineglutamate exchanger	384	854.1	915.2	2.224219	2.383333
sorting nexin 10 (SNX10)	109	240.1	256.2	2.202752	2.350459
SRY (sex determining region Y)-box 20 (SOX20)	477.4	923.9	1118.1	1.935274	2.342061
Homo sapiens p35srj (MRG1)	57.2	88.7	133.9	1.550699	2.340909
Sm protein F (LSM6),	67.8	130.1	158.3	1.918879	2.334808
CCAATenhancer binding protein (CEBP), delta (CEBPD)	113.5	216	260.7	1.903084	2.296916
polycystic kidney disease 2 (autosomal dominant) (PKD2)	58	130	133.1	2.241379	2.294828
protein phosphatase 1, regulatory subunit 10 (PPP1R10)	96.1	139.7	220.5	1.453694	2.294485
lymphocyte adaptor protein (LNK)	49	100.5	109.5	2.05102	2.234694
breast cancer anti-estrogen resistance 3 (BCAR3)	60.1	85.9	133.7	1.429285	2.224626
target of myb1 (chicken) homolog-like 1 (TOM1L1)	246.2	507.1	542.2	2.059708	2.202275
bifunctional ATP sulfurylaseadenosine 5-phosphosulfate kinase	131.7	226.1	289.3	1.716781	2.196659
crystallin, zeta (quinone reductase) (CRYZ)	278.3	580.8	604.2	2.086957	2.171038
signal transducing adaptor molecule (SH3 domain and ITAM motif) 1 (STAM)	220.7	446.8	460	2.024468	2.084277
photolyase	85.6	159.3	178.2	1.860981	2.081776
thioredoxin	1971.3	3618.8	4094.8	1.835743	2.077208
serinethreonine protein phosphatase catalytic subunit (LOC51723)	1604.5	3276	3295.3	2.041758	2.053786
ring finger protein 5	154	290.3	316	1.885065	2.051948
non-ocogenic Rho GTPase-specific GTP exchange factor (proto-LBC)	177.7	256.9	363.4	1.445695	2.04502
period (Drosophila) homolog 2 (PER2)	79.3	152.4	160.7	1.921816	2.026482

Genes down-regulated in cells overexpressing Ets-1	Hybridization signal			Expression ratios	
	A: 2008	B: 2-5B	C: 2-5B+t	B/A	C/A
Human 56k cytoskeletal type II keratin mRNA.	4288.8	279.2	187.5	0.0651	0.043719
Homo sapiens keratin 13 (KRT13), mRNA	2327.6	285	199.6	0.122444	0.085754
Homo sapiens kallikrein 11 (KLK11), mRNA.	841.9	120.4	93.9	0.14301	0.111533
Homo sapiens EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1) transcript variant 1, mRNA.	316.8	80.6	54	0.254419	0.170455
Homo sapiens glioblastoma amplified sequence (GBAS)	511.4	262	93.2	0.512319	0.182245
Homo sapiens serumglucocorticoid regulated kinase (SGK)	852	344.6	165.6	0.40446	0.194366
nuclear receptor coactivator 3	243.5	72.1	49.7	0.296099	0.204107
Human soluble protein Jagged mRNA	412	146.9	92.7	0.356553	0.225
HOXA1 {alternatively spliced} human	422.4	153.3	98.2	0.362926	0.232481
Homo sapiens cathepsin B (CTSB)	456	170.2	106.4	0.373246	0.233333
Human Tis11d gene	797.7	220.1	184	0.275918	0.230663
Homo sapiens dystrophin	120.7	35.3	30.2	0.292461	0.250207
Homo sapiens reticulocalbin 2, EF-hand calcium binding domain (RCN2)	657.9	289.6	168.2	0.440188	0.255662
Homo sapiens RAB9	549.8	173.9	141.5	0.316297	0.257366
Homo sapiens, LIM domain only 4	251.5	113.6	67.3	0.45169	0.267594
Homo sapiens vascular endothelial growth factor mRNA,	354	169.9	99.2	0.479944	0.280226
Homo sapiens succinate dehydrogenase complex, subunit B, iron sulfur (Ip) (SDHB), nuclear gene encoding mitochondrial protein,	137.5	72.2	38.9	0.525091	0.282909
Homo sapiens asparagine synthetase (ASNS)	258.1	113.3	80.6	0.438977	0.312282
Homo sapiens CASK mRNA	632.4	266.3	199.9	0.421094	0.316097
Homo sapiens threonyl-tRNA synthetase (TARS)	1393.6	642.9	450.2	0.461323	0.323048
Homo sapiens serine carboxypeptidase 1 precursor protein (HSCP1)	312.2	139.5	101.7	0.446829	0.325753
nucleosome assembly protein 1-like 1	292.9	162.8	98.3	0.555821	0.335609
Homo sapiens amino acid transporter 2 (KIAA1382)	1831.8	841	615.4	0.459111	0.335954
Homo sapiens mRNA for L-type amino acid transporter 1	4351.1	1620.5	1483.5	0.372435	0.340948
Homo sapiens platelet derived growth factor C (PDGFC)	602.6	298.4	209.8	0.495188	0.348158
Homo sapiens argininosuccinate synthetase (ASS)	593.4	259.7	210.3	0.437647	0.354398
Homo sapiens DEADH (Asp-Glu-Ala-AspHis) box , polypeptide 1 (DDX1)	513.4	213.6	182.2	0.41605	0.354889
Homo sapiens ankyrin repeat domain 3 (ANKRD3	882.6	407.4	314.3	0.461591	0.356107
Homo sapiens HSC54 mRNA for heat shock cognate protein 54	747.8	556.9	270.6	0.744718	0.361861
Homo sapiens prion protein (p27-30)	647.2	295	235	0.45581	0.363103
Homo sapiens, nuclear cap binding protein subunit 2,	341.7	160.9	125.4	0.470881	0.366989
Homo sapiens ribonuclease H1 (RNASEH1)	208.3	125.7	79.3	0.603457	0.380701
Homo sapiens retinol dehydrogenase homolog (RDHL)	1116.7	592.5	439.3	0.530581	0.393391
Homo sapiens heat shock 40kD protein 1 (HSPF1)	616.7	362.8	243.8	0.588293	0.39533
Homo sapiens ornithine decarboxylase 1 (ODC1)	1194.1	552.9	474.4	0.463027	0.397287
Homo sapiens transcription factor-like 5 (basic helix-loop-helix) (TCFL5)	200.5	99.2	80.1	0.494763	0.399501
activating transcription factor 1	290.9	223.6	116.9	0.768649	0.401856
zinc finger protein 6 (CMPX1)	1408.8	627.1	567.3	0.445131	0.402683
Homo sapiens NADH dehydrogenase (ubiquinone) 1, alphabeta subcomplex,	1372.5	629.1	555.5	0.458361	0.404736
Homo sapiens fatty-acid-Coenzyme A ligase, long-chain 4 (FACL4)	92	50.3	37.3	0.546739	0.405435
mRNA for ABC transporter 7 protein	116.6	51.2	47.4	0.439108	0.406518

Genes down-regulated in cells overexpressing Ets-1(Continued)	Hybridization signal			Expression ratios	
	A: 2008	B: 2-5B	C: 2-5B+t	B/A	C/A
Interferon induced transmembrane protein 1 (9-27)	760.1	375.8	310	0.494409	0.407841
ATPase, H+ transporting, lysosomal (vacuolar proton pump) membrane sector associated protein	887.2	425	365	0.479035	0.411407
FK506 binding protein 12-rapamycin associated protein 1	1600.9	731	668	0.456618	0.417265
HIV TAT specific factor 1 (HTATSF1)	425.3	195.7	177.5	0.460146	0.417352
Similar to KIAA0266 gene product,	152.6	102.5	63.7	0.671691	0.417431
activated RNA polymerase II transcription cofactor 4 (PC4)	896.5	432.4	376.1	0.48232	0.41952
GTPase-activating protein (GAP) mRNA	243.5	171.6	103	0.704723	0.422998
A kinase (PRKA) anchor protein 1 (AKAP1	755	346.2	321.4	0.458543	0.425695
midline 1 (OpitzBBB syndrome) (MID1)	464.1	256.7	197.9	0.553114	0.426417
tyrosine 3-monooxygenasetryptophan 5-monooxygenase activation protein, theta polypeptide	2481.7	1371.9	1061	0.552807	0.42753
protein tyrosine phosphatase, receptor type, K (PTPRK)	1163.8	520	500.5	0.446812	0.430057
glutamate-ammonia ligase (glutamine synthase	1626.5	733.1	700	0.450722	0.430372
splicing factor, arginineserine-rich 2,(SFRS2IP	300.2	199.7	129.2	0.665223	0.43038
protein tyrosine phosphatase, non-receptor type 13 (APO-1CD95 (Fas)-associated phosphatase) (PTPN13)	127.7	85.9	55.7	0.67267	0.436179
Rho guanine nucleotide exchange factor (GEF) 3 (ARHGEF3)	611.8	300.8	267.2	0.491664	0.436744
FXYD domain-containing ion transport regulator 3 (FXYD3)	1366.1	753	597.4	0.551204	0.437303
methionine-tRNA synthetase	394.4	187.8	173.2	0.476166	0.439148
RAN binding protein 7	757.8	383	333.8	0.50541	0.440486
thymine-DNA glycosylase (TDG)	422.8	217.9	188.3	0.515374	0.445364
phosphoserine phosphatase-like (PSPHL)	179.4	95.1	79.9	0.5301	0.445373
ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide (ATP5B), nuclear gene encoding mitochondrial protein	1854.9	979.1	832.2	0.527845	0.44865
voltage-dependent anion channel 1	475.7	320	214.1	0.672693	0.450074
glutamate-cysteine ligase, catalytic subunit	288.6	168.6	130.4	0.5842	0.451836
Ferredoxin 1 (FDX1), nuclear gene encoding mitochondrial protein,	129.8	64.5	59.2	0.496918	0.456086
Golgi protein (GPP34)	1330.8	689.1	610.1	0.517809	0.458446
phosphoinositide-3-kinase, class 3 (PIK3C3	239.4	208.4	109.9	0.87051	0.459064
Homo sapiens succinate-CoA ligase, GDP-forming,) alpha subunit (SUCLG1	1561	781.9	720	0.500897	0.461243
replication protein A1 (70kD)	787.4	402.7	363.7	0.51143	0.4619
Fatty-acid-Coenzyme A ligase, long-chain 3 (FACL3)	258	175.4	119.6	0.679845	0.463566
inner membrane protein, mitochondrial (mitofilin) (IMMT),	757.9	490.4	351.5	0.647051	0.463782
Acid phosphatase 1, soluble (ACP1), transcript variant a	371.5	220.6	172.6	0.593809	0.464603
oxidized low-density lipoprotein receptor mRNA,	237.6	126.6	110.8	0.532828	0.46633
S-phase response (cyclin-related) (SPHAR),	140.5	87.6	65.9	0.623488	0.469039
annexin A4 (ANXA4)	1074.7	549.6	506.8	0.511399	0.471573
ribonucleotide reductase M2 polypeptide,	2727.4	1443.4	1287.2	0.529222	0.471951
NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5	721.3	399.4	341.9	0.553722	0.474005
DEADH (Asp-Glu-Ala-AspHis) box binding protein 1	142.4	83.4	67.7	0.585674	0.475421
similar to mouse neuronal protein 15.6 (FLJ20494)	172.9	96.8	82.4	0.559861	0.476576
ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1, cardiac muscle	2734.4	1800	1304.4	0.65828	0.477033
Contains the 3 part of the gene for Beta-1,4-galactosyltransferase	374.9	231.8	180.1	0.618298	0.480395
Ornithine aminotransferase (gyrate atrophy) (OAT), nuclear gene encoding mitochondrial protein,	2042.2	1268.5	984.5	0.621144	0.482078
cytochrome c-1 (CYC1),	339.5	276.5	164	0.814433	0.483063
tetraspan 3	894.8	573.3	432.8	0.640702	0.483684
CCAATenhancer binding protein (CEBP), beta	1165.1	664.8	567.4	0.570595	0.486997
hepatocyte nuclear factor 3, alpha (HNF3A),	270.7	179.3	132.1	0.662357	0.487994
mitosin mRNA,	93.9	55	46.1	0.585729	0.490948
pyruvate dehydrogenase (lipoamide) alpha 1 (PDHA1	564.1	293.1	278.8	0.519589	0.494239
farnesyltransferase, CAAX box, alpha	1129.3	715.5	558.3	0.633578	0.494377
CD9 antigen (p24) (CD9),	827.8	523.6	409.7	0.63252	0.494926
complement component 1, q subcomponent binding protein	1219.9	797.6	605.8	0.653824	0.496598
CDw44 antigen,	266.5	158.4	132.5	0.594371	0.497186
16.7Kd protein (LOC51142),	2994.7	1987	1494.1	0.663506	0.498915
thioredoxin peroxidase (antioxidant enzyme)	1003.7	614.2	501.2	0.611936	0.499352