A NOVEL PRO-OXIDANT ROLE OF GELSOLIN IN CANCER CELL INVASION

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(*M.Sc., University of Madras*)

A THESIS SUBMITTED

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHYSIOLOGY

YONG LOO LIN SCHOOL OF MEDICINE

NATIONAL UNIVERSITY OF SINGAPORE

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DECLARATION

I hereby declare that the thesis is my original work and it has been written by me in its entirety.

I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

LALCHHANDAMI TOCHHAWNG

10 January 2013

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Table of Contents

DECLARATION ..... **i**
ACKNOWLEDGEMENTS ..... **ii**
SUMMARY ..... **viii**
LIST OF TABLES ..... **x**
LIST OF FIGURES ..... **xi**
ABBREVIATIONS ..... **xiv**
CHAPTER 1 INTRODUCTION **1**
1.1 CANCER CELL INVASION 1
1.1.1 Invasion-metastatic cascade 1
1.1.2 Molecular mechanisms of cancer cell invasion 4
1.1.3 Cancer cell-extracellular matrix (ECM) interaction and ECM degradation 4
1.1.4 Loss of cell-to-cell adhesion 8
1.1.5 Actin cytoskeleton and cancer cell invasion 9
1.2 GELSOLIN 13
1.2.1 Introduction 13
1.2.2 Interaction between gelsolin and actin — structure to function relation 14
1.2.3 The role of gelsolin in cell motility: evidence from *in vivo* studies17
1.2.4 Gelsolin-mitochondrial association 18
1.2.5 Gelsolin in cancer 19
1.2.6 Gelsolin interacts with signalling proteins 24
1.3 REACTIVE OXYGEN SPECIES 26
1.3.1 Introduction 26
1.3.2 Sources of ROS 26
1.3.3 The antioxidant system 27
1.3.4 ROS – antioxidant imbalance and implications in cancer 28
1.3.5 ROS and cancer cell invasion 30
1.3.6 The actin cytoskeleton and ROS 33
1.4 PURPOSE AND OBJECTIVES OF THE STUDY 36
CHAPTER 2 MATERIALS AND METHODS **38**
2.1 MATERIALS 38

2.1.1. Chemicals and Reagents 38
2.2 METHODS 43
2.2.1 Cell culture 43
2.2.2 DNA transfection 43
2.2.3 siRNA Transfection 44
2.2.4 ROS measurements 44
2.2.5 Trypan Blue Exclusion 46
2.2.6 Matrigel Invasion Assay 46
2.2.7 Zymographic Assay 47
2.2.8 Bio-Rad Protein Assay 48
2.2.9 Superoxide dismutase (SOD) activity Assay 48
2.2.10 Western Blotting 50
2.2.11 Immunoprecipitation 52
2.2.12 Isolation of intact mitochondria and cytosolic fraction 53
2.2.13 Sandwiched Enzyme-linked immunosorbent assay (ELISA) 54
2.2.14 Real-time PCR 55
2.2.15 I*n situ* Proximity Ligation Assay 57
2.2.16 Microscopy 58
2.2.17 Statistical analysis 58
CHAPTER 3 GELSOLIN PROMOTES COLON CANCER CELL INVASION VIA MODULATING INTRACELLULAR LEVELS OF SUPEROXIDE (O_2^-) SPECIES 59
3.1 BACKGROUND 59
3.2 OBJECTIVES 61
3.3 RESULTS 62
3.3.1 Gelsolin expression modulates total intracellular superoxide (O_2^-) levels 62
3.3.2 Gelsolin expression influences mitochondrial superoxide (O_2^-) levels 65
3.3.3 Gelsolin expression does not affect the levels of other ROS species (H_2O_2, $^{\cdot}OH$ and HOCl) 68
3.3.4 Gelsolin overexpression promotes invasion of HCT116 cells 71
3.3.5 DPI treatment blocks O_2^- production and attenuates invasion in gelsolin-overexpressing cells 74

3.3.6 Increasing O_2^- levels by knockdown of Cu/Zn SOD rescues invasion in gelsolin-depleted HCT116 cells 78
3.3.7 Gelsolin expression modulates urokinase plasminogen activator (uPA) secretion and activity 81
3.3.8 DPI inhibits gelsolin-induced uPA secretion 85
3.4 DISCUSSION 87
3.4.1 Gelsolin modulates intracellular O_2^- levels 87
3.4.2 O_2^- is required for gelsolin-mediated cancer cell invasion 88
CHAPTER 4 GELSOLIN MODULATES INTRACELLULAR LEVELS OF SUPEROXIDE (O_2^-) SPECIES BY SUPPRESSING THE ACTIVITY OF THE ANTIOXIDANT COPPER/ZINC SUPEROXIDE DISMUTASE (CU/ZN SOD) 91
4.1 BACKGROUND 91
4.2 OBJECTIVES 93
4.3 RESULTS 94
4.3.1 Gelsolin overexpression suppresses the mRNA levels of antioxidant genes 94
4.3.2 Gelsolin does not affect the protein expression of Cu/Zn SOD and Mn SOD 96
4.3.3 Gelsolin suppresses total SOD activity 98
4.3.4 Gelsolin suppresses Cu/Zn SOD activity 100
4.3.5 Gelsolin does not affect protein expression of copper chaperone for Cu/Zn SOD (CCS) 109
4.3.6 Gelsolin co-immunoprecipitates with Cu/Zn SOD 111
4.3.7 Gelsolin and Cu/Zn SOD lie in close proximity to each other . 113
4.3.8 Gelsolin expression modulates the mRNA levels of Nox 115
4.4 DISCUSSION 117
4.4.1 Gelsolin suppresses the activity of Cu/Zn SOD 117
4.4.2 Gelsolin associates with Cu/Zn SOD 119
4.4.3 Gelsolin affects the mRNA levels of Nox isoforms 120
CHAPTER 5 GENERAL DISCUSSION, CONCLUSION AND FUTURE WORK 122
5.1 GENERAL DISCUSSION 122
5.1.1 Gelsolin as a pro-oxidant molecule 123
5.1.2 Gelsolin mediates invasion in an O_2^- dependent manner 123

5.1.3 Gelsolin suppresses Cu/Zn SOD activity possibly by interacting with it 124
5.1.4 Gelsolin increases mRNA levels of Nox 125
5.2 CONCLUSION AND CLINICAL IMPLICATION 127
5.3 FUTURE WORK 128
BIBLIOGRAPHY 130
APPENDIX I (SUPPLEMENTARY DATA) 153
APPENDIX II (PREPARATION OF BUFFERS) 160
APPENDIX III (PUBLICATIONS) 165

SUMMARY

Gelsolin is a multifunctional actin-binding protein that is known to play a crucial role in cancer cell motility and invasion. However, the mechanisms by which gelsolin promotes cancer cell invasion is poorly understood. Independent lines of studies have shown an association between the abnormal rise in Reactive Oxygen Species (ROS) levels with malignant growth and cancer cell invasion. In this study we have identified a new role of gelsolin in modulating the intracellular ROS levels, particularly the superoxide (O_2^-) species, which significantly contributes to gelsolin-mediated invasion. We show that HCT116 colon cancer cells that stably overexpress gelsolin have higher levels of O_2^- as well as enhanced invasion when compared to the empty vector control and wild-type HCT116 cells. Consistently, knockdown of endogenous gelsolin in two colon cancer cell lines HCT116 and RKO reduces the intracellular levels of O_2^- and attenuates invasion. Blockade of O_2^- generation with diphenyleneiodonium (DPI), an inhibitor of the O_2^- producing enzyme-NADPH oxidase (Nox) potently reduces the invasive capacities of gelsolin-overexpressing cells. Moreover, increasing the O_2^- levels in gelsolin-knockdown HCT116 cells by silencing the antioxidant enzyme Cu/Zn superoxide dismutase (Cu/Zn SOD) rescues invasion. These results strongly suggest a reliance on O_2^- for the gelsolin-mediated invasion process. We have previously shown that gelsolin-overexpressing cells secrete higher levels of the matrix degrading enzyme — urokinase plasminogen activator (uPA), thus facilitating invasion. Interestingly, treatment of gelsolin-overexpressing cells with DPI reduces uPA secretion providing evidence that uPA secretion by gelsolin-overexpressing cells is influenced by O_2^-. In addition, we also show

that gelsolin overexpression suppresses the activity of Cu/Zn SOD, an antioxidant enzyme that scavenges O_2^-. Gelsolin was found to co-immunoprecipitate with Cu/Zn SOD, suggesting that gelsolin may form a complex with Cu/Zn SOD and inhibit its O_2^- scavenging functions. The impairment of Cu/Zn SOD activity provides a mechanistic explanation for the elevated amount of intracellular O_2^- in gelsolin-overexpressing cells. In summary, our findings demonstrate a new insight into the role of gelsolin in cancer cell invasion through upregulation of O_2^- levels.

LIST OF TABLES

Table 1.1 Types of reactive oxygen species……………….……..…... 26

Table 1.2 Enzymatic and non-enzymatic antioxidant systems………… 28

Table 2.1 Real-time PCR primers……………….………………........... 38

Table S1. Interacting amino acid residues between Gelsolin and Cu/Zn SOD.. 158

LIST OF FIGURES

Figure 1.1 Schematic illustration of the invasion-metastatic cascade……………………………………………..…... 3

Figure 1.2 Cell-ECM interaction and ECM degradation………… 6

Figure 1.3 Acquisition of EMT-like phenotypes during cancer cell migration and invasion…………......................... 9

Figure 1.4 Actin-rich membrane protrusion-mediated cell migrations in different environment…………………. 12

Figure 1.5 Gelsolin structure to function domains………………. 15

Figure 1.6 Schematic diagram showing gelsolin binding, severing and capping of an actin filament…………… 17

Figure 1.7 Gelsolin interacts with signalling molecules that favour cell migration and invasion…………………… 25

Figure 1.8 Schematic diagram of ROS-triggered signalling pathways leading to cell migration and invasion…….. 32

Figure 3.1A&B Gelsolin overexpression increases total intracellular superoxide (O_2^-) levels……………………………….. 63

Figure 3.1C&D Gelsolin knockdown decreases total intracellular superoxide (O_2^-) levels……………………………….. 64

Figure 3.2A Overexpression of gelsolin expression elevates mitochondrial superoxide (O_2^-) levels……….......... 66

Figure 3.2B-D Knockdown of gelsolin reduces mitochondrial superoxide (O_2^-) levels…………………………......... 67

Figure 3.3A&B Fig 3.3A Gelsolin knockdown does not affect the levels other ROS ……….……………………………. 69

Figure 3.3C Overexpression of gelsolin expression does not alter H_2O_2 levels……………………………….…………... 70

Figure 3.4A	Gelsolin overexpression enhances cell invasion...........	72
Figure 3.4B&C	Gelsolin knockdown reduces invasion of HCT116 cells..	73
Figure 3.5A	DPI treatment reduces O_2^- levels……………………..	75
Figure 3.5B	DPI treatment reduces invasion………………………	76
Figure 3.5C	DPI has minimal effect on HCT116 cell viability……	77
Figure 3.6A&B	Increasing O_2^- levels by knockdown of Cu/Zn SOD rescues invasion in gelsolin depleted HCT116 cells………………………………………………...	79
Figure 3.6C	Increasing O_2^- levels by knockdown of Cu/Zn SOD rescues invasion in gelsolin-depleted HCT116 cells……………………………….………………..	80
Figure 3.7A	Gelsolin overexpression induces uPA secretion………………………………………………	82
Figure 3.7B&C	Gelsolin knockdown decreases uPA secretion……….	83
Figure 3.7D-F	Gelsolin expression modulates uPA activity…………	84
Figure 3.8	Gelsolin-induced uPA secretion is modulated by intracellular O_2^- levels……………………………….	86
Figure 4.1	Gelsolin overexpression downregulates mRNA levels of antioxidant genes…………………………………..	95
Figure 4.2A-D	Gelsolin expression does not affect the protein expression of Cu/Zn SOD and Mn SOD……………...	97
Figure 4.3A&B	Gelsolin expression modulates total SOD activity in HCT116 and RKO cells……………..........................	99
Figure 4.4A	Subcellular fractionation of cytosol and mitochondria in gelsolin-overexpressing cells………………………	102

Figure 4.4B&C	Overexpression of gelsolin suppresses Cu/Zn SOD activity in HCT116 cells………………………………...	103
Figure 4.4D&E	DDC inhibits total SOD activity………………..........	104
Figure 4.4F	DDC inhibits Cu/Zn SOD activity……………..........	105
Figure 4.4G	Subcellular fractionation of cytosol and mitochondria in gelsolin-knockdown cells.......................................	106
Figure 4.4 H&I	Knockdown of gelsolin increases Cu/Zn SOD activity in HCT116 and C1 cells………………………………	107
Figure 4.4 J&K	Knockdown of gelsolin does not affect Mn SOD activity in HCT116 and C1 cells……………………..	108
Figure 4.5A&B	Gelsolin expression does not affect protein expression of CCS………………………………………………..	110
Figure 4.6	Gelsolin co-immunoprecipitates with Cu/Zn SOD……………………………………….……….	112
Figure 4.7	Gelsolin and Cu/Zn SOD lie in close proximity to each other……………………………………………..	114
Figure 4.8A&B	Gelsolin expression affects mRNA levels of Nox……	116
Figure 5.1	Schematic illustration showing the summary of the key findings presented in this thesis………………….	126
Figure S1.1	3-dimenstional structure of Gelsolin-Cu/Zn SOD complex obtained using PatchDock………………….	157
Figure S1.2	Radius of gyration of gelsolin-Cu/Zn SOD ………….	159

ABBREVIATIONS

5-FU	: 5-fluorouracil
α-SMA	: Alpha-smooth muscle actin
AP-2	: Activator protein / Activating enhancer binding protein2
BrdU	: 5-bromo-2’-deoxyuridine
BSA	: Bovine serum albumin
CM – H_2DCFDA	: (5-(and-6) - chloromethyl-2’,7’ – dichlorodihydro-fluorescein diacetate acetyl ester
DDC	: Diethyldithiocarbamate
DEPC	: Diethylpyrocarbonate
DPI	: Diphenyleneiodonium
ECM	: Extracellular matrix
EDTA	: Ethylenediaminetetraacetic acid
EGF	: Epidermal growth factor
EGFR	: Epidermal growth factor receptor
EMT	: Epithelial-mesenchymal transition
GAPDH	: Glyceraldehyde 3-phosphate dehydrogenase
HDAC1	: Histone deacetylase-1
HRP	: Horse radish peroxidase
LPS	: Lipopolysaccharide
MAPK	: Mitogen-activated protein kinase
MMP	: Matrix metalloproteinases
mRNA	: Messenger ribonucleic acid
NFkB	: Nuclear factor kappa B
PAI	: Plasminogen activator inhibitor
PBS	: Phosphate buffered saline

PBST	: Phosphate buffered saline-tween
PCR	: Polymerase chain reaction
PIP_2	: Polyphosphoinositide 4,5-bisphosphate
PI3K	: Phosphoinositide 3-kinase
PIP5K 1α	: Phosphatidylinositol 4-phosphate 5-kinase 1α
PLC	: Phospholipase C
PLD	: Phospholipase D
PTP	: Protein tyrosine phosphate
PTP-PEST	: PTP-proline-glutamic acid- serine-threonine amino acid sequences
RTK	: Receptor tyrosine kinase
ROS	: Reactive oxygen species
SDS	: Sodium dodecyl sulfate
SDS-PAGE	: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	: Small interfering RNA
SOD	: Superoxide dismutase
Sp1	: Specificity protein 1
TBE	: Tris Borate EDTA
TEMED	: Tetramethylethylenediamine
TIMP	: Tissue nhibitor of metalloproteinases
uPA	: Urokinase-type plasminogen activator
uPAR	: Urokinase-type plasminogen activator receptor
VDAC	: Voltage-dependent anion channel

CHAPTER 1

INTRODUCTION

CHAPTER 1 INTRODUCTION

1.1 CANCER CELL INVASION

1.1.1 Invasion-metastatic cascade

Metastasis is one of the most life-threatening diseases which accounts for 90% of cancer death worldwide (Gupta & Massague, 2006; Mehlen & Puisieux, 2006; Weigelt *et al.*, 2005). The spread of tumour from the primary site to a distant region in the body is known as metastasis. Metastasis is a multifactorial process that involves a complex and an intricate interaction between the tumour cells and the surrounding environment which consists of the stroma, extracellular matrix (ECM), lymph vessels and the vascular systems.

The process of metastasis begins with detachment of tumour cells from the primary site and invasion through the nearby tissues. Invading tumour cells secrete proteolytic enzymes that are able to degrade the ECM and break away from the basement membrane. Degradation of ECM by proteases facilitates local invasion as well as migration of tumour cells out into the extracellular space. The tumour cells travel along the surface of other cells and force their way into the nearby vessels (lymph or blood vessels). Once the tumour cells reach the vessels, they degrade and invade through the epithelial lining of the blood vessels and infiltrate into the blood stream in a process called intravasation. Inside the circulation, the tumour cells move along the endothelial cell lining until they reach a point where they firmly attach to the endothelial cell walls. Once tumour cells strongly adhere to the endothelial

walls, they extravasate from the vessel to enter into the new tissue. If the new environment is suitable, these neoplastic cells proliferate and form new colonies called the secondary tumours. Secondary tumours often induce neoangiogenesis to ensure sufficient blood supply to the newly formed colonies (Chambers *et al.*, 2002; Friedl & Wolf, 2003; Steeg, 2003; Talmadge & Fidler, 2010; Yilmaz & Christofori, 2009). A schematic illustration of the invasion-metastatic cascade is shown in figure 1.1.

Metastasis is a tough and inefficient process from the point of view of a tumour cell because metastasizing cells encounter a number of adversities during their journey to the new site (Chambers *et al.*, 2002; Shibue & Weinberg, 2009; Valastyan & Weinberg, 2011). Migrating tumour cells may die while invading into the constricted spaces into the blood vessel walls; they may even succumb while in the blood circulation or be recognised and destroyed by the cells of the immune system. A significant fraction of migrating tumour cells that successfully reach a new location may still perish if the new environment is not suitable. Only the ones that survive throughout these adversities will proliferate to establish new colonies (Barbour & Gotley, 2003; Chambers *et al.*, 2002; Luzzi *et al.*, 1998; Shibue & Weinberg, 2009; Valastyan & Weinberg, 2011).

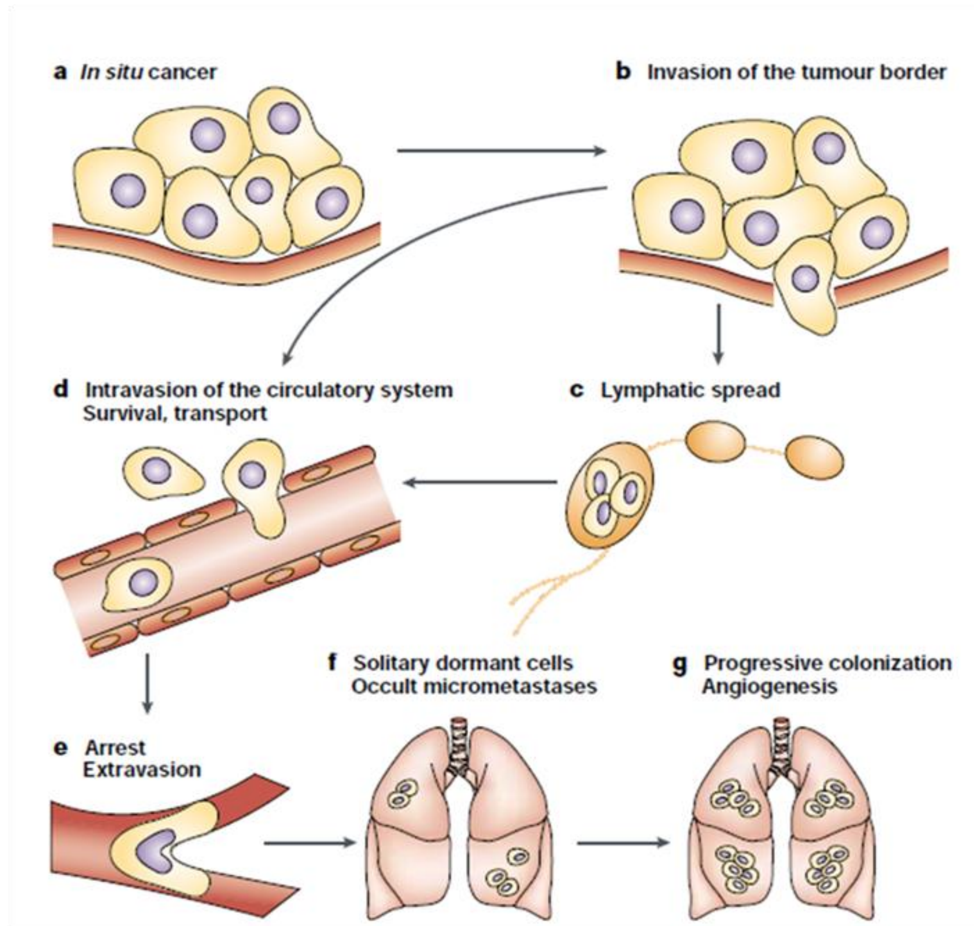


Figure 1.1. Schematic illustration of the invasion-metastatic cascade (a) *in situ* cancer cells outlined by an intact basement membrane. (b) Cancer cells acquire invasive phenotype which is characterised by loss of cell-cell adhesion, altered cell–extracellular matrix adherence, release of extracellular matrix-degrading proteases leading to fragmented basement and ECM, and increased motility (c) Cancer cells enter the lymphatic system and colonise at the lymph nodes, or (d) cancer cell directly enter the circulation system (e) Cancer cells that survive in the circulation travel to distant sites and attach to the endothelial walls and extravasate from the circulatory system (f) Formation of micrometatases at the secondary site which progresses from single cells and may remain dormant for years, to occult micrometastases or (g) Proliferation and formation of an angiogenic metastases. Figure is adapted from Steeg, 2003.

1.1.2 Molecular mechanisms of cancer cell invasion

Cancer cell motility and invasion constitutes one of the most critical steps during the course of metastasis. Cancer cell invasion involves a coordinated network of molecules that result in attachment to the matrix, degradation of the ECM, loss of intercellular adhesion, enhanced cell motility and resistance to death (Curran *et al.*, 2004; Curran & Murray, 2000; Friedl & Alexander, 2011; Kassis *et al.*, 2001; Leber & Efferth, 2009; Meyer & Hart, 1998; Sahai, 2005; Yilmaz & Christofori, 2009). During these processes a number of oncogenic factors are expressed in an orchestrated fashion allowing cells to acquire invasive and aggressive phenotype. Amongst the oncogenic factors are the oncogenes such as Rac, Ras, and Src, (Angers-Loustau *et al.*, 2004; Campbell & Der, 2004; Guarino, 2010; Zhuge & Xu, 2001), dysregulated actin cytoskeleton (Yamaguchi & Condeelis, 2007; Yamaguchi *et al.*, 2005; Yamaguchi *et al.*, 2006; Yilmaz & Christofori, 2009), as well as chemically reactive molecules such as the Reactive Oxygen Species (ROS) (Alexandrova *et al.*, 2006; Binker *et al.*, 2009; Nishikawa & Hashida, 2006). Depending on the molecular complex they associate with, these oncogenic factors may elicit different responses depending on the stimuli, the environment and the type of tumour.

1.1.3 Cancer cell-extracellular matrix (ECM) interaction and ECM degradation

Successful invasion of tissues requires cell-ECM interaction and breakdown of the components of the ECM and basement membrane. Integrin-ECM interaction instigate intracellular signalling cascade that regulate gene

expression required for actin cytoskeletal reorganisation and cell adhesion leading to enhanced invasion. To initiate cell adhesion, integrins assemble at the plasma membrane enabling cells to attach to the ECM at the focal adhesion point (Guan, 1997; Svineng *et al.*, 2008). Focal adhesion proteins, such as focal adhesion kinase (FAK), along with focal adaptor proteins — p130Cas and paxillin are important regulators of integrins (Ben Mahdi *et al.*, 2000; Price & Thompson, 2002). FAK associates with other signalling proteins such as Src kinase to modulate integrin-mediated cell migration by phosphorylating p130Cas and paxillin (Price & Thompson, 2002). Activated p130Cas and paxillin associates with other proteins involved in membrane ruffling, cell migration and invasion (Zhao & Guan, 2011).

Once invasive tumour cells adhere to and interact with the ECM, they infiltrate the surrounding ECM. The major constituents of the ECM are collagen, laminin and fibronectin that can be degraded by specific proteases. Of the most prominent proteases associated with cancer are the urokinase plasminogen activator (uPA) system and the matrix metalloproteinases (MMPs) (Brooks *et al.*, 2010; Meyer & Hart, 1998). Through their degradative actions, these proteases remodel the ECM, paving the way for invading cells to migrate and promote tumour cell invasion and metastasis (Baldassarre *et al.*, 2006; Yilmaz & Christofori, 2009). A schematic summary of the cell-ECM interaction as well as ECM degradation is presented in figure 1.2.

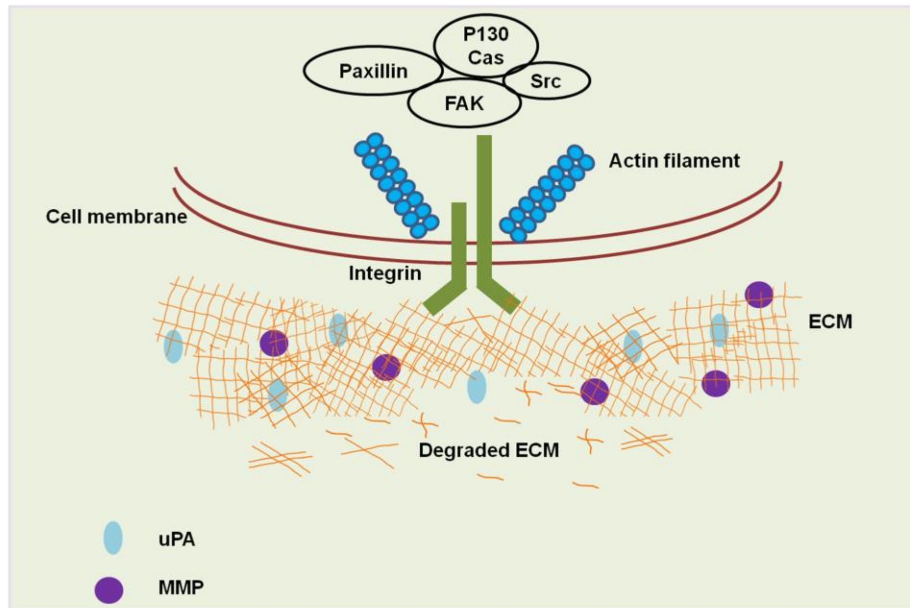


Figure 1.2. Cell-ECM interaction and ECM degradation. During cell adhesion, intracellular signalling molecules such as the focal adhesion kinase (FAK), Src, p130 Cas and paxillin associate with each other to activate integrin assembly at the plasma membrane. Activated integrins attach to the ECM at the focal adhesion point. Integrin-ECM interaction also triggers signalling cascade that is involved in cell invasion and migration. Once cancer cells are firmly adhered to ECM, they release proteolytic enzymes such as MMPs and uPA that degrade components of the ECM thereby facilitating cancer cell invasion.

Urokinase plasminogen activator (uPA) system

Tumour cells secrete the serine protease uPA as a soluble protein that binds to its specific cell surface receptor, the uPA receptor (uPAR). Binding of uPA to uPAR converts plasminogen to active plasmin, which can then degrade components of the basement membrane and ECM through its proteolytic activity (Brooks *et al.*, 2010; Duffy, 2004; Lijnen, 2001; Meyer & Hart, 1998; Syrovets & Simmet, 2004). Besides activation of plasmin, uPA can also induce the activation of other proteases involved in cancer cell invasion, such as the MMPs, highlighting the pivotal role of uPA in promoting cancer cell invasion (Legrand *et al.*, 2001; Taniguchi *et al.*, 1998; Zhao *et al.*,

2008). Increased levels of uPA have been implicated to correlate with invasive and aggressive phenotypes in tumours (Bauer *et al.*, 2005; Pyke *et al.*, 1991; Zhao *et al.*, 2008). In line with this, increased expression and activation of uPA was shown to enhance invasion (Zhang *et al.*, 2011) whilst downregulation of uPA reduced the invasive abilities of tumour cells (Mohanam *et al.*, 2002; Zhang *et al.*, 2011).

uPA expression is regulated by activation of the mitogen-activated protein kinase (MAPK) pathway and downstream activation of transcription factors including Nuclear Factor-kappa B (NF-κB), Activator Protein-2α (AP-2α) and Specificity protein 1 (Sp1) (Kim *et al.*, 2007b; Lee *et al.*, 2009; Tobar *et al.*, 2010b). ROS have also been shown to indirectly activate the uPA/uPAR system via activation of the MAPK pathway.

Matrix metalloproteinases

MMPs are a large family of endopeptidases that degrade a variety of substrates in the ECM. The different MMPs are categorised according to their specific substrates as collagenases, gelatinases, stromelysins and membrane-type MMPs (Lukaszewicz-Zając *et al.*, 2011). MMPs are first secreted as zymogens and the inactive pro-MMPs are activated by cleavage of the pro-peptide domain. MMPs can also be activated by serine proteases such as uPA, other active MMPs or by ROS oxidation (Nelson & Melendez, 2004; Rajagopalan *et al.*, 1996). The tissue inhibitors of metalloproteinases (TIMPs) are the endogenous inhibitors of MMPs. Often in invasive cancer cells, high amount of MMP activities are observed as opposed to lower activities of TIMPs (Curran *et al.*, 2004; Lukaszewicz-Zając *et al.*, 2011).

1.1.4 Loss of cell-to-cell adhesion

In several cancer types, the onset of tumour malignancy is often marked by dissolution of cell-to-cell contacts that results in cell scattering (Thiery, 2002). Cell scattering is induced when cell to cell adhesion molecules are deregulated which cause morphological changes to the cell. The alteration in cell shape closely resembles the switch observed during epithelial-mesenchymal transition (EMT) where epithelial cells lose their characteristics such as tight cell-cell adhesion and acquire a fibroblastic-like spindle-shaped morphology (Thiery, 2002) (figure 1.3). One of the hallmarks of EMT is the loss of cell-to-cell adhesion molecule E-cadherin with a concomitant increase in the expression of the mesenchymal marker N-cadherin, a process called the cadherin switch (Yilmaz & Christofori, 2009). Loss of epithelial markers (laminin 1, ZO-1, desmoplakin, cytokeratin, and collagen IV) and activation of mesenchymal markers (alpha-smooth muscle actin (α-SMA), vimentin) have also been observed during EMT-like changes. In most cases, loss of E-cadherin is a result of activation of transcriptional repressors of E-cadherin such as Snail, Slug, Zeb1, SIP1, E47 and Twist. These EMT-related features are associated with aggressive behaviour leading to the progression of invasion and the metastasis of many cancer types (Barnett *et al.*, 2011; Baum *et al.*, 2008; Thiery, 2002; Thiery, 2003; Thiery & Sleeman, 2006). It is however important to note that cancer cells can also migrate and invade using mechanism different from these EMT-like features.

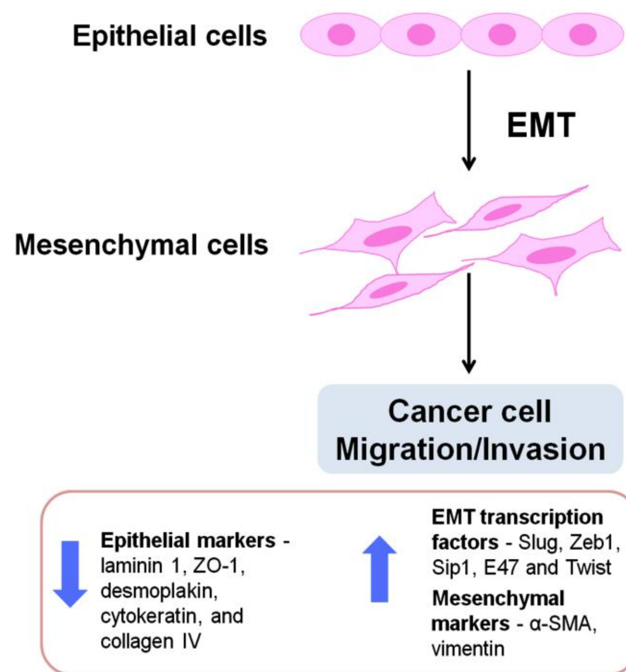


Figure 1.3. Acquisition of EMT-like phenotypes during cancer cell migration and invasion. The onset of EMT is characterised by the partial or complete loss of cell to cell adhesion molecules and induction of meshenchymal markers. These changes also involve cytoskeletal reorganisation leading to alteration in cell shape.

1.1.5 Actin cytoskeleton and cancer cell invasion

Although various factors are involved, regulation of the actin cytoskeleton is one of the most crucial determinants of cancer cell invasion. Many of the steps in cancer cell invasion depend on the structural changes and cell motility aspects which are driven by actin cytoskeletal dynamics. Changes in cell shape, particularly formation of actin rich protrusions such as lamellipodia, filopodia, invadopodia and podosomes have been illustrated during cell movement and invasion (Ridley, 2011). Activation of the small GTPases such as Rho, Rac and Cdc42 are responsible for transmitting signals to actin-binding proteins for the formation of actin-rich plasma membrane

protrusions necessary for migration and invasion (Kozma *et al.*, 1995; Nobes & Hall, 1995a; Nobes & Hall, 1995b; Nobes & Hall, 1999). Formation of these protrusive structures is a highly dynamic process tightly regulated by a network of actin-binding proteins. Key actin-binding proteins associated with the formation of protrusive structures include the Wiskott–Aldrich Syndrome Protein (WASP) family of proteins, the Arp2/3 complex, cofilin, cortactin, gelsolin and members of the gelsolin superfamily of proteins (Chellaiah *et al.*, 1998; Chellaiah *et al.*, 2000; Jones *et al.*, 2002; Yamaguchi & Condeelis, 2007; Yilmaz & Christofori, 2009).

Lamellipodia are flat, sheet-like membrane protrusions formed at the leading edge of migrating cells (Friedl & Gilmour, 2009; Yap *et al.*, 2005). Lateral branching of actin filaments and actin assembly in lamellipodia requires generation of free barbed ends of actin filaments. These functions are carried out by Arp2/3 complex, N-WASP, gelsolin and cofilin. Lamellipodia are known to generate the force required for cell migration. Formation of lamellipodia requires interaction of the cell with the ECM via cell surface receptors such as the integrins. A thin, rod-like projection composed of bundled, cross-linked actin filaments originate from the edge of lamellipodia. This projection is called the filopodium. Filopodia are believed to act as sensors for lamellipodia to detect chemoattractants or nutrients released from blood vessels. Metastatic cells are rich in filopodia-like structures which correlate with their invasiveness. However, the exact mechanisms underlying filopodia formation is not clearly understood. Lamellipodia and filopodia are mostly associated with cell movement on a 2-dimensional (2-D) surface

(Chellaiah *et al.*, 2000; Yamaguchi & Condeelis, 2007; Yilmaz & Christofori, 2009).

Invadopodia and podosomes are actin-rich ventral membrane protrusions associated with cancer cell invasion and metastasis. They are coupled with proteolytic cores that enable them to degrade the ECM. Secretion of proteases such as Membrane type 1-matrix metalloproteinase (MT1-MMP) by invadopodia provides advantage for degradation and migration in a dense rigid 3-dimensional (3-D) ECM environment (Chen, 1989; Ridley, 2011; Weaver, 2006). Formation of invadopodia and podosomes involves recruitment of a network of molecules including actin and actin regulatory proteins (cofilin, fascin, cortactin, WASP, gelsolin), signalling proteins (Rac1, Src), proteases (Membrane-type MMP-1) and the ROS-producing enzyme NADPH oxidase (Nox) (Chellaiah, 2006; Clark & Weaver, 2008; Clark *et al.*, 2007; Gianni *et al.*, 2010a; Li *et al.*, 2010; Machesky & Insall, 1998; Machesky & Li, 2010; Yamaguchi *et al.*, 2005; Yu *et al.*, 2012). Recently ROS have also been suggested to promote invadopodia formation (Diaz & Courtneidge, 2012; Diaz *et al.*, 2009; Gianni *et al.*, 2010a; Gianni *et al.*, 2010b). Cell migration and invasion driven by actin-rich protrusions on a 2-D and 3-D environment is illustrated in figure 1.4.

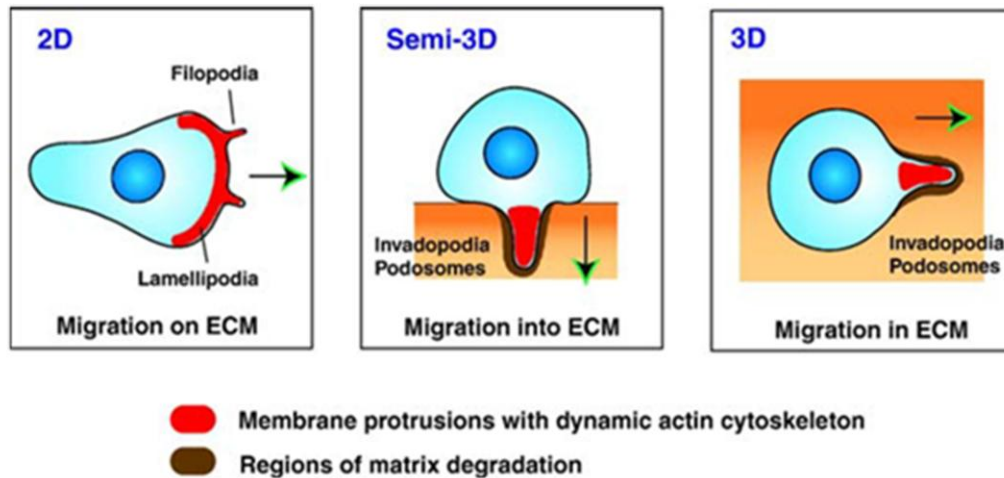


Figure 1.4. Actin-rich membrane protrusion-mediated cell migrations in different environment. Lamellipodia and filopodia are formed at the leading edge of migrating cells on a 2-D substrate. Invadopodia and podosomes are formed at the invading front on the ventral side of the cell. Invadopodia and podosomes are equipped with the ability to degrade the ECM which allows cells to enter and migrate through the 3-D ECM environment. Migration and invasion via membrane protrusions are directed by regulation of the actin cytoskeletal dynamics. Cancer cells express high levels of proteins involved in the formation of actin-rich protrusions associated with increased migratory and invasive potential. Arrows indicate the direction of migration. Figure is adapted from Yamaguchi and Condeelis, 2004.

1.2 GELSOLIN

1.2.1 Introduction

Gelsolin is a 82 kilodalton (kDa) multi-functional protein well known for its actin-binding property. Gelsolin is the founding member of the gelsolin superfamily of actin binding proteins which consist of six other members namely — capG, villin, supervillin, adseverin, advillin and flightless 1. All members of the gelsolin superfamily contain three or six homologous repeats of the gelsolin-like domains with binding sites for actin. Gelsolin is the best characterised actin-regulatory protein in this family (Silacci *et al.*, 2004). Gelsolin regulates actin dynamics by severing, capping and nucleating actin filaments (Burtnick *et al.*, 1997), thereby playing important roles in actin cytoskeletal organisation, cell migration, invasion, morphogenesis and apoptosis (Arora & McCulloch, 1996).

Three naturally occurring isoforms of gelsolin have been identified to date. These consist of two cytoplsmic forms (cytoplasmic gelsolin and gelsolin-3) and plasma gelsolin (Kwiatkowski *et al.*, 1986; Vouyiouklis & Brophy, 1997; Yin *et al.*, 1984). Cytoplasmic and plasma gelsolin are splice variants of the same gene. Plasma gelsolin contains an extra 25 amino acid at the N-terminus which serves as a signal peptide for secretion (Kwiatkowski *et al.*, 1986). Gelsolin-3 varies from cytoplasmic gelsolin in that it contains an additional 11 amino acid at the N-terminus (Vouyiouklis & Brophy, 1997).

Cytoplasmic gelsolin is the most abundant form of intracellular gelsolin with its functions being implicated in many cellular processes

including cell migration and invasion, apoptosis, platelet formation and development (Silacci *et al.*, 2004; Spinardi & Witke, 2007). Although cytoplasmic and plasma gelsolin differ in terms of localisation, they share a common principal function of regulating actin severing. In fact, gelsolin is one of the most potent and well characterised actin severing proteins identified to date. Plasma gelsolin plays an important function in scavenging actin debris from the circulation released during apoptosis or necrotic cell death. Mutated plasma gelsolin is one of the causes of amyloidosis, a disease induced by protein aggregation (Li *et al.*, 2012; Maury, 1991). Gelsolin-3 is found in the cytoplasm of oligodendrocytes in the brain, lungs, and testis. Gelsolin-3 is known to be associated with myelin formation and central nervous system (CNS) formation (Silacci *et al.*, 2004; Vouyiouklis & Brophy, 1997).

1.2.2 Interaction between gelsolin and actin — structure to function relation

The regulation of actin cytoskeletal dynamics is central for the maintenance of cell rigidity, locomotion and cellular homeostasis. Actin-binding proteins such as gelsolin are crucial in regulating the actin cytoskeletal dynamics. Gelsolin is activated by an intracellular rise in calcium (Ca^{2+}) concentration and negatively regulated by binding with polyphosphoinositide 4,5-bisphosphate (PIP_2) (Janmey & Stossel, 1987; Yin & Stossel, 1979; Yin & Stossel, 1980; Yin *et al.*, 1980). The actin-regulatory activity of gelsolin is attributable to its structural and domain rearrangement. Gelsolin consists of six domains which are arranged as two tandem homologous halves, each containing a 3-fold segmental repeats (Kwiatkowski *et al.*, 1986) (figure 1.5).

Domains G1-G3 constitute the N-terminal half, while domains G4-G6 make up the C-terminal half. The N (G1-3) and C (G4-6)-terminals are connected by a linker that contains cleavage site for caspase-3. Isolated N-half has been shown to sever actin in the absence of Ca^{2+} activation. However, Ca^{2+} is essential for activation of full length gelsolin that involves a fine cooperation between the N-and C-terminal halves (Burtnick *et al.*, 1997; Burtnick *et al.*, 2004).

Protein crystallography studies suggested that each domain in gelsolin possesses calcium binding site allowing the recriumtment of up to six Ca^{2+}ions (Burtnick *et al.*, 1997; Choe *et al.*, 2002; McLaughlin *et al.*, 1993; Pope *et al.*, 1995). However, due to the relatively lower concentration of Ca^{2+} inside the cell, it has been proposed that binding of two to three Ca^{2+} ions may be sufficient to induce the intracellular actin-severing ability of gelsolin (Burtnick *et al.*, 1997).

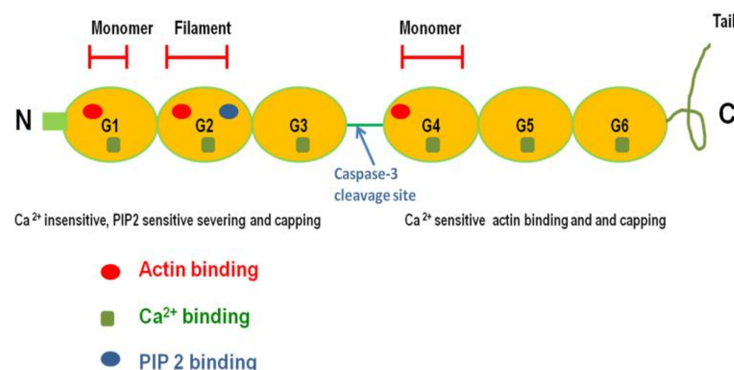


Figure 1.5. Gelsolin structure to function domains. Gelsolin consists of six domains, G1-G6. Gelsolin is regulated by Ca^{2+} (green squares) and PIP_2 (blue ovals) binding. The isolated domain G1 contains severing activity, while domains G1-3 possess the capping activity. Studies have identified three actin-binding sites (red ovals), six Ca^{2+} binding sites, one PIP_2 binding site and a caspase-3 cleavage site (blue arrow). Figure is adapted and modified from Spinardi and Witke, 2007; and Sun *et al.*, 1999.

At resting stage, gelsolin forms a compact closed structure that opens upon Ca^{2+} activation, allowing gelsolin to bind to actin filament (figure 1.6). The domain G6 of the C-terminus contains a short helix tail that latches back to G2 of the N-terminus that blocks the actin binding region of the N-half, this model is known as the "tail latch hypothesis" (Burtnick *et al.*, 2004; Choe *et al.*, 2002). However, Ca^{2+} binding induces a conformational change that opens up gelsolin and releases the G6 tail bound to the N-half, exposing the actin binding regions on the N-half of G2. This domain rearrangement can then direct the binding and positioning of gelsolin to actin to induce severing and subsequently cap the fast growing end. Capping makes severing very effective because it prevents annealing of the filament. Furthermore, severing creates a number of polymerisation-competent ends that act as sites for actin polymerisation once gelsolin is released from the barbed ends (Sun *et al.*, 1999). Polymerisation resumes when PIP_2 interacts with and detaches gelsolin from the barbed end of the actin filament (Janmey & Stossel, 1987). Uncapping of actin leads to rapid reassembly of actin without a lag time and enhancing actin polymerisation. Thus, gelsolin modulates actin filament assembly and disassembly which constitutes an important part in the dynamic regulation of the actin cytoskeleton. In addition, it has been suggested that gelsolin can promote actin nucleation by binding to two actin monomers to create a nuclei (Ditsch & Wegner, 1994; Tellam & Frieden, 1982).

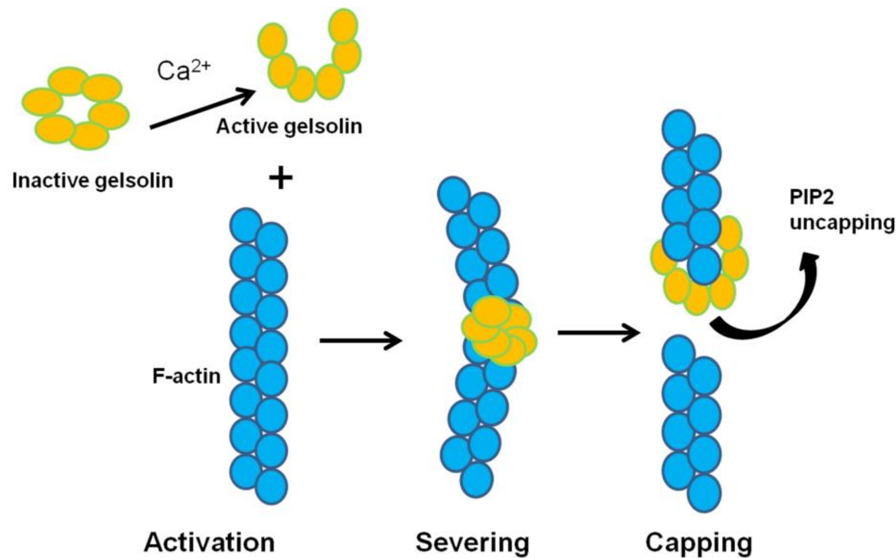


Figure 1.6. Schematic diagram showing gelsolin binding, severing and capping of an actin filament. Gelsolin forms a compact closed structure at resting stage. Upon Ca^{2+} trigger, gelsolin opens up and binds to and severs actin filament. After severing, gelsolin caps the fast growing end and prevent it from polymerisation. However, when PIP_2 binds to gelsolin, it induces a conformational change and releases gelsolin from actin. Figure is adapted and modified from Sun *et al.*, 1999.

1.2.3 The role of gelsolin in cell motility: evidence from *in vivo* studies

Studies using gelsolin null animals have established the importance of gelsolin in motility, survival and several other fundamental cellular processes (Azuma *et al.*, 1998; Endres *et al.*, 1999; Lu *et al.*, 1997; Witke *et al.*, 1995). Gelsolin-null fibroblasts showed reduced cell motility and increased actin stress fibers, a phenotype related to defective actin cytoskeletal remodelling (Azuma *et al.*, 1998). Gelsolin has been shown to function as a downstream effector of Rac, a regulator of membrane ruffle formation. Rac overexpression has been observed as a compensatory mechanism for the loss of gelsolin. Translocation of Rac is required for the formation of membrane ruffles and membrane protrusions. Although Rac translocates to the

membrane in response to epidermal growth factor (EGF), cell motility, membrane ruffle and lamellipodia formation are impaired in the absence of gelsolin suggesting that Rac requires gelsolin to carry out its function in cell motility (Azuma *et al.*, 1998). Gelsolin knockout animals also showed defects associated with reduced chemotaxis, wound healing, blood clotting, and impaired neurite retraction (Lu *et al.*, 1997; Maniatis *et al.*, 2009; Oikonomou *et al.*, 2009; Witke *et al.*, 1995).

In addition, evidence from animal models also demonstrates the importance of gelsolin in survival. Gelsolin-knockout mouse in a pure strain background died during birth or shortly after birth indicating the importance of gelsolin for survival (Kwiatkowski, 1999). However, gelsolin-null animals in a mixed background survive without major phonotypic effects which suggests the presence of compensatory mechanisms for survival.

1.2.4 Gelsolin-mitochondrial association

In addition to its localisation in the cytosol, gelsolin has been detected in an isolated mitochondrial fraction (Koya *et al.*, 2000; Kusano *et al.*, 2000). By interacting with the mitochondria, gelsolin can function as a negative regulator of apoptosis. Overexpression of full length gelsolin has been shown to confer anti-apoptotic properties by blocking cytochrome c release from the mitochondria and preventing mitochondrial membrane potential loss. Gelsolin also physically binds to voltage dependant anion channel (VDAC), thereby blocking VDAC activity as well as its subsequent downstream activities. Besides interacting with mitochondrial components, gelsolin in complex with PIP_2 was also found to inhibit apoptosis by inhibition of caspase-3 and -9

activities (Azuma *et al.*, 2000; Klampfer *et al.*, 2005; Kusano *et al.*, 2000; Ohtsu *et al.*, 1997). The exact mechanism by which gelsolin translocates to the mitochondria and how it regulates mitochondrial apoptotic pathways are not well understood. Gelsolin has also been shown to have a neuroprotective role against Alzheimer's disease by enhancing the mitochondrial activity (Antequera *et al.*, 2009). Overexpression of gelsolin enhances the activity of complex IV of the mitochondrial electron transport chain (ETC) leading to a reduction in nitric oxide and reduced cell death. On the other hand, due to the presence of the caspase-3 celavage site, gelsolin also serves as an effector molecule for caspase-3 mediated apoptosis. The N-terminal half of gelsolin loses the Ca^{2+} regulatory component acquiring an unregulated actin-severing capacity. Uncontrolled actin severing disrupts the integrity of the actin cytoskeleton, thus facilitating the apoptotic cell death signalling cascade (Geng *et al.*, 1998; Kothakota *et al.*, 1997).

1.2.5 Gelsolin in cancer

The role of gelsolin in cancer has been long been a topic of debate. Gelsolin has often been regarded as a tumour suppressor due to its downregulation in several cancer whilst overexpression of gelsolin leading to aggressive behaviours is also observed in a number of studies (Li *et al.*, 2012).

Tumour-suppressive functions of gelsolin

Gelsolin is found to be downregulated during the carcinogenesis of breast (Asch *et al.*, 1996; Winston *et al.*, 2001), colon (Gay *et al.*, 2008), stomach (Moriya *et al.*, 1994), bladder (Tanaka *et al.*, 1995), prostate (Lee *et al.*, 1999b), lung (Dosaka-Akita *et al.*, 1998) as well as in transformed human

fibroblast and epithelial cells (Vandekerckhove *et al.*, 1990). Studies have suggested that epigenetic regulations may play a role in the loss of gelsolin in breast cancer (Mielnicki *et al.*, 1999). The mRNA transcript of gelsolin was found to be downregulated in breast cancer carcinoma. The downregulation of gelsolin could be due to transcriptional repression whereby activating transcription factor 1 (ATF-1) repressed gelsolin promoter by binding to gelsolin negative regulatory element site (Dong *et al.*, 2002). Consistent with this, the transcription factor Sp1 has also been implicated to play a role in downregulating the expression of gelsolin in human bladder cancer cells (Haga *et al.*, 2004). Transcriptional repressors such as histone deactelyase 1 (HDAC-1) and DNA methylatransferases have been suggested to participate in silencing gelsolin expression in certain types of cancer including breast (Mielnicki *et al.*, 1999), cervix (Han *et al.*, 2000), gastric (Kim *et al.*, 2004), and urinary bladder (Haga *et al.*, 2004). In line with these findings, upregulation of gelsolin expression has been observed in several human cancer cells after treatment with HDAC inhibitors (Hoshikawa *et al.*, 1994; Mielnicki *et al.*, 1999; Noske *et al.*, 2005).

Post-translational modification of gelsolin protein has also been found to downregulate gelsolin protein levels in pancreatic cancer cells (Ni *et al.*, 2008). Gelsolin undergoes ubiquitin-mediated proteosomal degradation leading to decreased gelsolin levels. Interestingly, when pancreatic cancer cells were treated with proteosome inhibitor lactacycti, it induces gelsolin expression. Moreover, analyses of human pancreatic tissues show high correlation between high gelsolin poly-ubiquitination and lower gelsolin

expression. Thus, this study shows that proteosomal degradation accounts for the loss of gelsolin in human pancreatic cancer (Ni *et al.*, 2008).

Although no major chromosome rearrangement or mutations were observed in the studies conducted Asch *et al.*., (Asch *et al.*, 1996), point mutation at codon 321 of gelsolin has been identified in the Ras-transformed NIH3T3 cells (Mullauer *et al.*, 1993). This mutation occurred due to the replacement of cytosine by alanine, leading to a single amino acid substitution of proline to histidine referred to as the gelsolin variant His321. Gelsolin His321 variant is found in domain G3 of the N-half region of gelsolin. As the N-half is important for actin severing, it is conceivable that this point mutation may cause impaired actin severing and contributes to tumour suppression.

An important point to note is that in gelsolin-null mouse, loss of gelsolin itself does not lead to tumour formation. Therefore, the tumour suppressive role of gelsolin may result from other upstream factors that regulate gelsolin such as epigenetic alterations, post translational modifications and mutations in the gelsolin gene (Spinardi & Witke, 2007).

Tumour-promoting functions of gelsolin

Although gelsolin has been implicated to be a tumour suppressor, a significant amount of studies have also suggested the tumour-promoting functions of gelsolin. In the clinical set up, high gelsolin expression has been linked to bigger tumour size, invasive phenotype and poor patient outcome. Moreover, the expression of gelsolin in cancer has been proposed to display a biphasic expression pattern where gelsolin was found to be downregulated at the premalignant stage and upregulated at the more aggressive and malignant

stage (Gay *et al.*, 2008; Rao *et al.*, 2002; Shieh *et al.*, 2006; Shieh *et al.*, 1999; Thompson *et al.*, 2007; Thor *et al.*, 2001). In oral squamous cell carcinoma, high gelsolin expression in patients correlates with metastatic disease (Shieh *et al.*, 2006). Consistent with this, gelsolin expression was found to be low in the primary colon adenocarcinoma (Gay *et al.*, 2008) whereas gelsolin expression was observed to be high at the invasive front of liver metastases (Zhuo *et al.*, 2012). In a subset of breast cancer gelsolin expression was found to be associated with the overexpression and activation of the receptor tyrosine kinases epidermal growth factor receptor (EGFR) and erbB-2 and this association was further correlated to aggressive phenotype (Thor *et al.*, 2001). Gelsolin has also been found to be highly expressed in cervical cancer tissue when compared with the adjacent normal tissue (Liao *et al.*, 2011).

Gelsolin in cancer cell invasion

It has been established that changes in gelsolin levels have profound effects on cell motility. *In vitro* and *in vivo* experiments have shown that overexpression of gelsolin enhanced cell migration and invasion whilst loss of gelsolin resulted in decreased motility and invasion (Azuma *et al.*, 1998; Cunningham *et al.*, 1991; De Corte *et al.*, 2002; Van den Abbeele *et al.*, 2007). Because gelsolin is an actin regulatory protein, a number of gelsolin-mediated motility is linked to its function in modulating actin cytoskeletal dynamics (Litwin *et al.*, 2009; Litwin *et al.*, 2012; Radwanska *et al.*, 2012). In line with this, a recent study suggests the involvement of gelsolin in enhancing cancer cell migration and invasion in the colon adenocarcinoma cell line LS180 (Litwin *et al.*, 2012). Here, the authors show that increased gelsolin

expression leads to its translocation to the membrane. Once at the membrane, gelsolin associates with the actin cytoskeleton, leading to a decrease in the actin filament to monomeric actin ratio, which could result in the increased migratory ability observed (Litwin *et al.*, 2012). Another member of the gelsolin superfamily, supervillin is also known to play a role in invasion. However it has been observed that the supervillin exhibits its pro-invasive function in a gelsolin-dependent manner (Crowley *et al.*, 2009) which further evidences the centrality of gelsolin in invasion.

As noted earlier, motile cells acquire structural changes such as lamellipodia and invadopodia (Ridley, 2011). Gelsolin has been shown to play an important role in the EGF-induced cell migration by participating in the formation of lamellipodia (Chou *et al.*, 2002). The involvment of gelsolin in other forms of protrusions such as podosomes have been evidenced. Indeed, gelsolin has been shown to be crucial for podosome formation in osteoclasts, whereby deficiency of gelsolin inhibits podosomes formation (Chellaiah *et al.*, 2000). Invadopodia and podosomes are structurally related membrane protrusions that have been implicated in enhancing the invasion process by secretion of proteases (Chen, 1989; Ridley, 2011). It is therefore tempting to speculate that gelsolin at the invading front may participate in proteolysis. We have previously shown that gelsolin overexpression leads to the increased production and secretion of uPA thereby promoting invasion in colon cancer cells (Zhuo *et al.*, 2012). However, the mechanism by which gelsolin contributes to protease secretion is unclear.

1.2.6 Gelsolin interacts with signalling proteins

Gelsolin has been documented to associate with several molecules involved in signal transduction pathway that favours cell migration and invasion. The small GTPases, Ras and Rac are important mediators of cell motility that have been linked with gelsolin during migratory signalling events. Invasion mediated by gelsolin rely on the upstream signalling events triggered by Ras that acts through the Phosphatidylinositide 3-kinases (PI3K) – Rac pathway (De Corte *et al.*, 2002). Gelsolin-mediated membrane ruffling and regulation of collagen phagocytosis is also influenced by signalling pathways triggered in response to extracellular stimuli such as the EGF (Arora *et al.*, 2004; Azuma *et al.*, 1998; Chou *et al.*, 2002). Moreover, gelsolin has also been shown to play a role in enhancing cell migration following erbB-2/EGFR activation (Chen *et al.*, 1996). EGF enhances PIP_2 hydrolysis by Phospholipase C γ (PLCγ), releasing gelsolin from its bound state to participate in actin cytoskeletal rearrangement. Gelsolin is also involved in lipid metabolism and lipid signalling via its interactions with phospholipids such as Phospholipase C γ1 (PLCγ1) and phospholipase D (PLD) (Banno *et al.*, 1992; Chellaiah *et al.*, 1998; Chellaiah & Hruska, 1996; Finkelstein *et al.*, 2010).

Formation of a focal contact point is a crucial step during cell adhesion. Gelsolin has been found to form complexes with several focal adhesion proteins including the oncogenic Src, PI3K, p130Cas, focal adhesion kinase (FAK), vinculin, talin, paxillin and integrin α_v β_3. As mentioned earlier, gelsolin participates in the formation of podosomes and podosomes are known to play important roles during cell adhesion. Through its interaction

with focal adhesions proteins, gelsolin is involved in the remodelling of the actin cytoskeleton and podosome formation. At the focal adhesion site, gelsolin has been shown to associate with the protein tyrosine phosphatase (PTP)-proline-glutamic acid-serine-threonine amino acid sequences (PEST), PTP-PEST. As PTP-PEST is a negative regulator of receptor tyrosine kinase (RTK), (Chellaiah *et al.*, 2001), association between gelsolin and PTP-PEST may result in activation of signalling pathways downstream of RTK.

The dynamic nature of gelsolin shows its potential to interact with various other proteins that are important in fundamental cellular processes. Understanding the mechanisms of how gelsolin interact with these proteins as well as exploring other interacting partners of gelsolin may provide a better insight into how gelsolin promotes cancer cell migration and invasion.

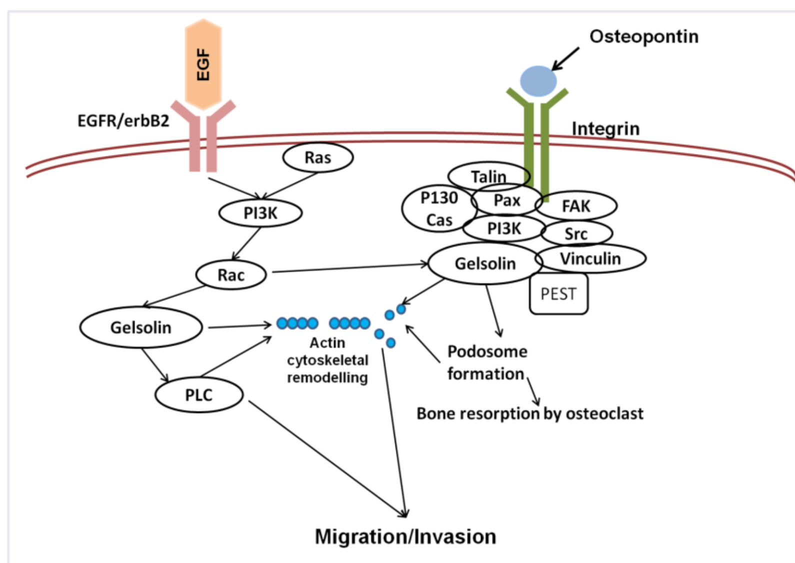


Figure 1.7. Gelsolin interacts with signalling molecules that favour cell migration and invasion. Gelsolin is a downstream effector of the Ras-Rac PI3K pathway and also respond to EGF trigger. Gelsolin is also found to form complexes with focal adhesion proteins that mediate the actin-rich podosome formation. Figure is adapted and modified from Chellaiah *et al.*,2001 and Li *et al.*, 2012.

1.3 REACTIVE OXYGEN SPECIES

1.3.1 Introduction

Reactive oxygen species are formed when molecular oxygen is incompletely reduced during cellular enzymatic reactions or during aerobic respiration. In aerobic cells, ROS are mainly produced as a by-product of metabolic reactions (D'Autréaux & Toledano, 2007; Halliwell, 1996; Novo & Parola, 2008). ROS comprise of several species that are either free radicals or non radicals as shown in Table 1.

Reactive Oxygen Species

Radicals	Non-radicals
Superoxide (O_2^{-})	Hydrogen peroxide (H_2O_2)
Hydroxyl ($^{\cdot}OH$)	Hypochlorous acid (HOCl)
Peroxyl ($RO_2.$)	Ozone (O_3)
Alkoxy ($RO^{\cdot}$)	Singlet oxygen ($^{1}\Delta g$)
Hydroperoxyl ($HO_2.$)	

Table 1.1 Types of reactive oxygen species. Adapted from Halliwell, 1996.

1.3.2 Sources of ROS

As noted earlier, cellular ROS are generated by enzymatic or by non-enzymatic reactions. One of the principal enzymes that produces ROS is the nicotinamide adenine dinucleotide phosphate-oxidase, NADPH oxidase (Nox). Phagocytic or non-phagocytic Nox are membrane bound enzymes that generates ROS using cytosolic nicotinamide adenine dinucleotide phosphate

(NADPH) as an electron donor. Nox is a multi-complex protein that consists of membrane subunits (gp91phox and p22phox) and cytosolic proteins (p47phox, p67phox, p40phox). Nox also consists of regulatory subunits such as small guanosine triphosphate (GTP)-binding protein Rac 1 and 2 (Moldovan *et al.*, 2006; Pervaiz & Clement, 2007). Other enzymatic sources include xanthine oxidases (XO), cyclooxygenases (COX), and lipoxygenases (LOX) (Gloire *et al.*, 2006).

Another important source of ROS is the mitochondrial ETC. The ETC comprise of a number of four protein complexes (I-IV) in which a number of oxidation-reduction reactions take place. ROS are mainly generated from complex I and III of the ETC. Free electrons transferred across the ETC from complex I to IV is used in the reduction of O_2 to water. However, during the course of electron transfer, O_2^- are generated when electrons leak and react with ambient O_2 (Cadenas & Davies, 2000). O_2^- is then converted to H_2O_2 by manganese superoxide dismutase (Mn SOD) in the mitochondria and by copper zinc superoxide dismutase (Cu/Zn SOD) in the cytosol (Fridovich, 1995). Subsequent reactions lead to the formation of other types of ROS.

1.3.3 The antioxidant system

Redox balance is necessary to maintain cellular homeostasis. Because abnormal increase in intracellular ROS levels can impede the normal functioning of the cell, cells are equipped with antioxidant defense systems to eliminate excess ROS. In mammalian cells, the antioxidant system include enzymatic (glutathione peroxidase, catalase, Cu/Zn SOD, Mn SOD, thioredoxin peroxidase and glutaredoxin) and non-enzymatic systems

(glutathione, carotenoids, vitamins C and E, and flavonoids) (Halliwell, 1996) as shown in Table 1.2. After O_2^- conversion to H_2O_2 by SODs, H_2O_2 is further detoxified to water by catalase, glutathione and thioredoxin peroxidases (Fridovich, 1978; Fridovich, 1995).

Enzymatic antioxidant defense system	
Cu/Zn SOD (Copper Zinc SOD)	$2\ O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2$
Mn SOD (Manganese SOD)	$2\ O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2$
Glutathione peroxidase	$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$
Catalase	$2H_2O_2 \rightarrow 2H_2O + O_2$
Thioredoxin (Trx) peroxidase	$Trx(SH)_2 + H_2O_2 \rightarrow TrxS2 + 2H_2O$
Non-Enzymatic antioxidant defense system	
Glutathione (GSH) – main thiol antioxidant and redox buffer of the cells	
Carotenoids	
α - tocopherol (Vitamin E)	
Ascorbic acid (Vitamin A)	
Flavoniods	

Table 1.2 Enzymatic and non-enzymatic antioxidant systems. Compiled from Halliwell, 1996 and Sies, 1997.

1.3.4 ROS – antioxidant imbalance and implications in cancer

Under physiological conditions, cells maintain a fine balance between ROS levels and efficient antioxidant systems. However, in pathological conditions including cancer, disturbance in the balance between ROS generation and antioxidant activities are often observed leading to a state of

oxidative stress (Halliwell, 2006; Sies, 1997). Increased oxidative stress conditions have been observed in most cancer cells when compared to their normal cell counterparts (Kumar *et al.*, 2008; Lee *et al.*, 1999a; Szatrowski & Nathan, 1991). Depending on the level of oxidative stress, cancer cells are differentially regulated. At a milder sub-lethal conditions of oxidative stress, signalling pathways that offer growth proliferation, migration and invasion advantages are activated. However, heightened oxidative stress levels fuels the cell death signalling pathways (Benhar *et al.*, 2002; Gloire *et al.*, 2006; Kang & Pervaiz, 2012; Mellier & Pervaiz, 2012; Nishikawa, 2008).

Downregulation of the three major antioxidants Cu/Zn SOD, Mn SOD and catalase was detected in prostate cancer (Bostwick *et al.*, 2000), whereas overexpression of Cu/Zn SOD, Mn SOD and catalase has been shown to inhibit growth and reduce aggressive behaviours in breast cancer cells (Glorieux *et al.*, 2011; Weydert *et al.*, 2006). Studies using animal models have shown the importance of antioxidants during cancer development. Mn SOD null animals die shortly after birth due to severe oxidant insult (Li *et al.*, 1995; Melov *et al.*, 2001). Although, heterozygous animals with 50% normal Mn SOD and a Cu/Zn SOD knockout mice could survive, these animals were susceptible to increased rates of liver cancer, lymphomas, adenocarcinomas and pituitary adenomas (Elchuri *et al.*, 2005; Van Remmen *et al.*, 2003).

1.3.5 ROS and cancer cell invasion

ROS have been well known to participate in signalling mechanisms that lead to cancer cell migration, invasion and EMT by activating the MAPK pathway (Cannito *et al.*, 2010; Wu, 2006a). Intracellular ROS levels may rise due to aberrant mitochondrial action, impaired antioxidant activities or activation of cell surface receptors by growth factors (such as the receptor tyrosine kinase, RTK). Binding of integrin to the ECM has also been shown to cause a mild oxidative burst by activation of LOX (Chiarugi *et al.*, 2003; Taddei *et al.*, 2007; Werner & Werb, 2002). It is not well understood how activation of growth factor receptors lead to increased ROS levels. This could likely be through activation of signalling molecules such as Rac, key regulator of non-phagocytic Nox enzymes (Brown & Griendling, 2009). Signalling pathways that trigger ROS generation and the activation of downstream molecules leading to cancer cell migration, invasion and EMT are represented in figure 1.8.

Protein kinase C (PKCs), an important activator of the MAPK pathway and the negative regulator of RTKs - the protein tyrosine phosphatases (PTPs) are two important signalling molecules targeted by ROS (Wu, 2006; Wu *et al.*, 2006). The critical cysteine residues present in both PKCs and PTPs renders them susceptible to oxidative modification by ROS. Oxidative modification of PKC activates the kinase whereas PTP is inactivated by ROS oxidation (Boivin *et al.*, 2010; den Hertog *et al.*, 2008; Salmeen & Barford, 2005). ROS-activated MAPK pathway can trigger the downstream transcriptional activation of AP-1, Ets-1, Snail, HIF-1α and NF-κB. Activation of these transcriptional factors induces EMT-like features such as loss of cell polarity

and cell-to-cell contact that facilitate cancer cell dissemination as well as cancer cell invasion (Tochhawng *et al.*, 2013). In addition, activation of redox sensitive transcription factors also elevates the expression of the ECM-degrading proteases, uPA and MMPs, thus enhancing cancer cell invasion (Kim *et al.*, 2007b; Nelson & Melendez, 2004; Tobar *et al.*, 2010b). ROS have been shown to upregulate several MMPs and uPA in a number of cancer cells (Binker *et al.*, 2009; Chiu *et al.*, 2010; Khoi *et al.*, 2012a; Kim *et al.*, 2007a; Pelicano *et al.*, 2009).

ROS have also been implicated in the enzymatic activation of MMPs via oxidative modification of the cysteine residue in the MMP molecule (Nelson & Melendez, 2004; Saari *et al.*, 1992); however, whether ROS also regulate the enzymatic activity of uPA is not known. In addition to increased gene transcription, ROS also aids in the stability of uPA and uPAR mRNAs (Tran *et al.*, 2003a). ROS have been shown to enhance the interaction between the uPA/uPAR mRNAs and the mRNA-stabilising Hu family of RNA-binding proteins (HuR) indicating the role of ROS in the post-transcriptional control of uPA and uPAR. Although the downstream targets and pathways of the ROS-mediated cancer cell invasion have been identified, little is known about the upstream regulators of ROS in cancer cells.

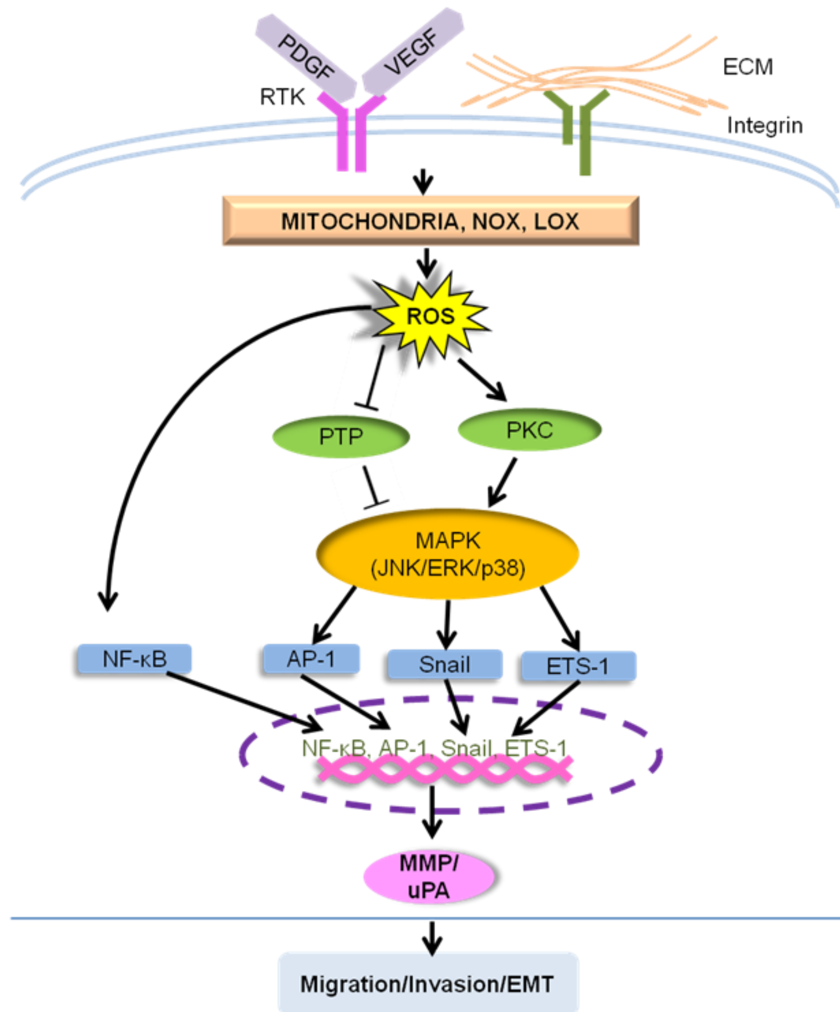


Figure 1.8. Schematic diagram of ROS-triggered signalling pathways leading to cell migration and invasion. Intracellular ROS may be generated from several sources including mitochondria, Nox and LOX as a result of interactions between growth factors - platelet-derived growth factor (PDGF)/ Vascular endothelial growth factor (VEGF) and cell surf ace receptors such as RTK and integrin assembly. The ROS produced can activate the MAPK signalling cascade and NF-κB pathways leading to activation of target genes such as uPAs and MMPs. Figure adapted and modified from (Tochhawng *et al.*, 2013).

1.3.6 The actin cytoskeleton and ROS

Evidence from several lines of studies have illustrated the relationship between the actin cytoskeleton and ROS. ROS have often been implicated in the regulation of cellular processes that involve the actin cytoskelal dynamics leading to enhanced migration and invasion (San Martin & Griendling, 2010; Tochhawng *et al.*, 2013). Increased O_2^- have been shown to stimulate Rac1 activity resulting in enhanced actin polymerisation. Accordingly, when O_2^- was scavenged by the antioxidants, diphenyleneiodonium (DPI) and MnTM PyP (manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin) growth of actin filament was abrogated (Moldovan *et al.*, 1999). These data suggest the participation of ROS in actin cytoskeletal dynamics.

ROS have been shown to play a role during the formation of actin rich cell protrusions (Diaz & Courtneidge, 2012; Kim *et al.*, 2009). Actin-rich protrusions including lamellipodia, filopodia and invadopodia are induced by signalling molecules such as Rac, Rho and cdc42 (Kozma *et al.*, 1995; Nobes & Hall, 1995a; Nobes & Hall, 1995b; Nobes & Hall, 1999). Rac1 regulates Nox activity to generate ROS (Cheng *et al.*, 2006; Miyano *et al.*, 2006), demonstrating a possible regulation of actin cytoskeleton by ROS. The actin binding protein cofilin is an actin depolymerising factor that plays an important role during lamellipodia formation (Lai *et al.*, 2008; Yap *et al.*, 2005). ROS have been suggested to activate cofilin during lamellipodia formation by stimulating the upstream regulators of cofilin, SSH and Slingshot 1L (SSHL1) proteins (Kim *et al.*, 2011; Kim *et al.*, 2009). ROS have also been found to be an important component of invadopodia (Diaz *et al.*, 2009; Gianni *et al.*, 2010a). As mentioned earlier, Nox participates in invadopodia-

induced cancer cell invasion in which ROS have been shown to play a critical role. Tyrosine phosphorylation of two Src substrates, tyrosine kinase substrates 4 and 5 (Tks4 and Tks5) regulate the Nox-induced ROS production. The ROS generated in turn modulate Src activation, suggesting the existence of a positive feedback mechanism which regulates invadopodial formation (Diaz *et al.*, 2009; Gianni *et al.*, 2010a).

ROS have been shown to directly oxidise monomeric (G-actin) as well as polymerized (F-actin) (DalleDonne *et al.*, 1995; DalleDonne *et al.*, 1999). Induction of ROS from integrin assembly has been shown to oxidise actin resulting in actin glutathionylation. The resulting oxidised actin leads to cell spreading via regulating the actin-mysoin contractility (Fiaschi *et al.*, 2006). Recently, a multidomain redox enzyme Molecule Interacting with CasL (Mical) has also been implicated in oxidising actin filaments leading to reduced actin polymerization (Giridharan *et al.*, 2012; Hung *et al.*, 2011).

Although studies have shown the ROS regulation of actin cytoskeleton, other studies have also evidenced that actin assembly may be required for the generation of ROS. Actin assembly has been shown to be required for O_2^- generation in eosinophils (Suzuki *et al.*, 2003). Nox activation requires the recruitment and assembly of its cytosolic components to the plasma membrane. Actin-binding proteins such as cortactin (Usatyuk *et al.*, 2007), WAVE1 (Wu *et al.*, 2003) and moesin, a member of the ezrin/radixin/moesin (ERM) family (Zhan *et al.*, 2004) have been shown to mediate the membrane translocation of the cytosolic component of Nox, p47 phox. In addition, actin cytoskeleton was shown to stabilise Nox protein complex at the plasma membrane by acting as a scaffold (Granfeldt & Dahlgren, 2001). The

involvement of actin and actin-associated proteins in ROS generation is largely understudied to date. As ROS and the actin cytoskeleton and its associated proteins play important roles in cell migration and invasion, understanding the interaction between them would provide better insights into the roles of ROS in cancer cell migration and invasion.

1.4 PURPOSE AND OBJECTIVES OF THE STUDY

Actin cytoskeletal proteins including gelsolin are known to regulate cancer cell invasion (Azuma *et al.*, 1998; Cunningham *et al.*, 1991; De Corte *et al.*, 2002; Van den Abbeele *et al.*, 2007). However, the mechanisms by which gelsolin promotes invasion have been poorly demonstrated. Interestingly, independent lines of studies have provided evidence that cancer cells generate increased levels of ROS as compared to their normal cell counterparts. It has been shown that the increase in ROS levels observed in cancer cells could transform cells to become more malignant and possess invasive phenotypes (Kumar *et al.*, 2008; Lee *et al.*, 1999a; Szatrowski & Nathan, 1991). Because ROS act as a second messenger in relaying signals and can interact with a number of other signalling molecules, ROS have been shown to promote tumourigenesis by amplification of tumour promoting signals (Wu, 2006b). Amongst the many molecules that ROS interacts with, ROS have been shown to directly oxidise actin that in turn contributes to cell spreading, for instance, by increasing the acto-myosin contractility (Fiaschi *et al.*, 2006). On the other hand, evidence also suggests the requirement of actin assembly for ROS generation (Suzuki *et al.*, 2003). It is therefore conceivable that actin or actin-binding proteins such as gelsolin be involved in ROS generation and contributes to cancer cell invasion. In an attempt to dissect the mechanisms by which gelsolin promotes cancer cell invasion, we propose that gelsolin may be an important factor that modulates intracellular ROS levels thereby enhancing invasion.

The aims of this project are:

1. To investigate whether gelsolin modulates intracellular ROS levels.
2. To investigate if gelsolin promotes cancer cell invasion by modulating ROS levels in cancer cells.
3. To determine if modulation of ROS levels by gelsolin influences extracellular matrix-degrading elements that promote invasion.
4. To dissect the mechanisms by which gelsolin modulates intracellular ROS levels.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1. Chemicals and Reagents

All chemicals and reagents were obtained from the following sources:

Abcam

- Mouse monoclonal anti-gelsolin (ab11081)

AIT Biotechnologies

Table 2.1. Real-time PCR primers

Nox1	forward	5'tatgaagtggctgtgctggt3'
	reverse	3'gaggttgtggtctgcacactg5'
Nox2	forward	5'tgttcagctatgaggtggtga3'
	reverse	3'tcagattggtggcgttattg5'
Nox4	forward	5'gctggaggcattggagtaac3'
	reverse	3'accaacggaaggactggata5'
Nox5	forward	5'actatctggctgcacattcg3'
	reverse	3'acactcctcgacagcctctt5'
Gapdh	forward	5'tcaagaaggtggtgaagcag3'
	reverse	3'tcgctgttgaagtcagagga5'

Amresco

- EDTA (0322)

Applied Biosystems

- Taqman PCR primers for Cu/Zn SOD (Hs00533490_m1), Mn SOD (Hs00167309_m1), Glulatredoxin 2 (Hs00375015_m1), Catalase (Hs00156308_m1), Antioxidant Protein 1 (Hs00187841_m1) and GAPDH (Hs99999905_m1)

BD Biosciences

- Mouse Monoclonal anti-MnSOD (611580), BD Matrigel basement membrabe matrix (354234)

BDH

- Sucrose (10274), Mannitol (29148)

Bioworld

- Dithiothreitol (DTT) (40400120)

Biorad

- Biorad protein assay dye reagent (500-0006), Coomassie brilliant blue dye (161-0400)

Calbiochem

- Amiloride Hydrochloride (2016-88-8)

Cell signalling technologies

- Mouse monoclonal anti-Cu/Zn SOD (4266), Rabbit polyclonal anti-VDAC (4866)

Enzo life Sciences

- SOD activity assay kit (ADI-900-157)

Fisher

- Glycine (G/0800/60)

Gibco

- Geneticin G418 (10131-027), Opti-MEM (31985)

Hyclone

- Fetal bovine serum (SV30160.03)

Invitrogen/ Molecular probes

- CM – H_2DCFDA (5-(and-6) - chloromethyl-2’,7’ – dichlorodihydro-fluorescein diacetate acetyl ester) (C6827), Mitosox Red probe (M36008), Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (A22188), Lipofectamine RNAiMAX transfection reagent (13778150), Lipofectamine 2000 (11668-019)

Merck

- Glycerol, Potassium hydroxide (KOH) (UN1813)

Olink Biosciences

- *In situ* Proximity Ligation Assay kit

PAA Laboratories

- Trypsin EDTA (L11-003)

Qiagen

- siRNA against Cu/Zn SOD (SI02623481)

QREC

- Potassium chloride (KCl) (7447407)

R&D Systems

- uPA ELISA kit (DY1310)

Roche

- Cell proliferation ELISA, BrdU kit (colorimetric) (11647229001), Complete Protease inhibitor cocktail tablets (05-872-988-001), Phosphatase inhibitor cocktail (04-906-837-001)

Santa Cruz Biotechnologies

- Rabbit polyclonal anti-copper chaperone for Cu/Zn SOD (sc-20141), Mouse monoclonal anti-GAPDH (sc-32233), Goat anti-rabbit IgG conjugated with HRP (sc-2004), Goat anti-mouse IgG conjugated with HRP (sc-2005), Protein A/G Plus Agarose Beads (sc-2003)

Sigma Aldrich

- Mouse monoclonal anti-β-actin (A2228), Diethylthiocarbamate (DDC) (71481), Dimethylsulfoxide (DMSO) (D2650), Diphenyleneiodonium chloride (DPI) (D2926), Bis-N-methylacridinium (Lucigenin) (M8010), Somatic ATP releasing reagent (FLSAR), Triton X-100, McCoy's 5A modified medium (M4892), RPMI 1640 (R4130),

Dulbecco's Modified Eagle's Medium (D1152), Sodium deoxycholate (D6750), Sodium bicarbonate ($NaHCO_3$) (S6297), Bovine serum albumin (A9418), Trypan blue dye (72-57-1), Pipes (P6757), Ammonium per sulfate (A3678), Crystal violet (C3886)

Thermo Scientific

- Super signal, West Dura, extended duration substrate (34076), stripping buffer (21059)

Vivantis

- Tris-Base (PR0612), 10X PBS (PB0344)

1st Base

- siRNA against gelsolin, biotechnology grade water (Buf1180), Tris-HCl (B10-1500), 10% SDS (Buf 2051)

2.1.2 Cell lines

HCT116, DLD-1, Caco-2 and RKO are human colon cancer cell lines obtained from the laboratories of Prof. Shing Chuan Hooi and Dr Richie Soong.

2.2 METHODS

2.2.1 Cell culture

HCT116 was cultured in McCoy's 5A modified medium (Sigma-Aldrich); DLD-1 was cultured in RPMI 1640 (R4130, Sigma-Aldrich) and RKO was cultured in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich). All media were supplemented with 10% fetal bovine serum (FBS) (Hyclone). Stable HCT116 cell lines overexpressing gelsolin C1 and C8 as well as empty vector control cells were grown in McCoy's 5A medium with 500μg/mL Geneticin G418 (Gibco). All cell lines were maintained at 37°C in a humidified incubator supplemented with 5% CO_2.

2.2.2 DNA transfection

Stable transfection

Stable cell lines overexpressing gelsolin have previously been derived from HCT116 cells in our laboratory (Zhuo *et al.*, 2012). Briefly, gelsolin cDNA was inserted into a plasmid encoding enhanced green fluorescence protein (EGFP) as well as Kanamycin resistance genes. The plasmids were then transfected to HCT116 cells and subjected to antibiotic Genecitin (G418) selection. Vector control cells were prepared by transfecting a plasmid encoding EGFP without gelsolin cDNA.

2.2.3 siRNA Transfection

The following siRNA duplex oligoribonucleotide were used for silencing target gene expressions:

- siRNA gelsolin – target sequence
  AAACGUCCAAUCUUGUUGGAGCAGG (Invitrogen)
- siRNA Cu/Zn SOD –target sequence
  CCATGCAGGTCCTCACTTTA (Qiagen)

10nM of stealth siRNA duplex oligoribonucleotide were used to silence gelsolin and Cu/Zn SOD. The siRNA complex was prepared by combining the desired amount of siRNA with Lipofectamine RNAiMAX transfection reagent (Invitrogen) in Opti-MEM reduced serum media (Gibco). The siRNA complex was incubated for 20 minutes at room temperature before transfection. The siRNA complex was then added to the cells in a drop-wise manner and incubated for four hours at 37 ℃. After the incubation period, transfection media were removed and replaced with complete media supplemented with 10% FBS. Non-targeting siRNA control with medium GC content (Invitrogen) was used as a negative control for gelsolin and All star negative control siRNA (Qiagen) was used as control for Cu/Zn SOD.

2.2.4 ROS measurements

2.2.4.1 Lucigenin Assay

Total intracellular superoxide (O_2^-) was measured using the chemiluminescence-based lucigenin method as described (Clement & Stamenkovic, 1996). Briefly, cells were cultured in complete media under

normal growth conditions. Cells were then harvested and cell pellets were lysed with 420 µL of ATP-releasing agent. 400 µL of lysate was immediately transferred to a glass tube chemiluminescence was monitored using a Berthold Sirius Luminometer. Data were described as Relative Light Units/second/milligram of protein (RLU/s/mg protein). The remaining 20 µL lysate was used for Bradford Protein assay and used for normalizing RLU reading.

2.2.4.2 Mitosox Red staining

Mitochondrial O_2^- was detected using Mitosox red, a fluorescent probe targeted to the mitochondria and highly susceptible to oxidation by O_2^-. Cells were stained with Mitosox Red probe with a final concentration of 5 µM at 37 ℃ for 30 minutes. Excess dye was washed off with 1X PBS. Cell pellet was then resuspended in serum free media and measurement was immediately carried out with flow cytometry at PE-Texas Red (510nm excitation) channel. Data were analysed using the Summit software 4.3.

2.2.4.3 CM-H_2DCFDA staining

General ROS were assessed using CM-H_2DCFDA probe. Cells were stained with CM-H_2DCFDA probe with a final concentration of 10 µM at 37 ℃ for 30 minutes. Excess dye was washed off with 1X PBS. Cell pellet was then resuspended in serum free media and measurement was immediately carried out with flow cytometry at FL-1 (488nm excitation) channel. Data were analysed using the Cell Quest Pro software.

2.2.4.4 Amplex Red

Cells were harvested and whole cell lyatse was prepared using Triton X-100-based lysis buffer. 20 μL of cell lysate was dispensed in a well of 96-well plate (black plate) and Amplex Red reagent mix was added to the samples. Fluorometric reading was immediately determined using Varioskan fluorometric reader. Data was normalized to protein concentration.

2.2.5 Trypan Blue Exclusion

$4x10^5$ Cells were seeded in a 12-well plate with or without without 5 μM Diphenyleneiodonium (DPI) in medium containing 1% FBS for 24 hours. Cells were detached from the wells and appropriate dilutions were made for cell counting. The Trypan Blue dye and the diluted cell suspension was then with mixed in equal ratio. 10 μL of this mixture was loaded onto a haemocytometer and the number of cells that did not take up the dye were counted.

2.2.6 Matrigel Invasion Assay

Matrigel invasion assay was performed using the BD Matrigel Basement Membrane Matrix (BD Biosciences). Matrigel was thawed on ice overnight and cells for invasion were serum starved overnight in serum free media. 40 μL of diluted matrigel (0.33mg/mL, diluted in chilled serum free media) was coated on 8μm pore size 6.5 mm diameter transwell filter membrane (Corning Costar). The matrigel was allowed to polymerise at 37°C for 2 hours. 2×10^5 cells with or without 5 μM DPI were seeded on the upper chamber of the transwell on top of the matrigel layer. Complete media containing 10% FBS was added to the lower chamber as a chemoattractant.

24 hours after incubation at 37°C, cells on the upper chamber were removed by cotton swabs while cells that have invaded through the matrigel and come out on the under-side of the membrane were fixed with 70% ethanol for 20 minutes at room temperature and stained with 0.2% crystal violet for 30 minutes at room temperature for visualisation. Invaded cells were captured using Canon powershot A640 camera at 20X magnification for counting and 2.5X magnification was used to represent the distribution of invaded cells in the entire well. 10 representative fields were captured per membrane and the numbers of invaded cells were manually counted and quantified using Metamorph or ImageJ softwares.

2.2.7 Zymographic Assay

Enzymatic activities of secreted uPA were determined by fibrin-plasminogen zymography. Cells were grown in serum-free medium with or without DPI for the desired time-points. The conditioned media were normalised using cellular lysate and then combined with non-reducing 5X sample loading buffer (0.2M Tris-HCL pH 6.8, 30% Glycerol, 10% SDS and 0.05% Bromophenol blue) and loaded onto 12% SDS-PAGE gel containing 730 μg/mL of human fibrinogen (Sigma-aldrich) and 20 μg/mL human plasminogen (Sigma-aldrich). After electrophoresis, gels were rinsed with distilled water twice and washed twice with chilled wash buffer (2.5% Triton X-100 and 50mM Tris-HCl pH 8.0) for 25 minutes with agitation. The gels were then incubation at 37°C with incubation buffer (0.1M glycine buffer, pH 8.0) for 16 hours with gentle agitation. The gels were stained with Coomassie blue (0.05% Coomassie dye, 40% methanol, 10% acetic acid) for 2 hours at

room temperature, and destained with de-staining solution (30% Methanol and 10% acetic acid) for 2 hours. Areas of lysis appear as zones of clearance against the Coomassie blue-stained background. Gels were placed on top of a light box and gel pictures were captured. Gel pictures were further processed using Adobe Photoshop CS6.

2.2.8 Bio-Rad Protein Assay

Protein concentration was determined using the Bio-Rad Protein dye reagent, using Bovine serum albumin (BSA) (sigma) as the standard. One part of the dye reagent was diluted with four parts of water and filtered. 10 μL of protein sample diluted at 10-30X were incubated with 200 μL of diluted dye reagents in a 96-well plate at room temperature for 5 minutes. Absorbance was measured at 595nm using an ELISA plate reader.

2.2.9 Superoxide dismutase (SOD) activity Assay

SOD activity was measured using the calorimetric based SOD activity kit from ENZO Life Sciences (ADI-900-157).

Principle

Superoxide (O_2^-) is generated by the oxidation of xanthine by xanthine oxidase. O_2^- can reduce the tetrazolium salt WST-1 to WST-1 formazon, which gives a coloured product that is detectable at 450nm. SOD in the sample dismutased O_2^- generated, thereby reducing the conversion rate of WST-1 to WST-1-formazan. Therefore, reduction of WST-1-formazan is a measure of SOD activity in the samples where the coluoration observed is inversely proportional to the SOD activity (Enzo SOD activity kit).

Protocol

2 x 10^6 cells were washed with 1X PBS. Cells were detached by gentle trypsinization and transferred to a 15mL falcon tube and centrifuged at 1500 rpm for 5 minutes. Supernatants were discarded and cell pellets were washed with 1mL of ice-cold 1X PBS and transferred to 1.5mL Axygen microtubes on ice. Cells were again centrifuged and supernatant was discarded. Cells were suspended in 100 μL of cold 1X Cell Extraction Buffer (Triton x-100, SOD buffer, and protease inhibitor). Cell suspensions were incubated on ice for 30 minutes with periodic vortexing (every 10 minutes). The disrupted cell suspensions were micro centrifuged at 10,000g for 10 minutes at 4℃ to remove insoluble material. The supernatants were recovered in a fresh tube pre-chilled on ice. Protein concentration of the cleared cell lysate was determined using Bradford Protein assay. Cell lysates were either stored in small aliquotes at -80℃ or immediately used for assaying SOD activity. 20 μg protein was used for per well which was diluted with distilled water to make up to a volume of 25μL. Each sample was pipetted in triplicate to a clear bottom 96-well plate. SOD standards from 0 Unit (buffer alone) to 20 Units were prepared by diluting purified SOD protein in SOD buffer, and 25μL of each standard unit was pipette in duplicate to the bottom of the well. 150μL of Master Mix (WST-1 and Xanthine oxidase) was pipetted into each well using a multichannel pipette. The reaction was then initiated by adding 25μL of 1x Xanthine Solution to all the wells using a multichannel pipet. The plate was immediately transferred to a microtiter plate reader and absorbance reading was obtained at 450nm every minute for 10 minutes at room temperature.

2.2.10 Western Blotting

2.2.10.1 Whole cell lysate preparation

Cells were harvested by gentle trypsinisation. Cell were washed once with chilled 1X PBS and pelleted followed by lysis with 1X radioimmunoprecipitation assay (RIPA) buffer (50mL Tris-HCL pH 8.0, 150mM NaCl, 0.5% Sodium deoxycholate and 1% NP-40) supplemented with protease inhibitors (Roche Complete protease inhibitor cocktail). Lysis was carried out by gently rocking the tubes at 4℃ for 30 minutes and microcentrifuged at 14,000g for 12 minutes at 4℃ to remove insoluble material. The supernatants were recovered in a fresh tube pre-chilled on ice. Cell lysates were aliquoted and stored at -20℃. Protein concentration was determined using Bradford Protein Assay. Equal amount of samples were loaded into each well for Western blot analysis. Housekeeping protein, β–actin or GAPDH were used to assess equal loading.

2.2.10.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gel electrophoresis was carried out using the Bio-Rad Powerpack system. The resolving gels used in the study (10, 12 and 15%) were prepared with 1.5M Tris HCl, pH 8.8, 10% SDS, 30% bis - acrylamide, and were polymerised by adding ammonium persulphate (APS) (the source of free radical) and N, N, N', N'- tetramethyl – ethylenediamine (TEMED) (the catalyst that catalyses the decomposition of the persulphate ion to generate free radical). A 4% stacking gel was used and was prepared with 1.0M Tris – HCl, pH 6.8, 10% SDS (w/v), 30% bis - acrylamide and polymerised with

APD and TEMED. For routine protein gel electrophoresis, protein samples were mixed with ¼ parts of 5X SDS sample loading buffer and were heated at 95 ℃ for 5 min before loading into the gels. For non-reducing gel electrophoresis (Zymography), protein samples were mixed with SDS sample loading buffer without the reducing agent. Protein samples were heated at 95 ℃ for 5 minutes before loading into the gel. Electrophoresis was carried out as described above.

SDS – PAGE resolved proteins were transferred onto activated polyvinylidene difluoride (PVDF) membrane (dipped in 100% methanol for 15 seconds followed by incubation in distilled water for 2 minutes with agitation at room temperature. Electroblotting was conducted using the Bio-Rad Powerpac system at 350mA for 1 hour at 4 ℃.

2.2.10.3 Immunodetection

After transfer, PVDF membrane was incubated with blocking buffer, 5% w/v milk (Blocking grade milk, Bio-Rad) in 1X TBST (20mM Tris – HCl, pH 7.6, 137mM NaCl, 0.1% Tween 20) for 1 hour at room temperature. After blocking, the membrane was incubated with the relevant primary antibodies (diluted in 5% milk w/v in 1X TBST or 5% BSA w/v in 1X TBST) and incubated overnight at 4 ℃ with gentle shaking. Unbound primary antibodies were washed off with 1X TBST (repeated 3 times, 7 minutes each with vigorous shaking). The membrane was incubated further with the respective horse radish peroxidase conjugated secondary antibodies in 5% milk with gentle shaking for 1 hour at room temperature. Unbound secondary antibodies were again washed off with 1x TBST (repeated 3 times, 7 minutes each with

vigorous shaking). The resulting immunocomplexes formed were detected using chemiluminescence substrate (Thermo Scientific, West Dura, Super signal extended duration substrate). For re-probing of the same membrane for different proteins, the membrane was incubated with stripping buffer for 5 minutes at room temperature. Membranes were rinsed once with 1X PBST for 5 minutes with agitation at room temperature. Stripped membranes were re-probed with the respective primary and secondary antibodies as mentioned.

2.2.11 Immunoprecipitation

Cells were harvested from 70-80% confluent 10cm plates. Plates were washed with chilled 1X PBS for 3 times, and cells were lysed on the plate using 80 μL of modified RIPA buffer (5M Nacl, 1M Tris-HCL, pH 8.0, 0.5M EDTA, 1% Triton X-100, 0.1% Sodium deoxycholate and protease inhibitors). Cells were then detached from the plate by gently scraping and detached cells were then transferred to a pre-chilled 1.5mL eppendorf tubes. Lysis was carried out by incubating the tube on a 360 °rotator at 4 ℃ for 30 mins and the cleared lysate was obtained by centrifugation at 10,000g at 4 ℃ for 10 minutes. 500 μg of the protein lysate was then subjected to pre-clearing.

Pre-clearing

500 μg of lysate was mixed with 20 μL of beads (Protein A/G PLUS Agarose Beads, Santa cruz) in a 1.5mL eppendorf tube. The volume was adjusted to 150 μL with the modified RIPA buffer and incubated on a 360 ° rotator at 4 ℃ for 1 hour. Pre-cleared lysate was then obtained by spinning down at 10,000g at 4 ℃ for 10 minutes. The supernatant that contains the pre-cleared lysate was retained and mixed with 20 μL of beads that have been

conjugated with 2 μg of primary antibodies (Gelsolin or Cu/Zn SOD) or 2 μg of Mouse IgG control antibody (used as a negative control).

Beads-antibody conjugation

20 μL of beads was mixed with 2 μg of primary antibodies and volume was made up to 150 μL with RIPA buffer and incubated on a rocker at 4 ℃ for 4 hours. Beads conjugated with antibody were obtained by spinning down 10,000g at 4 ℃ for 10 minutes.

The pre-cleared lysate and the antibody conjugated to the beads were combined in a 1.5mL epperdorf tube. The final volume was adjusted to 500 μL with the modified RIPA buffer and the mixture was incubated on a 360 ° rotator at 4 ℃ overnight. The pulled down material was then washed for four times using the modified RIPA buffer. After the final wash, the beads containing the pulled down material was boiled with sample loading buffer and spun down at 10,000g for 2 minutes. The supernatant was loaded onto a SDS-PAGE gel.

2.2.12 Isolation of intact mitochondria and cytosolic fraction

Cells were grown to 70% confluence in two 10cms tissue culture dishes to obtained approximately 25x106 cells. The dishes were washed twice with 10mL of cold 1xPBS. 150 μL of Mitochondrial extraction buffer (200mM mannitol, 68mM sucrose, 50mM Pipes-KOH (pH 7.4), 50mM KCl, 5mM EGTA, 2mM MgCl2 and1mM dithiothreitol), containing protease inhibitors (protease inhibitor cocktail, ROCHE) and was added to the dishes and cells were detached by scraping. The detached cells were transferred to 1.5mL Axygen tubes and incubated on ice for 20 minutes. After incubation,

the cells were homogenized with a dounce homogenizer and passaged for 30 – 40 strokes followed by centrifugation at 300 g for 10 minutes at 4 ℃. The supernatant was transferred to a fresh 1.5mL Axygen tubes and centrifuged at 12000g for 30 min at 4 ℃. The pellet contains the intact mitochondrial fraction. The supernatant was again centrifuged at 25,000g for 45 minutes to obtain a clear supernatant which contains the cytosolic fraction.

2.2.13 Sandwiched Enzyme-linked immunosorbent assay (ELISA)

Cells were plated at a density of 3 x 10^5 well in complete media in a 12 well-plate format. Cells were serum starved using serum free media with or without 5 μM DPI for the desired time-points. Conditioned media as well as cell lysates were harvested and stored at -20°C until analysis by ELISA. Amount of supernatants to be used for ELISA analysis was quantified by using an inverse ratio from the intracellular protein concentration (determined from the harvested lysate using Bradford Protein Assay). ELISA was performed following standard sandwiched ELISA methods using the uPA DuoSet ELISA kit (R&D Systems). A day before the experiment, ELISA plates were coated 100 μL capture antibody, plates were sealed and incubated overnight. Next day, wells were washed thoroughly (3-5 times) with 400 μL of wash buffer, followed by blocking the plates with 300 μL Reagent Diluent (R&D Systems) for 1 hour at room temperature. Wells were washed 3 times with 1X wash buffer (0.05% PBST). 100 μL of samples were dispensed to the wells and incubated for 2 hours at room temperature. Washing step was repeated for three more times. 100 μL of a working dilution of Streptavidin-HRP was added to each well; plates were covered and incubated for 20

minutes at room temperature. This was followed by adding 100μL of substrate solution and again incubated for 20 minutes at room temperature. The reaction was stopped by adding 50μL of stop solution. The optical density of each well was measured immediately, using ASYS UVM 340 microplate reader at 450nm, using 570nm as reference wavelength.

2.2.14 Real-time PCR

2.2.14.1 RNA Extraction

Total RNA was isolated from cells using RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. Samples were lysed and homogenised by passing through a 20-gauge needle for twenty times. Ethanol was added in a 1:1 ratio to provide ideal binding conditions before the lysates were loaded onto the silica membrane column. The column was washed a few times using washing buffer to remove contaminants. RNA was then eluted in 30-50μl of RNase-free water. Eluted RNA was treated with DNase to avoid DNA contamination. The RNA concentration and quality was determined using NanoDrop ND-1000 spectrophotometer. All RNA samples were stored at -80°C.

2.2.14.2 cDNA synthesis

RNA samples were converted to cDNA for Real-time PCR applications. 6μg of total RNA was converted to cDNA per 20μL reaction using ImProm-II Reverse Transcription System reagents (Promega). Oligo(dT) primer (2μg RNA in 1μL Oligo (dt)) was added to RNA in nuclease-free water. The reaction mix was heated at 70°C for 5 minutes for

target RNA and primer combination. This reaction mix was immediately chilled on ice for 5 minutes before adding reverse transcription reaction mix (ImProm-II5X Reaction Buffer, 3mM MgCl2, 0.5mM dNTP Mix, 20 units of Recombinant RNasein Ribonuclease Inhibitor and ImProm-II Reverse Transcriptase). The reaction mix was then incubated at 25°C for 5 minutes, 42°C for 50 minutes for annealing and extension step respectively. A no-template (RNA) control was included. The cDNAs were stored at -20°C until Real-time PCR analysis.

2.2.14.3 Real-Time PCR

Real-time PCR was performed using two platforms.

1. **ABI 7500 Fast Real-time PCR system**: Real-time PCR carried out on the ABI 7500 Fast Real-time PCR system. cDNA was added to a custom Taqman Gene Expression Assay comprising a primer and Taqman probe, and combined with Taqman Universal PCR Master Mix (ABI) according to manufacturer's instructions. The thermal cycling conditions were as follows: one cycle at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing extension at 60°C for 1 minute. The primers used include Cu/Zn SOD, (Hs00533490_m1), Mn SOD (Hs00167309_m1), Glulatredoxin 2 (Hs00375015_m1), Catalase (Hs00156308_m1), Antioxidant Protein 1 (Hs00187841_m1) and GAPDH (Hs99999905_m1) was included as internal control.
2. **Roche- LightCycler 480 system**: cDNAs was diluted with nuclease free water (1:3) and combined with LightCycler 480 SYBR Green

Master Mix. The thermal cycling conditions were as follows: one cycle at 95°C for 15 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds and annealing extension at 60°C for 30 seconds. Realtime PCR primers for Nox1, Nox2, Nox4, Nox5 and GAPDH were designed using Primer3 software (for sequence, refer to Table 2.1). All primers used here are obtained from AIT biotechnologies.

2.2.15 I*n situ* Proximity Ligation Assay

In situ Proximity Ligation Assay was performed using the 563 Duolink detection kit (OLINK, Uppsala, Sweden) according to manufacturer's instructions. Briefly, 1 x 10^5 cells were grown on a 12-cm coverslip for 24-36 hours. Cells were fixed with 4% parafolrmaldehyde for 20 mins followed by blocking using 5% BSA for 1 hour at room temperature. Cells were then incubated with primiary antibodies against gelsolin (1:200 dilution) and Cu/Zn SOD (1:400 dilution) at 4°C overnight. Primary antibodies were washed for 2x using the wash buffer A (provided in the kit). The cells were incubated with the PLA probes (secondary antibodies linked to oligonucleotides) at 37°C for 1 hour. PLA probes were washed off using wash buffer A for 2x. The oligonucleotides were then ligated using the ligation reaction mixture at 37°C for 30 minutes. After washing off the excess ligation mixture, ligated product was amplified using the red fluorescent amplification reagent at 37°C for 100 minutes. Cells were washed with Wash buffer B (provided in the kit) for 3x and the coverslip was mounted onto a glass slide using mounting media (provided in the kit) containing DAPI to counterstain the nuclei. Images were

captured using). Images were captured using Olympus DP72 microscope and cellSens software at 60x magnification.

2.2.16 Microscopy

Cell counting and images for Invasion assay were obtained using Carl Zeiss Axiovert 40 CFL - Inverted Microscope and images were processed Image J and Adobe photoshop CS6. Visualisation and images for the I*n situ* Proximity Ligation Assay were processed using Olympus DP72 microscope and cellSens software.

2.2.17 Statistical analysis

All statistical analyses were performed using a two tailed Student's t-test. Differences between sample means were considered statistically significant when p value < 0.05.

RESULTS

CHAPTER 3

CHAPTER 3 GELSOLIN PROMOTES COLON CANCER CELL INVASION VIA MODULATING INTRACELLULAR LEVELS OF SUPEROXIDE (O_2^-) SPECIES

3.1 BACKGROUND

Increased gelsolin expression in tissues positively correlates with invasive phenotype in many cancer types (Gay *et al.*, 2008; Rao *et al.*, 2002; Shieh *et al.*, 2006; Shieh *et al.*, 1999; Thompson *et al.*, 2007; Thor *et al.*, 2001). This correlation has been confirmed by several investigators using both cell lines and animal models. Many of the studies on the roles of gelsolin in cell migration and invasion have been linked to its calcium-regulated functions in actin dynamics, for instance, transfection of gelsolin in the colon cancer cell line LS180 resulted in increased migration by enhancing actin filament turnover (Litwin *et al.*, 2012). Downregulation of gelsolin was shown to counteract the invasive phenotypes of the breast cancer cell MDA-MB 231 as well as the cervical cancer cell line Hela (Van den Abbeele *et al.*, 2007). However, the mechanisms by which gelsolin promotes cancer cell invasion is not fully understood.

ROS play important roles as second messengers in migratory and invasive signalling pathways. When generated in response to growth factor stimulation of cancer cells, ROS have been shown to trigger migration and invasion signalling pathways (Wu, 2006a). ROS are involved the activation of MAPK pathway and transcriptional activation of genes involved in invasion such as MMPs and uPAs (Tochhawng *et al.*, 2013). ROS have also been suggested to directly interact and activate MMP via oxidation in nutrophils

(Saari *et al.*, 1992). Increased levels and activation of these proteases enhance invasion by digesting the ECM and paves the way for invading cells. In this way, ROS play an important role in facilitating the invasive process (Tochhawng *et al.*, 2013).

Although a number of studies have shown the downstream targets of ROS in invasion, the upstream regulators of ROS in cancer cell invasion is incompletely understood. Furthermore, how different molecules coordinate and interact with each to promote cancer cell invasion is still unclear. Therefore, it is necessary to delineate ROS regulation as well as its downstream targets. In this study we propose that gelsolin may act as an upstream molecule that modulates intracellular redox status and thereby enhance cancer cell invasion.

3.2 OBJECTIVES

To investigate the mechanisms by which gelsolin promotes invasion, we have the following objectives:

1. To determine whether gelsolin expression modulates ROS levels, and to identify the dominant ROS species involved.
2. To demonstrate if ROS contributes to gelsolin-induced cell invasion.
3. To determine whether alteration of the intracellular redox state by gelsolin contributes to upregulation of extracellular matrix-degrading enzymes.

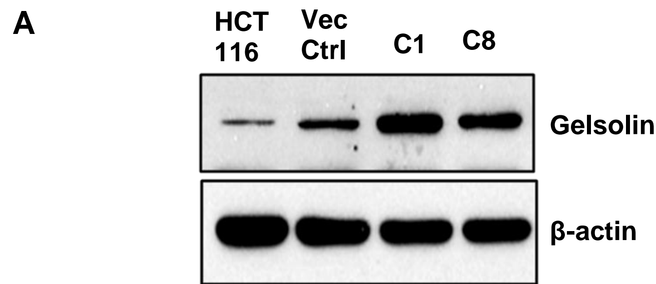
Study model

The study was conducted using a gain-of-function model by gelsolin overexpression, as well as loss-of-function model using siRNA-mediated knockdown of gelsolin. Our laboratory has previously derived stable clones that overexpress gelsolin from the colorectal cancer cell line HCT116 (Zhuo *et al.*, 2012). Two gelsolin-overexpressing stable clones, namely C1 and C8 were compared against the empty vector control and wild-type HCT116 cells. Loss-of-function studies were performed using siRNA-mediated knockdown of endogenous gelsolin in the colorectal cancer cell lines HCT116, RKO, Caco-2 and DLD-1. Gelsolin siRNA-treated cells were compared with the control siRNA-treated cells.

3.3 RESULTS

3.3.1 Gelsolin expression modulates total intracellular superoxide (O_2^-) levels

In order to characterise the ROS species modulated by gelsolin expression, the levels of total intracellular O_2^- were first assessed using the chemiluminescence-based Lucigenin assay (as described under materials & methods). Two gelsolin-overexpressing HCT116 clones C1 and C8 were compared with the empty vector control and wild-type HCT116 cells. Overexpression of gelsolin was confirmed by western blot as shown in figure 3.1A. Under normal growth conditions in complete media (McCoy 5A with 10% FBS), total O_2^- levels were elevated by three-fold in gelsolin-overexpression C1 and C8 cells when compared to the empty vector control and wild-type HCT116 cells (Fig.3.1B). We also investigated the role of endogenous gelsolin in modulating O_2^- levels by siRNA-mediated knockdown of gelsolin in HCT116 and RKO cells. Knockdown of gelsolin is evident from the western blot as shown in figure 3.1C. When gelsolin was silenced, the intracellular O_2^- levels also dropped significantly (Fig.3.1D). These data indicate that gelsolin is important in modulating intracellular O_2^- levels.



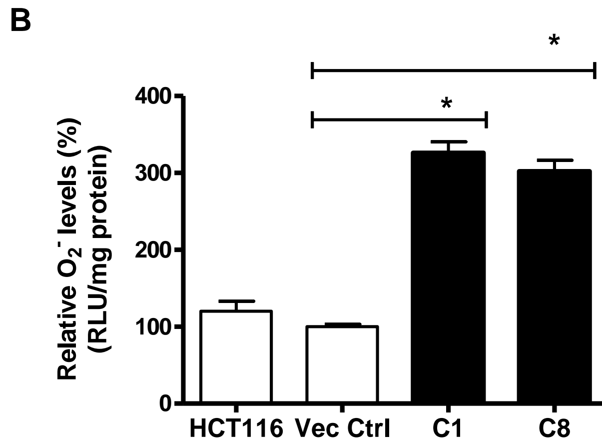


Figure 3.1A&B. Gelsolin overexpression increases total intracellular superoxide (O_2^-) levels. (A) Western blot showing overexpression of gelsolin. (B) Gelsolin overexpression increases total intracellular O_2^- levels in HCT116 cells: Total intracellular O_2^- levels was measured by the chemiluminescent-based lucigenin assay. Gelsolin-overexpressing cells show significantly higher levels of O_2^- compared to the vector control and wild-type HCT116 cells. Data shown are mean ± SD of at least three independent experiments. *p-value <0.05 (Two tailed Student's t- test).

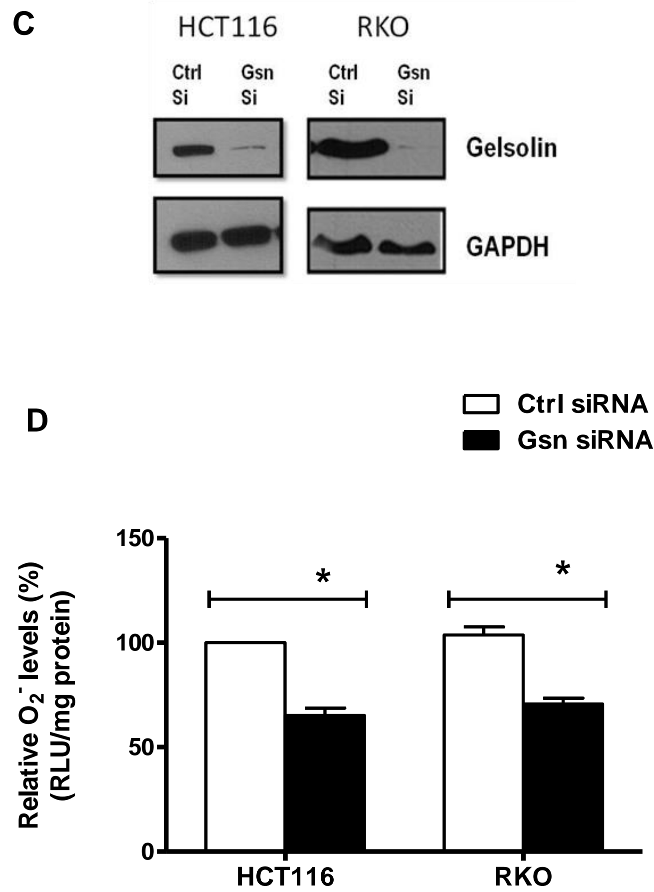


Figure 3.1C&D. Gelsolin knockdown decreases total intracellular superoxide (O_2^-) levels. (C) Western blot showing gelsolin knockdown in HCT116 and RKO cells. (D) siRNA knockdown of gelsolin in HCT116 and RKO cells results in decreased levels of O_2^-. Data shown are mean ± SD of at least three independent experiments. *p-value <0.05 (Two tailed Student's t- test).

3.3.2 Gelsolin expression influences mitochondrial superoxide (O_2^-) levels

As mitochondria are the major sources of ROS in the cell, the contribution of mitochondrial O_2^- to the increase in total intracellular O_2^- levels following gelsolin overexpression was analysed. Mitosox red dye was used to detect intra-mitochondrial O_2^-. Cells were treated with Mitosox red probe, and mitochondrial O_2^- levels were measured by flow cytometry. Gelsolin overexpressing cells C1 and C8 were found to exhibit higher mitochondrial O_2^- levels when compared to the empty vector control and wild-type HCT116 cells (Fig. 3.2A). This is observed by a strong intensity of Mitosox red dye indicated by the shift in the histogram to the right. Consistent with this observation, knockdown of endogenous gelsolin in wild-type HCT116 and RKO cells reduced O_2^- levels as indicated by the shift in the histogram to the left (Fig. 3.2B&C). The efficiency of gelsolin knockdown in HCT116 and RKO cells were analysed by Western blot (Fig. 3.2D). Thus, from our results, we observe a positive correlation between gelsolin expression and intra-mitochondrial O_2^- levels.

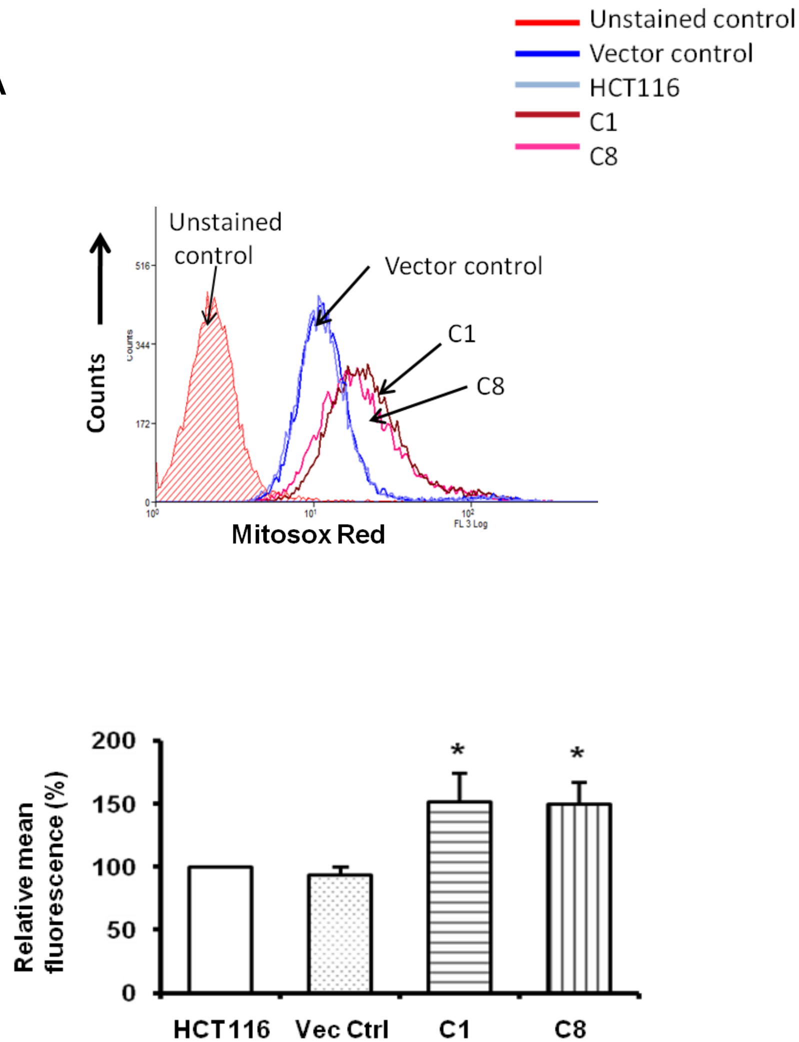


Figure 3.2A. Overexpression of gelsolin expression elevates mitochondrial superoxide (O_2^-) levels. (A) Overexpression of gelsolin showed higher mitochondrial O_2^- levels as compared to the empty vector control and wild-type HCT116 cells. **Upper panel**, histogram showing intensity of Mitosox Red dye measured using the PE-Texas Red log channel (shift towards right indicates higher intensity). **Lower panel**, bar charts showing mean fluorescence intensity of Mitosox red dye. Data shown are mean ± SD of at least three independent experiments. *p-value <0.05 (Two tailed Student's t- test).

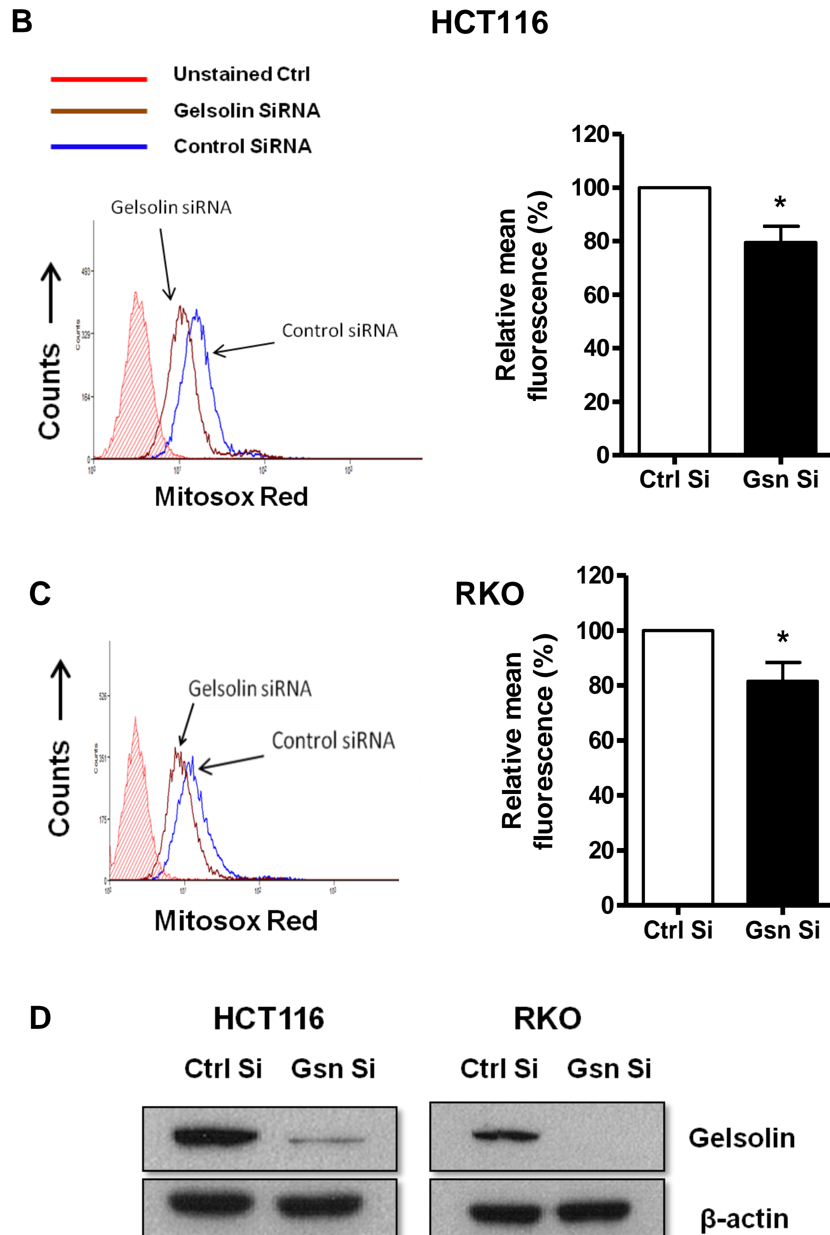


Figure 3.2B-D. Knockdown of gelsolin reduces mitochondrial superoxide (O_2^-) levels. Gelsolin was knocked down in HCT116 and RKO cells and mitochondrial O_2^- was measured by Mitosox red dye measured on a PE-Texas Red log scale. **Left panel**, histogram showing mitosox red staining in HCT116 and RKO following geloslin knockdown. Knockdown of gelsolin results in reduced O_2^- levels shown by a lower intensity of mitosox staining measured using the PE-Texas Red log channel (indicated by the left shift in the histogram). **Right panel**, bar charts showing mean fluorescence intensity of Mitosox Red dye. (D) Western blot analysis confirms the efficiency of gelsolin knockdown in HCT116 and RKO cells. Data shown are mean ± SD of at least three independent experiments. *p-value <0.05 (Two tailed Student's t- test).

3.3.3 Gelsolin expression does not affect the levels of other ROS species (H_2O_2, $^{\cdot}OH$ and HOCl)

As ROS consist of several species, we investigated whether gelsolin influence the intracellular levels of other species of ROS such as H_2O_2,$^{\cdot}OH$ and HOCl. An 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) oxidation assay was performed to examine if other ROS species are altered by gelsolin. Several ROS including H_2O_2,$^{\cdot}OH$ and HOCl can cause CM-H_2DCFDA oxidation (Myhre *et al.*, 2003; Royall & Ischiropoulos, 1993; Scott *et al.*, 1988; Zhu *et al.*, 1994). Gelsolin was silenced in different colon cancer cell lines including HCT116, RKO, DLD-1 and Caco-2 followed by CM-H_2DCFDA staining and green fluorescent intensity at FL-1 channel was assessed by flow cytometry. We observed no significant change in CM-H_2DCFDA intensity when gelsolin was silenced in these cell lines compared with the respective control siRNAs (Fig. 3.3A & B).

In addition, the Amplex Red hydrogen peroxide assay was used to detect H_2O_2 levels in gelsolin-overexpressing cells. The Amplex Red reagent reacts with H_2O_2 in a 1:1 stoichiometry. The resulting oxidation product, red fluorescent resorfin was assayed fluorometrically. As shown in figure 3.3C, no difference was observed in H_2O_2 levels when gelsolin expression was increased. These data suggest that gelsolin overexpression specifically modulates O_2^- levels and not the other species ROS.

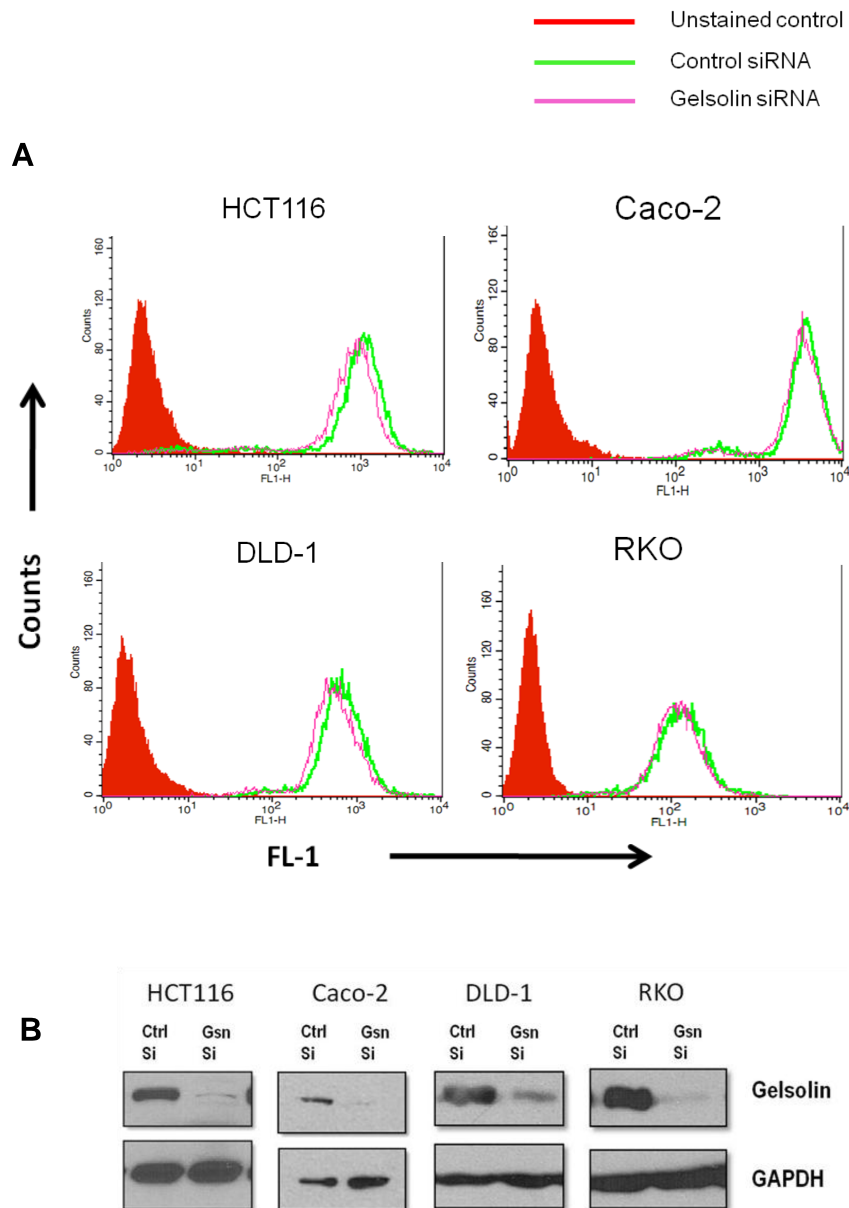


Figure 3.3A&B. Gelsolin knockdown does not affect the levels of other ROS (H_2O_2,$^{\cdot}$OH and HOCl). (A) Gelsolin was knocked down in HCT116, Caco-2, DLD-1 and RKO colorectal cancer cell lines and ROS levels were compared between gelsolin knockdown cells and control siRNA-treated cells using CM-H_2DCFDA probe. Histogram showing the intensity of CM-H_2DCFDA staining measured at FL-1 channel. (B) Western blots showing the efficiency of gelsolin knockdown in HCT116, Caco-2, DLD-1 and RKO cells. GAPDH was used as an internal loading control.

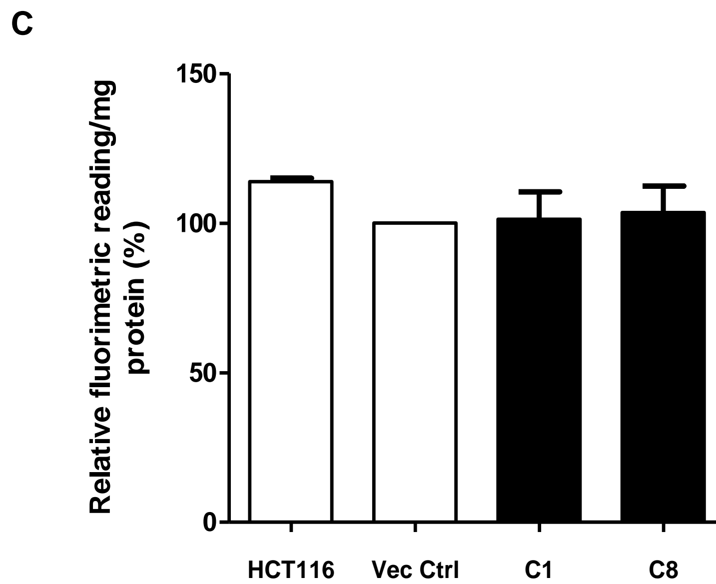
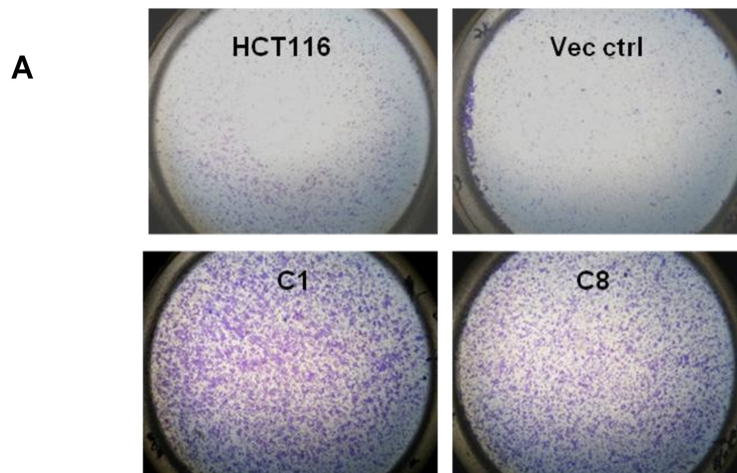


Figure 3.3C. Overexpression of gelsolin expression does not alter H_2O_2 levels. Amplex Red was used to assess H_2O_2 levels in gelsolin-overexpressing cells. Bar charts showing the relative fluorimetric reading of the Amplex Red dye. Data shown are mean ±SD of at least three independent experiments.

3.3.4 Gelsolin overexpression promotes invasion of HCT116 cells

To evaluate the role of increased gelsolin expression in cancer cell invasion, transwell invasion assay was performed. Gelsolin-overexpressing cells as well as empty vector control and wild-type HCT116 cells were allowed to invade through a matrigel matrix and 10% FBS containing media was used as a chemoattractant. Briefly, cells were suspended in serum-free media and seeded on the upper chamber. Cells were then allowed to invade for 24 hours towards the lower chamber that contains media with 10% FBS. Invaded cells were then fixed with 70% ethanol and stained with crystal violet and counted using Metamorph software. Gelsolin-overexpressing C1 and C8 cells were found to have enhanced invasion capacity when compared to the empty vector and wild-type HCT116 cells (Fig. 3.4A). Consistent with this, gelsolin knockdown results in a significant decrease in invasion of HCT116 cells (Fig. 3.4B). The efficiency of gelsolin knockdown in HCT116 cells were assessed by Western blot (Fig. 3.4C).

A



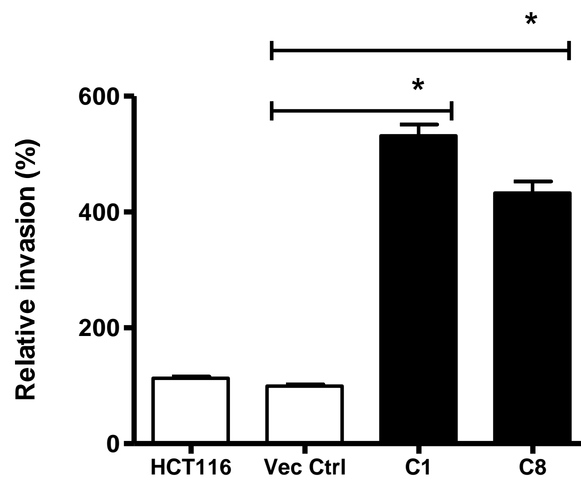


Figure 3.4A. Gelsolin overexpression enhances cell invasion. Cells with gelsolin overexpression (C1 & C8) have higher invasive capabilities when compared to the empty vector control and wild-type HCT116 cells. **Upper panel**, representative pictures of invaded cells are shown (2.5X magnification of the entire well). **Lower panel**, bar charts showing the quantification of invaded cell numbers. Data shown are mean ± SD of at least three independent experiments. *p-value <0.05 (Two tailed Student's t- test).

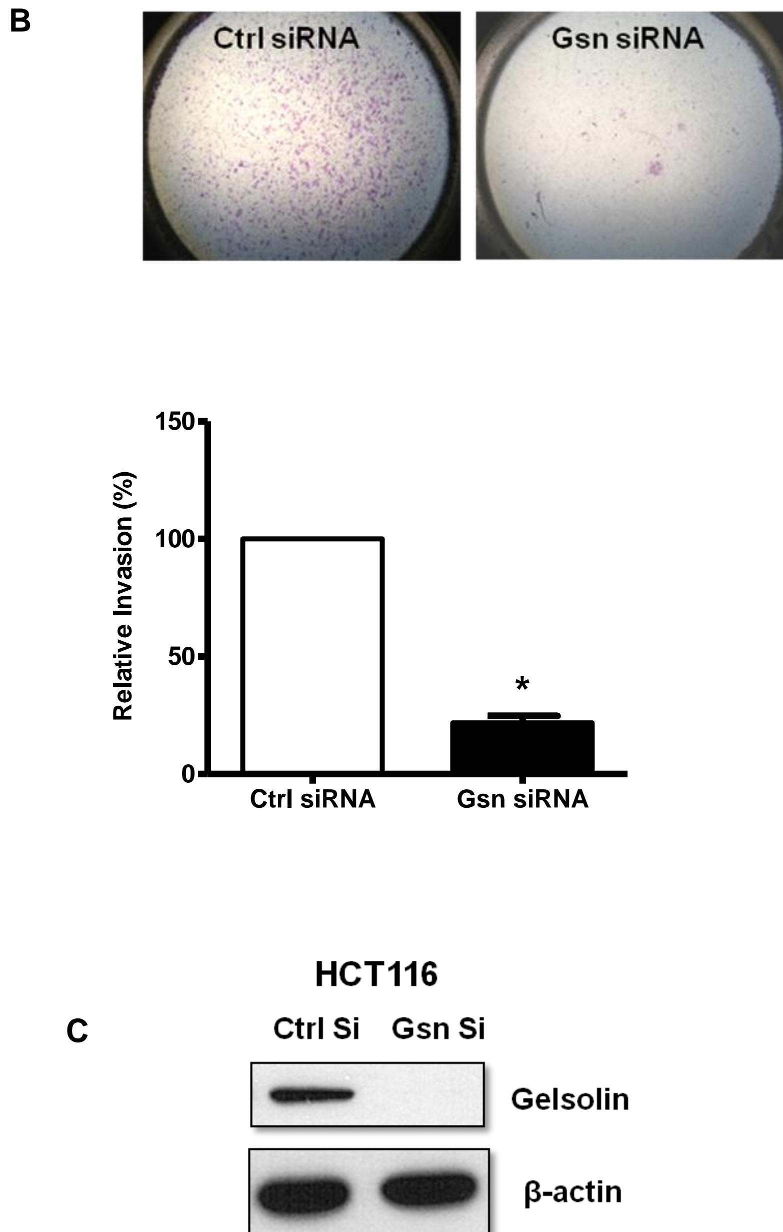


Figure 3.4B&C. Gelsolin knockdown reduces invasion of HCT116 cells. Gelsolin knockdown cells have lower invasive capacities compared to the control siRNA–treated cells. **Upper panel**, representative pictures of invaded cells are shown (2.5X magnification of the entire well). **Lower panel**, bar charts showing the quantification of invaded cell numbers. (C) Western blot showing gelsolin knockdown in HCT116 and RKO cells. Data shown are mean ± SD of at least three independent experiments. *p-value <0.05 (Two tailed Student's t- test).

3.3.5 DPI treatment blocks O_2^- production and attenuates invasion in gelsolin-overexpressing cells

Diphenyleneiodonium (DPI) is a potent inhibitor of the O_2^- producing enzyme NADPH oxidase (Nox) (Robertson *et al.*, 1990). In order to investigate whether O_2^- induced by gelsolin is required for gelsolin-mediated invasion, gelsolin-overexpressing cells were treated with 5 μM of DPI. DPI treatment significantly lowers the O_2^- levels of gelsolin-overexpressing C1 and C8 as well as empty vector control and wild-type HCT116 cells. However, C1 and C8 were more sensitive to DPI treatment and the reduction of O_2^- in C1 and C8 were brought down to a level comparable to that of the empty vector control cells (Fig. 3.5A). The role of O_2^- in gelsolin-mediated invasion was further assessed using a transwell invasion assay performed under similar conditions of DPI treatment. Consistent with the reduction in O_2^- levels, the invasive capacities of gelsolin-overexpressing cells significantly dropped following DPI treatment (Fig. 3.5B). To ensure that the reduction in invasion observed after DPI treatment is not due reduction in cell viability, we performed a cell viability assay using the Trpan Blue Excluson. We confirm that treatment of cells with 5 μM DPI for 24 hours has minimal effect on cell viability (Fig. 3.5C). These data therefore indicate that O_2^- is important in invasion and it may be an important molecule downstream of gelsolin that contributes to invasion.

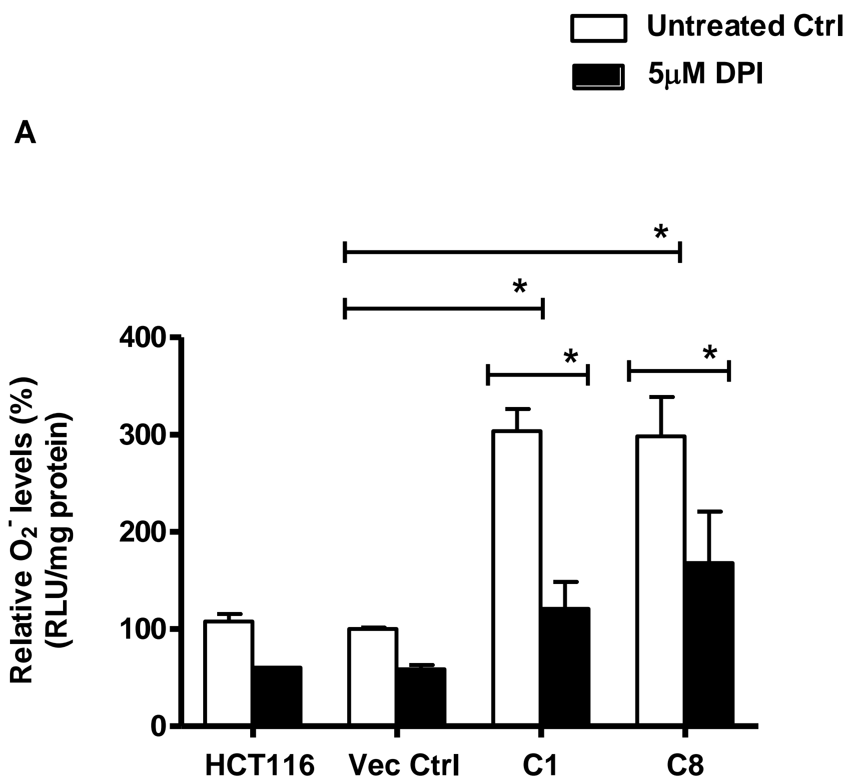


Figure 3.5A. DPI treatment reduces O_2^- levels. Treatment of cells with 5 μM DPI significantly lowered O_2^- levels. Data shown are mean ± SD of at least three independent experiments. *p-value <0.05 (Two tailed Student's t- test).

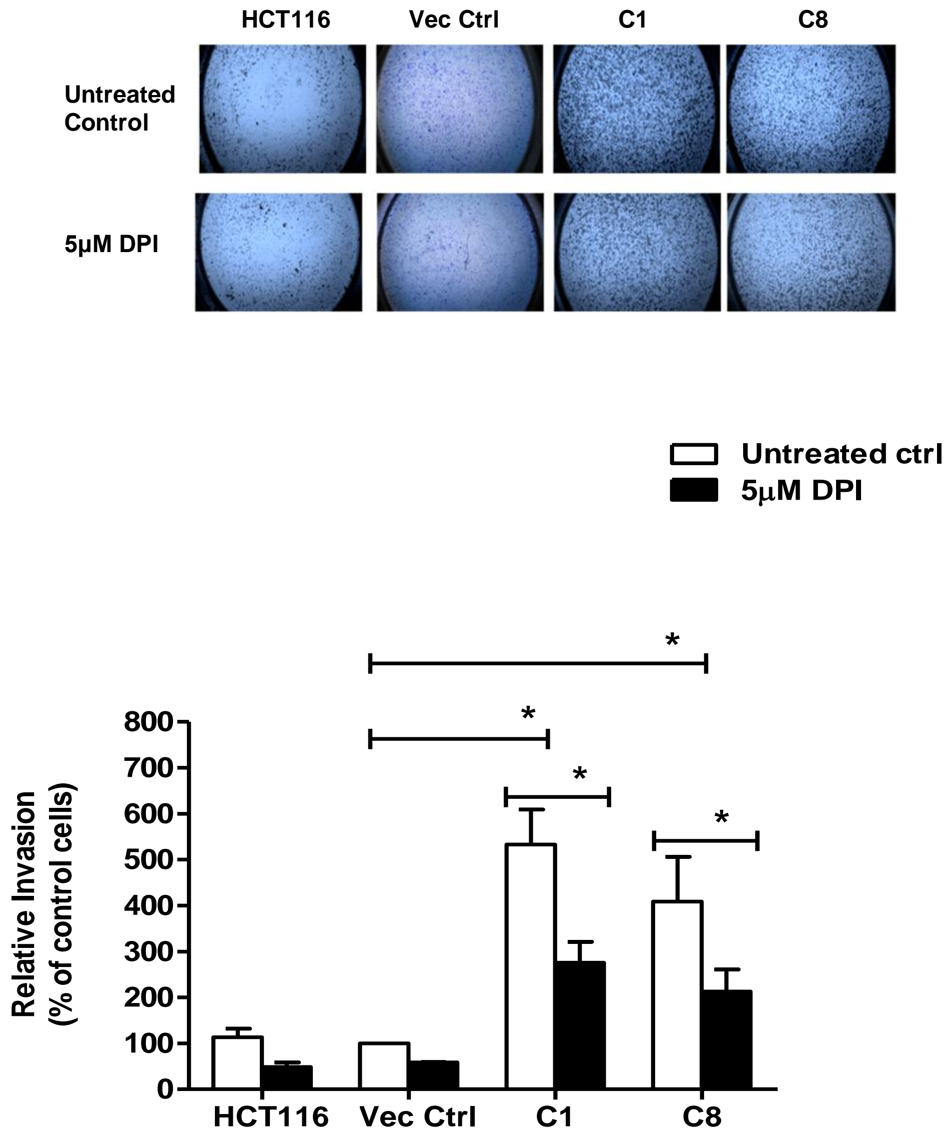


Figure 3.5B. DPI treatment reduces invasion. Treatment of cells with 5 µM DPI significantly lowered the invasive capacities of gelsolin overexpressing C1 & C8 cells. **Upper panel**, representative pictures of invaded cells with or without DPI treatments are shown (2.5X magnification of the entire well). **Lower panel**, bar charts showing the quantification of invaded cell numbers. Data shown are mean ± SD of at least three independent experiments. *p-value <0.05 (Two tailed Student's t- test).

C

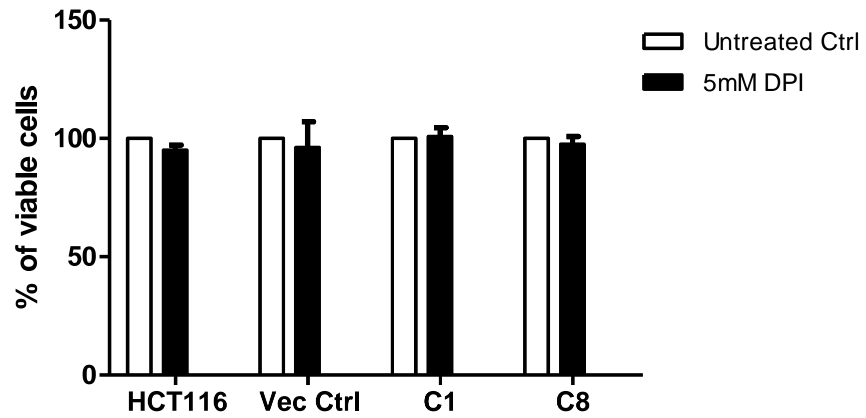


Figure 3.5C. DPI has minimal effect on HCT116 cell viability. To confirm the effect of DPI on gelsolin-induced cell invasion, gelsolin-overexpressing cells were treated with 5 μM DPI for 24h and cell viability was assessed using the Trypan Blue Exclusion cell counting method. No significant difference in cell viability was observed between control group and DPI treated cells. Data shown are mean ± SD of at least three independent experiments.

3.3.6 Increasing O_2^- levels by knockdown of Cu/Zn SOD rescues invasion in gelsolin-depleted HCT116 cells

As shown earlier knockdown of gelsolin reduces both O_2^- levels (refer to Fig. 3.1B) and invasion (refer to Fig. 3.4B). To confirm the requirement of O_2^- downstream of gelsolin for invasion, a rescue experiment was performed to increase O_2^- levels in gelsolin-knockdown cells. The antioxidant enzyme Cu/Zn SOD that catalyses the conversion of O_2^- to H_2O_2 was silenced in gelsolin-depleted HCT116. Silencing of Cu/Zn SOD abrogated the conversion of O_2^- to H_2O_2 thus leading to increased accumulation of O_2^- even in the absence of gelsolin. Knockdown of gelsolin and Cu/Zn SOD is evident from the western blots as shown in figure 3.6A. Knockdown of gelsolin results in decreased levels of O_2^-, however, knockdown of Cu/Zn SOD restores O_2^- in gelsolin-depleted cells (Fig 3.6B). Similarly, knockdown of gelsolin results in decreased invasion, however, simultaneous knockdown of Cu/Zn SOD together with gelsolin completely restores invasion in gelsolin-depleted cells (Fig. 3.6C). These data show the requirement of O_2^- in gelsolin-induced cancer cell invasion.

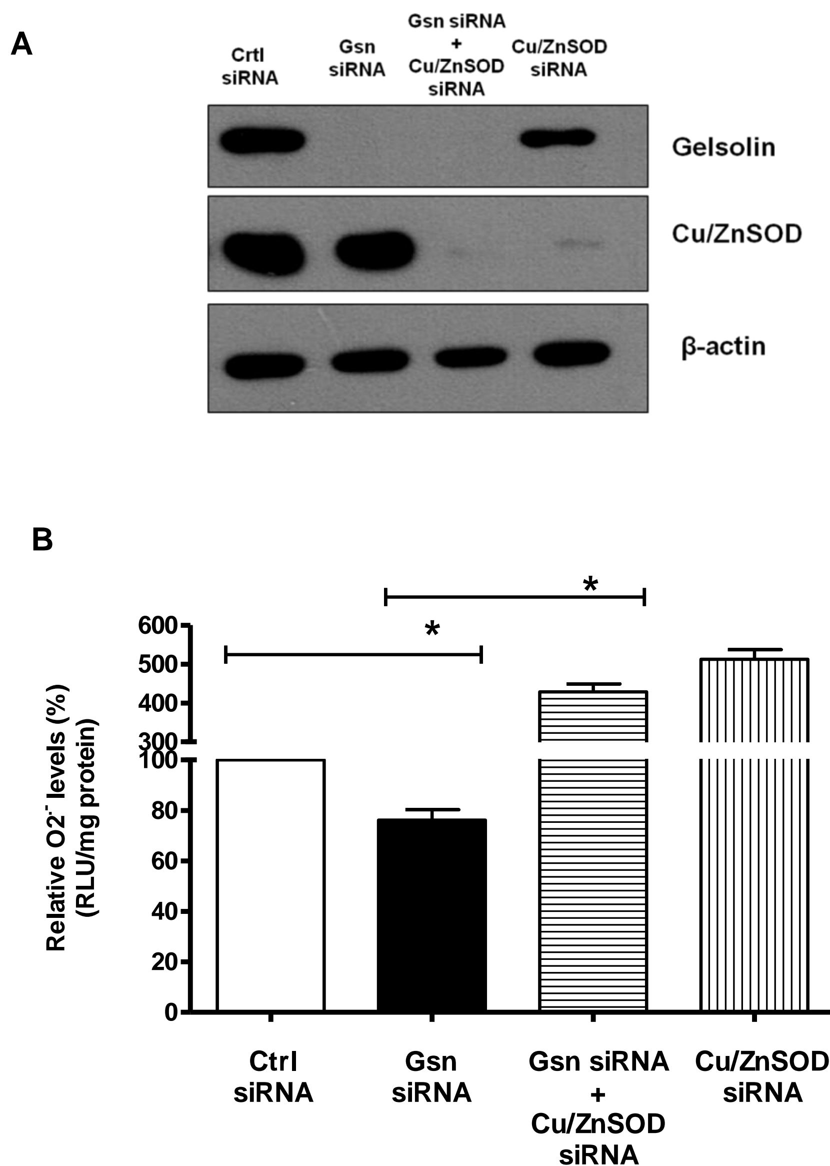


Figure 3.6A&B. Increasing O_2^- levels by knockdown of Cu/Zn SOD rescues invasion in gelsolin depleted HCT116 cells. (A) Western blot showing knockdown of gelsolin and Cu/Zn SOD. (B) Simultaneous knockdown of gelsolin and Cu/ZnSOD significantly increases O_2^- levels in gelsolin-depleted cells. Data shown are mean ± SD of at least three independent experiments. *p-value <0.05 (Two tailed Student's t- test).

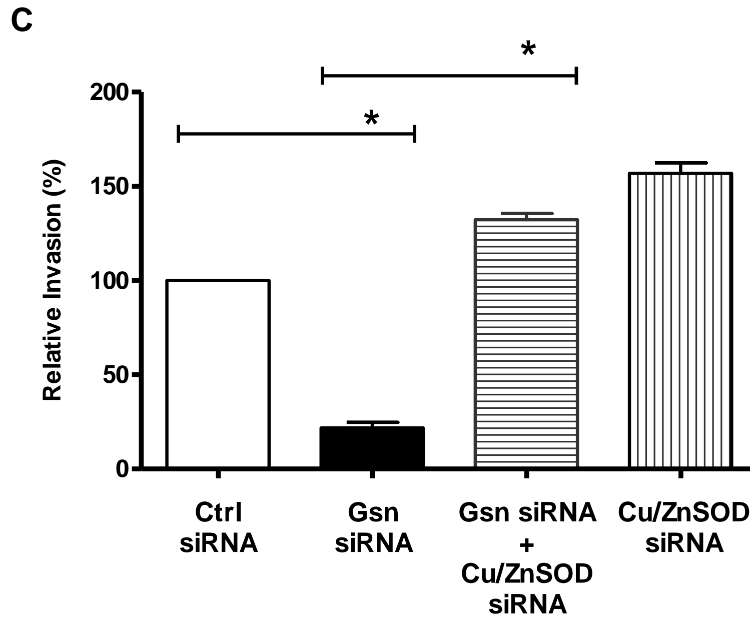


Figure 3.6C. Increasing O_2^- levels by knockdown of Cu/Zn SOD rescues invasion in gelsolin-depleted HCT116 cells. Simultaneous knockdown of gelsolin and Cu/ZnSOD significantly rescues the invasive capacity of gelsolin-depleted cells by increasing O_2^- levels. Data shown are mean ± SD of at least three independent experiments. *p-value <0.05 (Two tailed Student's t- test).

3.3.7 Gelsolin expression modulates urokinase plasminogen activator (uPA) secretion and activity

uPA is an extracellular serine protease that can activate the proenzyme plasminogen to active plasmin in the extracellular environment (Brooks *et al.*, 2010). Active plasmin can efficiently digest the ECM, thus facilitating cell invasion. uPA secretion was assessed using a double-sandwiched ELISA and the readings were normalised to protein concentrations. Cells were grown in serum-free media for 16 hours and the conditioned media were used to detect uPA secretion by ELISA. Figure 3.7A showed that uPA secretion is higher in gelsolin-overexpressing C1 and C8 cells when compared to the empty vector control and wild-type HCT116 cells — consistent with our previously published data (Zhuo *et al.*, 2012). In addition, when endogenous gelsolin was knocked down in several colon cancer cell lines including HCT116, Caco-2 and DLD-1 (Fig. 3.7B), reduced uPA secretion was observed (Fig. 3.7C). These data indicate that gelsolin modulates uPA secretion in these cells.

To understand the functional relevance of gelsolin-induced uPA secretion, a SDS-gel based enzymatic activity test was performed using zymography. Conditioned media mentioned above were loaded onto a SDS-PAGE gel containing uPA substrates (plasminogen and fibrinogen) and the gel was electrophoresed. The clear zone of lysis observed in gelsolin-overexpressing C1 and C8 cells were more pronounced when compared to the empty vector control and wild-type HCT116, indicating higher uPA activities conferred by gelsolin overexpression (Fig. 3.7D). Consistently, silencing of gelsolin in the gelsolin-overexpressing C1 cells and other colon cancer cell

lines including HCT116, DLD-1 and Caco-2 resulted in significant reduction of the proteolytic activity of uPA (Fig. 3.7E&F). Taken together, our data indicate that gelsolin modulates uPA secretion as well as the proteolytic activity of uPA on the degradation of the extracellular matrix.

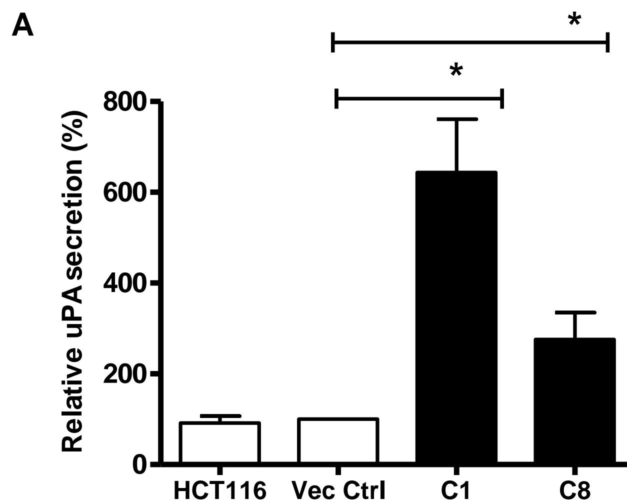


Figure 3.7A. Gelsolin overexpression induces uPA secretion. uPA secretion was assessed using double sandwiched ELISA. Overexpression of gelsolin in C1 and C8 cells augmented the secretion of uPA when compared to the empty vector control and wild-type HCT116 cells. Data shown are mean ± SD of at least three independent experiments. *p-value <0.05 (Two tailed Student's t-test).

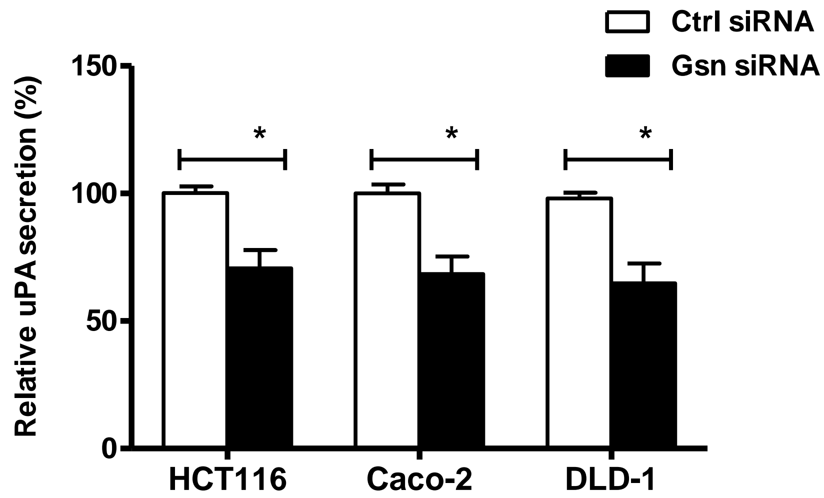


Figure 3.7B. Gelsolin knockdown decreases uPA secretion. (B) Knockdown of endogenous gelsolin in different colon cancer cell lines HCT116, Caco-2 and DLD-1 significantly reduced uPA secretion when compared to their respective control siRNAs. Data shown are mean ± SD of at least three independent experiments. *p-value <0.05 (Two tailed Student's t-test)

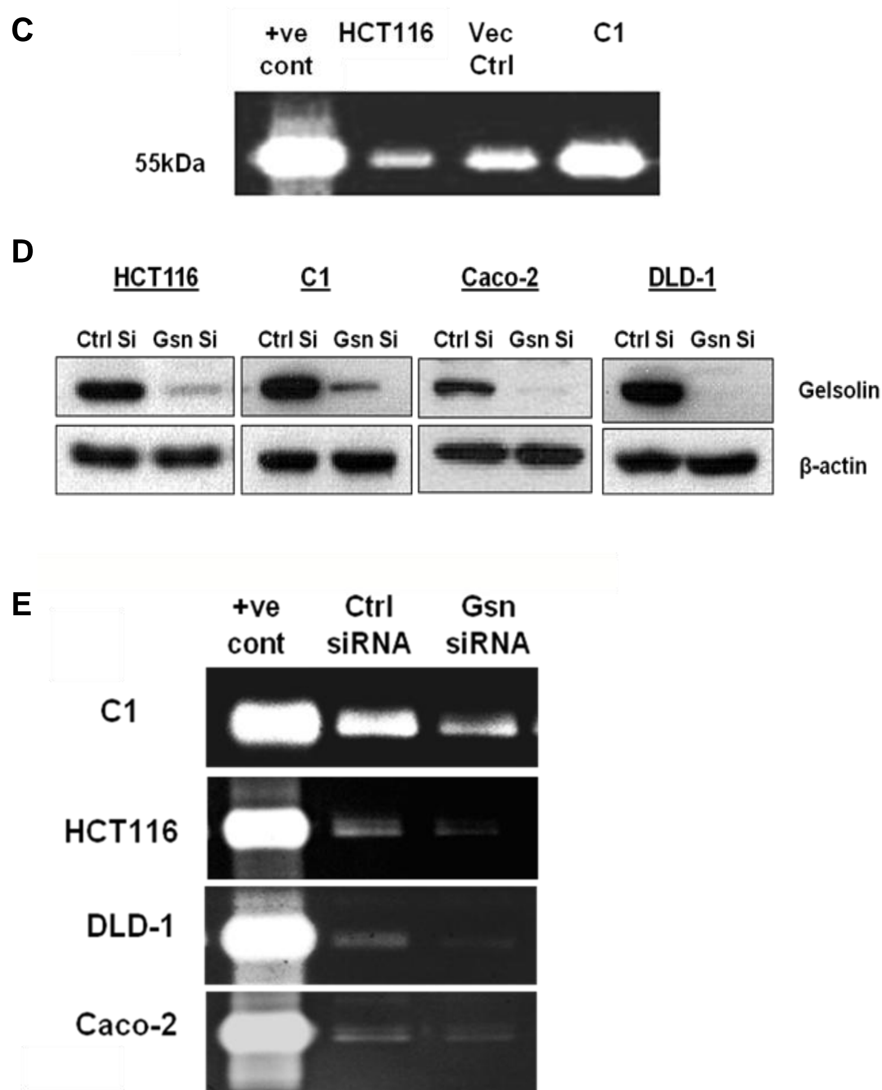


Figure 3.7C-E. Gelsolin expression modulates uPA activity. (C) uPA activity was determined by zymographic analysis using conditioned media from cells cultured in serum-free media for 16 hours. Gelsolin-overexpressing C1 have significantly higher uPA activity when compared to the empty vector control and wild-type HCT116 cells, as shown by the clear zone of lysis. (D) Western blot showing gelsolin knockdown in gelsolin-overexpressing C1, wild-type HCT116, Caco-2 and DLD-1 cells. (E) uPA activity was determined by zymographic analysis using conditioned media from gelsolin knockdown cells cultured in serum-free media for 3 hours. Knockdown of gelsolin in C1, HCT116, DLD-1 and Caco-2 showed decreased uPA activity. Data shown are mean ±SD of at least three independent experiments.

3.3.8 DPI inhibits gelsolin-induced uPA secretion

ROS have been implicated to play a role in cancer cell invasion by modulating the secretion and activities of ECM-degrading enzymes including uPA. To investigate whether O_2^- participate in enhancing uPA secretion induced by gelsolin, cells were serum-starved with 5 µM DPI or 50 µM Amiloride for 8 hours and the conditioned media were used to detect uPA by ELISA. Control cells were also serum starved without treatment for 8 hours and the conditioned media was used for detecting uPA by ELISA. Amiloride, an inhibitor of uPA was used as a positive control for efficiency of uPA inhibition. Amiloride has been shown to modulate uPA at the gene transcriptional level and post transcriptional level including blockade of secretion and activity (Vassalli & Belin, 1987; Wang *et al.*, 1995),. Treatment of cells with DPI significantly inhibited uPA secretion in gelsolin-overexpressing C1 and C8 cells when compared to the empty vector control and wild-type HCT116. Furthermore, the level of inhibition of uPA secretion by DPI in C1 and C8 cells were found to be comparable to the level of inhibition displayed by Amiloride, indicating an efficient inhibitory action by DPI treatment (Fig. 3.8). This data suggests that O_2^- plays a role in the gelsolin-induced uPA secretion.

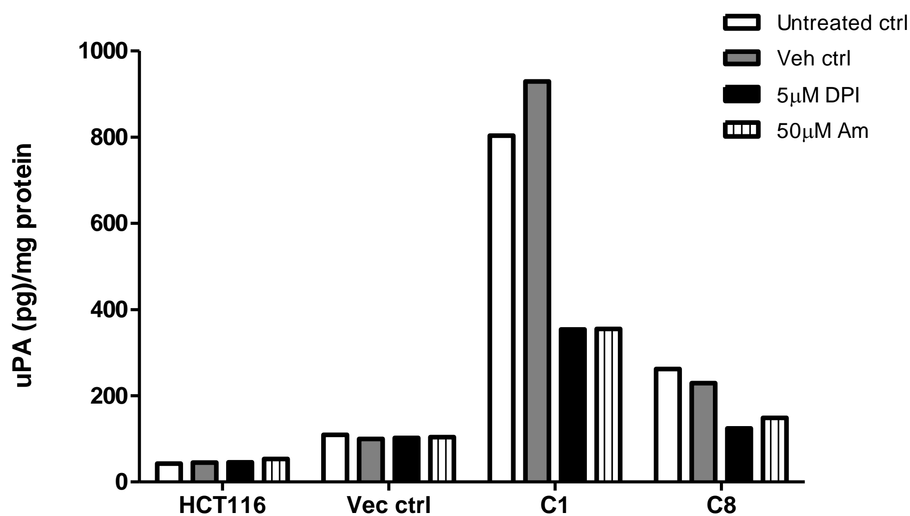


Figure 3.8. Gelsolin-induced uPA secretion is modulated by intracellular O_2^- levels. Cells were serum starved with or without 5 μM DPI for 8 hours and the conditioned media were used to detect uPA by ELISA. Amiloride (Am), an inhibitor of uPA was used as a positive control. Treatment of cells with 5 μM DPI significantly inhibited uPA secretion in gelsolin-overexpressing C1 and C8 cells whereas 5 μM DPI treatment did not show inhibitory effect of uPA secretion in the empty vector control and wild-type HCT116. Secreted uPA levels were normalised to protein concentration. Data shown here is a representative of at least three independent experiments.

3.4 DISCUSSION

3.4.1 Gelsolin modulates intracellular O_2^- levels

In this study we have demonstrated a novel role of gelsolin as a pro-oxidant molecule whose expression positively correlates with the intracellular levels of ROS particularly the O_2^- species. We found that overexpression of gelsolin increases intracellular O_2^- levels. Consistently, when gelsolin expression was silenced with siRNA targeted against gelsolin, a reduction in O_2^- levels was observed. As noted earlier in section 1.3.1, ROS consist of several other species including H_2O_2,$^{\cdot}OH$, HOCl etc., we assessed whether gelsolin expression could influence other ROS species besides O_2^-. We found that neither overexpression nor knockdown of gelsolin alters the levels of other species of ROS such as H_2O_2. Our results therefore indicate that gelsolin expression specifically modulates the total intracellular levels of O_2^-.

Since mitochondria are one of the major sources of O_2^- in the cell, we investigated whether mitochondrial O_2^- is affected by gelsolin expression. Our data showed that gelsolin overexpression increases mitochondrial O_2^- levels by 0.5 fold, suggesting that mitochondrial O_2^- contributes to the increase in total intracellular O_2^-. This increase in mitochondrial O_2^- could be a result of increased mitochondrial activity. Gelsolin overexpression has been implicated to modulate mitochondrial activity by increasing mitochondrial complex IV activity (Antequera *et al.*, 2009). However, mitochondrial O_2^- is likely to play a minor contribution in gelsolin's effects on O_2^- production, as gelsolin overexpression in HCT116 induced a 3-fold increase in the total intracellular O_2^- levels. This observation indicates that other extra-mitochondrial sources

of ROS including increased activities of the O_2^- producing enzymes such as Nox, or impaired activities of antioxidant enzymes such as the Cu/Zn SOD may participate in contributing to the total increase in O_2^- levels.

3.4.2 O_2^- is required for gelsolin-mediated cancer cell invasion

We and others have previously shown that gelsolin is important during cancer cell invasion (De Corte *et al.*, 2002; Van den Abbeele *et al.*, 2007; Zhuo *et al.*, 2012), and our data here also supports the role of gelsolin in promoting invasion. In addition, we show that gelsolin-induced invasion requires intracellular O_2^-. High levels of gelsolin results in increased O_2^- and invasion. The increase in invasion induced by gelsolin could be attenuated by lowering the levels of O_2^-. These data suggest that one of the mechanisms by which gelsolin enhances invasion is by increasing O_2^- levels. Consistent with this observation, knockdown of gelsolin reduces both O_2^- levels and invasion. The invasive capacities of HCT116 cells were rescued when O_2^- levels were increased in gelsolin-knockdown cells, consistent with an essential role for O_2^- downstream of gelsolin in cancer cell invasion. Taken together, these set of data indicate a reliance on O_2^- by gelsolin during the process of cancer cell invasion.

Mounting evidence has documented the role of ROS in promoting cancer cell invasion through enhancing ECM degradation. ROS have been shown to regulate ECM-degrading proteases such as the uPAs and the MMPs. Elevated levels of uPA and its receptor uPAR as well as MMPs are often observed in various cancer types, correlating with increased ROS levels and invasive phenotypes. The roles of ROS in upregulating uPAs and MMPs have

been mostly attributed to the involvement of ROS in triggering the MAPK pathways leading to activation of transcription factors that control the expression of uPAs and MMPs (Binker *et al.*, 2009; Chiu *et al.*, 2010; Khoi *et al.*, 2012a; Kim *et al.*, 2007a; Nelson & Melendez, 2004; Pelicano *et al.*, 2009). Besides transcriptional regulation, ROS have also been known to have the ability to induce the activation of MMPs (Saari *et al.*, 1992). However, whether this is true for uPA activity has not been documented. ROS induced by triggers such as *Helicobacter pylori* infection and nicotine treatment have also been shown to stimulate increased uPA and uPAR levels (Khoi *et al.*, 2012b; Kim *et al.*, 2007b). Furthermore, when cancer cells were treated with antioxidants, the effects of nicotine on uPAR expression were reversed, supporting the involvement of ROS in regulating uPA and uPAR expression. ROS induced by transforming growth factor beta (TGF-β) was also shown to upregulate uPA leading to enhanced cancer cell invasion (Tobar *et al.*, 2010a).

We have reported that overexpression of gelsolin upregulated the mRNA level, secretion level as well as enzymatic activities of uPA (Fig. 3.7) (Zhuo *et al.*, 2012). To determine whether O_2^- is involved in the induction of uPA in gelsolin-mediated invasion, gelsolin-overexpressing cells were treated with DPI. Treatment of gelsolin-overexpressing cells with DPI significantly attenuated the secretion of uPA, suggesting the role of O_2^- in enhancing uPA secretion. Thus, our result have suggested uPA as a downstream effector of O_2^- in gelsolin-medaited invasion. More importantly, our study provides a mechanistic insight into the regulation of uPA by gelsolin.

In most of the studies, H_2O_2 have been implicated in the gene regulation of uPA and uPAR via oxidation-induced activation of transcription factors such as NF-κB and AP-1. Although less is known about the role of O_2^- in the regulation of uPA gene expression, O_2^- has been shown to be crucial for the oxidation-mediated activation of the transcription factor HIF-1α (Wang *et al.*, 2004) and NF-kB (Marumo *et al.*, 1997). Therefore, it is plausible that O_2^- may participate in the upregulation of uPA gene expression following gelsolin overexpression.

Besides transcriptional regulation, ROS are also known to be involved in post-transcriptional as well as post-translational regulation of the ECM-degrading proteins. As noted earlier, (section 1.3.4) H_2O_2 has also been shown to stabilise the mRNAs of uPA and uPAR by facilitating the binding between uPA/uPAR mRNAs and the mRNA-stabilising Hu family of RNA-binding proteins (HuR) (Tran *et al.*, 2003b). In addition, H_2O_2, HOCl and $^{\cdot}OH$ activate MMPs through oxidation of the cysteine residue in the catalytic domain (Saari *et al.*, 1992). O_2^- also has the potential to modify proteins, for example, O_2^- stimulates PKC activity through thiol modification and alteration of zinc levels (Knapp & Klann, 2000). Thus, the role of O_2^- in activating uPA cannot be excluded. Conceivably, O_2^- induced by gelsolin could serve as an important determinant in the activation of ECM-degrading proteases such as uPA at the invasive edge.

In summary, our study has revealed a new mechanism by which gelsolin promotes cancer cell invasion. Gelsolin increases the intracellular levels of O_2^-, which acts to enhance invasion by facilitating extracellular matrix degradation through the induction of uPA secretion.

RESULTS

CHAPTER 4

CHAPTER 4 GELSOLIN MODULATES INTRACELLULAR LEVELS OF SUPEROXIDE (O_2^-) SPECIES BY SUPPRESSING THE ACTIVITY OF THE ANTIOXIDANT COPPER/ZINC SUPEROXIDE DISMUTASE (CU/ZN SOD)

4.1 BACKGROUND

Defects in the antioxidant system or upregulation of ROS-generating enzymes such as Nox can lead to increase in the intracellular levels of ROS. Antioxidants play important roles in maintaining the cellular redox homeostasis. Abnormal fluctuations in the antioxidant system results in disturbance in the ROS-antioxidant balance which can lead to pathological conditions such as cancer. The antioxidant systems consist of the SODs, catalase, glutathione, thioredoxins, glutaredoxins and antioxidant protein-1 (ATOX-1) (Halliwell, 2006; Sies, 1997). As excess ROS inside the cells can have harmful effects, the antioxidants play key roles in scavenging excess ROS by converting them to less harmful products. The efficiency of antioxidants is enhanced by their target specificity, for example, SODs scavenge O_2^- by dismutating them to H_2O_2 (Fridovich, 1978; Fridovich, 1995). H_2O_2 in turn is reduced to water by the action of catalase or other antioxidants. Defective functions of antioxidants thus result in accumulation of ROS in cells.

In most cases, O_2^- is the precursor ROS species; since SODs are the only enzymes responsible in eliminating O_2^-, they are often regarded as the first line in the antioxidant scavenging (Alscher *et al.*, 2002; Van Raamsdonk & Hekimi, 2012). Although the enzymatic functions of the two isoforms of SODs, the cytosolic Cu/Zn SOD and mitochondrial Mn SOD are similar, their

site of action is restricted to their cellular localisation. Whilst Cu/Zn SOD is responsible for eliminating O_2^- in the cytosol, Mn SOD scavenges O_2^- in the mitochondria (Halliwell, 2007).

In the previous chapter (chapter 3), we have shown that gelsolin overexpression correlates with an increase in the intracellular levels O_2^- species of ROS. Since defective antioxidants can result in accumulation of intracellular ROS levels, we hypothesize that gelsolin may affect the expression and activities of antioxidants. We therefore assessed the mRNA expression of a panel of antioxidant genes and determined the activities of SODs.

As the enzymatic regulation of Cu/Zn SOD depends on the metallochaperone, Copper chaperone for superoxide dismutase (CCS), we also examined the protein expression levels of CCS. CCS donates copper to Cu/Zn SOD as well as aids in the dimerisation of Cu/Zn SOD molecules (Banci *et al.*, 2012; Banci *et al.*, 2002; Furukawa *et al.*, 2004). Cu/Zn SOD activity can also be increased by increased gene and protein expression of Cu/Zn SOD. Gene expression of Cu/Zn SOD is regulated by activation of its upstream transcription factors such as AP-1, NF-kB and Sp1, Nuclear Factor E2-Related Factor2 and Early Growth Response-1 (Milani *et al.*, 2011).

Because NADPH oxidases (Nox) are the major ROS-producing enzymes in the mammalian cells, the mRNA levels of Nox were assessed to evaluate if gelsolin have effect on them and use it as a means to increase ROS levels in the cell.

4.2 OBJECTIVES

To investigate the mechanisms by which gelsolin induces increases in O_2^- , the following objectives were addressed:

1. Determine if gelsolin influences the expression of antioxidant genes.
2. Investigate the effect of gelsolin on the protein expression and the enzymatic activities of SODs.
3. Determine if gelsolin influences the expression of Nox genes.

4.3 RESULTS

4.3.1 Gelsolin overexpression suppresses the mRNA levels of antioxidant genes

To investigate whether gelsolin expression affects the gene expression of antioxidant genes, real-time PCR was employed to determine the mRNA levels of antioxidant genes including Cu/Zn SOD, Mn SOD, catalase, Glutaredoxin-2 (Glrx-2) and ATOX-1. Overexpression of gelsolin resulted in a reduction of the mRNA levels of the above-mentioned antioxidants. Gelsolin-overexpressing C1 and C8 have significantly lower gene expression levels of Cu/Zn SOD, Catalase and Glrx-2 when compared to the empty vector control and wild-type HCT116 cells (Fig. 4.1). This data suggests that gelsolin may downregulate the expression of antioxidant genes in cancer cells.

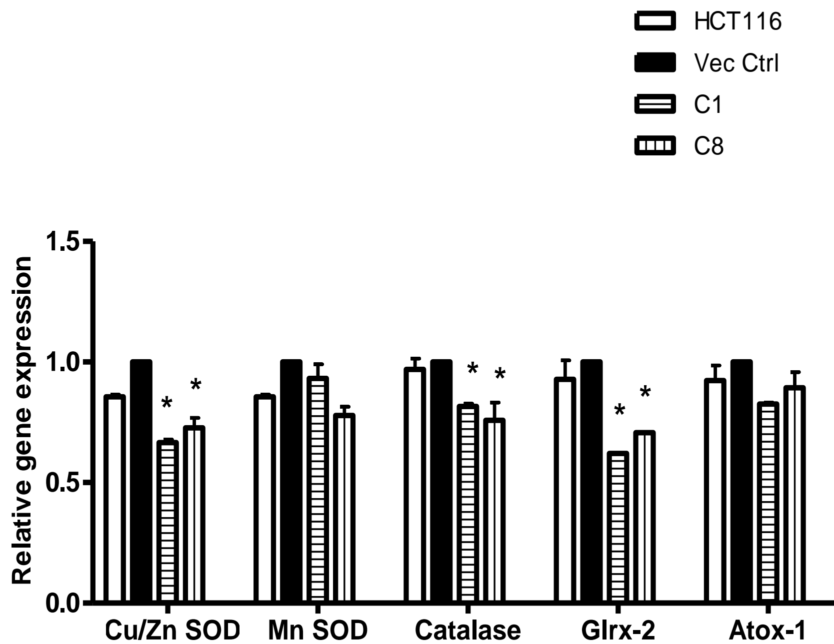


Figure 4.1. Gelsolin overexpression downregulates mRNA levels of antioxidant genes. Gelsolin-overexpressing cells shows significantly lower levels of Cu/Zn SOD, Catalase and Glrx-2 mRNA levels when compared to the vector control and wild-type HCT116 cells. Data shown are mean ± SD of at least three independent experiments. *p-value <0.05 (Two tailed Student's t-test).

4.3.2 Gelsolin does not affect the protein expression of Cu/Zn SOD and Mn SOD

In order to determine the underlying mechanisms by which gelsolin overexpression increases O_2^- levels, we assessed the protein levels of SODs as O_2^- is a specific target of SODs. Moreover, gelsolin-overexpression also showed reduced mRNA levels of Cu/Zn SOD and Mn SOD. Therefore, to investigate if the gene expression levels correlate to the protein expression levels, the protein levels of Cu/Zn SOD and Mn SOD were analysed by Western blot (Fig. 4.2). Differences in protein levels of Cu/Zn SOD and Mn SOD were not evident when compared between gelsolin-overexpressing C1 and C8 and empty vector control or wild-type HCT116 cells. Similarly, knockdown of gelsolin in HCT116 cells did not affect the protein expression of Cu/Zn SOD and Mn SOD when compared to the control siRNA-treated cells. These data show that although mRNA expression of Cu/Zn SOD and Mn SOD were reduced by gelsolin, the protein expression remained unaffected.

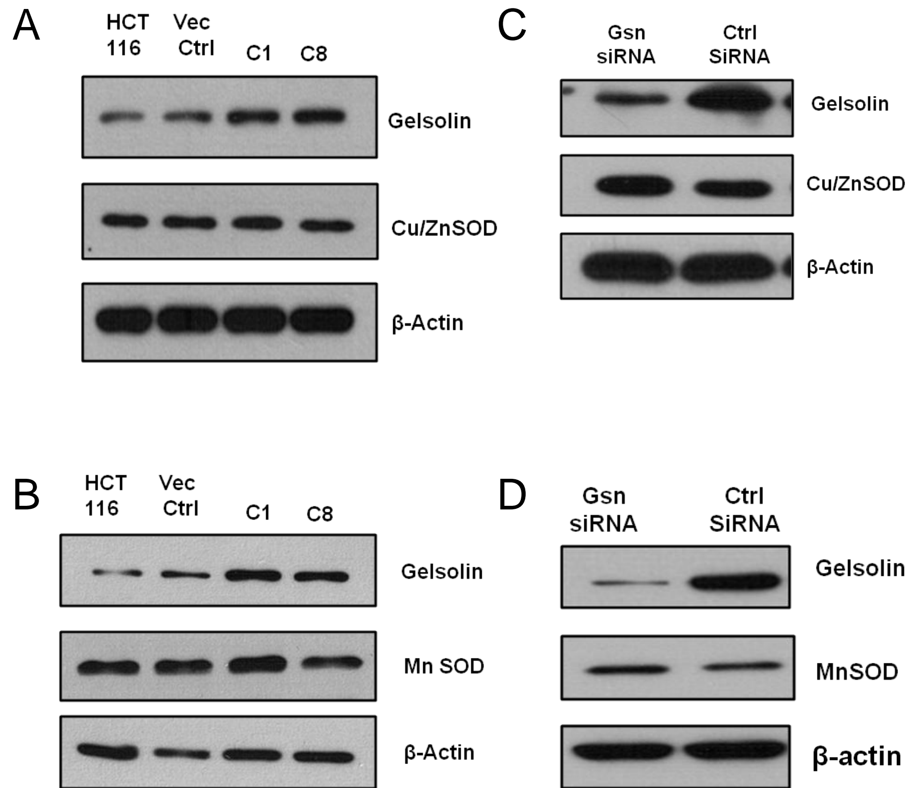


Figure 4.2A-D. Gelsolin expression does not affect the protein expression of Cu/Zn SOD and Mn SOD. (A) Cu/Zn SOD levels were not affected when gelsolin expression was altered. Comparison was made between gelsolin-overexpressing C1 &C8 cells with the empty vector control and HCT116 cells. (B) No change in Cu/Zn SOD levels were observed between siRNA knockdown of gelsolin when compared to siRNA control. (C) No significant change in Mn SOD levels were observed between gelsolin-overexpressing C1 and C8 cells when compared to empty vector control and HCT116 cells. (D) No significant change in Mn SOD levels were observed between siRNA knockdown of gelsolin when compared to siRNA control. β-actin and GAPDH were used as internal loading controls. The Western blots shown here are representatives of at least three independent experiments.

4.3.3 Gelsolin suppresses total SOD activity

The enzymatic activity of SOD was investigated in gelsolin-overexpressing HCT116 cell lines. Using total cell lysate, we determined the combined enzymatic activities of Cu/Zn SOD and Mn SOD (referred to as total SOD) using the SOD activity kit from Enzo. Gelsolin-overexpressing C1 and C8 cells were found to have lower total SOD activities compared to the empty vector control and wild-type HCT116 cells (Fig. 4.3A). Consistent with this observation, knockdown of endogenous gelsolin in wild-type HCT116 and RKO cells results in higher total SOD activity as compared to their control siRNA-treated counterparts (Fig. 4.3B). These data suggest that although the protein levels of SODs were not altered by gelsolin expression, gelsolin modulates the enzymatic activity SOD.

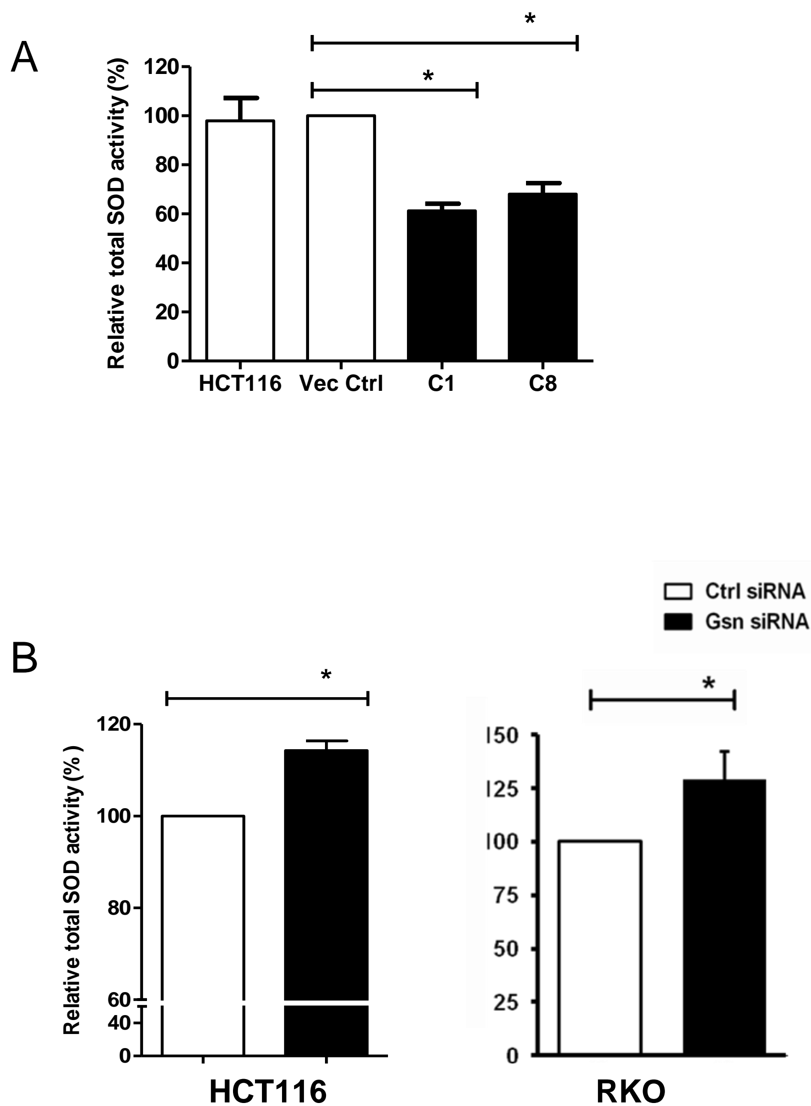


Figure 4.3A&B. Gelsolin expression modulates total SOD activity in HCT116 and RKO cells. Total intracellular superoxide dismutase (SOD) activity was analysed using total cell lysate. (A) Overexpression of gelsolin in C1 and C8 results in significantly lower SOD activities when compared to the empty vector control and wild-type HCT116 cells. (B) Knockdown of endogenous gelsolin in HCT116 and RKO cells results in increased total SOD activity. Data shown are mean ± SD of at least three independent experiments. *p-value <0.05 (Two tailed Student's t- test).

4.3.4 Gelsolin suppresses Cu/Zn SOD activity

In order to identify the specific SOD isoform affected by gelsolin, sub-cellular fractionation was performed to separate the cytosol and the mitochondria. Cu/Zn SOD and Mn SOD activities were assayed from the respective fractions. The validity of our fractionation was confirmed by the presence of Cu/Zn SOD and GAPDH in the cytosol and MnSOD and VDAC in the mitochondrial fractions, whilst β-actin was used as the internal loading controls for both fractions (Fig. 4.4A). Our result shows that gelsolin-overexpressing C1 and C8 cells have reduced Cu/Zn SOD activity (Fig 4.4B) when compared to empty vector control and wild-type HCT116 cells, whereas the activity of Mn SOD was minimally affected by gelsolin expression (Fig. 4.4C). This result suggests that Cu/Zn SOD is likely the isoform targeted by gelsolin.

Diethyldithiocarbamate (DDC) is a potent chelator of metals (iron or copper) by interacting with free and protein bound metal. DDC has been widely used as an agent to inhibit Cu/Zn SOD activity and increase intracellular O_2^- levels in cells (Didion *et al.*, 2001; Heikkila *et al.*, 1976). In this study, DDC was used as a positive control to determine the efficiency of Cu/Zn SOD inhibition observed in gelsolin-overexpressing cells. Gelsolin-overexpressing C1 cells and the empty vector control cells were both treated with 1mM DDC for an hour. Treatment with DDC significantly reduces total SOD activities in both C1 and the empty vector control cells (Fig. 4.4D). Reduction in total SOD activities was reflected by a significant increase in O_2^- levels in both C1 and vector control cells (Fig. 4.4E). Furthermore when cells

treated with 1mM DDC were subjected to sub-cellular fractionation, Cu/Zn SOD activity was significantly abrogated in C1 and vector control cells whereas Mn SOD activity was minimally affcted (Fig. 4.4F). The Mn SOD activity levels were comparable between C1 and vector control cells with or without DDC treatment. Therefore, these data indicate that the approach used is suitable for detection of the specific isoform of SOD following sub-cellular fractions. Our findings here support our observations in figures 4.4B&C that gelsolin overexpression suppresses Cu/Zn SOD activity but not Mn SOD.

Accordingly, siRNA-mediated silencing of gelsolin in HCT116 and C1 cells significantly increased Cu/Zn SOD activity whilst Mn SOD was nominally affected (Fig. 4.4G-K). Taken together, these data indicate that changes in gelsolin expression alter Cu/Zn SOD activity and not Mn SOD activity.

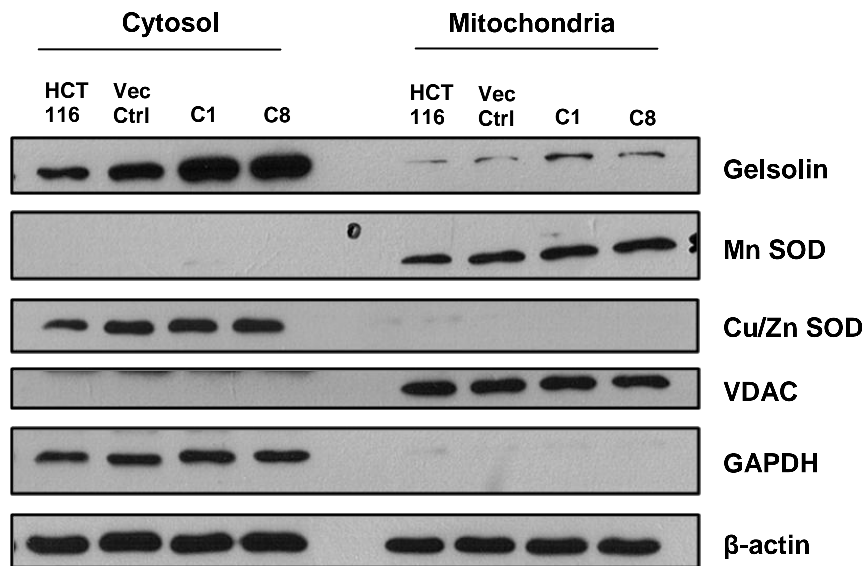
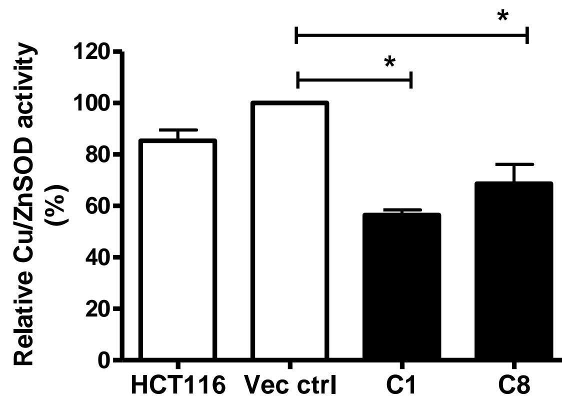


Figure 4.4A. Subcellular fractionation of cytosol and mitochondria in gelsolin-overexpressing cells. Cytoslic and mitochondrial fraction were isolated in gelsolin-overexpressing C1 and C8 as well as the empty vector and wild-type HCT116 cells. Fractionation was confirmed by the presence of Mn SOD and VDAC in the mitochondira and Cu/Zn SOD and GAPDH in the cytosol. β-actin was used as the internal loading control.

B



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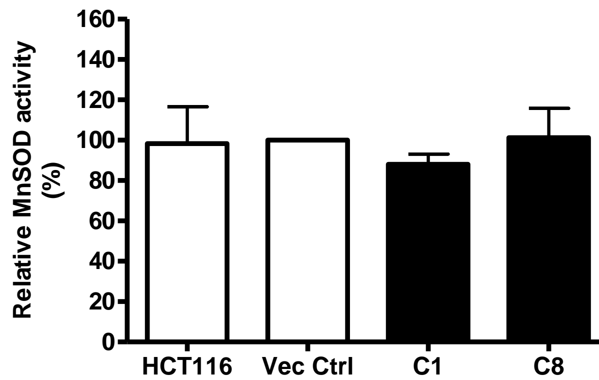
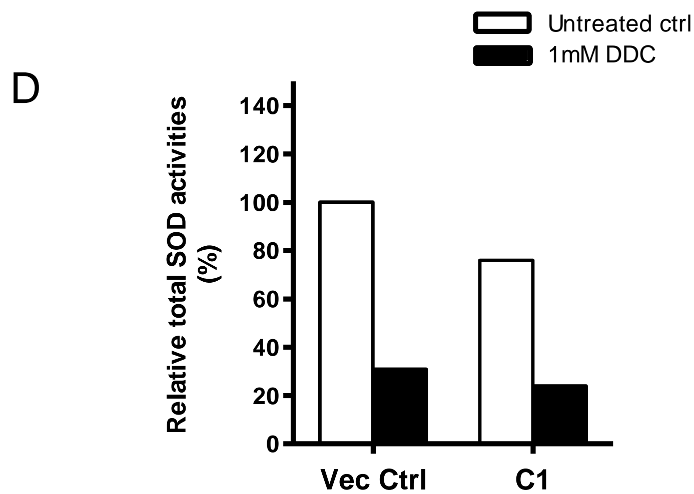


Figure 4.4B&C. Overexpression of gelsolin suppresses Cu/Zn SOD activity in HCT116 cells. (B) Cu/Zn SOD activity was measured from the cytosolic extract. Gelsolin overexpression results in suppression of Cu/Zn SOD activity when compared to the empty vector control and wild-type HCT116 cells. (C) Intact mitochondria were isolated and Mn SOD activity was determined. No significant change in MnSOD activity was observed upon gelsolin overexpression. Data shown are mean ± SD of at least three independent experiments. *p-value <0.05 (Two tailed Student's t-test).



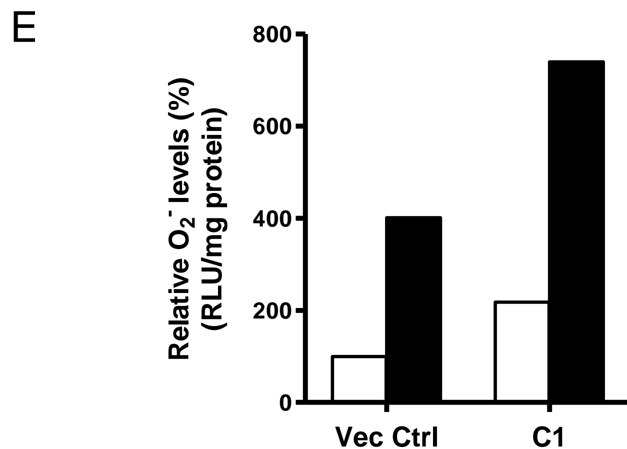


Figure 4.4D&E. DDC inhibits total SOD activity. Gelsolin-overexpressing C1 and empty vector control cells were both treated with 1mM of DDC for 1 hour. (D) Total SOD activities were reduced in both C1 and in the empty vector control cells following DDC treatment. (E) Concomitant with the drop in total SOD activity, 1mM DDC treatment results in increased O_2^- levels. Data shown here is representative of two independent experiments.

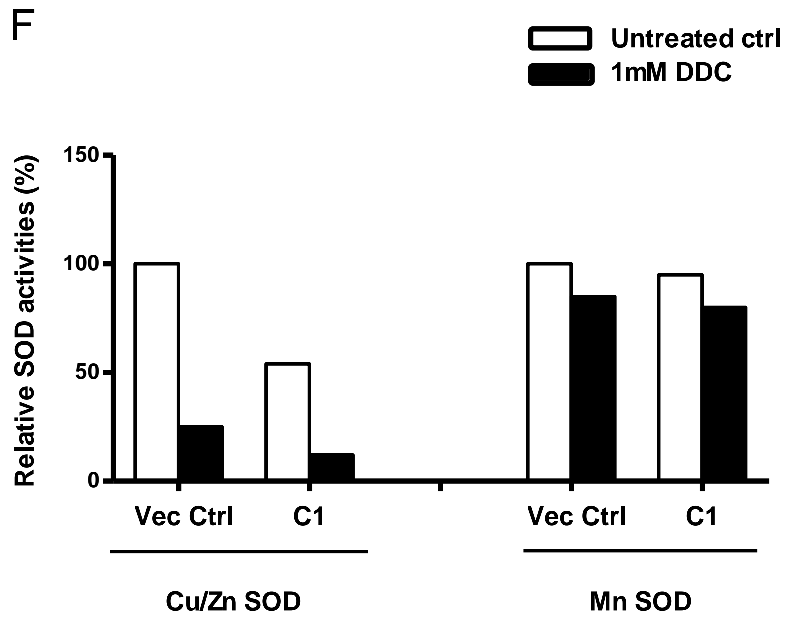


Figure 4.4F. DDC inhibits Cu/Zn SOD activity. Cells were treated with 1mM DDC for 1 hour and the cytosol and mitochondria was fractionated. Cu/Zn SOD and Mn SOD activites were assayed in from the respective fractions. Cu/Zn SOD activities were dramatically reduced following DDC treatment whilst Mn SOD activity was minimally affected. Data shown here is a representative of two independent experiments.

G

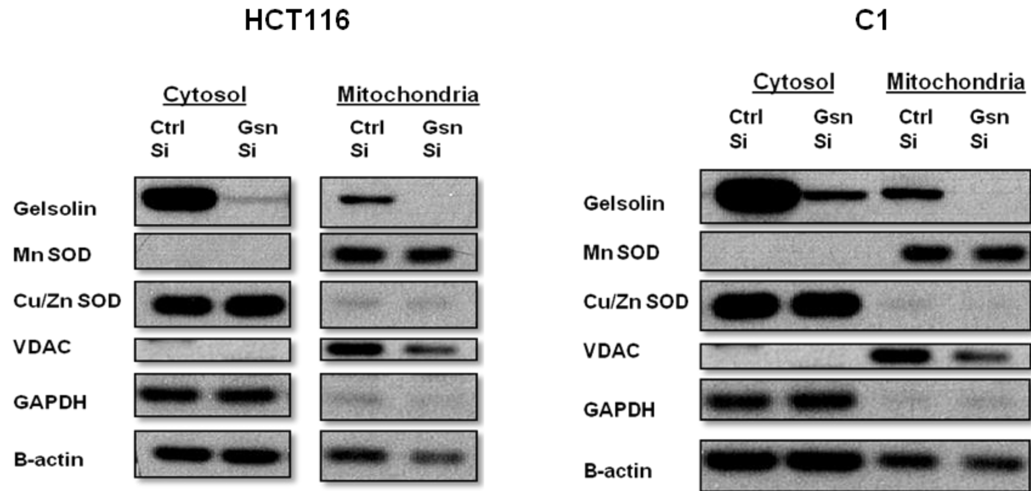


Figure 4.4G. **Subcellular fractionation of cytosol and mitochondria in gelsolin knockdown cells**. Gelsolin was silenced in HCT116 and C1 cells and cytosolic and mitochondrial fractions were isolated. Fractionation was confirmed by the presence of Mn SOD and VDAC in the mitochondria and Cu/Zn SOD and GAPDH in the cytosol. β-actin was used as the internal loading control.

CYTOSOLIC FRACTION

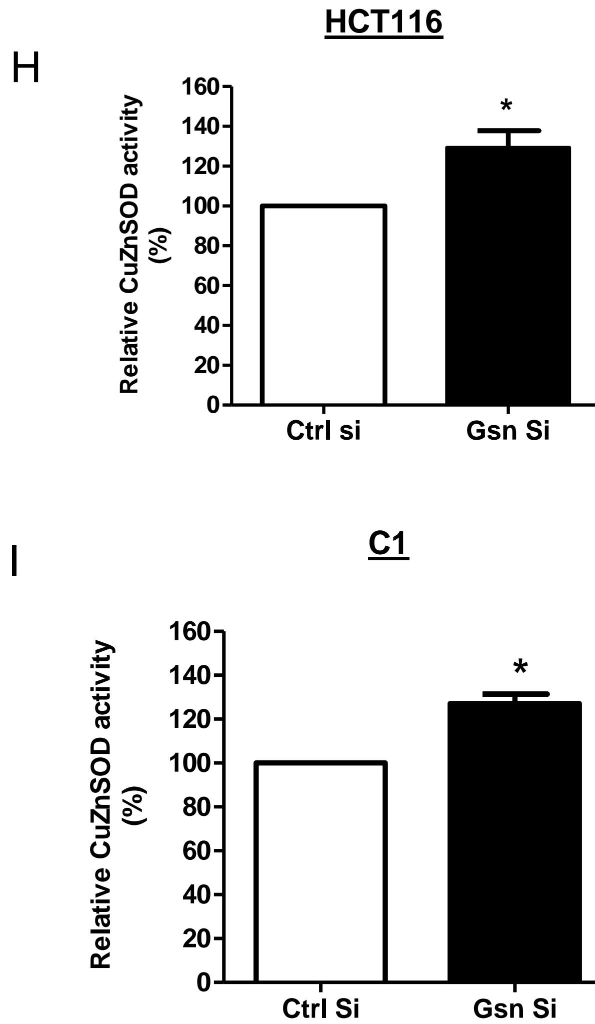


Figure 4.4H&I. Knockdown of gelsolin increases Cu/Zn SOD activity in HCT116 and C1 cells. Cu/Zn SOD activity was measured from the cytosolic extract in gelsolin knockdown HCT116 and C1 cells. Elevated Cu/Zn SOD activity was observed when gelsolin was knocked down in HCT116 and C1. Data shown are mean ± SD of at least three independent experiments. *p-value <0.05 (Two tailed Student's t-test).

MITOCHONDRIAL FRACTION

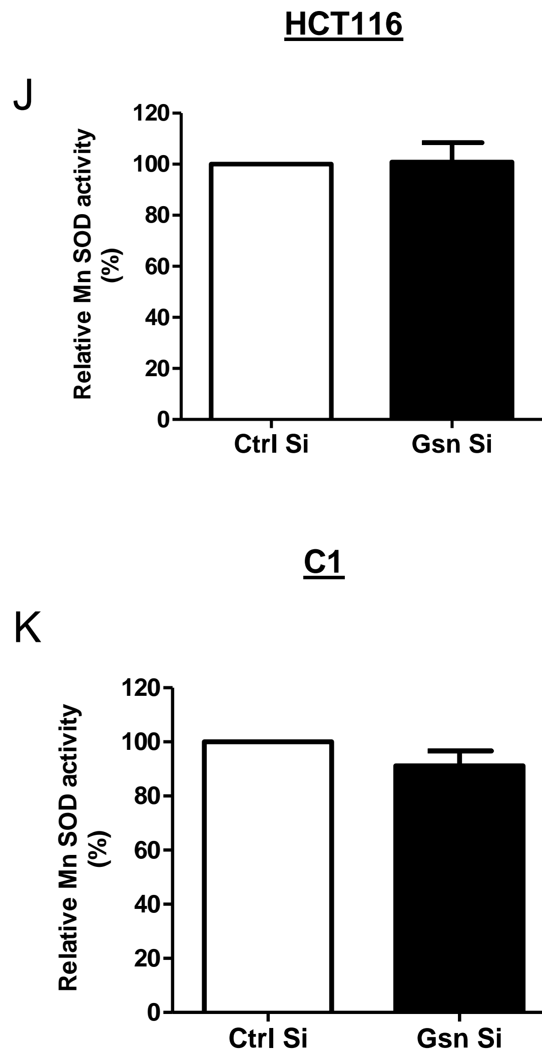


Figure 4.4J&K. Knockdown of gelsolin does not affect Mn SOD activity in HCT116 and C1 cells. Mn SOD activity was measured from the mitochondrial extract in gelsolin knockdown HCT116 and C1 cells. Mn SOD activity remains unchanged when gelsolin was knocked down in HCT116 and C1. Data shown are mean ± SD of at least three independent experiments. *p-value <0.05 (Two tailed Student's t-test).

4.3.5 Gelsolin does not affect protein expression of copper chaperone for Cu/Zn SOD (CCS)

CCS is a chaperone protein that physically interacts with Cu/Zn SOD and donates copper to it. CCS is the most potent regulator of Cu/Zn SOD activity. CCS facilitates Cu/Zn SOD dimerisation and also helps in maintaining its structural integrity that is required for efficient enzymatic action (Banci *et al.*, 2012; Banci *et al.*, 2002; Furukawa *et al.*, 2004). In an attempt to investigate the mechanisms by which gelsolin suppresses Cu/Zn SOD activity, we assessed the protein levels of CCS in both gelsolin-overexpressing as well as gelsolin knockdown cells. Our results showed that the protein levels of CCS remained unaltered when gelsolin expression was modulated either by overexpression or siRNA knockdown (Fig. 4.5A & B). These data indicate that gelsolin does not interfere with the protein levels of CCS and that the regulation of Cu/Zn SOD could be via a different mechanism.

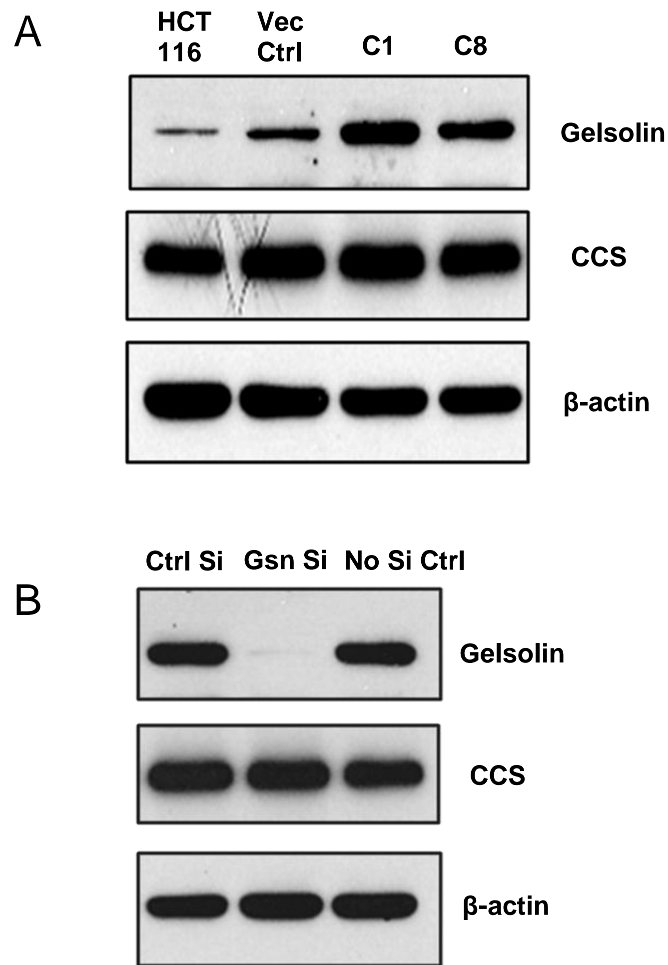


Figure 4.5A&B. Gelsolin expression does not affect protein expression of CCS. (A) The levels of CCS protein expression levels were found to be comparable between gelsolin-overexpressing C1 and C8 cells when compared to empty vector control and HCT116 cells. (B) Knockdown of gelsolin in C1 cells did not alter the protein expression levels of CCS as compared to the control siRNA and a no siRNA control cells. β-actin was used as an internal loading control.

4.3.6 Gelsolin co-immunoprecipitates with Cu/Zn SOD

To determine how gelsolin suppresses the enzymatic activity of Cu/Zn SOD, we investigated whether these two proteins can interact with each other. Endogenous Cu/Zn SOD was immunoprecipitated from the lysates of the gelsolin-overexpressing C1 cell line. The presence of gelsolin in the immunoprecipitate was detected by western blot (Fig. 4.6). A strong band of gelsolin in the Cu/Zn SOD lane was observed whereas no band was seen in the negative IgG control lane. This data indicate that gelsolin and Cu/Zn SOD are interacting partners.

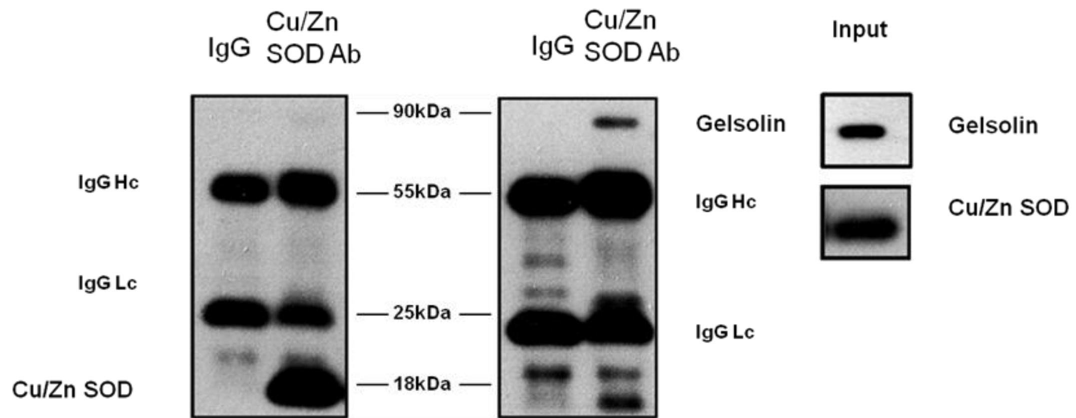


Figure 4.6 Gelsolin co-immunoprecipitates with Cu/Zn SOD. Endogenous Cu/Zn SOD was immunoprecipitated from lysates of C1 cells using Cu/Zn SOD antibody. Mouse IgG was used as negative control and lysates (input) were analysed for the expression of Cu/Zn SOD and gelsolin. **Upper panel left**: Blot showing the pulled down material, Cu/Zn SOD along with the IgG heavy chain (Hc) and IgG light chain (Lc). **Upper panel right**: Gelsolin was pulled down along with Cu/Zn SOD and the detected band is shown here. **Lower panel:** Blot showing the expression of gelsolin and Cu/Zn SOD from the C1 lysate. Data shown here is a representative of three independent experiments.

4.3.7 Gelsolin and Cu/Zn SOD lie in close proximity to each other

By employing the *in situ* Proximity Ligation Assay (PLA), we demonstrated the association between gelsolin and Cu/Zn SOD in the wild-type HCT116 cells. Cells were incubated with antibodies against gelsolin and Cu/Zn SOD and detection of red fluorescent signals indicates that these two proteins lie close to each other (Fig 4.7). The cells were also counterstained with DAPI to locate the nuclei. Negative controls such as isotype antibody and single antibody-treatment controls were included to confirm the specificity of the assay. No red fluorescent signals were detected in our negative control sets.

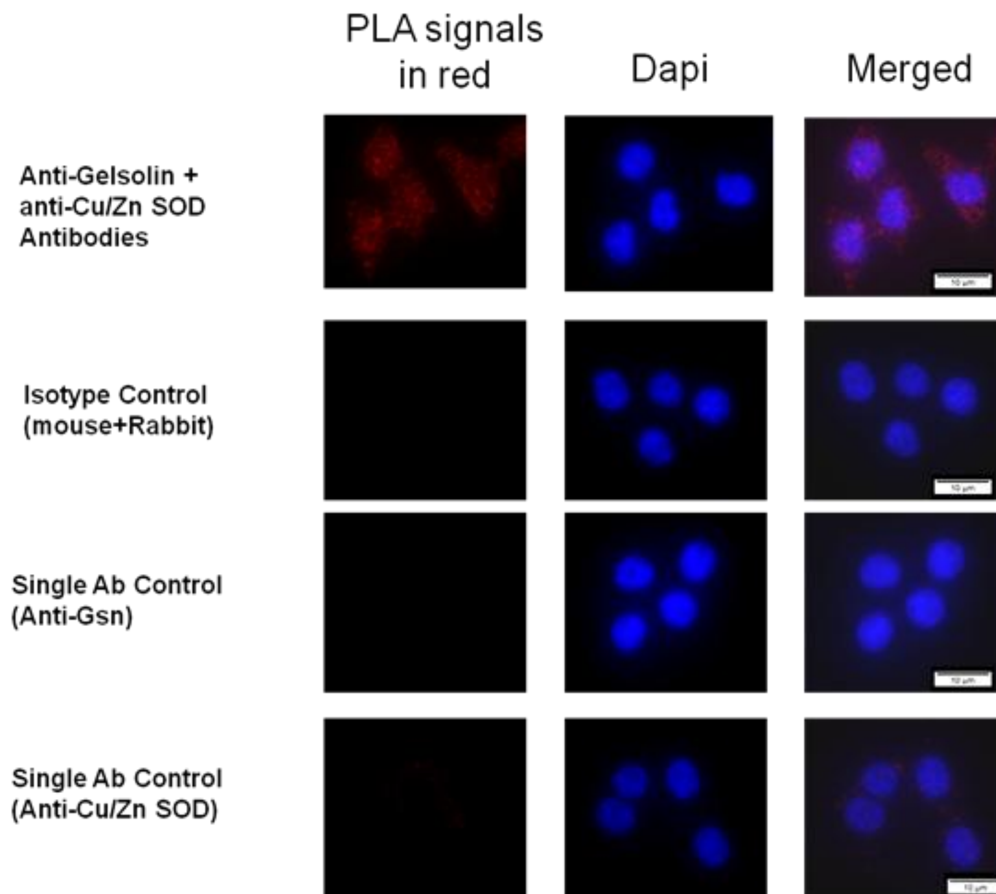


Figure 4.7 Gelsolin and Cu/Zn SOD lie in close proximity to each other. Proximity ligation assay was performed in HCT116 cells. PLA signals in red fluorescence were detected when cells were treated with both gelsolin and Cu/Zn SOD antibodies. PLA signals were not detected in isotype control cells as well as in single antibody-treated cells. Images were captured using Olympus DP72 microscope and cellSens software at 60x. Scale bar represent 20 μm. Data is a representative of three independent experiments.

4.3.8 Gelsolin expression modulates the mRNA levels of Nox

In an attempt to explore other mechanisms by which gelsolin leads to increased O_2^-, the gene expression levels of a panel of Nox isoforms were evaluated. As noted earlier NADPH oxidases (Nox) are the major ROS-producing enzymes in the mammalian cells. Five Nox isoforms (Nox1, Nox2, Nox3, Nox4, and Nox5) and two related enzymes Duox1 and Duox 2 have been identified to date (Katsuyama *et al.*, 2012). Here, Nox1, Nox2, Nox4 and Nox5 gene expression were screened as these are the isoforms that have been mostly implicated in cancer (Kamata, 2009). Out of the different isoforms of Nox screened, Nox 4 was undetectable in our study model. Overexpression of gelsolin increased the mRNA levels of Nox1 and Nox5, whilst Nox2 mRNA level was minimally affected (Fig. 4.8A). In accordance with this, knockdown of gelsolin in the gelsolin-overexpressing C1 cells reduced the mRNA levels of Nox1 and Nox5 but did not affect Nox2 (Fig. 4.8B). These data therefore suggest that gelsolin may be involved in modulating Nox1 and Nox5 mRNA expression.

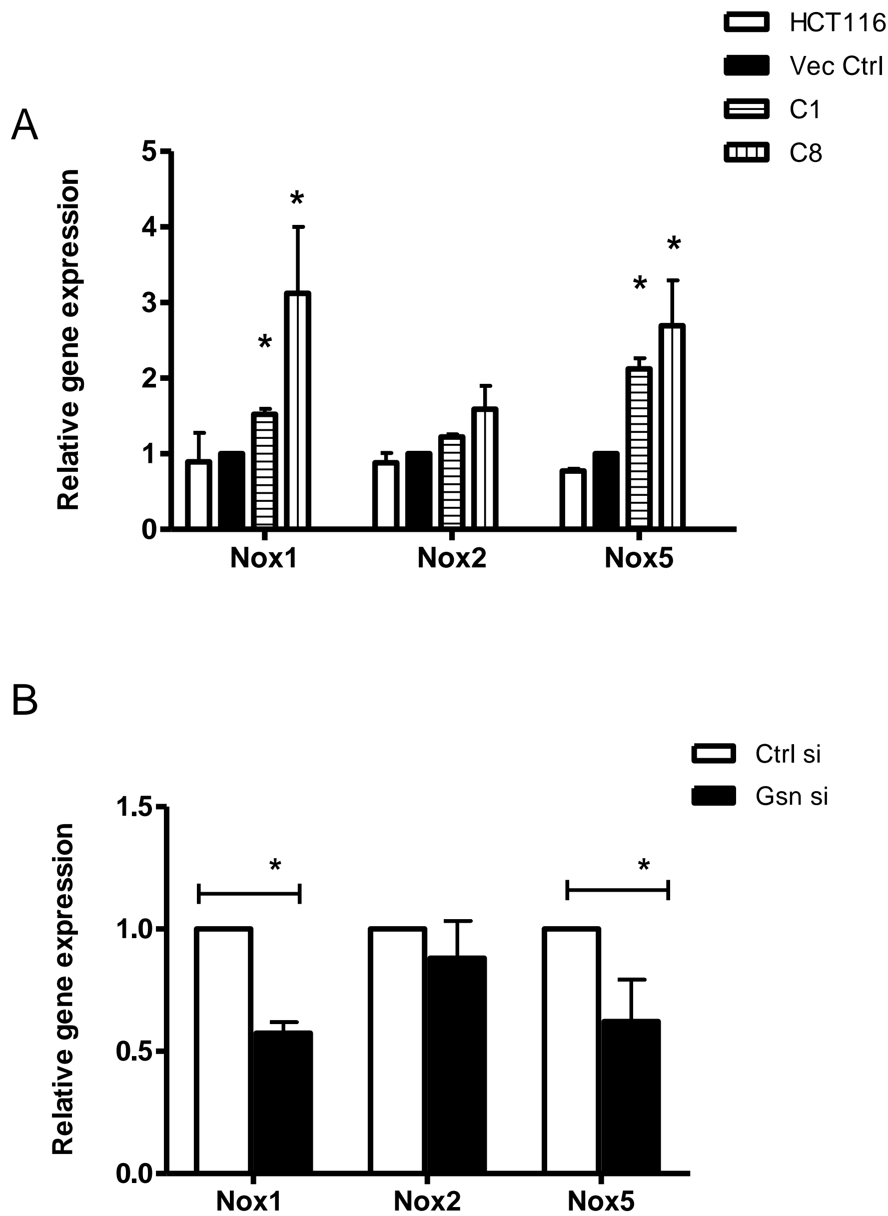


Figure 4.8A&B Gelsolin expression affects mRNA levels of Nox. (A) Overexpression of gelsolin in C1 and C8 leads to a significant increase in the gene expression of Nox1 and Nox5 when compared to the empty vector control and wild-type HCT116 cells. (B) Knockdown of gelsolin in C1 results in the downregulation of Nox1 and Nox5 when compared to the control siRNA. Data shown are mean ± SD of at least three independent experiments. *p-value <0.05 (Two tailed Student's t- test).

4.4 DISCUSSION

4.4.1 Gelsolin suppresses the activity of Cu/Zn SOD

SODs are directly responsible for the catalytic conversion of O_2^- to H_2O_2, thereby balancing the intracellular pool of O_2^-. Therefore, downregulation of SOD will lead to increase in O_2^- levels. To understand the mechanisms by which gelsolin modulates O_2^- levels, the mRNA and protein levels as well as SOD activities were determined. Although the mRNA level of Cu/Zn SOD was found to be reduced by gelsolin overexpression, the protein level remains unchanged. However, when the enzymatic activities of SODs were assayed, it was found that SOD activities were significantly attenuated following gelsolin overexpression.

Sub-cellular fractionation that separates the cytoplasm from intact mitochondria allowed us to determine which isoform of SOD is a target of gelsolin. SOD activity analysis from the cytoplasm shows a significantly reduced activity of SOD in gelsolin-overexpressing cells whereas no significant change in SOD activity was observed in the mitochondrial fraction. These data indicate that cytoplasmic Cu/Zn SOD is the specific target of gelsolin.

Several post-translational regulations are required to activate Cu/Zn SOD. Dimerisation of Cu/Zn SOD as well as efficient copper insertion is required for the enzymatic activation of this enzyme. Structural integrity is maintained by insertion of zinc ions, however, zinc can be replaced by other metal ions without affecting Cu/Zn SOD activity (Spagnolo *et al.*, 2004).

CCS is the most well known activator of the Cu/Zn SOD – by donating copper to Cu/Zn SOD, and also facilitates dimer formation, resulting in a catalytically active form of Cu/Zn SOD (Furukawa *et al.*, 2004). We assessed the protein expression levels of CCS following modulation of gelsolin expression to test whether gelsolin influences the protein expression of CCS to regulate Cu/Zn SOD. Our results show that protein expression of CCS was not altered by gelsolin expression. Therefore the suppression of Cu/Zn SOD activity by gelsolin could be via other channels other than modulation of CCS protein levels.

Glutaredoxins are antioxidants that catalyse the thiol-disulfide interchange reactions. Glutaredoxins (Glrx) are present in two forms in animals, Glrx-1 (cytosolic) and Glrx-2 (mitochondrial) (Ferri *et al.*, 2010; Gladyshev *et al.*, 2001). The mRNA levels of Glrx-2 was significantly reduced by gelsolin overexpression. Studies have suggested a link between glutaredoxins and Cu/Zn SOD. Glrx-2 has been implicated in the copper activation pathway that leads to Cu/Zn SOD stability (Carroll *et al.*, 2006). A mutant form of Cu/Zn SOD has been found to aggregate in the mitochondria leading to the pathogenesis of the motor neuron disorder, amyotrophic lateral sclerosis (ALS) (Milani *et al.*, 2011). Glrx-2 has been shown to prevent the aggregation of mutant Cu/Zn SOD in the mitochondria and abrogates its toxicity (Ferri *et al.*, 2010). Therefore, downregulation of Glrx-2 by gelsolin overexpression may contribute to reduced Cu/Zn SOD activity, since Glrx-2 appears to possess a certain degree of regulatory potential over Cu/Zn SOD.

4.4.2 Gelsolin associates with Cu/Zn SOD

Gelsolin has been shown to interact and bind with several proteins including PI3K, c-Src (Chellaiah *et al.*, 1998), p53 (An *et al.*, 2011) and VDAC (Qiao & McMillan, 2007). Therefore, we sought to test if gelsolin interacts with Cu/Zn SOD through protein-protein interaction. Our results interestingly show that gelsolin could co-immunoprecipitate with Cu/Zn SOD which indicates that gelsolin and Cu/Zn SOD are binding partners. In addition, *in situ* PLA analysis shows gelsolin and Cu/Zn SOD lie close to each other in the cell. However, whether gelsolin and Cu/Zn SOD directly associates with each other is unclear and would require further investigation. Cu/Zn SOD has been shown to associate with actin and other cytoskeletal proteins (Zhang *et al.*, 2007). Cu/Zn SOD weakly interacts with actin and mutation in Cu/Zn SOD enhanced this actin-binding capacity in neuroblastoma cells (Takamiya *et al.*, 2005). Recently, Cu/Zn SOD was also shown to co-localise with actin filaments which could be disrupted by phosphorylation of Cu/Zn SOD (Hjornevik *et al.*, 2012). Due to the close association of both gelsolin and Cu/Zn SOD with actin, it is also plausible that gelsolin may interact with Cu/Zn SOD through actin.

Preliminary findings from *in silico* experiments suggest that gelsolin and Cu/Zn SOD may directly bind to each other (unpublished data provided by Dr Pugalenthi G, supplementary figures in Appendix 1). Moreover, molecular dynamics simulation that mimics physiological condition shows that gelsolin-Cu/Zn SOD complex is stable. Additionally, this study also

suggests that the C-terminal region of gelsolin may be important for binding to Cu/Zn SOD.

Whether gelsolin and Cu/Zn SOD are direct binding partners will require further investigations, however, it is possible that the interaction between gelsolin and Cu/Zn SOD leads to the loss of Cu/Zn SOD activity. Indeed, gelsolin has the potential to suppress the activities of other proteins, for example, interaction between gelsolin and p53 leads to inactivation of p53 in hepatocarcinoma cells (An *et al.*, 2011). Therefore the binding to gelsolin potentially have a inhibitory effect on Cu/Zn SOD activity, perhaps, by interfering with proper insertion of copper or disrupting dimerisation of Cu/Zn SOD.

4.4.3 Gelsolin affects the mRNA levels of Nox isoforms

In addition to defective antioxidants observed following gelsolin overexpression, we also studied the effect of gelsolin on the ROS-producing enzyme compex, the NADPH oxidases (Nox). We observe that Nox1, Nox2 and Nox5 genes are expressed in HCT116 and its derivative cell lines. Moreover, our result shows that gelsolin expression significantly upregulates the mRNA levels of both Nox1 and Nox5. Transcription factors including Signal Transducer and Activator of Transcription-1 (STAT-1), AP-1 and Sp1 have been suggested to regulate the transcription of Nox1 and binding of STAT-5 to Nox5 promoter region has been shown to induce Nox5 expression. Gelsolin may be involved in the transcriptional regulation of Nox1 and Nox5 as gelsolin and other members of the gelsolin family such as flightless 1 and supervillin have been implicated as atranscriptional co-regulators. Gelsolin

has been found to interact with the androgen receptor in prostate cancer cells and enhances its activity (Nishimura *et al.*, 2003). Furthermore immunohistochemistry analysis of gelsolin has also detected the presence of gelsolin in the nucleus in pancreatic cancer tissues (Thompson *et al.*, 2007). However little is known on how gelsolin translocates to the nucleus and how it acts as a transcriptional co-regulator opening an avenue for future investigation.

Thus far, we have revealed potential mechanisms by which gelsolin mediates its pro-oxidant nature. We show that gelsolin expression negatively correlates with the mRNA expression of antioxidants as well as suppressing the activity of the antioxidant Cu/Zn SOD. Gelsolin expression also correlates with an increase in the mRNA levels of the ROS-producing Nox enzymes. More significantly, we found a novel interaction between gelsolin and Cu/Zn SOD that seemingly result in the inactivation of Cu/Zn SOD activity leading to a rise in the intracellular levels of O_2^-.

CHAPTER 5 GENERAL DISCUSSION, CONCLUSION AND FUTURE WORK

CHAPTER 5 GENERAL DISCUSSION, CONCLUSION AND FUTURE WORK

5.1 GENERAL DISCUSSION

The actin cytoskeleton and its regulatory proteins influence cancer cell invasion. Invasive cells are characterised by formation of actin-rich membrane protrusions that facilitate invasion by attachment to and degradation of ECM as well as generating force required for cell movement. Gelsolin is one of the most prominent actin-binding proteins that has been widely implicated in promoting cancer cell invasion. The in-depth mechanisms by which gelsolin carries out invasive behaviour however remain poorly demonstrated.

The role of gelsolin in cancer itself has been an open question. Because gelsolin has been found to be downregulated in several cancers it is considered as a tumour suppressor (Li *et al.*, 2012). Downregulation of gelsolin appears to be a result of transcriptional repression of gelsolin (Dong *et al.*, 2002; Mielnicki *et al.*, 1999). Loss of function due to mutations also contributes to the tumour suppressive role of gelsolin (Mullauer *et al.*, 1993). On the other hand, accumulating evidence supports the idea that gelsolin significantly contributes to tumour aggressiveness mainly in two areas — by its abilities to enhance invasion and resistance to apoptosis. Gelsolin has been shown to be a downstream effector of Ras in the Rac-mediated invasion pathway (De Corte *et al.*, 2002). We have also recently shown that gelsolin promotes cancer cell invasion by elevating uPA levels as well as uPA secretion (Zhuo *et al.*, 2012). In this study we explore other potential

mechanisms by which gelsolin mediates invasion, such as through modulating intracellular ROS levels.

5.1.1 Gelsolin as a pro-oxidant molecule

Our results show that gelsolin expression modulates the intracellular levels of O_2^-. Overespression of gelsolin results in increased levels of O_2^- whereas knockdown of endogenous gelsolin decreases O_2^- levels. However, it has been shown that gelsolin expression was heightened when PC12 rat medulla cells were treated with H_2O_2, and gelsolin has been suggested to have an antioxidant property (Chauhan *et al.*, 2008; Ji *et al.*, 2008). Since our findings clearly point gelsolin as a pro-oxidant molecule, the induction of gelsolin expression by H_2O_2 is likely a result of an adaptive response to oxidative stress. Indeed, gelsolin expression has been shown to be upregulated by hypoxic stress (Greijer *et al.*, 2005) and genotoxic stress caused by 5- fluorouracil (5-FU) (Klampfer *et al.*, 2005). Thus, our study has demonstrated a novel pro-oxidant role of gelsolin, and importantly, we show that the increase in O_2^- confers invasive phenotypes in cancer cells.

5.1.2 Gelsolin mediates invasion in an O_2^- dependent manner

ROS have been shown to actively participate in the progression of cancer disease and promotes cancer cell invasion. By virtue of their ability to modify proteins, DNA and lipids, and on the basis of how much ROS is produced, ROS regulates cancer in various aspects. For example, mild increase in ROS levels favour growth, proliferation and invasion whereas high levels of ROS are toxic to the cells (Benhar *et al.*, 2002; Gloire *et al.*, 2006;

Nishikawa, 2008). Here, we show that the oxidative stress environment created by gelsolin is not toxic to cells but rather confers invasive phenotype. Gelsolin overexpression increased O_2^- levels and enhanced cancer cell invasion. The invasive abilities of gelsolin-overexpressing cells could be attenuated by DPI treatment. Thus, our data corroborates the importance of O_2^- in gelsolin-mediated invasion.

Accumulating evidence have reported that ROS can upregulate MMPs and uPA expression (Binker *et al.*, 2009; Chiu *et al.*, 2010; Khoi *et al.*, 2012a; Kim *et al.*, 2007a; Pelicano *et al.*, 2009). In line with this, we found that gelsolin overexpression upregulates uPA secretion which could be lowered by DPI treatment. Importantly, we have shown that uPA is critical in gelsolin-mediated invasion (Zhuo *et al.*, 2012). Therefore, gelsolin-induced O_2^- probably plays a significant role in the upregulation of uPA and thereby promotes invasion. In addition, whilst most studies have shown H_2O_2 as the dominant species involved in regulation of these proteases, our findings suggest an important role of O_2^- in modulating uPA secretion and invasion. The mechanisms by which O_2^- modulates uPA secretion however requires further studies.

5.1.3 Gelsolin suppresses Cu/Zn SOD activity possibly by interacting with it

Antioxidants play protective roles against cancer progression. One such antioxidant is the Cu/Zn SOD. Downregulation of Cu/Zn SOD is associated with malignant phenotypes in cancer. Indeed loss of Cu/Zn SOD in animals leads to increased risk of liver cancer development (Elchuri *et al.*,

2005; Van Remmen *et al.*, 2003). Our study has shown that gelsolin suppresses Cu/Zn SOD activity resulting in elevated levels of intracellular O_2^- pool. Interestingly, our co-immunoprecipitation study reveals that gelsolin can form a complex with Cu/Zn SOD. Therefore, it is possible that binding of gelsolin to Cu/Zn SOD interferes with Cu/Zn SOD activity leading to increase O_2^-. Although *in silico* analysis performed by Pugalenthi *et al.*., (unpublished data, personal communication) suggests that gelsolin binds directly to Cu/Zn SOD, more experimental evidences are required to substantiate the direct interaction.of gelsolin and Cu/Zn SOD.

5.1.4 Gelsolin increases mRNA levels of Nox

Besides the loss of Cu/Zn SOD, we sought to determine if ROS-generating sources were influenced by gelsolin. As Nox enzymes are the major O_2^- generating source, we assessed the mRNA expressions of Nox. We found that gelsolin-overexpression positively correlates with Nox1 and Nox 5 mRNA levels. Gelsolin has been implicated as a transcriptional co-regulator, supporting the potential role of gelsolin in the transcriptional control of these Noxes. An important note to consider, however, is that Nox activity is highly dependent on its interactions with several cytosolic subunits (Katsuyama *et al.*, 2012). Interestingly, actin and other actin-associated proteins have been shown to be involved in Nox assembly and stabilisation of Nox complex at the plasma membrane. Therefore it is possible that gelsolin may also be involved in Nox activation via participating in Nox assembly.

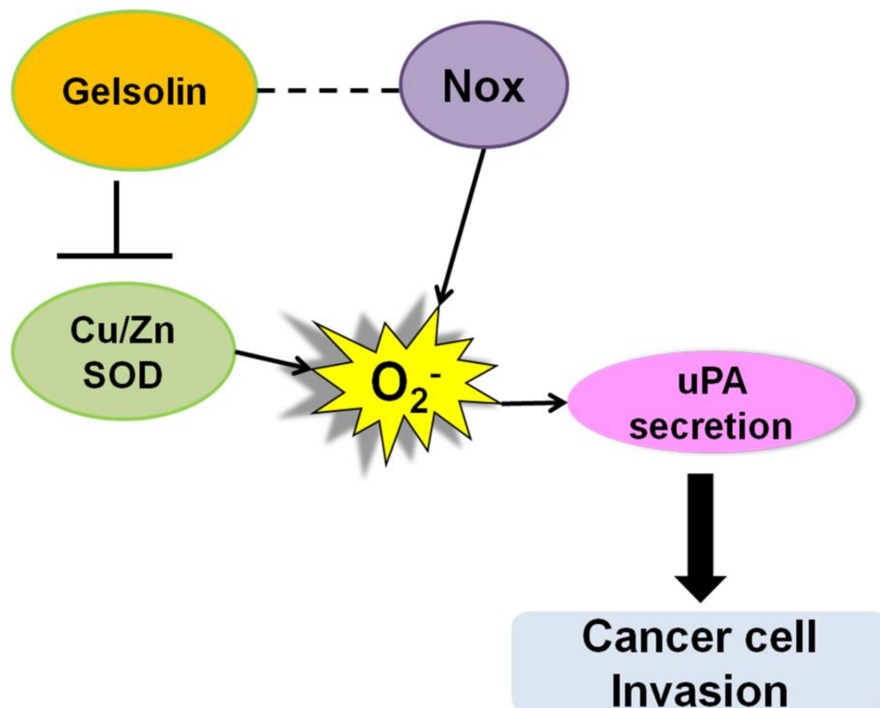


Figure 5.1 Schematic illustration showing the summary of the key findings presented in this thesis. Overexpression of gelsolin suppresses Cu/Zn SOD activity, thereby increasing O_2^- levels in the cell. The mRNA levels of Nox genes were also found to be upregulated by gelsolin overexpression which may contribute to increased O_2^- levels. O_2^- induced by gelsolin participates in uPA secretion resulting in enhanced cancer cell invasion.

5.2 CONCLUSION AND CLINICAL IMPLICATION

In this study, we have delineated a novel role of gelsolin in creating a pro-oxidant milieu that favours cancer cell invasion. The pro-oxidant nature of gelsolin triggers proteases such as uPA to degrade the ECM which facilitates the process of cancer cell invasion. We further demonstrate a novel interaction between gelsolin and the antioxidant Cu/Zn SOD and this interaction possibly renders Cu/Zn SOD inactive, leading to the increase in O_2^- levels. Disruption of the gelsolin-Cu/Zn SOD complex that restores Cu/Zn SOD activity will bring down the intracellular ROS levels and could at least in part attenuate the progression of cancer cell invasion. Therefore, a better understanding of the interaction between gelsolin and Cu/Zn SOD is required. Targeting the gelsolin-Cu/Zn SOD complex will aid in lowering the intracellular ROS levels that can be used as a tool for therapeutic regime against cancer.

5.3 FUTURE WORK

Key areas of interest for future investigations are:

- **To elucidate whether gelsolin and Cu/Zn SOD are direct binding partners and to identify the binding region**: Our findings have provided evidence that gelsolin suppresses Cu/Zn SOD activity, however, the mechanisms remain unclear. We show that gelsolin co-immunoprecipitates with Cu/Zn SOD and preliminary findings using *in silico* analysis suggest that the C-terminal half of gelsolin may be involved in this interaction (Appendix I, supplementary figure 1). Our study can be extended to determine the nature of gelsolin-Cu/Zn SOD interaction. Furthermore gelsolin mutant constructs, such as C-terminal truncate or domain deletion mutants can be employed to determine the region of gelsolin important in the binding and regulation of Cu/Zn SOD.
- **To study the role of gelsolin and O_2^- in invadopodia**: Formation of invadopodia is an important mechanism by which cancer cells promote invasion. Actin cytoskeleton and its regulatory protein are important in invadopodia. The role of gelsolin and O_2^- can be explored which may provide a mechanistic insight into the role of gelsolin-induced invasion.
- **Role of gelsolin-induced O_2^- in uPA regulation:** Although we have shown that O_2^- is involved in upregulating uPA secretion, the mechanism is not known. This study can be extended by assessing whether uPA transcription is influenced by O_2^- induced by gelsolin.

Moreover, it would also be interesting to study if O_2^- is involved in the activation step of uPA. Understanding the mechanisms by which O_2^- participates in upregualting uPA secretion will enhance our understanding of the role of ROS-mediated invasion.

BIBLIOGRAPHY

BIBLIOGRAPHY

Alexandrova AY, Kopnin PB, Vasiliev JM, Kopnin BP (2006) ROS up-regulation mediates Ras-induced changes of cell morphology and motility. *Exp Cell Res* **312:** 2066-2073

Alscher RG, Erturk N, Heath LS (2002) Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *Journal of experimental botany* **53:** 1331-1341

An JH, Kim JW, Jang SM, Kim CH, Kang EJ, Choi KH (2011) Gelsolin negatively regulates the activity of tumor suppressor p53 through their physical interaction in hepatocarcinoma HepG2 cells. *Biochem Biophys Res Commun* **412:** 44-49

Angers-Loustau A, Hering R, Werbowetski TE, Kaplan DR, Del Maestro RF (2004) SRC regulates actin dynamics and invasion of malignant glial cells in three dimensions. *Mol Cancer Res* **2:** 595-605

Antequera D, Vargas T, Ugalde C, Spuch C, Molina JA, Ferrer I, Bermejo-Pareja F, Carro E (2009) Cytoplasmic gelsolin increases mitochondrial activity and reduces Abeta burden in a mouse model of Alzheimer's disease. *Neurobiology of disease* **36:** 42-50

Arora PD, Glogauer M, Kapus A, Kwiatkowski DJ, McCulloch CA (2004) Gelsolin mediates collagen phagocytosis through a rac-dependent step. *Mol Biol Cell* **15:** 588-599

Arora PD, McCulloch CA (1996) Dependence of fibroblast migration on actin severing activity of gelsolin. *J Biol Chem* **271:** 20516-20523

Asch HL, Head K, Dong Y, Natoli F, Winston JS, Connolly JL, Asch BB (1996) Widespread loss of gelsolin in breast cancers of humans, mice, and rats. *Cancer Res* **56:** 4841-4845

Azuma T, Koths K, Flanagan L, Kwiatkowski D (2000) Gelsolin in complex with phosphatidylinositol 4,5-bisphosphate inhibits caspase-3 and -9 to retard apoptotic progression. *J Biol Chem* **275:** 3761-3766

Azuma T, Witke W, Stossel TP, Hartwig JH, Kwiatkowski DJ (1998) Gelsolin is a downstream effector of rac for fibroblast motility. *EMBO J* **17:** 1362-1370

Baldassarre M, Ayala I, Beznoussenko G, Giacchetti G, Machesky LM, Luini A, Buccione R (2006) Actin dynamics at sites of extracellular matrix degradation. *Eur J Cell Biol* **85:** 1217-1231

Banci L, Bertini I, Cantini F, Kozyreva T, Massagni C, Palumaa P, Rubino JT, Zovo K (2012) Human superoxide dismutase 1 (hSOD1) maturation through interaction with human copper chaperone for SOD1 (hCCS). *Proc Natl Acad Sci U S A* **109:** 13555-13560

Banci L, Bertini I, Cramaro F, Del Conte R, Viezzoli MS (2002) The solution structure of reduced dimeric copper zinc superoxide dismutase. The structural effects of dimerization. *European journal of biochemistry / FEBS* **269:** 1905-1915

Banno Y, Nakashima T, Kumada T, Ebisawa K, Nonomura Y, Nozawa Y (1992) Effects of gelsolin on human platelet cytosolic phosphoinositide-phospholipase C isozymes. *J Biol Chem* **267:** 6488-6494

Barbour A, Gotley DC (2003) Current concepts of tumour metastasis. *Annals of the Academy of Medicine, Singapore* **32:** 176-184

Barnett P, Arnold RS, Mezencev R, Chung LW, Zayzafoon M, Odero-Marah V (2011) Snail-mediated regulation of reactive oxygen species in ARCaP human prostate cancer cells. *Biochem Biophys Res Commun* **404:** 34-39

Bauer TW, Liu W, Fan F, Camp ER, Yang A, Somcio RJ, Bucana CD, Callahan J, Parry GC, Evans DB, Boyd DD, Mazar AP, Ellis LM (2005) Targeting of urokinase plasminogen activator receptor in human pancreatic carcinoma cells inhibits c-Met- and insulin-like growth factor-I receptor-mediated migration and invasion and orthotopic tumor growth in mice. *Cancer Res* **65:** 7775-7781

Baum B, Settleman J, Quinlan MP (2008) Transitions between epithelial and mesenchymal states in development and disease. *Semin Cell Dev Biol* **19:** 294-308

Ben Mahdi MH, Andrieu V, Pasquier C (2000) Focal adhesion kinase regulation by oxidative stress in different cell types. *IUBMB Life* **50:** 291-299

Benhar M, Engelberg D, Levitzki A (2002) ROS, stress-activated kinases and stress signaling in cancer. *EMBO Rep* **3:** 420-425

Binker MG, Binker-Cosen AA, Richards D, Oliver B, Cosen-Binker LI (2009) EGF promotes invasion by PANC-1 cells through Rac1/ROS-dependent secretion and activation of MMP-2. *Biochem Biophys Res Commun* **379:** 445-450

Boivin B, Yang M, Tonks NK (2010) Targeting the reversibly oxidized protein tyrosine phosphatase superfamily. *Sci Signal* **3:** pl2

Bostwick DG, Alexander EE, Singh R, Shan A, Qian J, Santella RM, Oberley LW, Yan T, Zhong W, Jiang X, Oberley TD (2000) Antioxidant enzyme expression and reactive oxygen species damage in prostatic intraepithelial neoplasia and cancer. *Cancer* **89:** 123-134

Brooks SA, Lomax-Browne HJ, Carter TM, Kinch CE, Hall DM (2010) Molecular interactions in cancer cell metastasis. *Acta Histochem* **112:** 3-25

Brown DI, Griendling KK (2009) Nox proteins in signal transduction. *Free Radic Biol Med* **47:** 1239-1253

Burtnick LD, Koepf EK, Grimes J, Jones EY, Stuart DI, McLaughlin PJ, Robinson RC (1997) The crystal structure of plasma gelsolin: implications for actin severing, capping, and nucleation. *Cell* **90:** 661-670

Burtnick LD, Urosev D, Irobi E, Narayan K, Robinson RC (2004) Structure of the N-terminal half of gelsolin bound to actin: roles in severing, apoptosis and FAF. *EMBO J* **23:** 2713-2722

Cadenas E, Davies KJ (2000) Mitochondrial free radical generation, oxidative stress, and aging. *Free Radic Biol Med* **29:** 222-230

Campbell PM, Der CJ (2004) Oncogenic Ras and its role in tumor cell invasion and metastasis. *Seminars in cancer biology* **14:** 105-114

Cannito S, Novo E, di Bonzo LV, Busletta C, Colombatto S, Parola M (2010) Epithelial-mesenchymal transition: from molecular mechanisms, redox regulation to implications in human health and disease. *Antioxid Redox Signal* **12:** 1383-1430

Carroll MC, Outten CE, Proescher JB, Rosenfeld L, Watson WH, Whitson LJ, Hart PJ, Jensen LT, Cizewski Culotta V (2006) The effects of glutaredoxin and copper activation pathways on the disulfide and stability of Cu,Zn superoxide dismutase. *J Biol Chem* **281:** 28648-28656

Chambers AF, Groom AC, MacDonald IC (2002) Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer* **2:** 563-572

Chauhan V, Ji L, Chauhan A (2008) Anti-amyloidogenic, anti-oxidant and anti-apoptotic role of gelsolin in Alzheimer's disease. *Biogerontology* **9:** 381-389

Chellaiah M, Fitzgerald C, Alvarez U, Hruska K (1998) c-Src is required for stimulation of gelsolin-associated phosphatidylinositol 3-kinase. *J Biol Chem* **273:** 11908-11916

Chellaiah M, Hruska K (1996) Osteopontin stimulates gelsolin-associated phosphoinositide levels and phosphatidylinositol triphosphate-hydroxyl kinase. *Mol Biol Cell* **7:** 743-753

Chellaiah M, Kizer N, Silva M, Alvarez U, Kwiatkowski D, Hruska KA (2000) Gelsolin deficiency blocks podosome assembly and produces increased bone mass and strength. *J Cell Biol* **148:** 665-678

Chellaiah MA (2006) Regulation of podosomes by integrin alphavbeta3 and Rho GTPase-facilitated phosphoinositide signaling. *Eur J Cell Biol* **85:** 311-317

Chellaiah MA, Biswas RS, Yuen D, Alvarez UM, Hruska KA (2001) Phosphatidylinositol 3,4,5-trisphosphate directs association of Src homology 2-containing signaling proteins with gelsolin. *J Biol Chem* **276:** 47434-47444

Chen P, Murphy-Ullrich JE, Wells A (1996) A role for gelsolin in actuating epidermal growth factor receptor-mediated cell motility. *J Cell Biol* **134:** 689-698

Chen WT (1989) Proteolytic activity of specialized surface protrusions formed at rosette contact sites of transformed cells. *The Journal of experimental zoology* **251:** 167-185

Cheng G, Diebold BA, Hughes Y, Lambeth JD (2006) Nox1-dependent reactive oxygen generation is regulated by Rac1. *J Biol Chem* **281:** 17718-17726

Chiarugi P, Pani G, Giannoni E, Taddei L, Colavitti R, Raugei G, Symons M, Borrello S, Galeotti T, Ramponi G (2003) Reactive oxygen species as essential mediators of cell adhesion: the oxidative inhibition of a FAK tyrosine phosphatase is required for cell adhesion. *J Cell Biol* **161:** 933-944

Chiu WT, Shen SC, Chow JM, Lin CW, Shia LT, Chen YC (2010) Contribution of reactive oxygen species to migration/invasion of human glioblastoma cells U87 via ERK-dependent COX-2/PGE(2) activation. *Neurobiology of disease* **37:** 118-129

Choe H, Burtnick LD, Mejillano M, Yin HL, Robinson RC, Choe S (2002) The calcium activation of gelsolin: insights from the 3A structure of the G4-G6/actin complex. *Journal of molecular biology* **324:** 691-702

Chou J, Stolz DB, Burke NA, Watkins SC, Wells A (2002) Distribution of gelsolin and phosphoinositol 4,5-bisphosphate in lamellipodia during EGF-induced motility. *Int J Biochem Cell Biol* **34:** 776-790

Clark ES, Weaver AM (2008) A new role for cortactin in invadopodia: regulation of protease secretion. *Eur J Cell Biol* **87:** 581-590

Clark ES, Whigham AS, Yarbrough WG, Weaver AM (2007) Cortactin is an essential regulator of matrix metalloproteinase secretion and extracellular matrix degradation in invadopodia. *Cancer Res* **67:** 4227-4235

Clement MV, Stamenkovic I (1996) Superoxide anion is a natural inhibitor of FAS-mediated cell death. *EMBO J* **15:** 216-225

Crowley J, Smith T, Fang Z, Takizawa N, Luna E (2009) Supervillin reorganizes the actin cytoskeleton and increases invadopodial efficiency. *Mol Biol Cell* **20:** 948-962

Cunningham CC, Stossel TP, Kwiatkowski DJ (1991) Enhanced motility in NIH 3T3 fibroblasts that overexpress gelsolin. *Science* **251:** 1233-1236

Curran S, Dundas SR, Buxton J, Leeman MF, Ramsay R, Murray GI (2004) Matrix metalloproteinase/tissue inhibitors of matrix metalloproteinase phenotype identifies poor prognosis colorectal cancers. *Clin Cancer Res* **10:** 8229-8234

Curran S, Murray GI (2000) Matrix metalloproteinases: molecular aspects of their roles in tumour invasion and metastasis. *Eur J Cancer* **36:** 1621-1630

D'Autréaux B, Toledano MB (2007) ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat Rev Mol Cell Biol* **8:** 813-824

DalleDonne I, Milzani A, Colombo R (1995) H2O2-treated actin: assembly and polymer interactions with cross-linking proteins. *Biophys J* **69:** 2710-2719

DalleDonne I, Milzani A, Colombo R (1999) The tert-butyl hydroperoxide-induced oxidation of actin Cys-374 is coupled with structural changes in distant regions of the protein. *Biochemistry* **38:** 12471-12480

De Corte V, Bruyneel E, Boucherie C, Mareel M, Vandekerckhove J, Gettemans J (2002) Gelsolin-induced epithelial cell invasion is dependent on Ras-Rac signaling. *EMBO J* **21:** 6781-6790

den Hertog J, Ostman A, Böhmer FD (2008) Protein tyrosine phosphatases: regulatory mechanisms. *FEBS J* **275:** 831-847

Diaz B, Courtneidge SA (2012) Redox signaling at invasive microdomains in cancer cells. *Free Radic Biol Med* **52:** 247-256

Diaz B, Shani G, Pass I, Anderson D, Quintavalle M, Courtneidge SA (2009) Tks5-dependent, nox-mediated generation of reactive oxygen species is necessary for invadopodia formation. *Sci Signal* **2:** ra53

Didion SP, Hathaway CA, Faraci FM (2001) Superoxide levels and function of cerebral blood vessels after inhibition of CuZn-SOD. *American journal of physiology Heart and circulatory physiology* **281:** H1697-1703

Ditsch A, Wegner A (1994) Nucleation of actin polymerization by gelsolin. *European journal of biochemistry / FEBS* **224:** 223-227

Dong Y, Asch HL, Ying A, Asch BB (2002) Molecular mechanism of transcriptional repression of gelsolin in human breast cancer cells. *Exp Cell Res* **276:** 328-336

Dosaka-Akita H, Hommura F, Fujita H, Kinoshita I, Nishi M, Morikawa T, Katoh H, Kawakami Y, Kuzumaki N (1998) Frequent loss of gelsolin expression in non-small cell lung cancers of heavy smokers. *Cancer Res* **58:** 322-327

Duffy MJ (2004) The urokinase plasminogen activator system: role in malignancy. *Current pharmaceutical design* **10:** 39-49

Elchuri S, Oberley TD, Qi W, Eisenstein RS, Jackson Roberts L, Van Remmen H, Epstein CJ, Huang TT (2005) CuZnSOD deficiency leads to persistent and widespread oxidative damage and hepatocarcinogenesis later in life. *Oncogene* **24:** 367-380

Endres M, Fink K, Zhu J, Stagliano NE, Bondada V, Geddes JW, Azuma T, Mattson MP, Kwiatkowski DJ, Moskowitz MA (1999) Neuroprotective effects of gelsolin during murine stroke. *J Clin Invest* **103:** 347-354

Ferri A, Fiorenzo P, Nencini M, Cozzolino M, Pesaresi MG, Valle C, Sepe S, Moreno S, Carri MT (2010) Glutaredoxin 2 prevents aggregation of mutant SOD1 in mitochondria and abolishes its toxicity. *Human molecular genetics* **19:** 4529-4542

Fiaschi T, Cozzi G, Raugei G, Formigli L, Ramponi G, Chiarugi P (2006) Redox regulation of beta-actin during integrin-mediated cell adhesion. *The Journal of biological chemistry* **281:** 22983-22991

Finkelstein M, Etkovitz N, Breitbart H (2010) Role and regulation of sperm gelsolin prior to fertilization. *J Biol Chem* **285:** 39702-39709

Fridovich I (1978) Superoxide radicals, superoxide dismutases and the aerobic lifestyle. *Photochemistry and photobiology* **28:** 733-741

Fridovich I (1995) Superoxide radical and superoxide dismutases. *Annu Rev Biochem* **64:** 97-112

Friedl P, Alexander S (2011) Cancer invasion and the microenvironment: plasticity and reciprocity. *Cell* **147:** 992-1009

Friedl P, Gilmour D (2009) Collective cell migration in morphogenesis, regeneration and cancer. *Nat Rev Mol Cell Biol* **10:** 445-457

Friedl P, Wolf K (2003) Tumour-cell invasion and migration: diversity and escape mechanisms. *Nat Rev Cancer* **3:** 362-374

Furukawa Y, Torres AS, O'Halloran TV (2004) Oxygen-induced maturation of SOD1: a key role for disulfide formation by the copper chaperone CCS. *EMBO J* **23:** 2872-2881

Gay F, Estornes Y, Saurin JC, Joly-Pharaboz MO, Friederich E, Scoazec JY, Abello J (2008) In colon carcinogenesis, the cytoskeletal protein gelsolin is down-regulated during the transition from adenoma to carcinoma. *Hum Pathol* **39:** 1420-1430

Geng YJ, Azuma T, Tang JX, Hartwig JH, Muszynski M, Wu Q, Libby P, Kwiatkowski DJ (1998) Caspase-3-induced gelsolin fragmentation contributes to actin cytoskeletal collapse, nucleolysis, and apoptosis of vascular smooth muscle cells exposed to proinflammatory cytokines. *Eur J Cell Biol* **77:** 294-302

Gianni D, Taulet N, DerMardirossian C, Bokoch GM (2010a) c-Src-mediated phosphorylation of NoxA1 and Tks4 induces the reactive oxygen species (ROS)-dependent formation of functional invadopodia in human colon cancer cells. *Mol Biol Cell* **21:** 4287-4298

Gianni D, Taulet N, Zhang H, DerMardirossian C, Kister J, Martinez L, Roush WR, Brown SJ, Bokoch GM, Rosen H (2010b) A novel and specific NADPH oxidase-1 (Nox1) small-molecule inhibitor blocks the formation of functional invadopodia in human colon cancer cells. *ACS chemical biology* **5:** 981-993

Giridharan SS, Rohn JL, Naslavsky N, Caplan S (2012) Differential regulation of actin microfilaments by human MICAL proteins. *J Cell Sci*

Gladyshev VN, Liu A, Novoselov SV, Krysan K, Sun QA, Kryukov VM, Kryukov GV, Lou MF (2001) Identification and characterization of a new mammalian glutaredoxin (thioltransferase), Grx2. *J Biol Chem* **276:** 30374-30380

Gloire G, Legrand-Poels S, Piette J (2006) NF-kappaB activation by reactive oxygen species: fifteen years later. *Biochemical pharmacology* **72:** 1493-1505

Glorieux C, Dejeans N, Sid B, Beck R, Calderon PB, Verrax J (2011) Catalase overexpression in mammary cancer cells leads to a less aggressive phenotype and an altered response to chemotherapy. *Biochemical pharmacology* **82:** 1384-1390

Granfeldt D, Dahlgren C (2001) An intact cytoskeleton is required for prolonged respiratory burst activity during neutrophil phagocytosis. *Inflammation* **25:** 165-169

Greijer AE, van der Groep P, Kemming D, Shvarts A, Semenza GL, Meijer GA, van de Wiel MA, Belien JA, van Diest PJ, van der Wall E (2005) Up-regulation of gene expression by hypoxia is mediated predominantly by hypoxia-inducible factor 1 (HIF-1). *J Pathol* **206:** 291-304

Guan JL (1997) Role of focal adhesion kinase in integrin signaling. *Int J Biochem Cell Biol* **29:** 1085-1096

Guarino M (2010) Src signaling in cancer invasion. *J Cell Physiol* **223:** 14-26

Gupta GP, Massague J (2006) Cancer metastasis: building a framework. *Cell* **127:** 679-695

Haga K, Fujita H, Nomoto M, Sazawa A, Nakagawa K, Harabayashi T, Shinohara N, Takimoto M, Nonomura K, Kuzumaki N (2004) Gelsolin gene silencing involving unusual hypersensitivities to dimethylsulfate and KMnO4 in vivo footprinting on its promoter region. *International journal of cancer Journal international du cancer* **111:** 873-880

Halliwell B (1996) Antioxidants in human health and disease. *Annual review of nutrition* **16:** 33-50

Halliwell B (2006) Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. *Plant physiology* **141:** 312-322

Halliwell B (2007) Oxidative stress and cancer: have we moved forward? *The Biochemical journal* **401:** 1-11

Han JW, Ahn SH, Park SH, Wang SY, Bae GU, Seo DW, Kwon HK, Hong S, Lee HY, Lee YW, Lee HW (2000) Apicidin, a histone deacetylase inhibitor, inhibits proliferation of tumor cells via induction of p21WAF1/Cip1 and gelsolin. *Cancer Res* **60:** 6068-6074

Heikkila RE, Cabbat FS, Cohen G (1976) In vivo inhibition of superoxide dismutase in mice by diethyldithiocarbamate. *J Biol Chem* **251:** 2182-2185

Hjornevik LV, Fismen L, Young FM, Solstad T, Fladmark KE (2012) Nodularin Exposure Induces SOD1 Phosphorylation and Disrupts SOD1 Co-localization with Actin Filaments. *Toxins* **4:** 1482-1499

Hoshikawa Y, Kwon HJ, Yoshida M, Horinouchi S, Beppu T (1994) Trichostatin A induces morphological changes and gelsolin expression by inhibiting histone deacetylase in human carcinoma cell lines. *Exp Cell Res* **214:** 189-197

Hung RJ, Pak CW, Terman JR (2011) Direct redox regulation of F-actin assembly and disassembly by Mical. *Science* **334:** 1710-1713

Janmey PA, Stossel TP (1987) Modulation of gelsolin function by phosphatidylinositol 4,5-bisphosphate. *Nature* **325:** 362-364

Ji L, Chauhan A, Chauhan V (2008) Cytoplasmic gelsolin in pheochromocytoma-12 cells forms a complex with amyloid beta-protein. *Neuroreport* **19:** 463-466

Jones GE, Zicha D, Dunn GA, Blundell M, Thrasher A (2002) Restoration of podosomes and chemotaxis in Wiskott-Aldrich syndrome macrophages following induced expression of WASp. *Int J Biochem Cell Biol* **34:** 806-815

Kamata T (2009) Roles of Nox1 and other Nox isoforms in cancer development. *Cancer Sci* **100:** 1382-1388

Kang J, Pervaiz S (2012) Crosstalk between Bcl-2 family and Ras family small GTPases: potential cell fate regulation? *Frontiers in oncology* **2:** 206

Kassis J, Lauffenburger DA, Turner T, Wells A (2001) Tumor invasion as dysregulated cell motility. *Seminars in cancer biology* **11:** 105-117

Katsuyama M, Matsuno K, Yabe-Nishimura C (2012) Physiological roles of NOX/NADPH oxidase, the superoxide-generating enzyme. *Journal of clinical biochemistry and nutrition* **50:** 9-22

Khoi PN, Park JS, Kim NH, Jung YD (2012a) Nicotine stimulates urokinase-type plasminogen activator receptor expression and cell invasiveness through mitogen-activated protein kinase and reactive oxygen species signaling in ECV304 endothelial cells. *Toxicol Appl Pharmacol* **259:** 248-256

Khoi PN, Park JS, Kim NH, Jung YD (2012b) Nicotine stimulates urokinase-type plasminogen activator receptor expression and cell invasiveness through mitogen-activated protein kinase and reactive oxygen species signaling in ECV304 endothelial cells. *Toxicol Appl Pharmacol*

Kim JH, Choi YK, Kwon HJ, Yang HK, Choi JH, Kim DY (2004) Downregulation of gelsolin and retinoic acid receptor beta expression in gastric cancer tissues through histone deacetylase 1. *Journal of gastroenterology and hepatology* **19:** 218-224

Kim JS, Bak EJ, Lee BC, Kim YS, Park JB, Choi IG (2011) Neuregulin induces HaCaT keratinocyte migration via Rac1-mediated NADPH-oxidase activation. *J Cell Physiol* **226:** 3014-3021

Kim JS, Huang TY, Bokoch GM (2009) Reactive oxygen species regulate a slingshot-cofilin activation pathway. *Mol Biol Cell* **20:** 2650-2660

Kim MH, Yoo HS, Kim MY, Jang HJ, Baek MK, Kim HR, Kim KK, Shin BA, Ahn BW, Jung YD (2007a) Helicobacter pylori stimulates urokinase plasminogen activator receptor expression and cell invasiveness through reactive oxygen species and NF-kappaB signaling in human gastric carcinoma cells. *Int J Mol Med* **19:** 689-697

Kim MH, Yoo HS, Kim MY, Jang HJ, Baek MK, Kim HR, Kim KK, Shin BA, Ahn BW, Jung YD (2007b) Helicobacter pylori stimulates urokinase plasminogen activator receptor expression and cell invasiveness through reactive oxygen species and NF-kappaB signaling in human gastric carcinoma cells. *Int J Mol Med* **19:** 689-697

Klampfer L, Swaby L, Huang J, Sasazuki T, Shirasawa S, Augenlicht L (2005) Oncogenic Ras increases sensitivity of colon cancer cells to 5-FU-induced apoptosis. *Oncogene* **24:** 3932-3941

Knapp LT, Klann E (2000) Superoxide-induced stimulation of protein kinase C via thiol modification and modulation of zinc content. *J Biol Chem* **275:** 24136-24145

Kothakota S, Azuma T, Reinhard C, Klippel A, Tang J, Chu K, McGarry TJ, Kirschner MW, Koths K, Kwiatkowski DJ, Williams LT (1997) Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. *Science* **278:** 294-298

Koya R, Fujita H, Shimizu S, Ohtsu M, Takimoto M, Tsujimoto Y, Kuzumaki N (2000) Gelsolin inhibits apoptosis by blocking mitochondrial membrane potential loss and cytochrome c release. *J Biol Chem* **275:** 15343-15349

Kozma R, Ahmed S, Best A, Lim L (1995) The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. *Mol Cell Biol* **15:** 1942-1952

Kumar B, Koul S, Khandrika L, Meacham RB, Koul HK (2008) Oxidative stress is inherent in prostate cancer cells and is required for aggressive phenotype. *Cancer Res* **68:** 1777-1785

Kusano H, Shimizu S, Koya R, Fujita H, Kamada S, Kuzumaki N, Tsujimoto Y (2000) Human gelsolin prevents apoptosis by inhibiting apoptotic mitochondrial changes via closing VDAC. *Oncogene* **19:** 4807-4814

Kwiatkowski DJ (1999) Functions of gelsolin: motility, signaling, apoptosis, cancer. *Curr Opin Cell Biol* **11:** 103-108

Kwiatkowski DJ, Stossel TP, Orkin SH, Mole JE, Colten HR, Yin HL (1986) Plasma and cytoplasmic gelsolins are encoded by a single gene and contain a duplicated actin-binding domain. *Nature* **323:** 455-458

Lai FP, Szczodrak M, Block J, Faix J, Breitsprecher D, Mannherz HG, Stradal TE, Dunn GA, Small JV, Rottner K (2008) Arp2/3 complex interactions and actin network turnover in lamellipodia. *EMBO J* **27:** 982-992

Leber MF, Efferth T (2009) Molecular principles of cancer invasion and metastasis (review). *International journal of oncology* **34:** 881-895

Lee AC, Fenster BE, Ito H, Takeda K, Bae NS, Hirai T, Yu ZX, Ferrans VJ, Howard BH, Finkel T (1999a) Ras proteins induce senescence by altering the intracellular levels of reactive oxygen species. *J Biol Chem* **274:** 7936-7940

Lee HK, Driscoll D, Asch H, Asch B, Zhang PJ (1999b) Downregulated gelsolin expression in hyperplastic and neoplastic lesions of the prostate. *The Prostate* **40:** 14-19

Lee KH, Kim SW, Kim JR (2009) Reactive oxygen species regulate urokinase plasminogen activator expression and cell invasion via mitogen-activated protein kinase pathways after treatment with hepatocyte growth factor in stomach cancer cells. *J Exp Clin Cancer Res* **28:** 73

Legrand C, Polette M, Tournier JM, de Bentzmann S, Huet E, Monteau M, Birembaut P (2001) uPA/plasmin system-mediated MMP-9 activation is implicated in bronchial epithelial cell migration. *Exp Cell Res* **264:** 326-336

Li A, Dawson JC, Forero-Vargas M, Spence HJ, Yu X, Konig I, Anderson K, Machesky LM (2010) The actin-bundling protein fascin stabilizes actin in invadopodia and potentiates protrusive invasion. *Curr Biol* **20:** 339-345

Li GH, Arora PD, Chen Y, McCulloch CA, Liu P (2012) Multifunctional roles of gelsolin in health and diseases. *Medicinal research reviews* **32:** 999-1025

Li Y, Huang TT, Carlson EJ, Melov S, Ursell PC, Olson JL, Noble LJ, Yoshimura MP, Berger C, Chan PH, Wallace DC, Epstein CJ (1995) Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nature genetics* **11:** 376-381

Liao CJ, Wu TI, Huang YH, Chang TC, Wang CS, Tsai MM, Hsu CY, Tsai MH, Lai CH, Lin KH (2011) Overexpression of gelsolin in human cervical carcinoma and its clinicopathological significance. *Gynecologic oncology* **120:** 135-144

Lijnen HR (2001) Elements of the fibrinolytic system. *Ann N Y Acad Sci* **936:** 226-236

Litwin M, Mazur AJ, Nowak D, Mannherz HG, Malicka-Blaszkiewicz M (2009) Gelsolin in human colon adenocarcinoma cells with different metastatic potential. *Acta biochimica Polonica* **56:** 739-743

Litwin M, Nowak D, Mazur AJ, Baczynska D, Mannherz HG, Malicka-Blaszkiewicz M (2012) Gelsolin affects the migratory ability of human colon adenocarcinoma and melanoma cells. *Life sciences* **90:** 851-861

Lu M, Witke W, Kwiatkowski DJ, Kosik KS (1997) Delayed retraction of filopodia in gelsolin null mice. *J Cell Biol* **138:** 1279-1287

Lukaszewicz-Zając M, Mroczko B, Szmitkowski M (2011) Gastric cancer - The role of matrix metalloproteinases in tumor progression. *Clin Chim Acta* **412:** 1725-1730

Luzzi KJ, MacDonald IC, Schmidt EE, Kerkvliet N, Morris VL, Chambers AF, Groom AC (1998) Multistep nature of metastatic inefficiency: dormancy of solitary cells after successful extravasation and limited survival of early micrometastases. *The American journal of pathology* **153:** 865-873

Machesky LM, Insall RH (1998) Scar1 and the related Wiskott-Aldrich syndrome protein, WASP, regulate the actin cytoskeleton through the Arp2/3 complex. *Curr Biol* **8:** 1347-1356

Machesky LM, Li A (2010) Fascin: Invasive filopodia promoting metastasis. *Communicative & integrative biology* **3:** 263-270

Maniatis NA, Harokopos V, Thanassopoulou A, Oikonomou N, Mersinias V, Witke W, Orfanos SE, Armaganidis A, Roussos C, Kotanidou A, Aidinis V (2009) A critical role for gelsolin in ventilator-induced lung injury. *Am J Respir Cell Mol Biol* **41:** 426-432

Marumo T, Schini-Kerth VB, Fisslthaler B, Busse R (1997) Platelet-derived growth factor-stimulated superoxide anion production modulates activation of transcription factor NF-kappaB and expression of monocyte chemoattractant protein 1 in human aortic smooth muscle cells. *Circulation* **96:** 2361-2367

Maury CP (1991) Gelsolin-related amyloidosis. Identification of the amyloid protein in Finnish hereditary amyloidosis as a fragment of variant gelsolin. *J Clin Invest* **87:** 1195-1199

McLaughlin PJ, Gooch JT, Mannherz HG, Weeds AG (1993) Structure of gelsolin segment 1-actin complex and the mechanism of filament severing. *Nature* **364:** 685-692

Mehlen P, Puisieux A (2006) Metastasis: a question of life or death. *Nat Rev Cancer* **6:** 449-458

Mellier G, Pervaiz S (2012) The three Rs along the TRAIL: resistance, re-sensitization and reactive oxygen species (ROS). *Free radical research* **46:** 996-1003

Melov S, Doctrow SR, Schneider JA, Haberson J, Patel M, Coskun PE, Huffman K, Wallace DC, Malfroy B (2001) Lifespan extension and rescue of spongiform encephalopathy in superoxide dismutase 2 nullizygous mice treated with superoxide dismutase-catalase mimetics. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **21:** 8348-8353

Meyer T, Hart IR (1998) Mechanisms of tumour metastasis. *Eur J Cancer* **34:** 214-221

Mielnicki LM, Ying AM, Head KL, Asch HL, Asch BB (1999) Epigenetic regulation of gelsolin expression in human breast cancer cells. *Exp Cell Res* **249:** 161-176

Milani P, Gagliardi S, Cova E, Cereda C (2011) SOD1 Transcriptional and Posttranscriptional Regulation and Its Potential Implications in ALS. *Neurology research international* **2011:** 458427

Miyano K, Ueno N, Takeya R, Sumimoto H (2006) Direct involvement of the small GTPase Rac in activation of the superoxide-producing NADPH oxidase Nox1. *J Biol Chem* **281:** 21857-21868

Mizuguchi K, Deane CM, Blundell TL, Johnson MS, Overington JP (1998) JOY: protein sequence-structure representation and analysis. *Bioinformatics* **14:** 617-623

Mohanam S, Chandrasekar N, Yanamandra N, Khawar S, Mirza F, Dinh DH, Olivero WC, Rao JS (2002) Modulation of invasive properties of human glioblastoma cells stably expressing amino-terminal fragment of urokinase-type plasminogen activator. *Oncogene* **21:** 7824-7830

Moldovan L, Irani K, Moldovan NI, Finkel T, Goldschmidt-Clermont PJ (1999) The actin cytoskeleton reorganization induced by Rac1 requires the production of superoxide. *Antioxid Redox Signal* **1:** 29-43

Moldovan L, Mythreye K, Goldschmidt-Clermont P, Satterwhite L (2006) Reactive oxygen species in vascular endothelial cell motility. Roles of NAD(P)H oxidase and Rac1. *Cardiovasc Res* **71:** 236-246

Moriya S, Yanagihara K, Fujita H, Kuzumaki N (1994) Differential expression of hsp90, gelsolin and gst-pi in human gastric-carcinoma cell-lines. *International journal of oncology* **5:** 1347-1351

Mullauer L, Fujita H, Ishizaki A, Kuzumaki N (1993) Tumor-suppressive function of mutated gelsolin in ras-transformed cells. *Oncogene* **8:** 2531-2536

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. *Biochemical pharmacology* **65:** 1575-1582

Nelson KK, Melendez JA (2004) Mitochondrial redox control of matrix metalloproteinases. *Free Radic Biol Med* **37:** 768-784

Ni XG, Zhou L, Wang GQ, Liu SM, Bai XF, Liu F, Peppelenbosch MP, Zhao P (2008) The ubiquitin-proteasome pathway mediates gelsolin protein downregulation in pancreatic cancer. *Mol Med* **14:** 582-589

Nishikawa M (2008) Reactive oxygen species in tumor metastasis. *Cancer Lett* **266:** 53-59

Nishikawa M, Hashida M (2006) Inhibition of tumour metastasis by targeted delivery of antioxidant enzymes. *Expert Opin Drug Deliv* **3:** 355-369

Nishimura K, Ting HJ, Harada Y, Tokizane T, Nonomura N, Kang HY, Chang HC, Yeh S, Miyamoto H, Shin M, Aozasa K, Okuyama A, Chang C (2003) Modulation of androgen receptor transactivation by gelsolin: a newly identified androgen receptor coregulator. *Cancer Res* **63:** 4888-4894

Nobes CD, Hall A (1995a) Rho, rac and cdc42 GTPases: regulators of actin structures, cell adhesion and motility. *Biochem Soc Trans* **23:** 456-459

Nobes CD, Hall A (1995b) Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* **81:** 53-62

Nobes CD, Hall A (1999) Rho GTPases control polarity, protrusion, and adhesion during cell movement. *J Cell Biol* **144:** 1235-1244

Noske A, Denkert C, Schober H, Sers C, Zhumabayeva B, Weichert W, Dietel M, Wiechen K (2005) Loss of Gelsolin expression in human ovarian carcinomas. *Eur J Cancer* **41:** 461-469

Novo E, Parola M (2008) Redox mechanisms in hepatic chronic wound healing and fibrogenesis. *Fibrogenesis Tissue Repair* **1:** 5

Ohtsu M, Sakai N, Fujita H, Kashiwagi M, Gasa S, Shimizu S, Eguchi Y, Tsujimoto Y, Sakiyama Y, Kobayashi K, Kuzumaki N (1997) Inhibition of apoptosis by the actin-regulatory protein gelsolin. *EMBO J* **16:** 4650-4656

Oikonomou N, Thanasopoulou A, Tzouvelekis A, Harokopos V, Paparountas T, Nikitopoulou I, Witke W, Karameris A, Kotanidou A, Bouros D, Aidinis V (2009) Gelsolin expression is necessary for the development of modelled pulmonary inflammation and fibrosis. *Thorax* **64:** 467-475

Pelicano H, Lu W, Zhou Y, Zhang W, Chen Z, Hu Y, Huang P (2009) Mitochondrial dysfunction and reactive oxygen species imbalance promote breast cancer cell motility through a CXCL14-mediated mechanism. *Cancer Res* **69:** 2375-2383

Pervaiz S, Clement M (2007) Superoxide anion: oncogenic reactive oxygen species? *Int J Biochem Cell Biol* **39:** 1297-1304

Pope B, Maciver S, Weeds A (1995) Localization of the calcium-sensitive actin monomer binding site in gelsolin to segment 4 and identification of calcium binding sites. *Biochemistry* **34:** 1583-1588

Price JT, Thompson EW (2002) Mechanisms of tumour invasion and metastasis: emerging targets for therapy. *Expert Opin Ther Targets* **6:** 217-233

Pyke C, Kristensen P, Ralfkiaer E, Grondahl-Hansen J, Eriksen J, Blasi F, Dano K (1991) Urokinase-type plasminogen activator is expressed in stromal cells and its receptor in cancer cells at invasive foci in human colon adenocarcinomas. *The American journal of pathology* **138:** 1059-1067

Qiao H, McMillan JR (2007) Gelsolin segment 5 inhibits HIV-induced T-cell apoptosis via Vpr-binding to VDAC. *FEBS letters* **581:** 535-540

Radwanska A, Litwin M, Nowak D, Baczynska D, Wegrowski Y, Maquart FX, Malicka-Blaszkiewicz M (2012) Overexpression of lumican affects the migration of human colon cancer cells through up-regulation of gelsolin and filamentous actin reorganization. *Exp Cell Res* **318:** 2312-2323

Rajagopalan S, Meng XP, Ramasamy S, Harrison DG, Galis ZS (1996) Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases in vitro. Implications for atherosclerotic plaque stability. *J Clin Invest* **98:** 2572-2579

Rao J, Seligson D, Visapaa H, Horvath S, Eeva M, Michel K, Pantuck A, Belldegrun A, Palotie A (2002) Tissue microarray analysis of cytoskeletal actin-associated biomarkers gelsolin and E-cadherin in urothelial carcinoma. *Cancer* **95:** 1247-1257

Ridley AJ (2011) Life at the leading edge. *Cell* **145:** 1012-1022

Robertson AK, Cross AR, Jones OT, Andrew PW (1990) The use of diphenylene iodonium, an inhibitor of NADPH oxidase, to investigate the antimicrobial action of human monocyte derived macrophages. *Journal of immunological methods* **133:** 175-179

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

Saari H, Sorsa T, Lindy O, Suomalainen K, Halinen S, Konttinen YT (1992) Reactive oxygen species as regulators of human neutrophil and fibroblast interstitial collagenases. *Int J Tissue React* **14:** 113-120

Sahai E (2005) Mechanisms of cancer cell invasion. *Current opinion in genetics & development* **15:** 87-96

Salmeen A, Barford D (2005) Functions and mechanisms of redox regulation of cysteine-based phosphatases. *Antioxid Redox Signal* **7:** 560-577

San Martin A, Griendling KK (2010) Redox control of vascular smooth muscle migration. *Antioxid Redox Signal* **12:** 625-640

Schneidman-Duhovny D, Inbar Y, Nussinov R, Wolfson HJ (2005) PatchDock and SymmDock: servers for rigid and symmetric docking. *Nucleic acids research* **33:** W363-367

Scott JA, Homcy CJ, Khaw BA, Rabito CA (1988) Quantitation of intracellular oxidation in a renal epithelial cell line. *Free Radic Biol Med* **4:** 79-83

Shibue T, Weinberg RA (2009) Integrin beta1-focal adhesion kinase signaling directs the proliferation of metastatic cancer cells disseminated in the lungs. *Proc Natl Acad Sci U S A* **106:** 10290-10295

Shieh DB, Chen IW, Wei TY, Shao CY, Chang HJ, Chung CH, Wong TY, Jin YT (2006) Tissue expression of gelsolin in oral carcinogenesis progression and its clinicopathological implications. *Oral oncology* **42:** 599-606

Shieh DB, Godleski J, Herndon JE, 2nd, Azuma T, Mercer H, Sugarbaker DJ, Kwiatkowski DJ (1999) Cell motility as a prognostic factor in Stage I nonsmall cell lung carcinoma: the role of gelsolin expression. *Cancer* **85:** 47-57

Sies H (1997) Oxidative stress: oxidants and antioxidants. *Experimental physiology* **82:** 291-295

Silacci P, Mazzolai L, Gauci C, Stergiopulos N, Yin HL, Hayoz D (2004) Gelsolin superfamily proteins: key regulators of cellular functions. *Cellular and molecular life sciences : CMLS* **61:** 2614-2623

Spagnolo L, Toro I, D'Orazio M, O'Neill P, Pedersen JZ, Carugo O, Rotilio G, Battistoni A, Djinovic-Carugo K (2004) Unique features of the sodC-encoded superoxide dismutase from Mycobacterium tuberculosis, a fully functional copper-containing enzyme lacking zinc in the active site. *J Biol Chem* **279:** 33447-33455

Spinardi L, Witke W (2007) Gelsolin and diseases. *Sub-cellular biochemistry* **45:** 55-69

Steeg PS (2003) Metastasis suppressors alter the signal transduction of cancer cells. *Nat Rev Cancer* **3:** 55-63

Sun HQ, Yamamoto M, Mejillano M, Yin HL (1999) Gelsolin, a multifunctional actin regulatory protein. *J Biol Chem* **274:** 33179-33182

Suzuki M, Kato M, Hanaka H, Izumi T, Morikawa A (2003) Actin assembly is a crucial factor for superoxide anion generation from adherent human eosinophils. *The Journal of allergy and clinical immunology* **112:** 126-133

Svineng G, Ravuri C, Rikardsen O, Huseby NE, Winberg JO (2008) The role of reactive oxygen species in integrin and matrix metalloproteinase expression and function. *Connective tissue research* **49:** 197-202

Syrovets T, Simmet T (2004) Novel aspects and new roles for the serine protease plasmin. *Cellular and molecular life sciences : CMLS* **61:** 873-885

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

Taddei ML, Parri M, Mello T, Catalano A, Levine AD, Raugei G, Ramponi G, Chiarugi P (2007) Integrin-mediated cell adhesion and spreading engage different sources of reactive oxygen species. *Antioxid Redox Signal* **9:** 469-481

Takamiya R, Takahashi M, Park YS, Tawara Y, Fujiwara N, Miyamoto Y, Gu J, Suzuki K, Taniguchi N (2005) Overexpression of mutated Cu,Zn-SOD in neuroblastoma cells results in cytoskeletal change. *Am J Physiol Cell Physiol* **288:** C253-259

Talmadge JE, Fidler IJ (2010) AACR centennial series: the biology of cancer metastasis: historical perspective. *Cancer Res* **70:** 5649-5669

Tanaka M, Mullauer L, Ogiso Y, Fujita H, Moriya S, Furuuchi K, Harabayashi T, Shinohara N, Koyanagi T, Kuzumaki N (1995) Gelsolin: a candidate for suppressor of human bladder cancer. *Cancer Res* **55:** 3228-3232

Taniguchi T, Lemoine NR, Kakkar AK (1998) Urokinase receptor is a key player in tumour progression. *Gut* **43:** 739-740

Tellam R, Frieden C (1982) Cytochalasin D and platelet gelsolin accelerate actin polymer formation. A model for regulation of the extent of actin polymer formation in vivo. *Biochemistry* **21:** 3207-3214

Thiery JP (2002) Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* **2:** 442-454

Thiery JP (2003) Epithelial-mesenchymal transitions in development and pathologies. *Curr Opin Cell Biol* **15:** 740-746

Thiery JP, Sleeman JP (2006) Complex networks orchestrate epithelial-mesenchymal transitions. *Nat Rev Mol Cell Biol* **7:** 131-142

Thompson CC, Ashcroft FJ, Patel S, Saraga G, Vimalachandran D, Prime W, Campbell F, Dodson A, Jenkins RE, Lemoine NR, Crnogorac-Jurcevic T, Yin HL, Costello E (2007) Pancreatic cancer cells overexpress gelsolin family-capping proteins, which contribute to their cell motility. *Gut* **56:** 95-106

Thor AD, Edgerton SM, Liu S, Moore DH, 2nd, Kwiatkowski DJ (2001) Gelsolin as a negative prognostic factor and effector of motility in erbB-2-positive epidermal growth factor receptor-positive breast cancers. *Clin Cancer Res* **7:** 2415-2424

Tina KG, Bhadra R, Srinivasan N (2007) PIC: Protein Interactions Calculator. *Nucleic acids research* **35:** W473-476

Tobar N, Villar V, Santibanez JF (2010a) ROS-NFkappaB mediates TGF-beta1-induced expression of urokinase-type plasminogen activator, matrix metalloproteinase-9 and cell invasion. *Molecular and cellular biochemistry* **340:** 195-202

Tobar N, Villar V, Santibanez JF (2010b) ROS-NFkappaB mediates TGF-beta1-induced expression of urokinase-type plasminogen activator, matrix metalloproteinase-9 and cell invasion. *Mol Cell Biochem* **340:** 195-202

Tochhawng L, Deng S, Pervaiz S, Yap CT (2013) Redox regulation of cancer cell migration and invasion. *Mitochondrion* **13:** 246-253

Tran H, Maurer F, Nagamine Y (2003a) Stabilization of urokinase and urokinase receptor mRNAs by HuR is linked to its cytoplasmic accumulation induced by activated mitogen-activated protein kinase-activated protein kinase 2. *Mol Cell Biol* **23:** 7177-7188

Tran H, Maurer F, Nagamine Y (2003b) Stabilization of urokinase and urokinase receptor mRNAs by HuR is linked to its cytoplasmic accumulation induced by activated mitogen-activated protein kinase-activated protein kinase 2. *Mol Cell Biol* **23:** 7177-7188

Usatyuk PV, Romer LH, He D, Parinandi NL, Kleinberg ME, Zhan S, Jacobson JR, Dudek SM, Pendyala S, Garcia JG, Natarajan V (2007) Regulation of hyperoxia-induced NADPH oxidase activation in human lung endothelial cells by the actin cytoskeleton and cortactin. *J Biol Chem* **282:** 23284-23295

Valastyan S, Weinberg RA (2011) Tumor metastasis: molecular insights and evolving paradigms. *Cell* **147:** 275-292

Van den Abbeele A, De Corte V, Van Impe K, Bruyneel E, Boucherie C, Bracke M, Vandekerckhove J, Gettemans J (2007) Downregulation of gelsolin family proteins counteracts cancer cell invasion in vitro. *Cancer Lett* **255:** 57-70

Van Der Spoel D, Lindahl E, Hess B, Groenhof G, Mark AE, Berendsen HJ (2005) GROMACS: fast, flexible, and free. *Journal of computational chemistry* **26:** 1701-1718

Van Raamsdonk JM, Hekimi S (2012) Superoxide dismutase is dispensable for normal animal lifespan. *Proc Natl Acad Sci U S A* **109:** 5785-5790

Van Remmen H, Ikeno Y, Hamilton M, Pahlavani M, Wolf N, Thorpe SR, Alderson NL, Baynes JW, Epstein CJ, Huang TT, Nelson J, Strong R, Richardson A (2003) Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging. *Physiological genomics* **16:** 29-37

Vandekerckhove J, Bauw G, Vancompernolle K, Honore B, Celis J (1990) Comparative two-dimensional gel analysis and microsequencing identifies gelsolin as one of the most prominent downregulated markers of transformed human fibroblast and epithelial cells. *J Cell Biol* **111:** 95-102

Vassalli JD, Belin D (1987) Amiloride selectively inhibits the urokinase-type plasminogen activator. *FEBS letters* **214:** 187-191

Vouyiouklis DA, Brophy PJ (1997) A novel gelsolin isoform expressed by oligodendrocytes in the central nervous system. *Journal of neurochemistry* **69:** 995-1005

Wang FS, Wang CJ, Chen YJ, Chang PR, Huang YT, Sun YC, Huang HC, Yang YJ, Yang KD (2004) Ras induction of superoxide activates ERK-dependent angiogenic transcription factor HIF-1alpha and VEGF-A expression in shock wave-stimulated osteoblasts. *J Biol Chem* **279:** 10331-10337

Wang Y, Dang J, Liang X, Doe WF (1995) Amiloride modulates urokinase gene expression at both transcription and post-transcription levels in human colon cancer cells. *Clin Exp Metastasis* **13:** 196-202

Weaver AM (2006) Invadopodia: specialized cell structures for cancer invasion. *Clin Exp Metastasis* **23:** 97-105

Weigelt B, Peterse JL, van 't Veer LJ (2005) Breast cancer metastasis: markers and models. *Nat Rev Cancer* **5:** 591-602

Werner E, Werb Z (2002) Integrins engage mitochondrial function for signal transduction by a mechanism dependent on Rho GTPases. *J Cell Biol* **158:** 357-368

Weydert CJ, Waugh TA, Ritchie JM, Iyer KS, Smith JL, Li L, Spitz DR, Oberley LW (2006) Overexpression of manganese or copper-zinc superoxide dismutase inhibits breast cancer growth. *Free Radic Biol Med* **41:** 226-237

Winston JS, Asch HL, Zhang PJ, Edge SB, Hyland A, Asch BB (2001) Downregulation of gelsolin correlates with the progression to breast carcinoma. *Breast cancer research and treatment* **65:** 11-21

Witke W, Sharpe AH, Hartwig JH, Azuma T, Stossel TP, Kwiatkowski DJ (1995) Hemostatic, inflammatory, and fibroblast responses are blunted in mice lacking gelsolin. *Cell* **81:** 41-51

Wu RF, Gu Y, Xu YC, Nwariaku FE, Terada LS (2003) Vascular endothelial growth factor causes translocation of p47phox to membrane ruffles through WAVE1. *J Biol Chem* **278:** 36830-36840

Wu WS (2006) The signaling mechanism of ROS in tumor progression. *Cancer Metastasis Rev* **25:** 695-705

Wu WS, Tsai RK, Chang CH, Wang S, Wu JR, Chang YX (2006) Reactive oxygen species mediated sustained activation of protein kinase C alpha and extracellular signal-regulated kinase for migration of human hepatoma cell Hepg2. *Mol Cancer Res* **4:** 747-758

Yamaguchi H, Condeelis J (2007) Regulation of the actin cytoskeleton in cancer cell migration and invasion. *Biochim Biophys Acta* **1773:** 642-652

Yamaguchi H, Lorenz M, Kempiak S, Sarmiento C, Coniglio S, Symons M, Segall J, Eddy R, Miki H, Takenawa T, Condeelis J (2005) Molecular mechanisms of invadopodium formation: the role of the N-WASP-Arp2/3 complex pathway and cofilin. *J Cell Biol* **168:** 441-452

Yamaguchi H, Pixley F, Condeelis J (2006) Invadopodia and podosomes in tumor invasion. *Eur J Cell Biol* **85:** 213-218

Yap CT, Simpson TI, Pratt T, Price DJ, Maciver SK (2005) The motility of glioblastoma tumour cells is modulated by intracellular cofilin expression in a concentration-dependent manner. *Cell Motil Cytoskeleton* **60:** 153-165

Yilmaz M, Christofori G (2009) EMT, the cytoskeleton, and cancer cell invasion. *Cancer Metastasis Rev* **28:** 15-33

Yin HL, Kwiatkowski DJ, Mole JE, Cole FS (1984) Structure and biosynthesis of cytoplasmic and secreted variants of gelsolin. *J Biol Chem* **259:** 5271-5276

Yin HL, Stossel TP (1979) Control of cytoplasmic actin gel-sol transformation by gelsolin, a calcium-dependent regulatory protein. *Nature* **281:** 583-586

Yin HL, Stossel TP (1980) Purification and structural properties of gelsolin, a Ca2+-activated regulatory protein of macrophages. *J Biol Chem* **255:** 9490-9493

Yin HL, Zaner KS, Stossel TP (1980) Ca2+ control of actin gelation. Interaction of gelsolin with actin filaments and regulation of actin gelation. *J Biol Chem* **255:** 9494-9500

Yu X, Zech T, McDonald L, Gonzalez EG, Li A, Macpherson I, Schwarz JP, Spence H, Futo K, Timpson P, Nixon C, Ma Y, Anton IM, Visegrady B, Insall RH, Oien K, Blyth K, Norman JC, Machesky LM (2012) N-WASP coordinates the delivery and F-actin-mediated capture of MT1-MMP at invasive pseudopods. *J Cell Biol* **199:** 527-544

Zhan Y, He D, Newburger PE, Zhou GW (2004) p47(phox) PX domain of NADPH oxidase targets cell membrane via moesin-mediated association with the actin cytoskeleton. *J Cell Biochem* **92:** 795-809

Zhang F, Strom AL, Fukada K, Lee S, Hayward LJ, Zhu H (2007) Interaction between familial amyotrophic lateral sclerosis (ALS)-linked SOD1 mutants and the dynein complex. *J Biol Chem* **282:** 16691-16699

Zhang J, Sud S, Mizutani K, Gyetko MR, Pienta KJ (2011) Activation of urokinase plasminogen activator and its receptor axis is essential for macrophage infiltration in a prostate cancer mouse model. *Neoplasia* **13:** 23-30

Zhao X, Guan JL (2011) Focal adhesion kinase and its signaling pathways in cell migration and angiogenesis. *Advanced drug delivery reviews* **63:** 610-615

Zhao Y, Lyons CE, Jr., Xiao A, Templeton DJ, Sang QA, Brew K, Hussaini IM (2008) Urokinase directly activates matrix metalloproteinases-9: a potential role in glioblastoma invasion. *Biochem Biophys Res Commun* **369:** 1215-1220

Zhu H, Bannenberg GL, Moldeus P, Shertzer HG (1994) Oxidation pathways for the intracellular probe 2',7'-dichlorofluorescein. *Archives of toxicology* **68:** 582-587

Zhuge Y, Xu J (2001) Rac1 mediates type I collagen-dependent MMP-2 activation. role in cell invasion across collagen barrier. *J Biol Chem* **276:** 16248-16256

Zhuo J, Tan EH, Yan B, Tochhawng L, Jayapal M, Koh S, Tay HK, Maciver SK, Hooi SC, Salto-Tellez M, Kumar AP, Goh YC, Lim YC, Yap CT (2012) Gelsolin induces colorectal tumor cell invasion via modulation of the urokinase-type plasminogen activator cascade. *PloS one* **7:** e43594

APPENDICES

APPENDIX I (SUPPLEMENTARY DATA)

In silico analysis of gelsolin and Cu/Zn SOD interaction

(These data have been analysed and kindly provided by Dr Ganesan Pugalenthi, King Abdullah University of Science and Technology, Saudi Arabia)

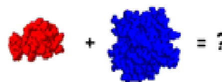
Aims

1. To investigate whether gelsolin and Cu/Zn SOD can directly bind to each other
2. To determine the stability of gelsolin-Cu/Zn SOD complex

Docking

To investigate whether gelsolin and Cu/Zn SOD are direct binding partners, docking was performed. Crystal structures of gelsolin and Cu/Zn SOD were obtained from the protein database (PDB). Gelsolin-SOD docking was then performed between pdb: 3FFN, chain A (gelsolin) and pdb: 1PU0 Chain A (Cu/Zn SOD) using PatchDock (Schneidman-Duhovny *et al.*, 2005). Briefly, PatchDock is a geometry-based molecular docking algorithm. It is aimed at finding docking transformations that yield good molecular shape complementarity.

PATCHDOCK



Molecular Docking Algorithm Based on Shape Complementarity Principles

[About PatchDock] [Web Server] [Download] [Help] [FAQ] [References]

Receptor	Ligand	Complex Type	Clustering RMSD	User e-mail
3FFNA.pdb	1PU0A.pdb	Default	4.0	gpugal@gmail.com

Solution No	Score	Area	ACE	Transformation
1	17160	2582.00	337.33	-0.07 0.14 -2.68 61.76 91.21 -27.20
2	16034	2550.10	102.12	-0.56 -0.31 2.67 102.23 46.55 17.73
3	16034	2254.40	204.37	3.03 0.15 -2.18 92.94 99.71 0.87
4	15740	2831.80	475.52	-2.32 0.33 -1.47 43.87 97.75 5.48
5	15584	2373.40	215.73	-3.01 -0.19 1.52 -7.26 -20.33 23.67
6	15530	2600.20	279.78	2.49 -1.36 -0.07 15.01 73.63 37.22
7	15172	2751.90	251.44	-0.67 -0.56 2.60 96.07 48.56 33.41
8	15066	2744.40	482.62	0.84 0.77 1.76 54.74 21.76 -79.00
9	15016	1919.70	209.59	2.91 0.08 2.99 88.20 -27.98 -12.55
10	14932	2043.20	336.56	0.77 0.13 2.23 80.41 -2.06 -58.45
11	14854	1977.10	412.79	-3.05 0.50 1.99 26.55 -41.67 -17.55
12	14846	1919.20	426.19	-2.19 0.27 -1.23 23.50 93.98 11.13
13	14674	1931.60	362.32	2.68 0.70 2.07 13.54 -31.15 -48.80
14	14666	1981.80	452.68	-0.34 -1.11 -1.40 -34.71 96.78 42.45
15	14602	1945.20	482.70	-3.00 0.30 -0.96 31.96 99.76 -12.06
16	14568	2145.00	344.95	2.11 -0.61 2.67 77.19 -34.08 -3.67
17	14518	2635.70	490.01	-1.98 0.49 -1.52 33.90 101.17 0.04
18	14510	2082.90	476.05	2.83 0.64 -0.89 38.14 91.85 -42.06
19	14454	2054.50	411.92	-1.77 -0.56 2.14 57.56 13.80 62.14
20	14406	2057.10	447.02	-0.92 -0.07 -0.50 -35.61 18.39 15.76

Molecular dynamics

To determine the stability of the docked gelsolin-Cu/Zn SOD complex, molecular dynamics simulations were performed under conditions that would mimic physiological conditions. For molecular dynamics simulations the GROMACS version 4.5.3 with OPLS force field (Van Der Spoel *et al.*, 2005) was employed. The heterodimer model was placed in a cubic box with the box-edges at least 10 nm apart from the protein surface. The system was solvated with Simple Point Charge (SPC216) water molecules and appropriate number of counterions were added in the box to neutralise the system. In order to remove the possible clashes between atoms, the energy minimisation was set to run for 50000 steps or until convergence to machine precision. After energy minimisation, simulations were performed for 100 ps at constant temperature and pressure with periodic boundary conditions, particle-mesh Ewald summation, and a 2-fs time step to heat and equilibrate the system. Then the system was subjected to production MD simulations for 10 nano

seconds. Structures were saved every 2 pico second for analysis. The output files from the GROMACS 4.5.3 was analysed using XMGRACE software. The overall stability of the dimer was measured by estimating the following root mean square deviation (RMSD) of the molecule and its radius of gyration (Rg)

Radius of gyration: Radius of gyration of a protein is a measure of its compactness. If a protein is stably folded, it will likely maintain a relatively steady value of Rg. If a protein unfolds, its Rg will change over time.

Root Mean Square Displacement (RMSD): to evaluate the deviation of the structure from the original starting structure over the course of the simulation.

Interactions were analysed (hydrogen bonds and hydrophobic forces) at the interface in each simulated structure. Interaction between hydrophobic side chains are identified using a distance cutoff of 5 Angstrom between apolar groups in the apolar side chains (Tina *et al.*, 2007). The hydrogen bonds formed between subunits are identified using HBOND program which is a part of JOY suite (Mizuguchi *et al.*, 1998). The interactions that exist in at least 60% of the simulated structures were considered as dynamically stable and used for the interpretation of stability.

Result and Discussion

In section 4.6, we have shown that gelsolin co-immunoprecipitates with Cu/Zn SOD, however, it is unclear whether these two proteins directly bind to each other. In order to predict the mode of interaction between gelsolin and Cu/Zn SOD, docking analysis using Patchdock followed by

molecular dynamic simulations were performed. Docking between pdb: 3FFN, chain A (gelsolin) and pdb: 1PU0 Chain A (Cu/Zn SOD) shows that gelsolin and Cu/Zn SOD are potential direct binding partners as shown in Appendix Figure 1. Further analysis using Patchdock provide details of the interacting residues as well as the interaction type with the main chains and side chains involved in the interaction. Analysis of the residues involved in the interaction reveals that the C-terminal region of gelsolin is mostly likely the important region for its binding with Cu/Zn SOD. Furthermore, to test the stability of the docked gelsolin-Cu/Zn SOD complex, molecular dynamics simulation that mimics physiological conditions was performed. Molecular dynamics simulation result show that the gelsolin-Cu/Zn SOD complex shows that the complex structure is intact up to 10 nano seconds (Appendix, Fig S1.2). Taking together, these results indicate that Gelsolin and Cu/Zn SOD can form a complex together and the complex thus formed is stable. This finding ties in with our previous result (Section 4.4), that gelsolin suppresses the activity of Cu/Zn SOD. If gelsolin-Cu/Zn SOD complex is maintained under physiological conditions, it is plausible that this interaction alters the activity of Cu/Zn SOD.

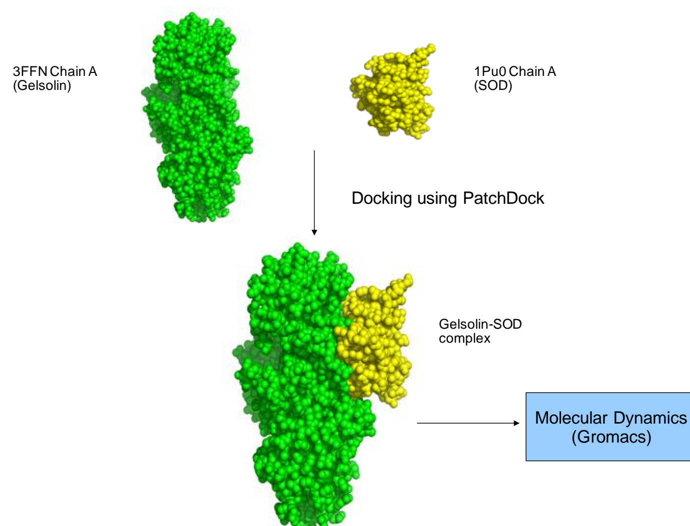


Figure S1.1. 3-dimenstional structure of Gelsolin-Cu/Zn SOD complex obtained using PatchDock. The green structure corresponds to gelsolin (pdb id – 3FFN) and the yellow structure represents the Cu/Zn SOD (pdb id – 1Pu0).

Gelsolin		SOD		Interaction type	Observed (%)
Residue #	Residue	Residue #	Residue		
133	TYR	54	THR	Hbond-SO	80
222	ASP	143	ARG	Hbond-SN	66
223	ASN	55	ALA	Hbond-SO	52
223	ASN	58	THR	Hbond-SS	95
389	ASN	114	GLY	Hbond-SO	69
403	SER	109	ASP	Hbond-SS	64
662	MET	50	PHE	Hydrophobic	73
736	TRP	68	SER	Hbond-SO	98
737	ASP	136	LYS	Salt-bridge	99
739	ASP	136	LYS	Hbond-SO	97
752	GLU	122	LYS	Sal-bridge	92

Hbond-SO – hydrogen bond between side chain and main chain oxygen
Hbond-SN – hydrogen bond between side chain and main chain nitrogen

Table S1. Predicited interacting amino acid residues between Gelsolin and Cu/Zn SOD

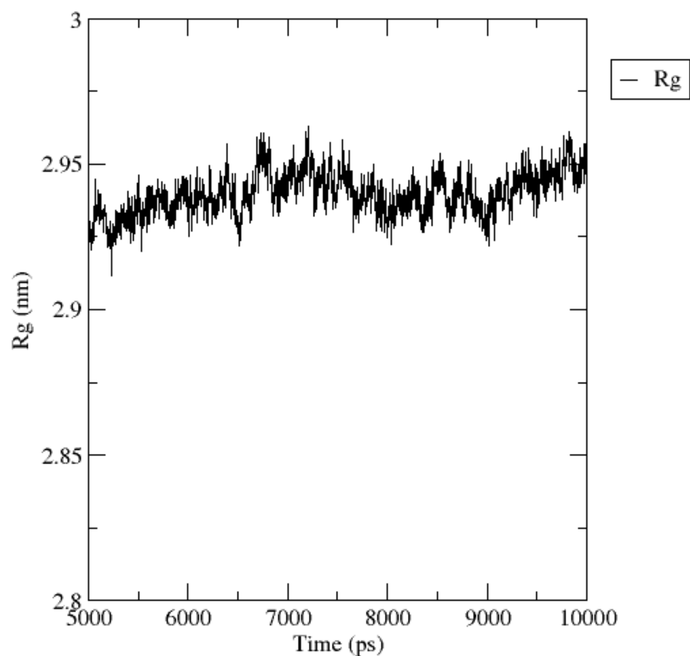


Figure S1.2. The radius of gyration of the complex structure of gelsolin-Cu/Zn SOD measured upto 10 nano seconds. The radius of gyration of a protein is a measure of its compactness. If a protein is stably folded, it will likely maintain a relatively steady value of Rg. If a protein unfolds, its Rg will change over time. The above curve shows a steady value of Rg, indicating that stability of the gelsolin-Cu/Zn SOD complex structure is maintained for upto 10 nano seconds.

APPENDIX II (PREPARATION OF BUFFERS)

CELL CULTURE MATERIALS

McCoy's 5A Medium (1L)

1 bottle of McCoy's 5A powder (M4892, Sigma-aldrich)

2.2g $NaHCO_3$,

100mL Fetal Bovine Serum

Top up to 900mL MiliQ water and sterile-filter through 0.22 μm filter membrane.

Dulbecco's Modified Eagle Medium (DMEM) (1L)

1 bottle of DMEM powder (D1152, Sigma-aldrich)

3.7 $NaHCO_3$,

100mL Fetal Bovine Serum

Top up to 900mL MiliQ water and sterile-filter through 0.22 μm filter membrane.

RPMI-1640 (1L)

1 bottle of RPMI-1640 powder (R4130, Sigma-aldrich)

2g $NaHCO_3$,

100mL Fetal Bovine Serum

Top up to 900mL MiliQ water and sterile-filter through 0.22 μm filter membrane.

Freezing media

95% FBS

5% DMSO

ELISA MATERIALS

1X PBST (Wash solution)

0.05% PBS-Tween

WESTERN BLOT AND IMMUNOPRECIPITATION MATERIALS

RIPA buffer

50mL Tris-HCL pH 8.0

150mM NaCl

0.5% Sodium deoxycholate

1% NP-40

Modified RIPA buffer for IP

5M Nacl

1M Tris-HCL, pH 8.0

0.5M EDTA

1% Triton X-100

0.1% Sodium deoxycholate

5X SDS Gel loading buffer

0.2M Tris-HCL pH 6.8,

30% Glycerol,

10% SDS

0.05% Bromophenol blue)

Resolving gel for SDS-PAGE

Distilled water

30% bis-acrylamide

1.5M Tris (pH 8.8)

10% SDS

10% APS

TEMED

4% Stacking gel for SDS-PAGE

Distilled water

30% bis-acrylamide

1M Tris (pH 6.8)

10% SDS

10% APS*

TEMED

SDS-PAGE running buffer (10Xstock)

25mM Tris base

192mM Glycine

0.1% SDS

Transfer buffer for Sds-Page (10X stock)

25mM Tris base,

192mM Glycine

0.025% SDS

For 1x working solution, freshly add 20% ethanol.

Phosphate Buffered Saline (PBS) (10X Stock, 1L)

1.37M NaCl

81mM Sodium phosphate dibase anhydrous Na_2HPO_4

27.6mM Potassium chloride KCl

14.7mM Potassium dihydrogen phosphate KH_2PO_4

<u>INVASION ASSAY MATERIALS</u>

Crystal Violet Stain

0.2% Crystal Violet (w/v)

0.9% NaCl

10% ethanol

<u>ZYMOGRAPHIC ASSAY MATERIALS</u>

Wash Buffer

2.5% Triton X-100

20mM Tris-HCl, pH 8.0

Incubation Buffer for uPA/Plasmin activity

0.1M Glycine, pH 8.0

Coomassie Blue Stain

0.1% Coomassie R-250

40% Methanol

10% Acetic acid

Destain Solution

10% Acetic acid

30% Methanol

MITOCHONDRIAL FRACTONATION BUFFER

200mM mannitol

68mM sucrose

50mM Pipes-KOH (pH 7.4)

50mM KCl

5mM EGTA

2mM MgCl2

1mM dithiothreitol

APPENDIX III (PUBLICATIONS)

LIST OF PUBLICATIONS

- **Tochhawng L**, Deng S, Pervaiz S and Yap CT. (2012). *Redox Regulation of Cancer Cell Migration and Invasion***.** Mitochondrion. 13(3):246-53.

- Zhuo J, Tan EH, Yan B, **Tochhawng L**, Jayapal M, *et al.* (2012). Gelsolin Induces Colorectal Tumour Cell Invasion via Modulation of the Urokinase-Type Plasminogen Activator Cascade. PLoS ONE 7(8): e43594.

- **Tochhawng L**, Pervaiz S and Yap CT. *Gelsolin promotes colon cancer cell invasion via modulating intracellular superoxide levels*. (In preparation)

COMMUNICATIONS PUBLISHED IN ABSTRACT FORM

- **Tochhawng L**, Jingli Zhuo, Pervaiz S, Yap CT. (2012). *A novel pro-oxidant role of gelsolin in colon cancer cell invasion.* Cold Spring Harbor Laboratory meeting on models and mechanisms of cancer Proceedings, pg-243 (Cold Spring Harbor, New York, August 14-18, 2012).

- **Tochhawng L**, Pervaiz S, Yap CT. (2012). Gelsolin promotes colon cancer cell invasion via increasing intracellular reactive oxygen species level. Yong Loo Lin school of Medicine 2nd Annual Graduate Scientific Congress Proceedings, pg-31, (Singapore, 15 February 2012). ***Oral presentation.***

- **Tochhawng L**, Pervaiz S, Yap CT. (2011). Gelsolin acts as a novel pro-oxidant molecule and promotes colon cancer cell invasion by modulating the intracellular levels of reactive oxygen species. Microscopy Society of Singapore Annual General and Scientific Meeting Proceedings, pg-24 (Singapore, 7 to 8 April 2011). ***Oral presentation***.

- **Tochhawng L**, Pervaiz S, Yap CT. (2011). Elucidating the interactions between gelsolin and reactive oxygen species in cancer cell invasion. Models of Physiology and diseases Proceedings, pg-22, (Singapore 29 to 30 September 2011). ***Oral presentation***.

- **Tochhawng L**, Deng S, Pervaiz S and Yap CT (2012). The role of gelsolin in tumour cell survival. International Cell Death Society, Cell death: Aging, metabolism and ramifications for therapeutics and drug development Proceedings, pg-68 (Singapore, 29 June to 2 July 2012, pg-68).

- **Tochhawng L**, Zhuo J, Pervaiz S and Yap CT (2012). Gelsolin promotes colon cancer cell invasion via increasing intracellular reactive oxygen species level. The 5th International Epithelial-Mesenchymal Transition Meeting Proceedings, pg-116 (Singapore, 10 to 13 October 2011).

- Huang BH, Zhuo J, Dinh TD, Yan B, Yap YL, Ooi CH, **Tochhawng L**, Gopinadhan S, Jiong T, Salto-Tellez M, Tan P, Soong R, So JBK, Yap CT (2011). Gelsolin is a novel negative regulator of E-cadherin and contributes to the dissemination of gastric cancer. Singapore Gastric Cancer Consortium, 4th Annual Scientific Meeting Proceedings, pg-58 (Singapore, 4 to 5 July, 2011).