Mcl-1 in Breast Cancer: Regulation by the EGF Receptor Family and Role in Cell Survival and Drug Resistance

by

Evan Paul Booy

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry and Medical Genetics

Faculty of Medicine

University of Manitoba

Winnipeg, Manitoba, Canada

Abstract

Myeloid Cell Leukemia-1 (Mcl-1) is a widely expressed anti-apoptotic member of the Bcl-2 family that is elevated in a variety of tumour types including breast cancer. Mcl-1 promotes tumour cell survival and drug resistance and was a mechanism of resistance to first generation Bcl-2 family inhibitors. To determine the significance of Mcl-1 in breast cancer, we evaluated the regulation of Mcl-1 by signalling via the epidermal growth factor receptors (EGFRs). EGFR signalling is frequently deregulated in breast cancer and leads to increased proliferation and survival of tumour cells. We aimed to determine whether Mcl-1 is a critical downstream effector of this pathway and therefore an important therapeutic target. We found that Mcl-1 protein and messenger RNA levels were rapidly induced upon stimulation of breast cancer cells with epidermal growth factor. This induction was blocked by inhibitors of the Ras/Raf/Mek/Erk signalling cascade and was dependent upon activation of the transcription factor Elk-1. We found Mcl-1 to be an essential survival protein, as targeted knock-down with small interfering RNA alone was sufficient to induce apoptosis. Mcl-1 may be critical for the survival advantage conferred by EGFR activation, as prevention of its up-regulation by Mek/Erk inhibitors significantly reduced the drug resistance conferred by EGF. Furthermore, we found a correlation between phosphorylated Elk-1 and Mcl-1 protein levels in breast tumour samples. Therefore, we conclude that Mcl-1 is an important downstream effector of survival and drug resistance mediated by elevated EGF signalling, making it an important therapeutic target in breast cancer.

Acknowledgements

I owe a debt of gratitude to my first graduate program supervisor, Dr. Marek Los, for welcoming me into his lab and training me in the theory and practice of molecular biology and oncology research. Marek ensured my success as a graduate student by challenging and encouraging me to be a productive and effective researcher, which led to several publications early in my graduate program and translated into successful fellowships and scholarship applications.

I also would like to especially thank Dr. Spencer Gibson, who took me under his wing as a PhD student mid-way through my degree and provided me with a wonderful research environment in which I undertook this project which has become my doctoral thesis. I would also like to thank all of the members of the Gibson lab for welcoming me and the good times we could enjoy together over the past three years. I particularly would like to acknowledge Elizabeth Henson, who worked closely with me on many aspects of my thesis project.

I also would like to thank my advisory committee, Dr. Leigh Murphy, Dr. Bob Shiu and Dr. Michael Mowat for the advice and guidance they have provided over the years and particularly for the time spent in reviewing my thesis and offering wise guidance and suggestions. I would also like to thank the external examiner, Dr. Christine Pratt, for reviewing my thesis and coming to Manitoba to be present at my defence.

Finally I would like to thank my wife, Trisha, as well as my extended family for their love and encouragement over the course of my graduate studies.

Dedication

I dedicate this work to my daughter Eliana Grace, who came into this world shortly before I began the task of preparing this thesis and provided me with a wonderful distraction from reading and writing. As I end this chapter in my life as a student, I look forward with great expectation to the coming years where my most important role will be as a father. I look forward to many great experiences as we grow together as a family.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

°C	Degrees Celsius
7AAD	7-aminoactinomycin D
A	Adenine
AIF	Apoptosis inducing factor
Apaf-1	Apoptotic protease activating factor 1
ATCC	American type culture collection
ATF2	Activating transcription factor 2
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
BAC	Bacterial artificial chromosome
BAD	Bcl-2 associated death promoter
Bak	Bcl-2 associated antagonist killer
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2 associated X-protein
Bcl-2	B-cell lymphoma 2
Bcl-X_L	Basal cell lymphoma - extra large
BH	Bcl-2 homology
Bid	BH3 interacting domain death agonist
Bik	Bcl-2 interacting killer
Bim	Bcl-2 interacting mediator of cell death
bp	base pairs
BRCA1	Breast cancer 1 early onset
BRCA2	Breast cancer type 2 susceptibility protein
BSA	Bovine serum albumin
C	Cytosine
CAD	Caspase activated DNase
Caspase	Cysteine-directed aspartate protease
CBP	Creb binding protein
CD95	Cluster of Differentiation 95
CDK	Cyclin dependent kinase
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CLL	Chronic lymphocytic leukima
Cox2	Cyclooxygenase 2
Creb	cAMP response element binding protein
CugBP2	CUG binding protein 2
DAG	Diacylglycerol
DAPI	4'6-diamidino-2-phenylindole
ddH_2O	Double distilled water
DED	Death effector domain
DIABLO	Direct IAP Binding Protein with Low PI
DISC	Death inducing signalling complex

DMEM	Dulbecco's modified essential medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
DR4	Death receptor 4
DR5	Death receptor 5
E. coli	Escherichia coli
ECL	Enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGCG	Epigallocatechin gallate
EGF	Epidermal Growth Factor
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eIF2α	Eukaryotic initiation factor 2 alppha
EMSA	Electrophoretic mobility shift assay
ER	Estrogen receptor
Erk	Extracellular signal-regulated kinase
Ets	E-twenty-six
FADD	Fas associated with a death domain
Fas	apoptosis stimulating fragment
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FTI	Farnesyltransferase inhibitor
G	Guanine
GDP	Guanosine diphosphate
GGTI	Geranylgeranyltransferase inhibitor
GM-CSF	Granulocyte Macrophage colony stimulating factor
GPR30	G-protein coupled receptor 30
Grb2	Growth factor receptor bound protein 2
GSK-3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
HAT	Histone acetyl transferase
HB-EGF	Heparin-binding epidermal growth factor
HDAC	Histone deacetylase
Her	Human epidermal growth factor receptor
HPLC	High performance liquid chromatography
HSP90	Heat shock protein 90
Htra2	mammalian homolog of high temperature requirement protein 2
IAP	Inhibitor of apoptosis protein
iCAD	Inhibitor of caspase activated DNase
IF	Immunofluorescence
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL-3	Interleukin 3
iNOS	Inducible nitric oxide synthase

IP3	Inositol triphosphate
JAK	Janus kinase
Jnk	c-jun n-terminal kinase
kDa	kilodalton
LB	Luria-Bertani
MADS	MCM1, Agamous, Deficiens, SRF
MAPK	Mitogen activated protein kinase
MBTB	Manitoba Breast Tumour Bank
MCF-7	Michigan Cancer Foundation - 7
Mcl-1	Myeloid cell leukemia 1
Mcl-1ES	Mcl-1 extra short
Mcl-1s	Mcl-1 short
Mek	Mitogen activated Erk kinase
mRNA	messenger ribonucleic acid
MRTF	Myocardin related transcription factor
MSK	Mitogen and stress activated protein kinase
MTT	3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide
MULE	Mcl-1 ubiquitin ligase E3
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK-cell	Natural killer cell
NOXA	Phorbol-12-myristate-13-acetate-induced protein 1 (Latin for *damage*)
NP40	Nonident P-40
NRG	Neuregulin
ONPG	ortho-Nitrophenyl-β-galactoside
PAK	p21 activated kinase
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PEST	Proline, glutamic acid, serine, threonine
PgR	Progesterone Receptor
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PLAD	Pre-ligand assembly domain
PLC γ	Phospholipase C gamma
Poly dI-dC	Poly(deoxyinosinic-deoxycytidylic) acid
PS	Phosphatidylserine
PTB	phospho-tyrosine binding
PTEN	Phosphatase and tensin homolog
PUMA	P53 up-regulated modulator of apoptosis
PVDF	polyvinyl difluoride
Raf	Rapidly accelerated fibrosarcoma
Ras	Rat sarcoma
RB	Retinoblastoma

RIPA	Radioimmunoprecipitation assay
RLU	Relative luciferase units
RNA	Ribonucleic acid
rpm	Revolutions per minute
r_S	Spearman correlation coefficient
RT	Room temperature
RT-PCR	Real-time polymerase chain reaction
SDS/PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	Serine
SERM	Selective estrogen receptor modulator
SH2	Src homology 2
siRNA	Small interfering RNA
SK-BR-3	Sloan-Kettering breast carcinoma - 3
SMAC	Second mitochondrial activator of caspases
SOS	Son of sevenless
SRC	Sarcoma
SRE	Serum response element
SRF	Serum Response Factor
STAT	Signal transducer and activator of transcription
STR	Short tandem repeat
T	Thymine
tBid	Truncated Bid
TBP	TATA-binding protein
TBS	Tris buffered saline
TBS-T	Tris buffered saline with tween-20
TCF	Ternary complex factor
TCTP	Translationally controlled tumour protein
TGFα	Transforming growth factor receptor alpha
Thr	Threonine
TMA	Tissue microarray
TNF	Tumour necrosis factor
TNM Stage	Tumour, lymph node, metastasis staging
TP53	Tumour protein 53
TPA	12-*O*-Tetradecanoylphorbol-13-acetate
TRAIL	TNF-related apoptosis inducing ligand
tRNA	Transfer ribonucleic acid
Tyr	Tyrosine
U	Uracil
UV	Ultra-violet
VDAC	Voltage-dependent anion channel
VEGF	Vascular endothelial growth factor
Z-VAD-fmk	N-benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone
β-gal	Beta galactosidase
βTrCP	Beta-transducin repeat containing protein

Chapter 1 Introduction

1.1 Introductory remarks

The following is a description of research performed to elucidate the role of the Mcl-1 protein in breast cancer. The basis for this project was the initial identification of a relationship between the epidermal growth factor receptor ErbB2 and elevated Mcl-1 expression. This work expands upon that initial observation to identify the molecular pathways linking EGF receptor activation and Mcl-1 gene transcription in breast cancer. Furthermore, it assesses the contribution of Mcl-1 to cell survival and drug resistance in breast cancer cell line models and explores the implications of Mcl-1 and its upstream regulatory network on breast cancer patient survival and disease progression. The ultimate goal of this study is to establish the relevance of Mcl-1 to breast cancer and evaluate its potential as a drug target. While the work focuses on breast cancer, the results have implications on many tumour types that rely upon similar survival pathway activation.

1.2 Cancer

Cancer is a widespread disease that will afflict 40% of Canadian women and 45% of Canadian men over the course of their lifetime. Overall 1 in 4 Canadians is expected to die as a result of this disease (1). Cancer develops as a result of uncontrolled proliferation and accumulation of poorly differentiated cells in the human body. Cancer development is a multi-step process, initiated by acquired or inherited genetic mutations that allow cells to escape intrinsic regulatory mechanisms. Hanahan and Weinberg describe “six

essential alterations in cell physiology that collectively dictate malignant growth: self sufficiency in growth signals, insensitivity to growth-inhibitor (anti-growth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis." (2). While much effort has been put into studying and targeting all six of these cancer hallmarks, the sixth hallmark is arguably the most critical feature responsible for cancer mortality (3).

1.3 Breast Cancer

Breast cancer is the most common malignancy among Canadian women. Over the course of their lifetime, 1 in 9 Canadian women is expected to develop breast cancer and 1 in 28 will die from the disease (1). The majority of breast tumours originate in the glandular tissue of the breast and are therefore referred to as adenocarcinomas. These tumours primarily arise from the lining of the milk ducts and are termed ductal carcinoma. Ductal carcinoma accounts for up to 80% of breast cancers. The second most common form of breast cancer is lobular carcinoma. Lobular carcinoma describes a tumour that originates within the milk glands. Lobular carcinoma accounts for roughly 10% of breast tumours. In addition to ductal and lobular carcinoma, a number of other rare forms of breast cancer also exist (4). While many advances have been made in the diagnosis and treatment of breast cancer, there is clearly a need for more effective therapeutic options. New investigations into the molecular mechanisms behind breast cancer initiation and progression are paving the way for the development of targeted therapies. Targeted inhibition of both estrogen receptor signalling as well as signalling from the epidermal growth factor receptors has greatly enhanced treatment of the disease

(5-8). Expanding upon the currently available treatment options will be critical to overcome resistance and target a wider variety of breast tumour types.

1.3.1 Causes

While the majority of breast carcinomas appear to arise due to sporadic mutations, it is estimated that 5-10% of breast cancers are primarily a result of hereditary factors (9). These hereditary factors are exemplified by mutations in the BRCA1 and BRCA2 genes. BRCA1 and BRCA2 are both involved in the regulation of DNA repair through the homologous recombination pathway(10); in fact the majority of genes identified to contribute to hereditary breast cancer play some type of role in maintaining the genome (11). Loss of these genes leads to genomic instability and leaves cells more susceptible to spontaneous mutations. These spontaneous mutations can subsequently lead to tumour formation by contributing to one or more of the above-described hallmarks of cancer. Women carrying mutations in either the BRCA1 or BRCA2 genes, have as high as a 20-fold increased risk of developing breast cancer by the age of 60 (11). Mutations in other genes, including TP53, CHEK2, ATM and several others, have been identified to play a significant role in increasing susceptibility to breast cancer; however, the penetrance of these mutations individually is far less than is seen in the case of BRCA1/2 and in all likelihood the increased hereditary risk is due to a complex interplay of multiple genetic changes (12). In addition to an inherited predisposition towards the disease, a variety of environmental factors including the age of menarche, pregnancy, breastfeeding, the use of oral contraceptives, dietary habits, obesity, alcohol consumption, radiation exposure, and natural and synthetic chemical exposure can modify both positively and negatively the risk of developing breast cancer (13-17).

1.3.2 Classification and tumour grade

Breast cancer is a heterogeneous disease that has been classified into a number of different categories both morphologically through histological examination as well as by molecular characterization using high throughput microarray analyses (18-21). Histological examination of breast tumours allows for the classification into as many as 17 separate categories that have implications upon disease prognosis and treatment modality (22-23). More recently, the use of microarray technology has allowed for the classification of breast cancers into five distinct categories based on a molecular profile; luminal A, luminal B, normal breast-like, ErbB2+ and basal-like (18-19). These sub-types are distinguished from one another by variations in their expression profile of 456 separate genes. Each sub-type demonstrates significant variation in overall survival as well as progression free survival, indicating that the molecular characterization acts well as a prognostic indicator (19). In general, ErbB2+ and basal-like breast cancer are high grade tumours with the highest risk of recurrence. Luminal A breast cancer is the most common form and also has the most favourable prognosis. Luminal B tumours demonstrate more variable responses to treatment and generally have a higher risk of recurrence as compared to Luminal A (19, 24-25). The normal breast-like subtype has variable prognosis and it is hypothesized that this subtype may be due to a high amount of normal tissue contamination (24). Expression of the clinically relevant markers ERα, PgR and ErbB2 divides somewhat along the lines of the microarray subtypes with Luminal A/B tumours being ERα/PgR positive whereas both Luminal B and ErbB2+ demonstrate over-expression of ErbB2. The bulk of the basal-like subtype also constitute

the Triple negative classification of breast cancer (ERα-, PgR-, ErbB2-); however, ErbB2 expression is observed in a subset of the basal subtype (18-19).

Gene expression analysis coupled with tumour grading and staging provides valuable prognostic information (20-21). Breast tumours are graded to assess the degree to which the tumour cells vary from normal tissue. Grading is performed according to an assessment of tubule formation, nuclear pleomorphism and mitotic counts. Breast cancer is also staged to assess tumour progression. Staging is based on the TNM classification system, which evaluates tumour size, presence of cancer cells within local lymph nodes and tumour metastasis. The large degree of variation evident in breast tumours is indicative of the need for individualized treatment options. Currently, the hormone receptor status (estrogen receptor alpha (ERα) and progesterone receptor (PgR)) and human epidermal growth factor receptor 2 (Her2/ErbB2) status are of critical importance when designing patient care (26). In the future, further biomarkers may be used to tailor a patient's treatment course to the molecular profile of their disease.

1.3.3 Treatment

Treatment options for breast cancer consist of surgery, radiation therapy, chemotherapy, hormonal therapy and biological therapy (4). There are two main types of surgery for the treatment of breast cancer, lumpectomy and mastectomy. Lumpectomy is a breast-conserving technique that involves removal of only the breast tumour and some surrounding tissue. Mastectomy refers to complete surgical removal of the affected breast. Following surgery, radiation therapy is often used to prevent recurrence by

eliminating any remaining tumour cells. Radiation therapy is also frequently used following chemotherapy (4).

A variety of chemotherapeutic options are available for the treatment of breast cancer and these drugs are often administered in combination (27). These drugs include naturally occurring compounds and their derivatives such as the anthracyclines, (doxorubicin) as well as taxanes (paclitaxel). Anthracyclines act by preventing nucleic acid synthesis in dividing cells through intercalation between base pairs of DNA and RNA (28). Anthracyclines also act through inhibition of topoisomerase II and via the generation of reactive oxygen species (29). Topoisomerases are enzymes that play a role in the winding and unwinding of the DNA double helix during DNA replication or transcription (30). Interference with the activity of topoisomerases can result in DNA damage as well as stalling of critical cellular processes causing the cell to undergo cell death (31). Etoposide, a derivative of podophyllotoxin, is also used in the treatment of breast cancer (32). As its name suggests, etoposide also acts through the inhibition of topoisomerase II (33). The taxanes disrupt microtubule function and thereby inhibit cell division (34). Other drugs commonly administered include antimetabolites (5-fluorouracil, methotrexate) and alkylating agents (cyclophosphamide) (4).

In addition to standard chemotherapy, which is broadly cytotoxic, a number of targeted hormonal and biological therapies are used in the treatment of breast cancer. These treatment options, which include the estrogen receptor antagonist tamoxifen as well as the monoclonal antibody trastuzumab, have greatly improved survival in ERα+ and ErbB2+ breast tumours respectively (5-7). These treatments have resulted in a

significant decrease in breast cancer mortality over the past 20 years, even in the face of increasing prevalence of the disease in Western society. Targeted therapies, including tamoxifen and trastuzumab are discussed in further detail in Sections 1.4.3, 1.5 and 1.8.2.

1.4 Epidermal Growth Factor Receptors

The EGF receptor family is composed of four key membrane spanning receptors referred to as ErbB1-4 (Her1-4) (35), for which there are currently 11 known ligands (36). All four receptors share similar structure and function and exhibit sequence homology, with particularly high similarity within the kinase domain (37). The basic receptor structure is shown in Figure 1.1 and consists of four extracellular domains, a short transmembrane region and an intracellular region that contains a tyrosine kinase domain as well as several C-terminal phosphorylation sites. The four extracellular domains are referred to as L1, L2, S1 and S2. L1 and L2 are required for ligand binding while S1 forms interactions between dimerization partners and S2 regulates receptor dimerization. In the absence of ligand binding, S2 binds S1 preventing receptor dimerization. In the presence of ligand a conformational change displaces S2 from S1 facilitating interactions between the dimerization partners (38). ErbB2 and ErbB3 differ from the common structure in that ErbB2 lacks ligand binding ability and ErbB3 does not possess a functional tyrosine kinase domain. Signalling from the epidermal growth factors regulates a plethora of cellular functions including differentiation, growth, adhesion, migration and apoptosis (35-36, 39).

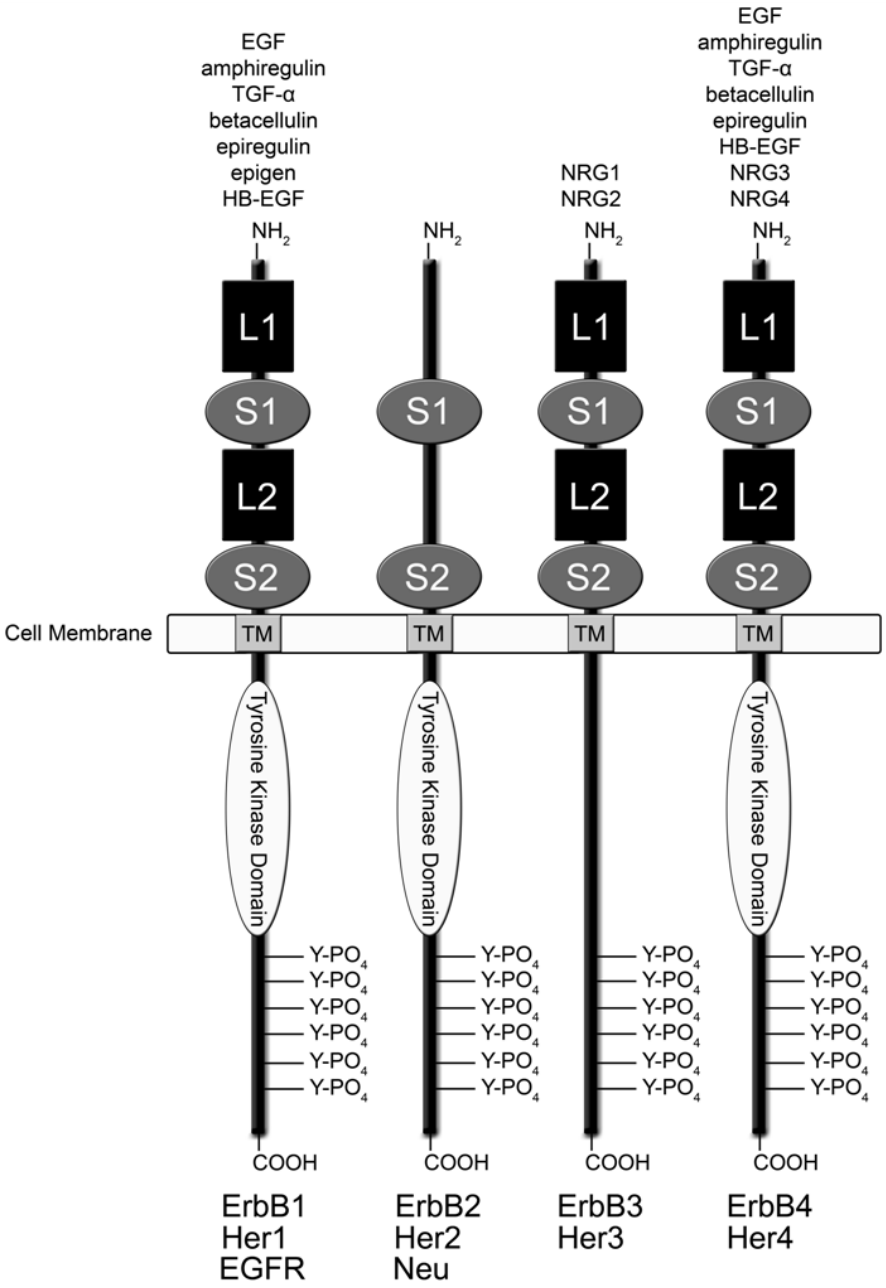

Figure 1.1 EGF receptor family domain structure

The EGF receptor family consists of four members that share a common domain structure. The extracellular ligand binding domains L1 and L2 dictate ligand binding affinity and specificity. The other two extracellular domains, S1 and S2, regulate receptor dimerization in response to ligand binding. A short hydrophobic transmembrane domain resides within the cell membrane. On the intracellular face, a tyrosine kinase domain exists that can transphosphorylate multiple tyrosine residues of the binding partner resulting in activation of downstream signalling. The ErbB2 receptor does not have any known ligand but can serve as a dimerization and activation partner for the other receptors. ErbB3 does not possess a functional tyrosine kinase domain. The known ligands for each receptor are indicated above the receptor and the commonly used receptor names are indicated below.

1.4.1 Receptor Activation and downstream signalling

The molecular mechanism behind activation of the receptors involves binding of the receptor by ligand followed by homo or heterodimerization of the 4 family members (40). Dimerization can also occur in the absence of ligand under conditions in which the receptors are highly over-expressed (41). Receptors can also be turned on constitutively by activating mutations (42-43). Following receptor dimerization, the EGF receptors are autophosphorylated at multiple tyrosine residues within the cytoplasmic domain of the receptor (36, 44). Tyrosine autophosphorylation occurs as a result of ligand-induced conformational changes that enhance the activity of the kinase domain. Receptor trans-phosphorylation is also facilitated by the close proximity of receptors upon dimerization (44). These phosphorylated tyrosines act as docking sites for proteins that contain a Src-homology-2 (SH2) domain or a phospho-tyrosine binding domain (PTB) (45). The most studied downstream signalling pathways of the EGF receptors include the mitogen activated protein kinase (MAPK) pathway, phosphatidylinositol 3-kinase pathway (PI3K), the Jak/Stat pathway, phospholipase Cγ (PLCγ) signalling and the Src kinase pathway (36, 46).

Proteins bound to the phospho-tyrosine residues via their SH2 or PTB domains can activate downstream signalling through catalytic activity, as is the case for PLCγ. In this example, PLCγ is not only activated by phosphorylation but is also, through binding to the receptor, brought into close proximity with its substrate phosphotidylinositol (4,5) bisphosphate (PIP_2) (47). Activated PLCγ hydrolyzes PIP_2 to generate the secondary messengers IP3 and diacylglycerol (DAG) resulting in calcium release and activation of protein kinase C (PKC) (47).

A second mode of downstream signalling involves recruitment of 'scaffolding' proteins that act as docking sites for the assembly of protein complexes that can thereby activate downstream signalling through catalytic function. A fitting example is the recruitment of the Grb2 adaptor protein. Grb2 binds to the activated EGF receptor and recruits the protein Son of sevenless (SOS). SOS activates the small GTP binding protein Ras through guanine nucleotide exchange of GDP to GTP. This triggers the recruitment of Raf to Ras and in doing so activates the mitogen activation protein kinase (MAPK) signalling cascade (36).

Another mechanism of receptor signalling that has more recently been described is receptor translocation to the nucleus following ligand binding and receptor internalization (48). To date, all four members of the EGF receptor family have been discovered within the nucleus under certain contexts (49). Elevated levels of EGFR in the nucleus has been correlated with poor survival in breast cancer (50). Recent studies have clarified the role of nuclear receptor tyrosine kinases. ErbB1 has been described to act as a transcriptional co-activator and has been implicated in the regulation of cyclin D1 as well as inducible nitric oxide synthase (iNOS) (48, 51). Furthermore, the ErbB2 receptor is able to bind to the Cox2 promoter and regulate gene transcription (52). The discovery of a nuclear role for the EGFR family has added another layer of complexity to an already elaborate signalling network.

1.4.2 EGF receptors in breast cancer

Members of the EGFR family have been implicated in the development and progression of breast cancer. The ErbB2 (Her2) receptor is amplified in as many as 25%

of breast cancer cases and correlates with poor prognosis (53). ErbB2 is over-expressed in both the Her2+ and Luminal B subtypes. In Luminal B breast cancer ErbB2 is over-expressed in as high as 50% of the cases. This over-expression may partially explain the poorer prognosis of Luminal B as compared to the Luminal A subtype (19, 24-25). The ErbB1 receptor is also increased in breast cancer but increased gene transcription rather than gene amplification appears to be the primary reason for over-expression (54). In contrast to normal tissue, the expression level of ErbB1 in breast cancer can be increased by as much as 20-fold (35). ErbB1 over-expression is frequently observed in basal like/triple negative breast cancer as well as the Luminal B subtype (22, 25). While less studied, ErbB3 is also found to be increased in 19-29% of breast cancer cases and this increase, like that of ErbB1, is not primarily due to a change in gene copy number (55). While the prognostic significance of ErbB3 in breast cancer is not as straightforward as for Her2, a general trend towards disease progression and poorer outcome has been reported (55). The case of ErbB4 is probably less clear than ErbB3, with both down- and up-regulation observed in breast tumour samples and potential roles as both an oncogene and a tumour suppressor described in cell line models (56). Despite some uncertainty as to the roles of ErbB3 and ErbB4, it has been demonstrated quite clearly that both ErbB1 and ErbB2 contribute to survival, resistance and metastasis in breast cancer and for these reasons these receptors have been considered valuable drug targets.

1.4.3 Targeting of the EGF receptors

Because the EGF receptors are key regulators of survival and proliferative signals in cancer, they have been actively targeted through the development of therapeutic monoclonal antibodies as well as small molecule tyrosine kinase inhibitors that disrupt

receptor function or target cells over-expressing the receptor for destruction (57). Trastuzumab (Herceptin®) is a monoclonal antibody that binds to the extracellular domain of the ErbB2 epidermal growth factor receptor and disrupts its function (58). The introduction of trastuzumab for the treatment of ErbB2 positive breast cancer has drastically improved the outcome for these patients (6, 59). While Herceptin does not result in a change in Her2 expression levels it blocks signalling from the receptor and also targets bound cells for destruction by antibody mediated cellular toxicity. As a single agent, Herceptin results in response rates of 35% and these rates increase to 50% when used in combination with chemotherapy. Herceptin has proven particularly beneficial in reducing mortality and recurrence in women with early stage Her2 positive breast cancer (8). Several monoclonal antibodies have also been developed that target the ErbB1 receptor. Cetuximab (Erbitux®) is a monoclonal antibody that binds to the extracellular domain of the ErbB1 receptor. Cetuximab has demonstrated clinical response in patients with metastatic colorectal cancer, head and neck cancer as well as non small cell lung carcinoma (60).

In addition to monoclonal antibody therapies, epidermal growth factor receptors have also been targeted through the use of small molecule tyrosine kinase inhibitors (61). These drugs have demonstrated success in a variety of tumour types. These inhibitors bind to the ATP binding site of the receptor's tyrosine kinase domain. Lapatanib (Tykerb®) is a cell permeable small molecule that binds to the tyrosine kinase domains of both ErbB1 and ErbB2. This binding inhibits the kinase activity of the receptors and prevents substrate phosphorylation. Lapatinib improves response rate when used in

combination with the chemotherapeutic drug capecitabine (Xeloda®) in ErbB2+ breast cancer (61).

Despite the existence of numerous targeted agents, many patients do not respond to these treatments (intrinsic resistance) and those that initially do often develop resistance (acquired resistance) over a short period of time (62). Resistance to agents targeting ErbB1, such as cetuximab, as well as small molecule tyrosine kinase inhibitors has been linked to activating mutations in the K-Ras gene (63), the B-Raf gene (64) as well as mutations in the catalytic subunit of PI3Kinase (65). In addition, over-expression of vascular endothelial growth factor (VEGF) was observed as a means of resistance in mouse models of squamous cell carcinoma (66). Resistance to agents targeting ErbB2 such as trastuzumab has been found to frequently involve loss of PTEN (67). Additionally, increased expression of the ligand transforming growth factor alpha (TGF-α) was found in tumours following developed resistance to ErbB family inhibitors (68). Additional resistance mechanisms, including the over-expression/activation of other receptor tyrosine kinases such as ErbB3, Met and insulin-like growth factor 1 receptor, have been implicated in resistance to ErbB1 and ErbB2 targeted therapies (69-71).

1.5 The ERK MAPK Pathway

The Ras-Raf-Mek-Erk signalling pathway is a well-studied signalling network that plays an essential role in a host of cellular functions including cell survival, metabolism, cell motility, proliferation, differentiation and apoptosis (72). The pathway is activated by a diverse set of stimuli that relay signals through receptor tyrosine kinases, G-protein coupled receptors and cell adhesion receptors (73). The downstream effectors

ultimately act through the modulation of nuclear substrates that regulate gene transcription as well as a variety of cytoplasmic and cytoskeletal targets that can affect cellular functions (72). The basic mitogen activated protein kinase (MAPK) pathway is diagrammed in Figure 1.2. The core of this signalling network involves a MAP kinase that is activated via phosphorylation by a MAP kinase kinase (MAP2K) that itself is activated by a MAP kinase kinase kinase (MAP3K) (74). The MAP3K is typically converted into an active state through binding of a small G-protein, but can also be activated via phosphorylation and oligomerization (75).

In the case of extracellular signal regulated kinase (Erk) signalling, the G-protein Ras resides at the top of the pathway. Activated receptor tyrosine kinases, such as the EGF receptor, recruit the protein Growth factor receptor bound protein 2 (Grb2), which binds phospho-tyrosine residues in the C-terminal domain of the receptor via its SH2 domain. Grb2 can either be recruited directly to the receptor or via interactions with receptor-bound Src homology 2 domain containing transforming protein 1 (SHC). Grb2 recruits the guanine nucleotide exchange factor Son of Sevenless (Sos), which induces the exchange of the guanosine diphosphate (GDP) molecule bound to Ras for guanosine triphosphate (GTP), converting Ras into its active state. Ras subsequently binds to and activates the MAP3K Raf, which in turn activates the MAP2Ks MAP kinase and Erk Kinase1 and 2 (Mek1/2) that ultimately phosphorylate the MAPKs Erk1and Erk2 (76). Erk1/2 are considered the key effector molecules of the pathway; however, a linear activation pathway simplifies a much more complicating signalling network involving many side branches both known and unknown (72). To date, more than 100 targets of Erk1/2 have been discovered (73); however, the means by which specificity is obtained

by a pathway that is activated by many stimuli and controls many cellular functions has remained elusive.

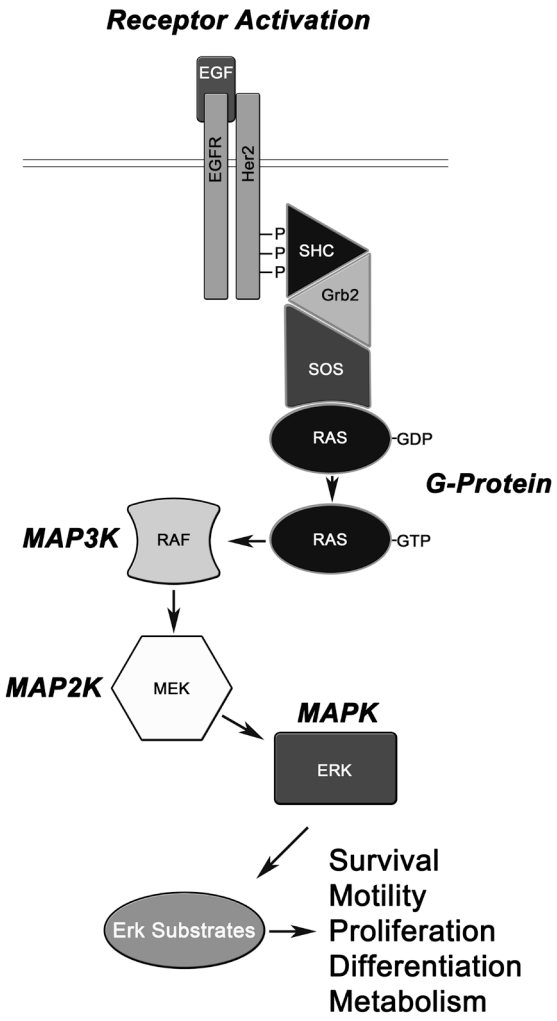

Figure 1.2 The MAPK signalling pathway

The MAPK signalling pathway is exemplified in the above figure by the Ras/Raf/Mek/Erk signalling cascade. The Ras-MAPK signalling pathway is activated by a variety of stimuli including receptor tyrosine kinases. Tyrosine phosphorylation of the receptors recruits the SH2 domain containing protein SHC. SHC subsequently recruits Grb2 which binds and recruits SOS. SOS is a guanine nucleotide exchange factor that activates Ras through the exchange of bound GDP for GTP. Activated Ras phosphorylates and activates the MAP3K Raf which subsequently phosphorylates and activates the MAP2K Mek. Mek phosphorylates the MAPKs Erk1/2 which then phosphorylate a wide variety of cellular substrates leading to increased cell survival and proliferation.

1.5.1 Ras

The Ras family includes 4 primary members encoded by three separate genes. H-Ras, N-Ras and K-Ras are each encoded by separate yet highly homologous genes. K-Ras can be alternatively spliced to generate two isoforms K4A and K4B (77). Ras proteins are subjected to prenylation, a process in which a hydrophobic lipid molecule is covalently linked to a protein; this modification confers membrane localization (78). Under unstimulated conditions, Ras is associated with the cell membrane in its GDP bound inactive state. Phosphorylated receptor tyrosine kinases recruit guanine nucleotide exchange factors (GEFs) that convert Ras into its active GTP bound state. GTP bound activated Ras then recruits Raf to the membrane where it is phosphorylated and transmits the signal downstream (79). Ras is converted back to the inactive GDP bound state through hydrolysis of GTP to GDP via its intrinsic GTPase activity. This GTPase activity is enhanced through interactions with GTPase activating proteins (GAPs) (80).

Mutations in codons 12, 13 or 61 of K-Ras and N-Ras are found in many human tumours. These mutations prevent conversion of Ras from its GTP bound state back to the inactive GDP bound state. As a result, Ras signalling remains constitutively on leading to improper activation of survival and proliferative signals (81). Mutations in Ras occur in approximately 30% of human tumours and for this reason Ras has been identified as an important therapeutic target (82). Several approaches have been pursued to target Ras with the primary focus being inhibitors of Ras prenylation (83). Inhibition of Ras prenylation prevents the post-translational modification of Ras with a lipid molecule and thereby prevents the localization of Ras at the cell membrane. Farnesyltransferase inhibitors (FTIs) were developed that showed great promise in cell

line and murine models; however the compounds met with disappointing clinical results (77). It was hypothesized that under conditions of treatment with FTIs, Ras was being modified by geranylgeranyltransferase (GGT), an enzyme that adds a 20 carbon isoprenoid group to Ras. The combination of FTIs and geranylgeranyltransferase inhibitors (GGTIs) demonstrated high toxicity in preclinical models and was abandoned as a treatment option (84).

1.5.2 Raf

The rapidly accelerated fibrosarcoma (Raf) family consists of 3 members referred to as A-Raf, B-Raf and C-Raf (85). All three proteins function as serine/threonine kinases; however, they are thought to have non-redundant mechanisms of action as all three isoforms are subjected to differing mechanisms of regulation, exhibit different expression patterns and also have unique downstream signalling targets (86). Raf proteins are recruited to the membrane through binding to activated Ras as well as through interactions between Raf and membrane lipids (87). Raf activation occurs through phosphorylation at serine and tyrosine residues by p21-activated kinase (PAK) and members of the SRC family of kinases. Phosphorylation, along with changes in association with 14-3-3 proteins, heat shock protein 90 (hsp90) and several other bound proteins causes a conformational change that allows Raf to bind to its substrates with high affinity (88-92). Raf then is able to bind and activate the downstream effectors Mek1/2 (81). Raf proteins have also been demonstrated to have functions that do not require active kinase activity and have been postulated to also act as scaffolding proteins (93).

While mutations in A-Raf and C-Raf are rare, mutations in B-Raf are seen in as many as 20% of all cancers including malignant melanomas as well as in a variety of other tumour types including colon cancer and ovarian cancer (94). These mutations can result in constitutive activation, but not all mutations result in increased enzymatic activity. It is thought that some mutations increase the affinity of B-Raf for C-Raf and that these heterodimers are critical for the observed downstream activation of the Erk pathway (95). Raf has been targeted through the development of small molecule inhibitors of its catalytic activity (77, 85). The most studied compound is Sorafenib, which prevents ATP binding of both C-Raf and B-Raf (96). This drug has shown success in the treatment of renal cell carcinoma; however, it is uncertain whether Raf is the primary target of the drug or whether other targets including the VEGF receptor are the primary mode of action (85).

1.5.3 Mek1/2

Mek1 and Mek2 are dual-specificity kinases that phosphorylate key threonine and tyrosine residues within their downstream targets Erk1/2 (97). Mek1/2 are activated by Raf through phosphorylation of two serine residues located within the activation loop of the proteins (98). Both B-Raf and C-Raf have the strongest ability to activate Mek1/2 whereas A-Raf only weakly activates Mek1 (87). Activated Mek1/2 go on to phosphorylate and activate Erk1/2, the primary downstream effectors of this pathway (99). Mek1/2 have very stringent substrate specificity and the only known targets of these kinases to date are Erk1/2 (100). While Mek1 and 2 share similar structure and function, it is believed that they are subjected to differential regulation and also exhibit differences in downstream function (101-102).

Mek mutations, while relatively rare, have been observed in lung, ovarian, colon and skin cancers (103-105). Despite the rarity of mutations (<1% of all cancers) , Mek1/2 are downstream effectors of a pathway that is deregulated in as many as 50% of all tumour types (99). For this reason, several Mek inhibitors have been developed that prevent the activity of Mek1/2 with a large degree of specificity. The first generation inhibitors such as PD98059 and U0126 demonstrated specific Mek inhibition; however, they did not display sufficient efficacy to gain any clinical interest (99). PD0325901, a second generation Mek inhibitor with 100-fold increased affinity for Mek1/2 displayed some promise, but increased levels of toxicity resulted in termination of future studies (106). Several new Mek inhibitors that exhibit fewer toxic side effects are currently in Phase I and II clinical trials with results yet to be published (99).

1.5.4 Erk1/2

Erk1 and Erk2 display 84% identity in amino acid sequence and are the key downstream effectors of the Erk MAPK pathway (107). Despite this high degree of similarity, mouse studies have clearly demonstrated differences between Erk1 and Erk2 knock-out phenotypes, indicating the roles are not completely redundant (108-109). The results of mouse knock-out models combined with data from several in-vitro studies analyzing Erk1 and Erk2 activity through loss and gain of function assays, suggests that Erk1 may act to inhibit the function of Erk2 (110). Despite possible differences in function, it is well established that activation of Erk1/2 by phosphorylation triggers these kinases to phosphorylate their substrates at a consensus Proline-X-Serine/Threonine-Proline motif, where X is any amino acid (111).

To date more than 100 targets have been identified for Erk1/2 indicating their involvement in a wide range of cellular processes. Erks act primarily on nuclear targets, but also phosphorylate other kinases, phosphatases, cytoskeletal proteins and proteins involved in the regulation of apoptosis. Upon activation, Erk1/2 transport to the nucleus where they phosphorylate a number of transcription factors including the Ets-family transcription factor Elk-1 (112).

While no mutations contributing to tumourigenesis have yet been discovered in Erk1/2, there has recently been some advancement in the development of inhibitors (113-115). While these compounds have demonstrated effectiveness in cell culture models it remains to be seen if they will make their way into clinical use.

1.6 Elk-1

Elk-1 is a member of the E twenty-six (Ets) transcription factor family. Ets family transcription factors were initially discovered due to their homology with the v-ets protein. The v-ets protein is encoded by the oncogenic E26 avian retrovirus, from which the Ets family derives its name (116). The Ets family of transcription factors play roles in development, differentiation and proliferation. There are greater than 25 Ets family transcription factors and these can be divided into as many as 10 sub-families based on sequence homology (117). The Ets family transcription factors have been implicated in cancer, where misregulation and Ets fusion proteins have been found to contribute to tumourigenesis (118).

Elk-1 belongs to the Ets-subfamily of ternary complex factors (TCFs) along with the closely related transcription factors Net and Sap-1 (119). All three TCFs form

complexes with serum response factor (SRF) dimers at serum response elements (SREs) of target genes. An Elk-1/SRF TCF bound to a serum response element is illustrated in Figure 1.3. Serum response elements are characterized by a 5' Ets DNA binding element in close proximity to a downstream SRF binding site (CarG box) (120). Elk-1 was initially discovered as a protein isolated from HeLa nuclear extracts that formed a ternary complex on the serum response element of c-fos with serum response factor. (121).

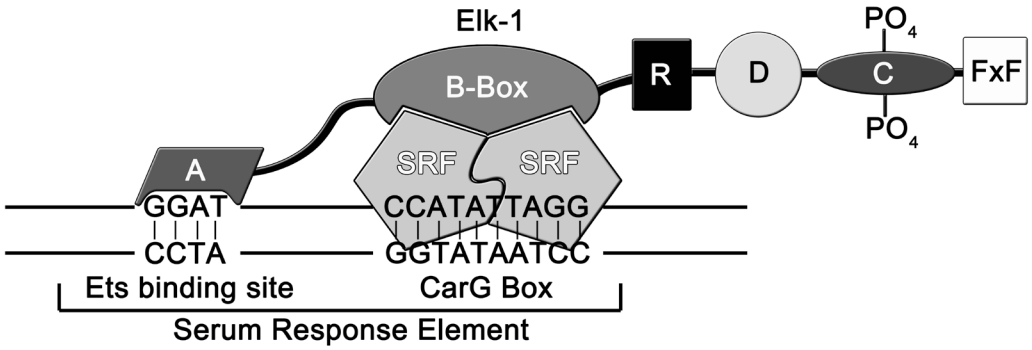

Figure 1.3 Binding of Elk-1 to the Serum Response Element
A serum response element is defined as a DNA sequence containing an Ets binding site in close proximity to the SRF binding CarG box. Elk-1 binds to the Ets binding site through protein-DNA interactions made by the A-domain. SRF binds to the CarG box as a dimer and recruits Elk-1 into a ternary complex through interactions with the Elk-1 B-Box domain. Additional domains in Elk-1 include the transcriptionally repressive R-domain, the MAP kinase binding D and FxF motifs as well as a domain subjected to multiple phosphorylation events termed the C-domain.

The TCF transcription factors contain 6 conserved domains, which are shown schematically for Elk-1 in Figure 1.3. The A-domain is the Ets DNA-binding domain and is found in all Ets family transcription factors (122). This domain facilitates DNA binding to Ets binding sites which consist of the core sequence 5'-GGA(A/T)-3' surrounded by additional DNA sequence that serves to dictate specificity amongst the Ets family members (119). The second domain is the B-box, which is critical for interactions with

SRF (123). The C-domain contains as many as 9 serine and threonine phosphorylation sites (124), of which Ser383 is critical for transcriptional activation. The specific functions of the other phosphorylation sites are yet to be determined (119). Two additional domains, the D-domain and the FxF motif have been found to play a role in the binding of Erk1/2 and other MAP kinases (125). A final motif found to play a role in the transcriptional repressive activity of Elk-1 is referred to as the R-motif (122).

The primary mechanism of regulation of Elk-1 involves phosphorylation at multiple residues within the C-domain. Elk-1 is phosphorylated by both the Erk and Jnk map kinases (126). Phosphorylation of these sites increases the affinity of Elk-1 for its DNA binding site as well as to its binding partner SRF (127). Furthermore, phosphorylation of Elk-1 reduces its affinity for the msin3A-histone deacetylase (HDAC) complex that binds to the Ets domain and represses transcription (128). While Elk-1 was initially discovered as part of a ternary complex with SRF, Elk-1 has been demonstrated to bind DNA autonomously to high affinity Ets binding sites as well as in conjunction with additional transcription factors (129-130). It is presumed that activation of Elk-1 involves recruitment of additional co-activators; however, the exact nature of these co-activators is yet to be clearly defined (119). Interaction between the ternary complex of Elk-1 and SRF with the histone acetyl-transferases (HATs) Creb binding protein (CBP) and p300 has been demonstrated, but does not appear to be dependent upon Elk-1 phosphorylation (131-132). Furthermore, the Srb mediator complex has been found to bind Elk-1 and may serve as a link between the transcription factor complex and the transcriptional machinery of the cell (133). More recently it has been determined that Elk-1 recruits the kinase MSK1 to serum response elements where it plays a role in the

phosphorylation of histone H3 resulting in a more open chromatin conformation that facilitates transcription (134).

The TCF sub-family of Ets domain transcription factors have been shown to regulate multiple immediate early genes such as c-fos, and jun-B contributing to cell survival and cell cycle progression (119). Furthermore, it has been shown that fusion of Elk-1 to a transcriptional repressive domain blocks cell growth and induces apoptosis (135). Taken together, the TCF subfamily of Ets domain transcription factors plays a critical role in the cellular response to survival and growth promoting stimuli.

1.7 SRF

SRF is a member of the MADS box family of transcription factors, a group characterized by the presence of a conserved 56 amino acid DNA binding domain. The family name is derived from the names of the four family members initially discovered: MCM1, Agamous, Deficiens and SRF (136). SRF was discovered in 1987 as a protein isolated from HeLa cells that bound with high affinity to DNA probes containing the serum response element (137). SRF binds specifically to the CarG box. The CarG box is a part of the serum response element and has the consensus DNA sequence 5'-CCATATTAGG-3' (138).

The SRF protein consists of three separate domains, a DNA binding domain (the MADs box), a transcriptional activation domain and a region subjected to phosphorylation (139-140). The SRF mRNA is also alternatively spliced to yield four different transcripts. Through Western blotting, it has been determined that these multiple transcripts result in the translation of the full length 67kDa protein as well as two smaller

splicing variants. (141-144). Phosphorylation of SRF occurs following stimulation of cells with serum and greatly increases the binding affinity of SRF for its DNA binding element (145). SRF acts in concert with two major groups of co-activators, the TCF family of Ets domain transcription factors and the myocardin-related transcription factors (MRTF) (146). Through these protein-protein interactions SRF enhances transcription of target genes through recruitment of additional co-activators and the RNA Polymerase II transcriptional machinery. In addition to its ability to activate transcription, SRF has also been determined to repress target genes while in the unstimulated state as well as following transient activation (140).

Since its discovery, SRF has been implicated in the regulation of over 30 genes. These genes include many immediate early genes, which are genes transcriptionally activated by serum in the absence of new protein synthesis. In addition to immediate early genes, SRF controls the expression of many muscle and neuronal specific genes (138). Knock-out of the SRF gene was embryonically lethal and mice demonstrated impaired mesoderm development (147). SRF has also been implicated in the progression of several cancers (148-151). These results suggest that SRF plays an important role in both development and disease progression.

1.8 Estrogen mediated signalling

The significance of estrogen in breast cancer has long been established and the molecular mechanisms by which estrogen elicits survival and proliferation are continuing to be unravelled. Estrogen signals through two key receptors of the nuclear receptor superfamily termed ERα and ERβ. Binding of estrogen to these receptors results in

signalling through both the classical genomic pathway as well as through more recently understood non-genomic mechanisms. The classical pathway involves binding of the ligand to the receptor in the cytoplasm and subsequent receptor dimerization and nuclear relocalization. Once in the nucleus, the receptors bind to estrogen responsive elements (EREs) in the promoters of target genes resulting in context-dependent activation or repression of transcription (152). More recently it has been determined that estrogen receptors can heterodimerize with other DNA binding transcription factors and thereby stimulate transcription of genes that do not contain a functional ERE (153). Estrogen has also been demonstrated to act through non-genomic mechanisms that involve activation of estrogen receptors located near the cell membrane and initiation of downstream signalling cascades. Additionally, binding of estrogen to non-classical receptors such as G Protein-coupled Receptor-30 (GPR30) is demonstrated to result in stimulation of the mitogen activated protein kinase (MAPK) pathway (154-155).

Regardless of the exact mechanism, the survival and proliferative effects elicited by estrogen signalling makes the pathway an important therapeutic target in breast cancer. Blockage of estrogen mediated signalling through anti-estrogens such as Tamoxifen and inhibition of estrogen synthesis by treatment with aromatase inhibitors has met with substantial clinical success. Despite the success of these approaches, resistance to treatment is a consistent problem and therefore additional means of targeting proliferative/survival signals in breast cancer need to be sought (156).

1.8.1 Crosstalk between estrogen and growth factor signalling

With an increased understanding of the intricacies of both estrogen and growth factor mediated signalling pathways, it has become apparent that there exists a large degree of interconnectedness between them. Both the EGFR and estrogen receptor signalling pathways have the capacity of influencing one another as well as compensating for each other. This cross-talk has been implicated as a key reason why resistance develops to targeted agents currently being used in the clinic (157). Several lines of evidence suggest that knock-down of estrogen receptor signalling results in transcriptional activation of the genes of the EGFR family (158). Additionally, ligand independent phosphorylation and activation of the estrogen receptor can occur through signalling elicited via the EGF receptors (159). This dynamic relationship between multiple signalling pathways calls for increased focus on combined treatments or drugs that have the capacity of inhibiting key components common to the survival and proliferative advantage conferred by both signalling networks.

1.8.2 Targeted approaches to inhibit estrogen mediated signalling in breast cancer.

Signalling elicited by estrogen stimulation has been targeted in breast cancer through the use of hormonal therapy. Hormonal therapy includes the use of aromatase inhibitors, selective estrogen receptor modulators (SERMs) and pure estrogen receptor antagonists and has met with a large degree of clinical success (160-161). Aromatase inhibitors, an example of which is letrozole, are drugs that block the synthesis of estrogen and thereby prevent the growth of hormone-responsive tumours (162). SERMs, such as tamoxifen and raloxifene, are synthetic ligands that bind to and act agonistically or antagonistically on the estrogen receptors in a tissue dependent manner (163). Pure ER

antagonists, such as fulvestrant, act to block estrogen receptor signalling in all known contexts and have demonstrated efficacy in tumours that have developed resistance to SERMs or aromatase inhibitors (160).

1.9 Overview of the NF-κB transcription factor and its role in cancer

NF-κB is a protein complex that regulates the transcription of a wide variety of target genes that perform diverse cellular functions. The NF-κB protein complex is comprised of homo or heterodimers of five subunits, all of which contain a Rel homology domain (RHD). This domain is key for protein-protein interactions as well as binding to DNA. The five subunits include NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), RelB and c-Rel (164). In the canonical pathway, NF-κB dimers reside in the cytoplasm sequestered in an inactive state by binding to members of the inhibitor of κB (IκB) family. Phosphorylation of IκBs by IκB kinase (IKK) targets IκB for ubiquitin mediated proteolytic degradation and release of activated NF-κB dimers. Activated NF-κB transports to the nucleus and activates transcription. This activation of NF-κB occurs in response to many stimuli including cytokines, growth factors as well as cellular stress (165).

Constitutive NF-κB activation is observed in a number of tumour types. In breast cancer, over-expression of the p100 and p65 NF-κB subunits has been reported (166-167). Increased nuclear localization was found in the ERα negative subset of breast tumours, particularly tumours over-expressing the EGF receptors ErbB1 and/or ErbB2 (168-169). This over-activation of NF-κB leads to increased expression of genes that play

a role in cancer progression through activation of cell migration, stimulation of angiogenesis, modulation of cell cycle control and regulation of apoptosis (164).

1.10 Overview of the Stat3 transcription factor and its role in cancer

The Stat transcription factor family consists of 7 members Stat1-5a, 5b and 6. Stats are activated by phosphorylation in response to varied extracellular stimuli. Phosphorylation can occur directly by growth factor receptors or by JAK and Src kinases. Following phosphorylation, homo and heterodimerization occurs amongst the Stat family members followed by nuclear translocation, DNA binding and activation of gene transcription. The Stat transcription factor family regulates both cell survival and proliferation and constitutive activation is observed in both solid and haematological malignancies (170). Of all Stat family members, Stat3 and Stat5 are the most frequently hyper-activated, with increased phosphorylation observed in a large number of malignancies (171).

Stat3 over-expression occurs in many tumour types and, when over-expressed, has been found to be sufficient to initiate tumour growth (172). Genes regulated by Stat3 are critical for both cell migration and invasion, two traits critical for tumour metastasis (173). In breast tumours, increased phosphorylation of Stat3 was observed in tumour cells as compared to normal tissue and elevated levels were associated with poorer treatment response (174). Increased activation of Stat3 in both breast cancer as well as other tumour types has led to the development of small molecule inhibitors. Inhibition of Stat3 has proven effective in mouse tumour models of hepatocellular carcinoma as well as non small cell lung cancer (175-176). In breast cancer, Stat3 inhibition prevented the growth

of breast cancer tumours in both mouse and chicken embryo xenograft models (177). These preliminary studies support the future implementation of Stat3 inhibitors in the treatment of multiple tumour types.

1.11 Programmed Cell Death

Cell death has been traditionally divided into two categories: necrosis and programmed cell death. Necrosis describes unregulated cell devastation in response to serious cellular damage and is not dependent upon energy in the form of adenosine triphosphate (ATP). Necrotic cell death results in cell swelling and loss of membrane integrity causing the release of cellular contents. The release of cellular contents by necrotic cells generates an inflammatory response (178). On the other hand, programmed cell death describes a controlled process by which the removal of unwanted, damaged or infected cells takes place. While programmed cell death was originally considered synonymous with apoptosis, programmed cell death has more recently been classified into three types: apoptosis, autophagy and programmed necrosis (179-180). The decision to die by programmed cell death rather than unregulated necrosis seems largely dependent upon the severity of cellular damage as well as the availability of energy in the form of ATP to carry out the necessary steps of programmed cell death (181-182). Apoptosis is the prototypical form of programmed cell death and will be described in detail further below.

Autophagy is a cellular response to starvation conditions such as glucose or amino acid starvation and/or low oxygen conditions (hypoxia). Autophagy involves engulfment and breakdown of cellular organelles and macromolecules to generate energy and

molecular building blocks such as amino acids (183). Under certain conditions, autophagy can switch from a survival mechanism to a mechanism of cell death that is distinct from apoptosis (184).

More recently, the notion that a controlled form of necrosis (often termed necroptosis) occurs in response to certain stimuli has gained recognition in the literature. Programmed necrosis can take place as a backup form of cell death in cells with impaired apoptotic machinery (185-187). An example of this occurs upon activation of the death receptors under conditions of ischemia or caspase inactivation/inhibition (180). Like apoptosis, programmed necrosis appears to depend on intact cellular signalling networks; the context of a particular cell appears to be a determining factor as to which cell death pathway is chosen.

1.11.1 Apoptosis

Apoptosis describes the process of controlled cellular degradation and is critical in development, tissue maintenance and homeostasis and in the eradication of damaged or diseased cells (188). Apoptosis is a tightly controlled process that involves cellular breakdown while maintaining membrane integrity. Exposure of the phospholipid phosphatidylserine (PS) to the exterior of the cell membrane signals for the engulfment of apoptotic cells by phagocytes (189). In addition to PS exposure, characteristic hallmarks of apoptosis include cell shrinkage, membrane blebbing, the generation of apoptotic bodies, and DNA fragmentation (190). Apoptosis is carried out by two main pathways, the extrinsic and intrinsic apoptotic pathway. The details of these two pathways are outlined in Figure 1.4. Both pathways rely on the activity of caspases to initiate and carry

out the program. The intrinsic pathway is regulated by the Bcl-2 family of proteins, whereas the Bcl-2 family only plays a role in the extrinsic pathway when cross-talk through Bid cleavage occurs (188). Both the intrinsic and extrinsic pathways will be described in detail further below.

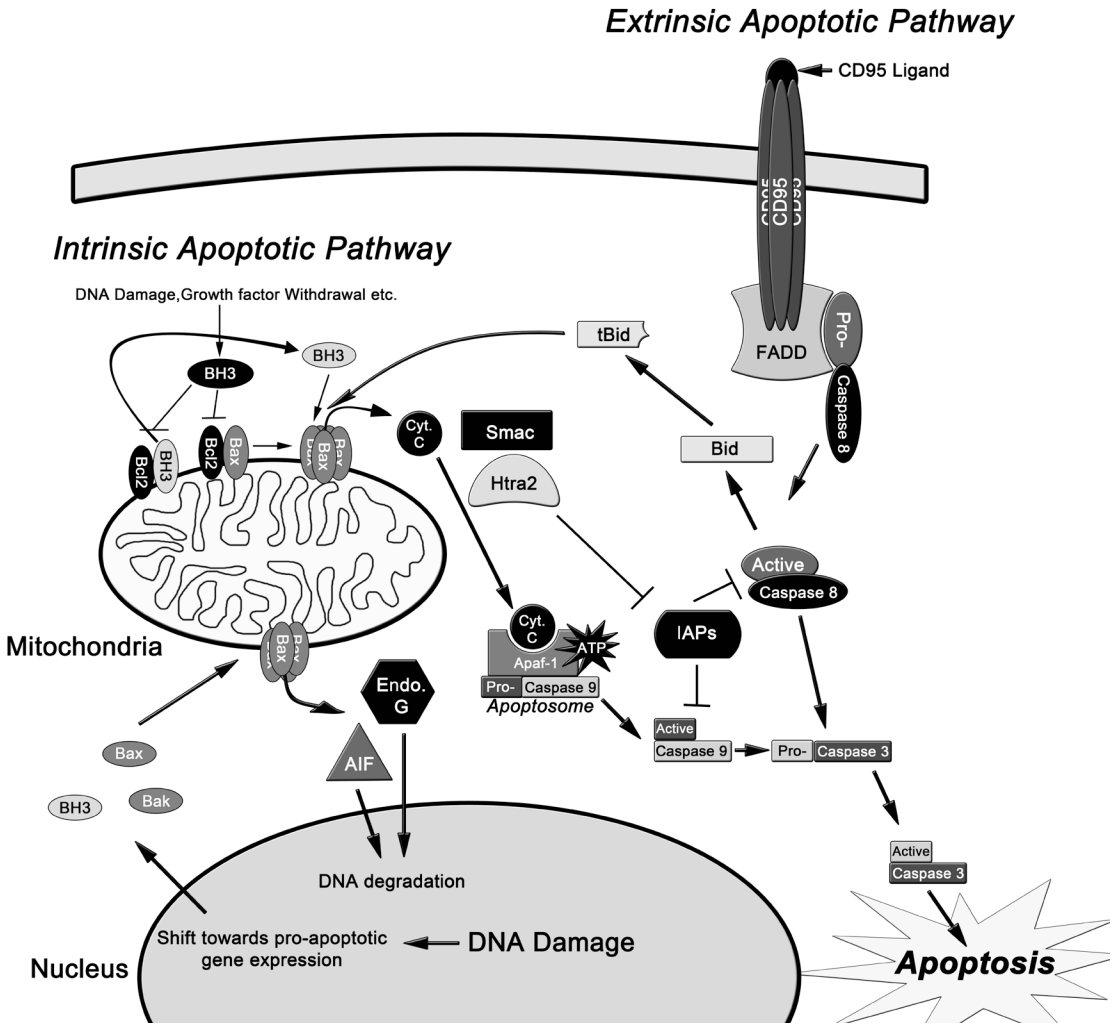

Figure 1.4 The extrinsic and intrinsic apoptotic pathways

The extrinsic and intrinsic apoptotic pathways signal the destruction of the cell through programmed cell death. The extrinsic apoptotic pathway is activated through binding of ligands of the TNF superfamily to a trimerized death receptor. Binding of ligand results in the recruitment of adapter proteins containing death domains such as fas-associated with a death domain (FADD). FADD recruits pro-caspase 8 which is cleaved to generate active caspase 8. Activate caspase 8 can cleave the effector caspase caspase-3 resulting in a cascade of proteolytic cleavage events the culminate in apoptosis. Pro-caspase 8 can cleave the Bcl-2 family member Bid to generate truncated Bid (tBid) which cross-talks with the intrinsic apoptotic pathway. The intrinsic apoptotic pathway is activated when a shift in the balance between pro- and anti-apoptotic Bcl-2 family members results in a loss of integrity of the mitochondrial outer membrane. This results in release of cytochrome c (Cyt. C) which forms a complex with Apaf-1 and pro-caspase 9 to form the apoptosome. Cleavage of pro-caspase 9 within the apoptosome forms active caspase-9 which cleaves caspase-3 and subsequently initiates apoptosis. Smac and Htra2 release from mitochondria relieve the repressive effect of inhibitor of apoptosis proteins on caspase activation. Furthermore, the proteins Endonuclease G and AIF are transported from the mitochondria to the nucleus where they partake in DNA degradation.

1.11.1.1 Caspases

The caspases (cysteine-dependent, aspartate-directed proteases) are a family of 14 enzymes that catalyze the proteolytic cleavage of their substrates at key aspartate residues (191). All caspases are synthesized as zymogens (pro-caspases) that require proteolytic cleavage for activation (192). The caspases involved in apoptosis can be divided into two main groups, the initiator caspases (caspases 2,8,9,10) that trigger the apoptotic cascade and the downstream effector caspases (caspases 3,6,7) that carry out the apoptotic process (193). Through their ability to enzymatically cleave a large number of target substrates, this family of proteins is critical for the initiation and process of apoptotic programmed cell death (194).

Activation of initiator caspases is highly regulated and involves the formation of an intricate multi-protein complex such as the death inducing signalling complex of the extrinsic pathway (195), the apoptosome of the intrinsic pathway (196), and the genotoxic stress induced piddosome (197). These multi-protein complexes are thought to activate the initiator caspases through induced-proximity and/or proximity-induced dimerization (198). Recruitment into these complexes results in caspase activation through autocatalytic cleavage. As cleavage alone is shown to be insufficient for full caspase activation, activation is hypothesized to depend upon additional molecular interactions that are currently poorly understood (199). The activation of effector caspases is better understood and involves proteolytic cleavage that removes inhibitor domains, such as inhibitor of apoptosis protein (IAP) binding sites, and results in a conformational change to the active state (193, 200). Once fully activated, the caspases commit the cell irreversibly to undergo programmed cell death.

The effector caspases are involved in cellular degradation as well as amplification of the apoptotic signal. This process is carried out by cleavage of >250 caspase substrates within the cell (201). This cleavage results in degradation of apoptosis inhibitory proteins, conversion of anti-apoptotic proteins to pro-apoptotic proteins and activation of additional apoptotic effectors (194). The many targets of the effector caspases include cyto/nucleoskeletal proteins (Lamin A, fodrin, gelsolin), anti-apoptotic Bcl-2 family members (Mcl-1, Bcl-2), pro-apoptotic Bcl-2 family members (Bid), proteins involved in DNA repair (PARP), proteins that prevent the function of apoptotic effectors (iCAD) and proteins involved in the cell cycle (RB) (194). The combined effect of all of these activities leads to the biochemical and morphological features of apoptosis.

1.11.1.2 Bcl-2 Family

The B-cell lymphoma 2 (Bcl-2) family of proteins are a group classified by the presence of at least 1 of 4 Bcl-2 homology (BH1-4) domains. The family comprises 25 members, all of which are implicated in the regulation of programmed cell death (202). The primary function of the Bcl-2 family is to govern the permeablization of the outer-mitochondrial membrane, a key event in the initiation of apoptosis (203). Bcl-2 proteins are divided into two sub-categories, those Bcl-2 family members that act to resist apoptosis (anti-apoptotic) and those that act to promote apoptosis (pro-apoptotic). The pro-apoptotic Bcl-2 family members can be further characterized by distinguishing those that have multiple BH domains (multi-domain pro-apoptotic) and those that only contain a single BH3 domain (BH3 only) (202). The structure and classification of the Bcl-2 family members is presented in Figure 1.5. Precise regulation of the Bcl-2 family, both in terms of protein expression and activity, is essential in the determination of cell fate. Loss

of regulation of members of the Bcl-2 family has been implicated in a variety of diseases and particularly in cancer, where over-expression of anti-apoptotic Bcl-2 family members is thought to occur in as many as 50% of total cases (204-205).

Figure 1.5 Domain structure of the Bcl-2 family

The Bcl-2 family consists of three distinct groups, anti-apoptotic Bcl-2 proteins, pro-apoptotic multi-domain Bcl-2 proteins and pro-apoptotic BH3-only proteins. Anti-apoptotic Bcl-2 family members typically contain all four Bcl-2 homology domains as well as a C-terminal transmembrane domain. Pro-apoptotic multi-domain Bcl-2 family members contain 3 Bcl-2 homology domains as well as a C-terminal transmembrane domain. The BH3 only domain Bcl-2 family members contain only the BH3 Bcl-2 homology domain and may or may not contain a C-terminal transmembrane domain.

1.11.1.2.1 Anti-apoptotic Bcl-2 family members

The anti-apoptotic Bcl-2 sub-family consists of six members: Bcl-2, Bcl-Xl, Mcl-1, Bcl-w, Bfl-1 and Bcl-B. With the exception of Mcl-1 and Bfl-1, which appear to lack a BH4 domain, the anti-apoptotic Bcl-2 family members contain all four Bcl-2 homology domains as well as a carboxy-terminal trans-membrane domain (206). The alpha-helical Bcl-2 homology domains form a hydrophobic pocket that is critical for protein-protein interactions, primarily via interaction with the amphipathic alpha helix of the binding partners BH3 domain (202, 207). The hydrophobic trans-membrane domain plays a role in the localization of these proteins to the membranes of both the mitochondria and endoplasmic reticulum (202).

The founding member of the family, Bcl-2, was discovered due to its translocation from chromosome 18 to 14 in non-Hodgkin's lymphoma. This translocation places Bcl-2 under the control of the immunoglobulin heavy chain enhancer, resulting in protein over-expression (208). Over-expression of Bcl-2 and other anti-apoptotic family members is seen in a variety of tumours in addition to lymphoma where they confer strong resistance to apoptosis (205). The anti-apoptotic Bcl-2 family members are located within the outer mitochondrial membrane as well as in the cytoplasm and endoplasmic reticulum. These proteins act to sequester both the BH3-only and the multi-domain pro-apoptotic Bcl-2 family members (Figure 1.6). Sequestration of pro-apoptotic Bcl-2 family members is critical for preventing their action and maintaining cell viability (207).

1.11.1.2.2 Pro-apoptotic Bcl-2 family members

The pro-apoptotic Bcl-2 family members are instrumental in executing the intrinsic apoptotic pathway. The two multi-domain family members, Bax and Bak, are the primary effectors of apoptosis. When activated, Bax and Bak form homo-oligomers that generate pores in the outer mitochondrial membrane (Figure 1.6). Permeablization of the outer mitochondrial membrane results in liberation of pro-apoptotic signalling molecules from the mitochondrial inter-membrane space (202). Once permeablization of the membrane occurs, the cell is at a point of no return and is committed to undergo apoptosis (209). The function, therefore, of both the anti-apoptotic Bcl-2 family members and the BH3-only domain Bcl-2 family members is to coordinate the activity of Bak and Bax (207).

The BH3-only family members represent the largest group of the Bcl-2 family. The reason for their abundance is that they play a key role in regulating the action of both the pro- and anti-apoptotic Bcl-2 family members in response to a large variety of stimuli (206). This group has been further refined into two categories, “direct activators” and “sensitizers/derepressors”. The BH3-only proteins that bind to and activate Bax/Bak are referred to as direct activators. Those that only bind to anti-apoptotic Bcl-2 family members are referred to as sensitizers/derepressors (202, 210). Under normal conditions, the expression of Bax/Bak is at a basal level and their activity is kept in check through direct binding and sequestration by anti-apoptotic Bcl-2 family members. Additionally, the direct-activator BH3 only proteins are also bound and prevented from interacting with Bax/Bak. Upon cellular stress, the expression levels of both the multi-domain and BH3-only domain pro-apoptotic Bcl-2 family members is elevated. When the level of sensitizers/derepressors is increased, they compete with Bax/Bak and the direct activators for binding to anti-apoptotic Bcl-2 family members (Figure 1.6). This causes a subsequent release and activation of Bax/Bak that results in disruption of the mitochondrial membrane (202-203, 207, 210). Post-translational modifications such as phosphorylation can both inhibit anti-apoptotic Bcl-2 family members and activate pro-apoptotic Bcl-2 family members shifting the balance even further towards cell death (211).

While the precise mechanism by which the interplay between Bcl-2 family members occurs and the exact nature of the pores generated in the mitochondrial outer membrane have been subject to debate, it is clear that the relative abundance of pro- and

anti-apoptotic Bcl-2 family members and their activation status determine whether a cell will continue living or execute the apoptotic program.

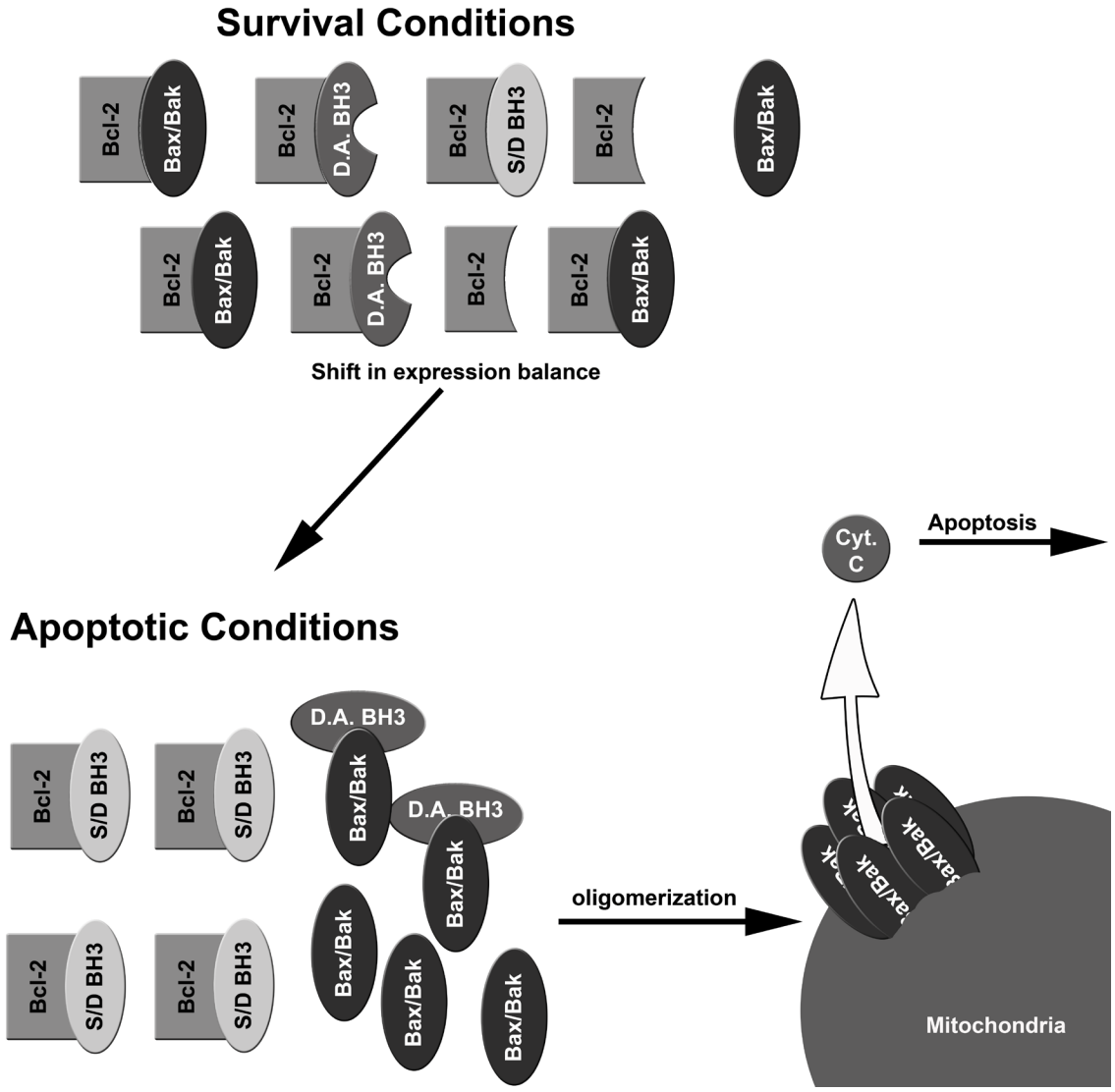

Figure 1.6 Mechanism of action of the Bcl-2 family

The Bcl-2 family consists of anti-apoptotic Bcl-2 family members, exemplified in this figure by Bcl-2, the pro-apoptotic multi-domain Bcl-2 family members Bax and Bak as well as two classes of BH3 only domain proteins, direct activators (D.A. BH3) and sensitizers/derepressors (S/D BH3). Under favourable conditions the cell expresses sufficient levels of anti-apoptotic Bcl-2 family members to prevent apoptosis induction by sequestering Bax/Bak. Under apoptotic conditions, expression and activity of pro-apoptotic Bcl-2 family members increases. Sensitizer/derepressor BH3 proteins displace Bax/Bak and direct activators from Bcl-2, resulting in activation of Bax/Bak, leading to oligomerization and pore formation in the outer mitochondrial membrane. The subsequent release of cytochrome C triggers apoptosis.

1.11.1.3 Extrinsic Apoptotic Pathway

The extrinsic apoptotic pathway is a key mechanism by which cells can be targeted to undergo programmed cell death. This pathway is particularly important throughout the process of development, in the maintenance of tissue homeostasis and also for tumour cell surveillance by the immune system (212-213). As shown in Figure 1.4, the extrinsic apoptotic pathway is activated via binding of cytokines of the tumour necrosis factor (TNF) ligand family to the death-receptors of the TNF-receptor super-family. Interaction of ligands with the receptors triggers a series of signalling events within the cell that subsequently activates both initiator and effector caspases causing the cell to rapidly undergo apoptosis (214). While this pathway has recently been explored as a means to eradicate cancer cells (215-216), the extrinsic pathway is also exploited by tumour cells as a means to evade destruction by the immune system (217-218). Furthermore, targeting of the death receptors is further complicated by recent studies that have uncovered a pro-survival/proliferative signalling function for the death receptors (219).

The TNF receptor super-family consists of several death receptors that include CD95/Fas, Tumour necrosis factor receptor (TNF-R1), Death receptor 4 (DR4) and Death receptor 5 (DR5). Receptors in the family contain up to six extracellular cysteine rich domains that dictate ligand binding specificity (212). An additional extracellular domain is the pre-ligand binding assembly domain (PLAD), which facilitates receptor-receptor interactions prior to activation by ligand (220). Within the cytosolic domain of the receptor, a conserved death domain mediates the intracellular interactions that lead to activation of apoptosis (221).

The death receptors are activated when bound by ligands of the TNF family, which include TNF, TNF related apoptosis inducing ligand (TRAIL) and CD95L/FasL (212). The ligands for the receptors are expressed as homotrimeric type-II transmembrane proteins but can also be proteolytically cleaved to generate soluble ligand. While soluble ligands retain the ability to activate the receptors, they typically do so with considerably less potency (222). The death receptors are present on the cell surface as a pre-formed trimer through interactions between the extracellular PLAD domains of adjacent receptors (220). Binding of ligand triggers a conformational change that results in recruitment of adapter proteins to the intracellular death domain to form the death inducing signalling complex (DISC) (223). In the case of the CD95 receptor, the adapter protein FADD (fas associated with death domain) binds to the activated receptor. FADD contains a death effector domain (DED) that binds to the DED of pro-caspase 8, recruiting it to the receptor. Elevated local concentrations of pro-caspase 8 at the receptor result in processing of caspase 8 to its active form through proximity induced activation and/or proximity induced dimerization. Activated caspase 8 can then cleave the effector caspase, caspase 3, resulting in proteolytic cleavage of the various effector caspase substrates and ultimately cell death (212). The extrinsic pathway can also cross-talk with the intrinsic apoptotic pathway through cleavage of the BH3 only Bcl-2 family member Bid resulting in activation of Bak/Bax (224).

The death receptor pathway is a logical target for the development of anti-cancer therapies. As the extrinsic apoptotic pathway can efficiently trigger apoptosis independent of the tumour suppressor p53, which is mutated in more than half of tumours, the pathway has been sought as an alternative to traditional chemotherapy (225).

Early attempts to use the cytokines TNFα and CD95L to induce apoptosis in tumours were abandoned due to a large extent of non-specific toxicity. More recently, the use of local administration and antibody-based targeting of the ligands to specific cell types has improved the likelihood of success for these types of therapy (226). Unlike TNFα and CD95L based therapies, TRAIL has received increased attention due to the fact that initial experiments in cell line and mouse models demonstrated enhanced sensitivity to TRAIL in tumour cells as compared to normal cells (227-228). While primary tumours demonstrated resistance to TRAIL, combined therapies have shown promise in tumour specific targeting (229). Currently, a large number of clinical trials are underway testing various TRAIL ligands as well as antibodies directed against the TRAIL receptors alone and in combination with various chemotherapeutic drugs (215, 226).

1.11.1.4 Intrinsic Apoptotic Pathway

The intrinsic apoptotic pathway, diagrammed in Figure 1.4, is activated by many cellular stresses including growth factor withdrawal, endoplasmic reticulum stress and DNA damage (188). This pathway hinges upon the regulation of mitochondrial membrane permeability by the Bcl-2 family of proteins (206). The expression level and activation status of both pro- and anti-apoptotic Bcl-2 family members is regulated by numerous signalling pathways and the balance between survival and death signals is pivotal in determining a cell's fate. (203) Once the balance is tipped in favour of cell death, the permeablization of the outer mitochondrial membrane results in an irreversible chain of events that ends with the destruction of the cell and engulfment of the remaining apoptotic bodies by phagocytes (207).

While the exact mechanisms are still incompletely understood, the release of pro-apoptotic signalling molecules from mitochondria is the critical step in the execution of this signalling cascade (203). The regulation of mitochondrial membrane permeabilization is governed by the Bcl-2 family of proteins, the details of which are described above. Once activated, the multi-domain pro-apoptotic Bcl-2 family members Bax and Bak trigger the release of pro-apoptotic signalling molecules from the mitochondrial inter-membrane space (230). This release occurs through the generation of pores in the mitochondrial membrane by Bax/Bak homo-oligomers (231) possibly with the involvement of voltage dependent anion channel (VDAC) (232-234). It is also hypothesized that Bax/Bak may cause a complete rupture of the outer mitochondrial membrane (231). Regardless of the precise mechanism, once the membrane has been compromised, the released proteins quickly and efficiently execute the apoptotic program.

The key apoptotic signalling component released from the mitochondria is cytochrome c. Cytochrome c normally plays a role as an electron carrier between complexes III and IV of the electron transport chain (230). When released from the mitochondria, cytochrome c forms a complex with apoptotic protease activating factor 1 (Apaf-1), pro-caspase 9 and ATP to form the apoptosome (235). Within the apoptosome, caspase 9 is activated and can subsequently cleave and activate the effector caspase, caspase 3 (203). Additional molecules released from the mitochondria include endonuclease G and apoptosis inducing factor (AIF), which are transported to the nucleus and participate in the degradation of cellular DNA (236-237). Smac/DIABLO is another protein released that plays a role in blocking the action of inhibitor of apoptosis proteins

(IAPs), preventing their ability to bind and inhibit caspase 9 (238). Htra2/Omi is a serine protease that also inhibits the action of IAPs through binding and proteolytic degradation (239). Several additional molecules have been detected that are released from mitochondria as well, but their exact functions remain unknown (230). While a complete understanding of the function of all molecules involved in the intrinsic apoptotic pathway remains to be elucidated, the basic biochemistry of this cell death pathway is currently well defined.

The intrinsic apoptotic pathway has recently been targeted through the development of small molecule Bcl-2 family inhibitors. These drugs are small compounds that mimic the structure of the BH3 domain and bind to the anti-apoptotic Bcl-2 family members (240). These drugs are particularly beneficial in the context of over-expression of Bcl-2, Bcl-xL and Mcl-1 where the strong resistance to apoptosis conferred by these molecules can be reversed (241). Binding of the inhibitors to the anti-apoptotic Bcl-2 family members displaces the pro-apoptotic Bcl-2 family members allowing them to participate in the activation of the intrinsic apoptotic pathway (240). Currently, several drugs, such as ABT-737 and GX15-070, are in clinical trials and have demonstrated a degree of success against a variety of tumour types (242).

1.11.2 The role of apoptotic regulators in breast cancer

The role of apoptotic regulators in development and progression of breast cancer has been the subject of considerable study. Analysis of the expression of anti-apoptotic Bcl-2 family members in breast cancer has yielded some unexpected results. As expected, Bcl-2 expression is widely observed in breast cancer, particularly within the LuminalA/B

subtypes, with elevated expression as compared to normal mammary tissue (243). Bcl-2 expression has been found to correlate with Estrogen receptor status (244) and the estrogen receptor directly regulates transcription of the Bcl-2 gene(245). Unexpectedly, initial studies demonstrated an association of elevated Bcl-2 expression with improved patient prognosis (246). These early observations have been confirmed by a recent analysis of 17 separate studies assessing the prognostic significance of Bcl-2 in breast cancer (247). While a concrete explanation for this paradoxical observation remains elusive, the possible roles of Bcl-2 as an inhibitor of cell cycle progression and its association with estrogen dependent tumours has been suggested as a possible reason (243). Similar results have been found for the expression of Bcl-X in breast tumours. Like Bcl-2, Bcl-X associates with estrogen receptor status and is a marker of favourable outcome (248). The prognostic significance of Mcl-1 has been less clear with some studies demonstrating correlation with poor prognosis (249-250) while others demonstrate no prognostic significance (251-252). Unlike the anti-apoptotic Bcl-2 family members assessed, the role of pro-Apoptotic Bax agrees well with the in-vitro data describing its cellular functions. While frame-shift mutations in Bax are common other cancer types, such as colorectal cancer (253), these mutations are not commonly observed in breast cancer. Nonetheless, loss of Bax expression is observed in a subset of metastatic breast tumours and correlates with poor prognosis (254). Study of the role of anti-apoptotic Bcl-2 family members in breast cancer has yielded some unexpected results. The conflicting data in the literature surrounding the role of Mcl-1 warrants further study which this thesis work aims to address.

1.12 Mcl-1

Mcl-1 was originally identified in a screen for immediate early genes induced upon phorbol ester (TPA) stimulated differentiation of the human myeloid cell leukemia cell line ML-1 (255). Upon sequence examination, Mcl-1 was found to have significant homology to the previously discovered Bcl-2, and it was speculated that Mcl-1 was acting to protect differentiating myeloblasts from apoptosis (255). Shortly after its initial discovery, the cell protective role of Mcl-1 was confirmed as, like Bcl-2, its over-expression prevented apoptosis in Chinese hamster ovary cells (256). These results were further confirmed in experiments where Mcl-1 expression prolonged viability in hematopoietic cells exposed to a wide range of cytotoxic stimuli (257). The same group confirmed these results in-vivo, demonstrating that transgenic over-expression of Mcl-1 in hematopoietic and lymphoid cells increased longevity (258). Since its initial discovery, Mcl-1 has been implicated as a survival gene and has now been accepted as an important contributor to tumour cell survival and cancer progression (259).

1.12.1 Physiological Roles of Mcl-1

Mcl-1 has a unique role amongst the anti-apoptotic Bcl-2 family members as knock-out of the Mcl-1 gene results in a markedly different phenotype than knock-out of Bcl-2, Bcl-XL, Bcl-W and Bfl-1 (260). The first observed phenotype of Mcl-1 deficiency in mice was peri-implantation lethality of the developing embryo (261). This defect was not associated with an increase in apoptosis, suggesting a novel role for Mcl-1 in the developing embryo. Further investigation into the physiological role of Mcl-1 was carried forth using temporal as well as tissue specific conditional knock-out mice. These experiments confirmed a critical role for Mcl-1 in the survival of hematopoietic and

lymphoid cells (262-263). Although targeted knock-out in the liver did not demonstrate an obvious phenotype (262), the expression profile of Mcl-1 is indicative of a role beyond the hematopoietic system (251). Mcl-1 demonstrates a unique expression profile as compared to Bcl-2 and is expressed in a wide range of tissue and cell types to varying degrees. Typically, high levels of Mcl-1 are observed in more differentiated epithelial cell layers in contrast to Bcl-2 that is preferentially expressed in the less differentiated stem-cell populations (251). Furthermore, the observed inverse relationship of Mcl-1 and Bcl-2 protein levels suggests cell-type specific roles for the anti-apoptotic Bcl-2 family members.

1.12.2 Structure

Mcl-1 is encoded on human chromosome 1q21 (264). Interestingly, this region of chromosome 1 is frequently rearranged or amplified in a number of cancers including breast cancer (265-266). The Mcl-1 transcript begins 80bp upstream of the ATG translation initiation site. The Mcl-1 gene does not appear to contain a typical consensus TATA box for transcriptional initiation (267), but a short sequence of GATAAA located 29 bp upstream of the transcription start site appears to function as a binding site for TBP (268). The pre-processed mRNA is shown in Figure 1.7. The Mcl-1 pre-mRNA transcript contains 2 introns and 3 exons, Exon 1 is 768 bp long and encodes the first 229 amino acids of the protein, exon 2 is 249 bp and encodes 83 amino acids and the final exon, exon 3 is 488 bp long and encodes the final 38 amino acids of the protein (267).

The full-length and most abundant form of Mcl-1 consists of 350 amino acid residues and has a predicted molecular weight of 37 kDa (269). The domain structure of

the Mcl-1 protein is shown in Figure 1.7. Mcl-1 contains three Bcl-2 homology domains, BH1-3, but appears to lack the BH4 domain observed in other anti-apoptotic Bcl-2 family members (255). The three BH domains form a hydrophobic cleft that plays an important role in the interaction of Mcl-1 with the pro-apoptotic Bcl-2 family members (270-271). In addition to the BH domains, Mcl-1 contains a C-terminal transmembrane domain as well as a PEST sequence at the N-terminus. The hydrophobic transmembrane domain confers membrane localization, particularly to the outer mitochondrial membrane (272). In addition to the mitochondrial membrane, Mcl-1 has been observed at the endoplasmic reticulum as well as the nuclear envelope (272). The PEST sequences are sequences rich in proline, glutamic acid, serine and threonine and are involved in the regulation of proteolytic degradation of the protein by the proteasome (273).

1.12.3 Splicing

The human Mcl-1 gene is subjected to alternative splicing that yields two major forms of the protein. Two independent groups discovered the presence of a smaller transcript by PCR from cDNA samples from human cell lines and tissues (274-275). The second smaller splice variant is generated as a result of skipping of the second exon of Mcl-1, resulting in a change of reading frame in exon 3 (274). The smaller version of Mcl-1 loses the C-terminal transmembrane domain as well as the BH1 and BH2 domains. The PEST sequences and BH3 domain are retained, resulting in a protein that resembles the BH3-only domain pro-apoptotic Bcl-2 family members. Over-expression of the shorter splice variant resulted in cell death, supporting the hypothesis that Mcl-1s possesses a function that is completely opposite to the full length protein (274). The shorter splice variant was found to exclusively interact with the full length Mcl-1 and

therefore suspected to act in a dominant negative fashion (275). More recently, a third splice variant, Mcl-1ES (Mcl-1 extra short), was discovered. This additional splice variant was found to be up-regulated upon exposure of cells to apoptotic stimuli. The Mcl-1ES transcript is generated by the splicing out of a large region of exon 1, removing the PEST sequences but retaining the BH3 domain. Like Mcl-1s, Mcl-1ES interacts with Mcl-1L and induces apoptotic cell death. The apoptotic effect of Mcl-1ES was enhanced in the presence of Mcl-1L over-expression; however, the mechanism explaining this phenomenon remains elusive. The Mcl-1ES transcript also contained three amino acid changes; however, the mechanism by which they are generated is unknown. It is speculated that these amino acid changes may occur through RNA editing (276).

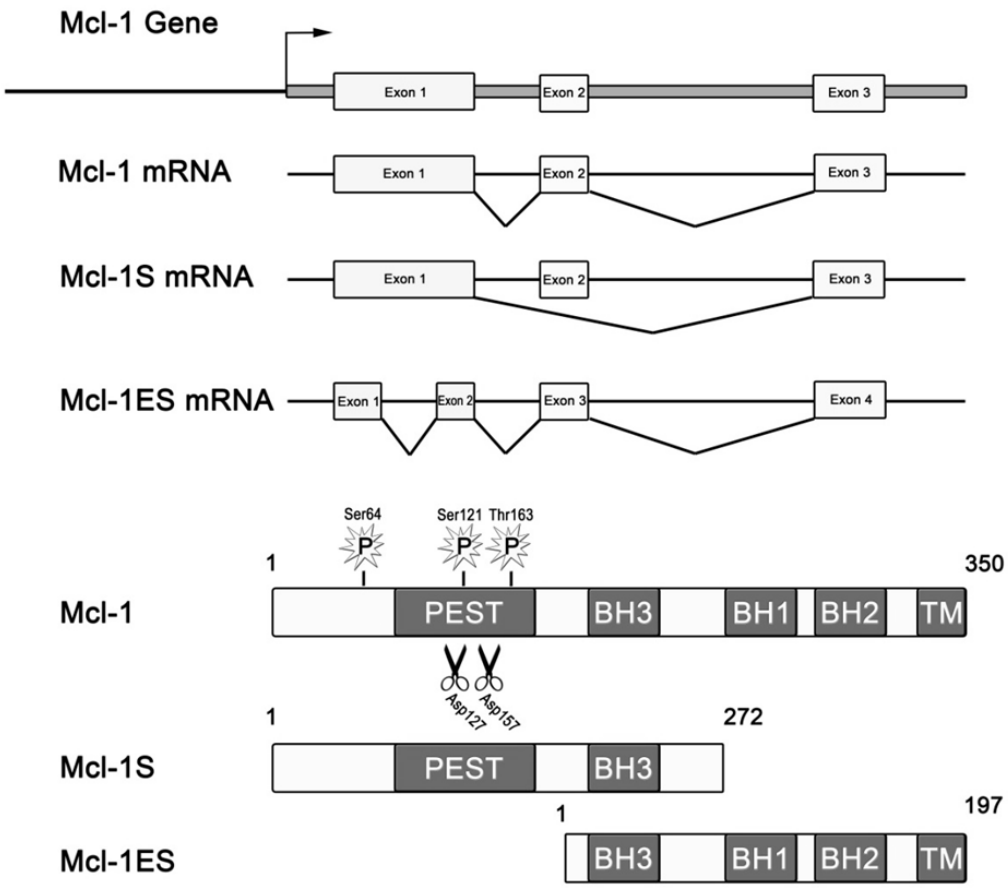

Figure 1.7 Mcl-1 mRNA and protein structure

The Mcl-1 gene consists of 3 exons and 2 introns. Transcription is initiated 29 bp upstream of the ATG start codon. The Mcl-1 mRNA can be alternatively spliced to generate 2 additional mature transcripts, Mcl-1S and Mcl-1ES. The full length Mcl-1 protein contains 3 Bcl-2 homology domains, BH1, BH2 and BH3. In addition to a C-terminal transmembrane domain, an N-terminal PEST sequence targets the protein for rapid turnover. Mcl-1 is post-translationally modified by phosphorylation at several residues, 3 of which are shown: Ser64, Ser 121 and Thr163. Additionally, the protein can be cleaved by caspases at Asp127 and Asp157. Mcl-1S lacks all but the PEST and BH3 domains whereas Mcl-1 ES loses the PEST sequences due to the splicing of an additional intron.

The modulation of Mcl-1 splicing is an attractive therapeutic target as it could potentially allow for the conversion of an over-expressed survival enhancing protein to a pro-apoptotic version. The activation of protein phosphatase 1 by the green tea extract EGCG as well as Ibuprofen has been found to alter the phosphorylation status of splicing factors at the Mcl-1 transcript. This treatment shifts the balance towards expression of the

shorter splice variant and sensitizes cells to apoptosis (277). Another group has used antisense morpholino oligonucleotides that target the Mcl-1 pre-mRNA and shifts the balance towards the smaller Mcl-1 splice variant. Treatment with these oligonucleotides alone is sufficient to induce apoptosis in a number of cancer cell lines of different origin (278).

1.12.4 mRNA regulation

The Mcl-1 mRNA has a short half-life and is another level at which Mcl-1 expression levels can be regulated. The Mcl-1 transcript is subjected to multiple modes of regulation including targeting by microRNA as well as translation inhibition by RNA binding proteins.

MicroRNAs are sequence specific regulators of gene expression that bind to and suppress the translation of mRNA targets (279-281). The mir-29b microRNA was discovered as a predicted Mcl-1 binding mRNA as the first nine nucleotides of the microRNA exhibit perfect complementarity to the 3' untranslated region of the Mcl-1 mRNA (282). mir-29b is down-regulated in cell lines that express high levels of Mcl-1 protein and transfection of the mir-29b precursor results in a dramatic reduction of Mcl-1 protein levels (282). The effect of mir-29b on Mcl-1 protein levels appears to be mediated through suppression of translation, as the levels of the Mcl-1 mRNA are not affected by over-expression of the microRNA. A second microRNA, mir-133a, regulates Mcl-1 in a similar manner as mir-29b. Mir-133a is down-regulated in lung cancer where Mcl-1 expression is elevated and reintroduction of the microRNA reduced Mcl-1 protein expression and sensitized cells to chemotherapy induced apoptosis (283).

In addition to regulation via microRNAs, Mcl-1 mRNA is affected by binding of the RNA binding protein CugBP2 to the 3' untranslated region. CugBP2 expression is increased when cells are exposed to cytotoxic stimuli. Binding of CugBP2 to the Mcl-1 mRNA prevents protein translation and coincides with induction of programmed cell death (284). This translational repression of Mcl-1 may occur with the cooperation of another RNA binding protein, HuR, which was shown in an independent study to bind to the Mcl-1 and Bcl-2 mRNA and is a known interacting partner of CugBP2 (285). Repression of Mcl-1 translation was also observed as a result of stress induced phosphorylation of eukaryotic translation initiation factor 2 (eIF2α) at Serine 51. eIF2α is necessary for the transfer of the initiating methionine tRNA to the ribosome and phosphorylation inhibits its function. The rapid down-regulation of Mcl-1 mediated by eIF2α phosphorylation was identified as a critical event in the induction of apoptosis by several cellular stresses such as ultra-violet light exposure or osmotic stress (286).

1.12.5 Post-translational Modifications

Mcl-1 is regulated post-translationally by several key mechanisms including caspase mediated proteolytic cleavage, phosphorylation at multiple residues and poly-ubiquitination. These protein modifications provide another level upon which the expression and activity of Mcl-1 can be finely tuned. Depending upon the cellular context, pro-survival modifications that enhance Mcl-1 activity can be favoured, resulting in resistance to apoptosis, or the activity of Mcl-1 can be rapidly quenched to facilitate the process of programmed cell death.

In response to apoptotic stimuli, Mcl-1 can be cleaved by caspases at two residues, Aspartate 127 or Aspartate 157 (287) (shown in Figure 1.7). This cleavage occurs in response to a variety of cytotoxic treatments including cisplatin, staurosporine, actinomycin D and imatinib (288). This cleavage generates either a 28kDa and 17kDa fragment or a 23kDa and 22kDa fragment. Identical cleavage patterns were observed when Mcl-1 was incubated with purified recombinant caspases and the cleavage was prevented by the inclusion of the pan-caspase inhibitor Z-VAD-fmk. While several caspases were demonstrated to cleave Mcl-1 in-vitro, the primary caspase responsible within the cell is caspase 3 (289). The caspase cleaved forms failed to protect HeLa cells from apoptosis, indicating that caspase cleavage essentially abolishes the activity of the Mcl-1 protein (287). Although one group only demonstrated a lack of activity of the cleaved Mcl-1 protein fragments (287), a second study demonstrated that transfection of the C-terminal fragments into a B-lymphoma cell line was sufficient to induce apoptosis whereas the N-terminal fragments demonstrated no activity (290).

As is common amongst the Bcl-2 family, an additional mechanism involved in the regulation of Mcl-1 is phosphorylation. Several phosphorylation sites on the Mcl-1 protein are shown in Figure 1.7. Phosphorylation of Mcl-1 occurs in response to activation of both pro-survival as well as pro-apoptotic signalling pathways. Mcl-1 was initially described to undergo two main types of phosphorylation, Erk depedendent phosphorylation upon TPA stimulation that did not cause a band shift in SDS/PAGE and phosphorylation induced by cytotoxic agents that was independent of Erk (291). The phosphorylation induced by cytotoxic drugs resulted in a shift to a more rapidly migrating band. This phosphorylation was monitored by ^{32}P incorporation and the

residues involved as well as functional consequences were not initially identified. Further studies with deletion mutants demonstrated that phosphorylation in response to TPA stimulation occurred at Thr163. This phosphorylation occurred within the PEST domain and coincided with a two-fold increase in the half-life of the protein, indicating that it may play a role in protein stabilization (292). This study also clarified that Erk was able to phosphorylate Mcl-1 directly in an in-vitro phosphorylation assay. The phosphorylation induced by cytotoxic agents was demonstrated to occur independently of an amino acid substitution at Thr163 indicating that the phosphorylation in this context occurs at a unique site. An additional study identified Serine 159 as a phosphorylation site for glycogen synthase 3 (GSK-3). Phosphorylation at this site occurred upon IL-3 withdrawal from IL-3 dependent cell lines and targeted Mcl-1 for rapid degradation by the ubiquitin-proteasome pathway (293). An additional study demonstrated that oxidative stress induced JNK and/or P38 dependent phosphorylation of Mcl-1 at Serine 121 and Threonine 163 (294). While this phosphorylation did not alter the half-life of the Mcl-1 protein, it did result in decreased activity through an as yet unknown mechanism. A further site, Serine 64, was found to be phosphorylated by CDK1, CDK2 and JNK1. Phosphorylation of this site did not have any effect on protein half-life but greatly increased the ability of Mcl-1 to counteract TRAIL induced apoptosis (295). Additional phosphorylation sites include Threonine 92, an Erk-1 target that enhances stability and Serine 155, a GSK-3 target that destabilizes the protein (296).

As previously discussed, protein turnover is an important aspect of Mcl-1 regulation. In fact, proteolytic degradation of Mcl-1 has been shown to be absolutely essential for apoptosis induced by UV irradiation (297). The rate of Mcl-1 turnover can

be modified by phosphorylation events that either increase or decrease the rate at which Mcl-1 is degraded by the proteasome (298). Proteasomal degradation of Mcl-1 is dependent upon ubiquitination of the protein at five conserved lysine residues within the N-terminal PEST domain (298). Ubiquitination at these residues was discovered to be dependent on a novel E3 ubiquitin ligase termed Mcl-1 ubiquitin ligase E3 (MULE) (299). MULE, contains a BH3 domain that specifically interacts with the BH3 domain of Mcl-1. Knock-down of MULE resulted in elevated Mcl-1 protein expression and enhanced survival of cells treated with DNA damaging agents (299). Another E3 ligase, β-TrCP was shown to be involved in the degradation of Mcl-1 following phosphorylation by GSK-3β (300). Although these studies have shown that ubiquitination plays a significant role in the turnover of Mcl-1, a recent paper has discovered that Mcl-1 can be efficiently degraded under conditions in which E1 ubiquitin activation enzyme activity is blocked and the potential ubiquitination sites in Mcl-1 are mutated (301). Therefore, while ubiquitin mediated degradation may play a strong role in the regulation of Mcl-1 turnover, in some contexts turnover can happen through alternative mechanisms.

1.12.6 Transcriptional Regulation

In accordance with the complex and diverse regulatory mechanisms that modify Mcl-1 protein and mRNA stability and activity, transcriptional regulation of the Mcl-1 gene involves multiple stimuli, signalling pathways and downstream transcription factors. Mcl-1 expression is regulated by many signalling molecules including interleukins, colony stimulating factors as well as growth factors. These stimuli activate cellular signalling pathways such as the Mek/Erk, Jak/Stat, PI3K/AKT and p38/MAPK cascades that activate several transcription factors including Stat3, Stat5, ATF6, Creb, PU.1, HIF-

1, Elk-1 and SRF, all of which have been shown to bind to the Mcl-1 promoter and regulate gene expression (259).

The Jak/Stat pathway has been identified as critical for Mcl-1 regulation in a number of contexts. Signal transducers and activators of transcription (Stats) comprise a family of transcription factors that are activated primarily by tyrosine phosphorylation by the upstream Janus kinases (JAKs). JAKs are activated following recruitment to activated growth factor/cytokine receptors by proximity induced autophosphorylation (302). Stat3 was shown to bind to the Mcl-1 promoter following activation by IL-6 in cholangiocarcinoma cells leading to elevated levels of Mcl-1 transcription (303). Another study demonstrated that IL-6 activated Stat3 in gliobastoma multiforme cells resulting in elevation of Mcl-1 protein levels (304). Additional evidence for the involvement of Stat3 in Mcl-1 transcriptional regulation was provided by Liu et al. in a study that demonstrated that constitutively activated Stat3 maintained Mcl-1 protein expression and promoted viability in macrophage cells (305). In NK-cell lymphoma, activated Stat3 showed a positive correlation with elevated Mcl-1 protein levels. Treatment of these cells with a specific JAK inhibitor reduced Mcl-1 protein expression and induced apoptosis (306). Finally, in breast cancer, high levels of active Stat3 demonstrated a positive correlation with Mcl-1 expression in tumour tissue microarray analysis (307).

The PI3K/AKT pathway is widely implicated to play a strong role in cell survival and proliferation. This pathway has been found to govern Mcl-1 expression in response to a number of different stimuli. In hepatocellular carcinoma, IL-6 stimulation led to PI3K/AKT dependent up-regulation of Mcl-1 that was independent of the JAK/STAT

and Mek/Erk pathways (308). Another study by Wang et al., found that IL-3 and GM-CSF activated the transcription factor CREB via the PI3K/AKT pathway leading to increased expression of Mcl-1 in pro-B-cells (309). The same group later identified an additional transcription factor, PU.1, which was activated via the p38MAPK pathway upon IL-3 stimulation and bound in concert with CREB to activate the Mcl-1 gene in pro-B-cells (310). Furthermore, the importance of the PI3K/AKT pathway in Mcl-1 regulation is supported by the requirement of AKT signalling for Mcl-1 up-regulation by activation of the B-cell receptor in CLL (311).

In addition to the above-mentioned pathways, it is well documented that the Mek/Erk signalling cascade is involved in transcriptional control of Mcl-1. The Mek/Erk pathway has been implicated to govern Mcl-1 transcription in pancreatic (312), esophageal (313) and hematopoietic cell lines (314-315). Following TPA stimulation, the activation of the transcription factors Elk-1 and SRF at the Mcl-1 promoter has been identified to occur dependent upon Erk1/2 in 293, Hela and several leukemic cell lines (129, 135, 314). Another group demonstrated that, in the hematopoietic cell line TF-1, transcriptional increases in Mcl-1 mRNA were dependent upon Mek/Erk, whereas translation of the Mcl-1 mRNA was regulated by PI3K/Akt (316). It is interesting to note that Erk signalling regulates Mcl-1 both at the transcriptional level via activation of Elk-1 and also phosphorylates Mcl-1 directly to increase the protein stability and activity.

1.12.7 Interactions

Mcl-1 primarily exerts its function through the binding to and inhibition of the pro-apoptotic Bcl-2 family member Bak (317). This is in contrast to other anti-apoptotic

Bcl-2 family members that appear to be able to prevent both Bak and Bax induced apoptosis to a similar extent. In addition to Bak, Mcl-1 has been demonstrated to bind multiple BH3-only domain Bcl-2 family members such as Bim, tBid, Bik, PUMA and NOXA (259). Binding of Mcl-1 by the BH3-only domain Bcl-2 family members is thought to activate apoptosis by displacing Bak from Mcl-1 resulting in Bak oligomerization (318).

In addition to Bcl-2 family members, Mcl-1 interacts with proliferating cell nuclear antigen (PCNA). This interaction occurs in the nucleus and appears to repress cell cycle progression (319). Mcl-1 has also been found to interact with translationally controlled tumour protein (TCTP), an interaction that increases Mcl-1 stability through prevention of ubiquitin mediated degradation (320). A yeast two-hybrid screen identified tankyrase 1 as an Mcl-1 interaction partner. Interaction of Mcl-1 with tankyrase1 appeared to result in a down-regulation of Mcl-1 protein levels independent of the poly(ADP-ribose) polymerase activity of tankyrase 1 (321). Finally, Mcl-1 also interacts with cyclin dependent kinase 1 (CDK1). This interaction took place exclusively between a proteolytic fragment of Mcl-1 and CDK1 in the nucleus and appeared to inhibit the kinase activity of CDK1 and thereby inhibit proliferation (322).

1.12.8 Methods of targeting Mcl-1 in cancer

The link between Mcl-1 and cancer was firmly established through the demonstration that Mcl-1 over-expression alone was sufficient to induce lymphoid tumours in a mouse model (323). The relationship between Mcl-1 and cancer has been further strengthened with Mcl-1 over-expression contributing to a variety of tumour types

including pancreatic (324), cervical (325), testicular (326), lung (327), breast (249), liver (328), melanoma (329-330), sarcomas (331), as well as a variety of leukemias and lymphomas (332-336). Due to the relevance of Mcl-1 to multiple types of cancer, several Mcl-1 specific therapeutic approaches have been explored. These approaches include targeting of Mcl-1 activity through small molecule Bcl-2 family inhibitors, targeting of its expression with kinase inhibitors and targeting its splicing through drug combinations as well as oligonucleotide approaches (259-260, 337).

Small molecule Bcl-2 family inhibitors based on the structure of the BH3 domain, such as ABT-737, are effective in a number of in-vitro systems and have shown promise in clinical trials (241, 338-339). First generation Bcl-2 family inhibitors lacked the ability to target Mcl-1 and as a result, Mcl-1 over-expression was frequently observed in resistant cells (339-340). In fact an unbiased study found that Mcl-1 expression was the only identified predictor of resistance to ABT-737 (340). More recently developed small molecule inhibitors such as GX15-070 (Obatoclax®) have demonstrated strong affinity for Mcl-1 and have overcome Mcl-1 mediated resistance to ABT-737 (341). In preclinical data, GX15-070 has shown effectiveness in a number of tumour types (342-344). In breast cancer cells, GX15-070 has demonstrated synergistic activity with inhibitors of the epidermal growth factor receptor family (345) and has also been found to overcome resistance to the tyrosine kinase inhibitor lapatinib (346). GX15-070 is currently being tested in clinical trials of chronic lymphocytic leukemia (CLL) (347), refractory leukemia (348), and small-cell lung cancer (349). To date only a small number of phase I clinical trials have been completed; however, they have produced optimistic results with the drug being well-tolerated and showing measureable responses.

In addition to direct targeting of Mcl-1 with small molecule inhibitors, several kinase inhibitors have demonstrated effectiveness at reducing Mcl-1 protein expression. While the targeting specificity and contribution of Mcl-1 to the drug response are difficult to ascertain, these drugs have shown effectiveness in sensitizing tumour cells to apoptosis. The kinase inhibitor sorafenib, which binds to and inhibits RAF1 (350), demonstrated effective anti-tumour activity in mouse models. The effect of Sorafenib on Mcl-1 expression occurs on multiple levels including down-regulation of gene transcription, inhibition of protein translation and modulation of stability/activity through changes in phosphorylation status (351-353). Another inhibitor, Roscovitine, which inhibits cyclin-dependent kinases has been shown to induce apoptosis in leukemic cells through a mechanism that in part relies on Mcl-1 down regulation. While Mcl-1 may play a significant role in the therapeutic benefit conferred by these molecules, the broad specificity in which they target entire signalling networks indicates that the survival benefit is likely due to a combined effect on multiple targets.

Mcl-1 has also been targeted through the use of synthetic oligonucleotides that affect either the Mcl-1 mRNA and protein expression level or the splicing preference of the Mcl-1 mRNA. siRNA mediated knock-down of the Mcl-1 gene has proven effective in several tumour cell lines as well as mouse tumour models (354-355). Knock-down of Mcl-1 alone is sufficient to induce apoptosis in multiple cell types and also increases sensitivity to radiation and chemotherapy induced apoptosis. Recently, antisense morpholino oligonucleotides have been employed to shift the splicing preference of Mcl-1. These oligonucleotides have shown the ability to sensitize tumour cells to apoptosis in cell line models (278). While these approaches are effective in cell-line and mouse

models, difficulties in drug stability and delivery have hampered efforts to bring RNA interference into human trials. Only recently have nucleic-acid based drugs made their way into clinical trials using liposome based delivery (356); however, this is still an emerging technology and much advancement will be required before its use becomes widespread (357).

1.13 The MCF-7 and SK-BR-3 cell line models

The two breast cancer cell lines MCF-7 and SK-BR-3 were used for the majority of the *in vitro* experimental work. MCF-7 cells are an estrogen receptor positive cell line that was isolated from a pleural effusion of a 69-year-old Caucasian woman with an invasive ductal carcinoma (358-359). The MCF-7 cell line can be stimulated to proliferate by stimulation with estrogen and is capable of producing estrogen dependent xenograft tumours in mice (359). The cells express the progesterone receptor and are negative for ErbB2 amplification. While negative for amplification of ErbB2, MCF-7 cells express detectable levels of all four EGF receptor family members (360). MCF-7 cells express a fully functional wild-type p53 (361-362) as well as wild-type PTEN (363). SK-BR-3 cells are an adenocarcinoma cell line that was also isolated from a pleural effusion . SK-BR-3 cells over-express ErbB2 and also express detectable levels of ErbB1 and ErbB3 (364). SK-BR-3 cells express mutant p53 (365). These cell lines were selected for this study because they are well characterized and provide representation of both estrogen dependent tumour cells as well as ErbB2 amplification. Both cell lines also demonstrated strong responses to EGF stimulation in preliminary experiments.

1.14 Study Rationale and Hypotheses

The basis for this study was a preliminary observation that the expression of Mcl-1 is tied to amplification of the EGF receptor ErbB2 in breast tumours. ErbB2 is a growth factor receptor over-expressed in a large portion of breast cancers. Both Mcl-1 and ErbB2 are known to confer a survival advantage to tumour cells; however, the relationship between ErbB2 amplification in breast cancer and Mcl-1 over-expression has not been studied. Furthermore, the specific contribution of Mcl-1 to cell survival in breast cancer is largely unknown. The goals of this study are to explore a possible signalling link between the EGF receptors and Mcl-1 in breast cancer, to determine the molecular biology governing the proposed relationship, and to assess the impact of this relationship in both cell line models of breast cancer and in a retrospective study of breast tumour samples. To that end we wished to test the following hypotheses:

- Activation of the EGF receptors confers a survival benefit to cell line models of breast cancer.
- Mcl-1 expression regulates sensitivity of breast cancer cells to drug induced apoptosis.
- Mcl-1 is transcriptionally controlled by the EGF receptor family.
- Mcl-1 induction is required for the protective effect conferred by EGF receptor activation.
- A correlation exists between EGF receptor status and Mcl-1 over-expression in breast tumours.
- A correlation exists between the specific activated kinases downstream of the EGF receptors and Mcl-1 protein expression in breast cancer.

- High levels of Mcl-1 expression are a negative prognostic indicator in breast cancer.
- Mcl-1 is an important drug target for the treatment of breast tumours.

Chapter 2 Experimental Procedures

2.1 Reagents

All standard laboratory chemicals were obtained from both Sigma-Aldrich (Oakville, ON) and Fisher Scientific (Ottawa, ON) and were of molecular biology grade. The origin and preparation of specific chemicals are described in the appropriate sections below. All standard buffers were prepared with ultrapure (resistance of 18.2 MΩ) double deionized water and were sterilized by autoclaving or 0.2μm filtration where appropriate.

2.2 EGF Preparation and Treatment

Recombinant human epidermal growth factor (EGF) was obtained from Sigma-Aldrich and dissolved in 0.22μm filter-sterilized 10 mM acetic acid with 0.1% bovine serum albumin (BSA) (Sigma-Aldrich). A 500mM stock solution of EGF was aliquoted and stored at -20°C. Once thawed, any remaining unused EGF stock was discarded. For all experiments EGF was added directly to the cell culture media at a final concentration of 1 μg/mL and the dishes were immediately swirled to mix. Control samples were treated in the same manner with an equal volume of 0.22 μm filter-sterilized 10 mM acetic acid with 0.1% BSA.

2.3 Drugs and Inhibitors

Etoposide and doxorubicin (Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 50 mM and stored at -20°C. The MEK inhibitor U0126 (Promega, Madison WI) was dissolved in DMSO at a concentration of 10 mM and stored at -20°C. The Erk inhibitor 3-(2-Aminoethyl)-5-((4-ethoxyphenyl)methylene)-

2,4-thiazolidinedione (Calbiochem now EMD Chemicals, Mississauga ON) was dissolved in DMSO at a concentration of 10 mg/mL and stored at -20° C. The Stat3 inhibitor Cucurbitacin I or JSI-124 (Calbiochem) was dissolved in DMSO at a concentration of 5 mg/mL and stored at -20°C. The Stat3 inhibitor S3I-201was dissolved in DMSO at a concentration of 10 mg/mL and stored at -20°C. The NF-κB inhibitor Bay 11-7082 (Calbiochem) was dissolved in DMSO at a concentration of 200 mg/mL and stored at 4°C. The pan-Bcl-2 family inhibitor GX15-070 (Obatoclax) was provided by Gemin X corporation. Serial dilutions of GX15-070 were dissolved in DMSO and used only as a fresh preparation. The treatment concentrations for all drugs and inhibitors is indicated within the appropriate results section. Drugs and inhibitors were pipetted directly into the cell culture media and the dishes were immediately swirled to mix. As a control, an identical set of samples was always treated with an equal volume of DMSO alone in a similar manner.

2.4 Antibodies

All antibodies were stored according the manufacturers specifications. Antibodies stored at -20°C were distributed into single use aliquots. Appropriate dilutions for each application to achieve optimum signal to noise ratio were determined by performing titration experiments. The specific details for each antibody used are indicated in the following tables.

Table 2.1 Primary antibodies for Western blot

Antigen	Species	Dilution	Source	Catalogue #
Mcl-1	Rabbit	1:8000	Sigma	M8434
Elk-1	Rabbit	1:2000	Abcam	Ab32106
Elk-1 phospho-Ser 383	Rabbit	1:500	Cell Signaling	9181
SRF	Mouse	1:1000	Millipore	MAB4369
Stat3	Rabbit	1:2000	Cell Signaling	9132
Stat3 phospho-Tyr705	Rabbit	1:1000	Cell Signaling	9145
Stat3 phospho-Ser727	Rabbit	1:1000	Cell Signaling	9134
NF-κB p65	Rabbit	1:4000	Abcam	Ab7970
P44/42 MAPK	Rabbit	1:1000	Cell Signaling	9102
P44/42 MAPK phospho-Thr202/Tyr204	Mouse	1:1000	Cell Signaling	9106
α-Tubulin	Mouse	1:20,000	Sigma	T6074
ErbB1	Rabbit	1:1000	Cell Signaling	4267
ErbB1 p-Tyr1068	Rabbit	1:1000	Cell Signaling	3777
ErbB2	Rabbit	1:500	Cell Signaling	2165

Table 2.2 Primary antibodies for chromatin immunoprecipitation

Antigen	Species	Quantity	Source	Catalogue #
Elk-1	Rabbit	5 ug/reaction	Abcam	AB32106
SRF	Mouse	5 ug/reaction	Millipore	MAB4369
Stat3	Rabbit	5 µg/reaction	Cell Signaling	9132
NF-κB p65	Rabbit	5 µg/reaction	Abcam	AB7970

Table 2.3 Primary antibodies for immunofluorescence

Antigen	Species	Dilution	Source	Catalogue #
Mcl-1	Rabbit	1:3000	Abcam	Ab32087
Elk-1 phospho-Ser 383	Rabbit	1:100	Cell Signaling	9181
Erk1/2 phospho-Thr202/Tyr204	Rabbit	1:500	Cell Signaling	9106
ErbB1	Mouse	1:1	Ventana	790-2988
ErbB2	Rabbit	1:500	Dako	A0485
ErbB3	Mouse	1:50	Dako	M7297

Table 2.4 Secondary antibodies

Antigen	Species	Dilution	Conjugate	Source	Catalogue #
Mouse IgG	Goat	1:10,000	HRP	Upstate	12-349
Rabbit IgG	Goat	1:20,000	HRP	Upstate	12-348
Mouse IgG	Goat	1:1000	Rhodamine Red	Invitrogen	R-6393
Rabbit IgG	Donkey	1:1000	Alexa Fluor 488	Invitrogen	A-21206
Biotin	Streptavidin	1:10,000	HRP	Sigma	S2438

2.5 Cell culture

The human breast adenocarcinoma cell lines MCF-7 (ATCC # HTB-22) and SK-BR-3 (ATCC # HTB-30) were obtained from the American Type Culture Collection (ATCC, Manassas VA). Cell identity was confirmed by the ATCC by short tandem repeat (STR) analysis. Cells were maintained in a humidified 5% CO_2 incubator at 37°C. MCF-7 cells were maintained in Dulbecco's modified eagle's medium (DMEM) medium

(Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) (Fisher Scientific) and 100 units/mL Penicillin and 100µg/mL Streptomycin (Invitrogen, Burlington ON). SK-BR-3 cells were maintained in McCoy's 5A medium (Invitrogen) with identical supplements.

2.5.1 Passaging of tumour cell lines

Cell lines were grown in T-75 flasks (Fisher Scientific) and were passaged twice weekly. To passage cells, the cell culture media was removed by aspiration and the cells were rinsed with 10 mL sterile pre-warmed phosphate buffered saline (PBS). Upon removal of the PBS, 3 mL of pre-warmed 0.025% Trypsin with .01% ethylenediaminetetraacetic acid (Trypsin-EDTA) (Invitrogen) was added to facilitate cell detachment. Cells were incubated at room temperature in the Trypsin-EDTA solution for 1-2 minutes until complete detachment was observed. Following detachment, 12 mL of pre-warmed cell culture media was added to the flask and the total volume was transferred to a 15 mL conical centrifuge tube (Fisher Scientific). The cell suspension was centrifuged at 1200 revolutions per minute (rpm) in an Eppendorf 5810R swing-bucket centrifuge for 5 minutes following which the supernatant was aspirated. The cell pellet was resuspended in cell culture media and then split at a ratio of 1:5 (MCF-7) or 1:3 (SK-BR-3) to carry. For all experiments, cells were counted with a haemocytometer and an equal number of cells appropriate for the dish size to attain ~70% confluence on the day of treatment were added to each well. The optimal cell number and plating volumes for each dish type used are indicated in Table 2.5. The plating numbers for MCF-7 cells were based on an estimated doubling time of 30 hr and the plating time for the SK-BR-3 cells were based on an estimated doubling time of 60 hr. Cell lines were

passaged no more than 20 times at which point early passage frozen stocks were recovered from cryopreservation in liquid nitrogen.

Table 2.5 Cell culture plating numbers and volumes

Dish Type	Cell Number	Plating Volume
12-well dish	400,000/well	1 mL
6-well dish	800,000/well	2 mL
10 cm dish	15,000,000/dish	20 mL
15 cm dish	30,000,000/dish	40 mL
T-75 Flask	15,000,000/flask	25 mL

2.5.2 Cell Freezing, storage and recovery.

For long-term storage cells were prepared for cryopreservation by detaching a confluent T-75 flask with Trypsin-EDTA. For each confluent flask, cells were pelleted at 1200 rpm for 5 minutes in an Eppendorf 5810R swing-bucket centrifuge. The supernatant was removed by aspiration and the cell pellet was resuspended in 2 mL freezing medium (80% FBS, 10% D-MEM, 10% DMSO). 1 ml aliquots were transferred to 1.5 mL cryovials and the cryovials were placed in a Nalgene Cryo freezing container in the -80°C freezer overnight. The following morning cells were transferred to liquid nitrogen for long term storage. To recover frozen cells, cryovials were thawed in warm water until the ice pellet could be dislodged and the contents were then transferred into a T-75 flask. Following attachment, cell culture media was replaced. Cells were passaged a minimum of three times prior to experimental use.

2.6 Cell Lysis

All whole-cell lysates were prepared with ice-cold RIPA buffer (50mM Tris pH 8.0, 150mM NaCl, 2mM EDTA, 1% nonident P-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (Complete mini, Roche, Laval QC) and phosphatase inhibitors (Phosphatase inhibitor cocktail 1 and 2, Sigma-Aldrich). Cells from a 6-well dish were detached with a cell scraper and transferred to a 2 mL snap-cap tube. Cells were pelleted by centrifugation at 3000 rpm for 5 minutes. Following centrifugation the supernatant was removed by aspiration and 2 mL cold PBS was added to each tube. Following a second centrifugation, the cells were resuspended in RIPA buffer by pipetting up and down 5-10 times. Once resuspended, cells were vortexed 3x10 seconds and then incubated with rocking at 4°C for 10 minutes. Following lysis, cell debris was eliminated by centrifugation at 10,000g for 10 minutes and cell lysate (supernatant) was transferred to a fresh tube. Total protein concentration was determined by performing the Bradford assay (366). Bradford assays were performed in 96-well plates by combining 200 µl Bradford reagent (Biorad, Mississauga ON) and 5 µl of protein lysate that was pre-diluted 10-fold in ddH_2O. To determine protein concentration, a standard curve was prepared with 0.1-1 µg/µl BSA and absorbance was measured in triplicate at 595nm with a Spectramax M5 spectrophotometer (Molecular Devices, Sunnyvale CA).

2.7 Lysis of Breast Tumour Tissue Samples

To extract total protein from breast tumours, the tumour samples were thawed on ice and then 400 µl SDS isolation buffer (50mM Tris pH 6.8, 20mM EDTA, 5% SDS,

5mM β-Glycerophosphate) supplemented with protease inhibitors (Complete mini, Roche) and phosphatase inhibitors (Phosphatase inhibitor cocktail 1 and 2, Sigma-Aldrich) was added. Tumour samples were vortexed for 30 seconds and then placed on ice for 30 minutes. Samples were then sonicated 10 times for 3 seconds each at output control 4, duty cycle 40 on a Branson sonifier 350 (Branson Sonic Power, Danbury CT). Following sonication, the samples were centrifuged at 13,000 rpm for 15 min at 4°C. Supernatants were transferred to a new tube and frozen at -80°C.

2.8 SDS/PAGE and Western blotting

Equal amounts of protein were resolved by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE). Cell lysates were combined with 5x SDS loading dye (0.313 M Tris-HCl pH6.8, 50% glycerol, 10% SDS 0.05% bromophenol blue) and loaded into the stacking gel. For the detection of phospho-Elk-1 60 µg protein lysate per lane was used for SDS/PAGE and Western blotting, for all other Western blots 30 µg total protein was used. Depending on target protein size, total protein was separated on 6-12% polyacrylamide gels immersed in 1x Tris-Glycine SDS running buffer (3.03g Tris-HCl, 14.4g Glycine, 1g SDS brought to 1 L) in a mini-protean 3 cell (Biorad). Gels were run at a constant voltage of 100V for migration through the stacking gel and once in the separating gel the voltage was increased to 175 volts. Following SDS/PAGE, proteins were transferred to polyvinyl difluoride (PVDF) membranes (Hybond P, GE Healthcare, Piscataway NJ) in a wet transfer apparatus (Biorad) at a constant voltage of 100V for 1 hr at 4°C. Membranes were blocked in Tris buffered saline containing 0.1% Tween-20 (TBS-T) and 5% skim milk powder for 1 hr at room temperature. Membranes were then incubated with primary antibodies overnight at

4°C in 5% milk TBS-T. Following incubation, membranes were washed three-fold in TBS-T and then incubated with the appropriate secondary antibody conjugated to horse radish peroxidise (HRP) for one hour at room temperature in 5% milk TBS-T. After a subsequent three washes with TBS-T, proteins were visualized by enhanced chemiluminescence (ECL Plus, GE Healthcare) using hyperfilm ECL (GE Healthcare).

2.9 RNA isolation and RT-PCR

Total RNA was isolated using the Qiagen RNeasy Plus mini kit (Qiagen, Mississauga ON) according to the manufacturer's protocol and RNA concentrations were quantified by measurement of absorbance at 260 nm with a spectrophotometer (SpectraMax M5, Molecular Devices, Sunnyvale CA). 100 ng total RNA was used as template for the real-time polymerase chain reaction (RT-PCR). One-step RT-PCR was performed using the iScript One-step RT-PCR kit (Biorad) and cycling and data collection were performed on an iCycler thermal cycler (Biorad) using the supplied software (iCycler IQ version 3.1, Biorad). The primers used for all RT-PCR experiments are indicated in Table 2.6. All experiments were standardized to the mRNA of the housekeeping gene cyclophilin. Primers were added to a final concentration of 200nM for all reactions. Prior to thermal cycling, a cDNA synthesis step was inserted for 10 minutes at 50°C. Following cDNA synthesis the following cycling conditions were used: 95°C for 5 min, then 40 cycles of 95°C for 10 seconds followed by the specific T_m indicated in Table 2.6 for 30 seconds (data collection step). Primer specificity was confirmed by visualizing DNA on an agarose gel following PCR. Data was analyzed according to the comparative C_t method (367).

Table 2.6 Primers for real-time PCR

Primer	Sequence	Tm	Product Size
MCL-1F	GCC AAG GAC ACA AAG CCA AT	55	377
MCL-1R	AAC TCC ACA AAC CCA TCC CA		
PS2-F	CAT CGA CGT CCC TCC AGA AGA G	55	104
PS2-R	CTC TGG GAC TAA TCA CCG TGC TG		
Cyclophilin-F	GCT GCG TTC ATT CCT TTG	55	354
Cyclophilin-R	CTC CTG GGT CTC TGC TTT G		

2.10 Mcl-1 promoter constructs

The Mcl-1 promoter was amplified by PCR from a BAC clone containing the appropriate region of Chromosome 1 (RP11-54A4, Invitrogen). Primers were designed to amplify the 5' promoter region (3974 base pairs downstream of the translation start site), as well as to generate seven large deletions from the 5' end. Primers used to generate each construct are indicated in Table 2.7. PCR was performed using the Platinum PFX DNA polymerase (Invitrogen) and accompanying 10x buffer. Final primer concentrations were 400nM. Each 50 µl PCR reaction contained 1 µl of a 100mM dNTP mix along with an additional 8 µl 25mM $MgCl_2$ as well as 1.5 µl DMSO and 10 µl 5M Betaine. The initial denaturation step of the PCR was at 98°C for 3 min followed by 30 cycles of 98°C for 40 seconds followed by a 58°C annealing temperature for 60 seconds and then a 72° C extension step for 4 minutes The primers incorporated restriction endonuclease cutting sites for HindIII and MluI and these enzymes were subsequently used to digest the DNA from the PCR reaction. PCR products were purified with the QIAquick PCR purification kit (Qiagen) and the purified DNA was incubated with 20 units of each restriction

endonuclease in a 50 µl reaction along with 5 µl NEBbuffer 2 10x restriction endonuclease cutting buffer (New England Biolabs, Pickering ON). Restriction digests were incubated for 1 hr at 37°C. In parallel, 5 µl of the PGL3 luciferase reporter vector (Promega Madison WI) was also digested under the same reaction conditions.. Following digestion, the cut DNA products were separated by electrophoresis on a 1.5% agarose gel and the appropriate bands were visualized under UV light and excised using a clean scalpel. DNA was purified from the agarose gel slices using Freeze and Squeeze DNA Gel extraction spin columns (Biorad). Purified promoter fragments were combined with 100ng PGL3 plasmid at a 3:1 molar ratio in a 20µl reaction along with 400 units T4 DNA ligase (New England Biolabs) and 2 µl 10x DNA ligase reaction buffer (New England Biolabs). Ligation reactions were incubated for 20 minutes at room temperature and 2 µl of each reaction was transformed into chemically competent One Shot TOP10F' E. Coli (Invitrogen). Transformed bacteria were plated onto Luria-Bertani (LB) agar plates containing 100µg/mL ampicillin and grown overnight at 37°C. Successful transformants were screened by plasmid isolation and restriction digestion. Positive clones, identified by a correct banding pattern on agarose gel following restriction digest, were further confirmed by sequencing from both the 5' and 3' ends using the GLprimer2 and RVprimer3 sequencing primers. Primer sequences are included in Table 2.8. Sequencing was performed by an in-house sequencing facility at the Manitoba Institute of Cell Biology.

Table 2.7 Primers used to generate Mcl-1 promoter constructs

Primer Name	Primer Sequence
Mcl-1P Reverse	GGTGGTAAGCTTTGCCAGTCGCCGCCGCCGCCTGGCTGAG
-3974	GGTGGTACGCGTCCTTGAGGACAGGAGTTGTAGACCATCCT GGATAACATAGCAAGACTTTG
-3593	GGTGGTACGCGTACAGGGCCAGTTGCAATGGCTCATGCC
-3013	GGTGGTACGCGTAGTTGCTGTACAAGATTAGACATTCCTTAA
-2463	GGTGGTACGCGTTTTTTGAGACCAAGTCTTGCTCTGTTG
-514	GGTGGTACGCGTTCAATGGTTGAAATTTTGATTTG
-333	GGTGGTACGCGTGTTACGTAACCGGCACTCAGAGCC
-204	GGTGGTACGCGTAACCCTCCGGAAGCTGCCGCCCCTTTC
-143	GGTGGTACGCGTAGTTCGCTGGCGCCACCCCGTAG

Table 2.8 Sequencing primers

Primer	Sequence
T7	TAATACGACTCACTATAGGG
GLprimer2	CTTTATGTTTTTGGCGTCTTCC
RVprimer3	CTAGCAAAATAGGCTGTCCC

2.11 Site Directed Mutagenesis

7-base pair deletions were introduced into the 3974 bp fragment of the Mcl-1 promoter using the Quikchange II site directed mutagenesis kit with slight modification of the manufacturer's protocol (Stratagene, now Agilent Technologies, Mississauga ON). The initial PCR denaturation temperature was increased from 95°C to 98°C and the denaturation time increased from 30 seconds to 2 minutes. Primers used to generate each

of the site directed mutations are shown in Table 2.9. Following site-directed deletion, the reaction products were transformed into one-shot Top10F' chemically competent E.Coli and bacteria were plated onto LB agar plates containing 100 µg/mL ampicillin. Plasmid DNA was purified from Ampicillin resistant clones and plasmids were screened by sequencing to verify successful deletion.

Table 2.9 Primers for site directed mutagenesis

Deletion site	Primer Sequence
Del. 1	GCTCGGAGCCGCCGTCCGGCACT
Del. 2	AGGTGCCGTGCGCCGGAAGCTGCC
Del. 3	GTGCGCAACCCTCTGCCGCCCTT
Del. 4	GCAACCCTCCGGAAGCCCTTTCCCCTTTTA
Del. 5	CCGGAAGCTGCCGCCCCTTTTATGGGAAT
Del. 6	GAAGCTGCCGCCCCTTTCATGGGAATACTTTTTTTA
Del. 7	AGCTGCCGCCCCTTTCCCCTTTTTACTTTTTTTAAAAAAAAA

2.12 Transfection of plasmid DNA using GenePorter 2 transfection reagent

For luciferase assays, plasmid DNA transfection was performed using the lipid-based transfection reagent GenePorter 2 (Genlantis, San Diego CA). To perform transient transfections using the GenePorter reagent, 400,000 cells/well were seeded into 12-well dishes 24 hours prior to transfection. On the day of transfection, the cell culture media was replaced with 500µl pre-warmed serum free media. Plates were placed back into the incubator while transfection reagents were assembled. For each well, 2 µg of DNA was combined with 50 µl GenePorter DNA Diluent B and mixed by pipetting. 0.2 µg of the

pCDNA3 vector containing the β-galactosidase cDNA was included in each transfection to control for efficiency of both transfection and cell harvesting. In a separate 1.5 mL centrifuge tube, 50 µl serum free media was combined with 7 µl GenePorter reagent. Following a five minute incubation the separate tubes were combined, mixed by pipetting and incubated a further five minutes. DNA/lipid complexes were then pipetted drop-wise into the appropriate well of the 12-well dish. Dishes were incubated for 4 hours at 37°C following which the media was replaced with 1mL media containing serum.

2.13 Electroporation of plasmid DNA and siRNA

Electroporation was performed using the nucleofector electroporation device and reagents (Lonza, Walkersville MD). Cells were passaged 3 days prior to electroporation and maintained in T-75 flasks. On the day of the experiment cells were detached with Trypsin-EDTA and resuspended in complete medium. Cells were counted using a haemocytometer and cell suspensions were centrifuged at 90g for 10 minutes to obtain a cell pellet. For MCF-7 cells, $1x10^6$ cells were used per transfection and for SK-BR-3 cells, $5x10^5$ cells were used per transfection. The supernatant was removed and cells were then resuspended in 100µl of the appropriate Nucleofector solution (solution V for MCF-7 and solution C for SK-BR-3) per transfection. For plasmid DNA transfections, 2 µg plasmid DNA was added to the cells suspended in nucleofector solution and cells were gently mixed by pipetting up and down with a 1 mL pipette. For siRNA transfections, 30 pmol siRNA was added to the cell suspension followed by gentle mixing. 100 µl of the cell/DNA or cell/siRNA suspension was then transferred to an electroporation cuvette and placed within the nucleofector device. Electroporation was performed using program P-020 for MCF-7 cells and program E-009 was used for SK-BR-3 cells. Following

electroporation, 500µl pre-warmed complete media was added to the cuvette and the cuvette contents were subsequently transferred to a 6-well dish containing 1.5 mL complete media.

2.14 Luciferase Assays

The Mcl-1 promoter luciferase reporter constructs were transfected into MCF-7 and SK-BR-3 cells using GenePorter transfection reagent (Genlantis) along with a plasmid containing the β-galactosidase cDNA to standardize results. Twenty-four hours after transfection, the cells were serum starved for an additional 24 hr and then treated with EGF or vehicle control. After 6 hr treatment, cells were lysed in 200 µl reporter lysis buffer (Promega) and luciferase activity was measured in 20µl lysate on an LMAX luminometer (Molecular devices) with 100 µl luciferase assay substrate (Promega). β-galactosidase activity was assessed by combining 50 µl lysate with 50 µl 2X β-gal buffer (200mM sodium phosphate pH 7.3, 2mM $MgCl_2$, 100mM β-mercaptoethanol, 1.33mg/mL ONPG) and measuring the absorbance at 450 nm.

2.15 Determination of potential Transcription Factor binding sites

To determine potential transcription factor binding sites, the sequence of the Mcl-1 promoter was analyzed by the TFsearch/TRANSFAC bioinformatics tools (368). A threshold score of 80.0 was employed. High scoring transcription factor binding sites identified within the region of interest established by luciferase assays were used as a basis for the generation of site-directed deletions within the Mcl-1 promoter.

2.16 Chromatin Immunoprecipitation (ChIP)

ChIP was performed to assess transcription factor binding to the Mcl-1 promoter. For each ChIP sample, 20 x 10^6 cells were plated in 15 cm dishes 2 days prior to the experiment. Following EGF stimulation, proteins were cross-linked to DNA by adding PBS containing 1% formaldehyde and incubating for 10 minutes with shaking at room temperature. The cross-linking reaction was stopped by the addition of 1.25M Glycine to a final concentration of 125 mM and incubating with shaking for 5 minutes. Cells were then rinsed twice in ice-cold PBS, transferred to a 50 mL tube and centrifuged for 5 min at 1000g. The pellet was then resuspended in 750 µl Lysis Buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl pH 8 and protease inhibitor cocktail) and sonicated 10x20 seconds at output control 4, duty cycle 40 on a Branson sonifier 350 (Branson Sonic Power). These sonication conditions yielded an average DNA fragment length of approximately 500 base pairs as determined by agarose gel electrophoresis. Following sonication, cell debris was pelleted by centrifugation and the supernatant was diluted 10 fold with RIPA buffer to reduce the SDS concentration. Lysates were pre-cleared for 1 hr with Protein-G Sepharose beads (GE Healthcare) and then 1 mL lysate was incubated with 5 µg antibody overnight at 4° C with rotation. The following day, 60 µl Dynabeads protein G (Invitrogen) were added to each immunoprecipitation and incubated 2 hr at 4°C with rotation. Beads were then washed 2x5 minutes with each of the following buffers: Low Salt Wash Buffer (0.1% SDS, 1% Triton-X-100, 2 mM EDTA, 20mM Tris-HCl pH 8.1, 150 mM NaCl), High Salt Wash Buffer (0.1 % SDS, 1% Triton-X-100, 2mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), LiCl Wash Buffer (250 mM LiCl, 1% NP40, 1% deoxycholate, 1mM EDTA, 10 mM Tris-HCl, pH 8.1), TE Buffer (10 mM Tris-HCl

pH 7.5, 1 mM EDTA). 125 µl elution buffer (1% SDS, 100mM $NaHCO_3$) was then added to the beads and the beads were incubated overnight at 65°C with shaking. The next day samples were incubated for 30 min at 37°C with 1 µg RNase A (Sigma-Aldrich) followed by 1 hr incubation at 55°C with 100 µg proteinase K (Sigma-Aldrich). DNA was then isolated using the QiaQuick PCR purification kit (Qiagen) and DNA concentration was determined using the PicoGreen dsDNA quantitation assay (Invitrogen). 0.1 ng ChIP DNA was amplified by RT-PCR using primers specific to the Mcl-1 promoter or the last exon of the Mcl-1 gene. Primer sequences are indicated in Table 2.10. To obtain fold enrichment values, the cT value of each ChIP sample was compared to the cT value of 0.1 ng Input DNA.

Table 2.10 Primers for chromatin immunoprecipitation

Primer Name	Sequence	Product Size
Mcl-1 Promoter CHIP Forward	TAG GTG CCG TGC GCA ACC CT	163
Mcl-1 Promoter CHIP Reverse	ACT GGA AGG AAG CGG AAG TGA GAA	
Mcl-1 Exon 3 CHIP Forward	TGT TGC TGG AGT AGG AGC TGG TTT	186
Mcl-1 Exon 3 CHIP Reverse	GCC ATA ATC CTC TTG CCA CTT GCT	

2.17 Streptavidin pull-down assay

To assess transcription factor binding to an Mcl-1 promoter specific probe, a streptavidin pull-down assay was performed with 20 x 10^6 cells per time point. The probe sequence is as follows:

CAACCCTCCGGAAGCTGCCGCCCCTTTCCCCTTTTATGGGAATACTTTTT. Both strands of the probe were labelled with biotin at the 5' ends and the probe was purified by high performance liquid chromatography (HPLC). Following treatment with EGF for the indicated times, cells were rinsed with 10 mL ice cold PBS and then scraped into 10 mL cold PBS and centrifuged at 1200 rpm for 5 minutes. To perform nuclear extraction, the cell pellet was resuspended in nuclear extract buffer 1 (25 mM Hepes pH 7.9, 5mM KCl, 0.5 mM $MgCl_2$ along with protease and phosphatase inhibitors). Once resuspended, 200 µl of nuclear extract buffer 2 (25 mM Hepes pH 7.9, 5mM KCl, 0.5 mM $MgCl_2$. 1% NP40 with protease and phosphatase inhibitors) was added. Tubes were then rotated at 4°C for 15 minutes and centrifuged at 2500 rpm for 1 min to pellet the nuclei. The supernatant (cytoplasmic fraction) was then discarded and the pellet washed in a 1:1 mixture of nuclear extract buffers 1 and 2. Following an additional centrifugation, nuclei were resuspended in nuclear extract buffer 3 (25 mM Hepes pH 7.9, 10% w/v sucrose, 350mM NaCl, 0.01% NP40 with protease and phosphatase inhibitors). Tubes were vortexed for 30 seconds and then rotated at 4°C for 1 hour. Samples were then spun at 13,000 rpm for 10 min to pellet debris and the supernatant (nuclear extract) was transferred to a new tube and protein concentration was quantified by Bradford assay.

Lysates were pre-cleared with 50 µl streptavidin agarose beads (Invitrogen) for 30 min at 4°C with rotation. Following pre-clearance, a binding reaction was prepared that

contained 500 µg nuclear extract, 50 ng/µl Poly dI-dC (Sigma-Aldrich), 1/5 volume 5x binding buffer (50mM Tris pH 7.5, 250 mM KCl, 5mM DTT), and 100 nM biotin labelled probe. Binding reactions were incubated for 30 min at room temperature at which point 50 µl streptavidin-agarose beads were added. Following an additional 30 minute incubation, beads were spun down at 3000 rpm for 1 min and washed 3x5 min in PBS. After washing, beads were resuspended in 50 µl 2X SDS Loading dye, boiled for 5 min and the isolated proteins were separated by SDS/PAGE and Western blot was performed to assess transcription factor binding. To perform cold competition, unlabelled probe was added 15 minutes prior to the addition of the biotin labelled Mcl-1 promoter probe.

2.18 Gene knock-down experiments

siRNA transfections were carried out using the Nucleofector (Amaxa) electroporation device as described in section 2.13. All experiments were performed 48 hr following transfection. Pre-validated siRNA duplexes were obtained from Invitrogen. The siRNAs used are shown in Table 2.11. The NF-κB siRNA was obtained from Santa Cruz Biotechnology. To control for specificity, all experiments were performed using an equal amount of the Medium GC StealthRNAi Universal Negative Control duplex (12935-300) from Invitrogen (Control siRNA).

Table 2.11 siRNA sequences

siRNA Target	siRNA sequence
Mcl-1	GAAAGUAUCACAGACGUUCUCGUAA
SRF	GCUACACGACCUUCAGCAAGAGGAA
Elk-1	GCGGCCAGAAGUUCGUCUACAAGUU
Stat3	GCAGUUUCUUCAGAGCAGGUAUCUU
NF-κB p65	Sequence unavailable: Product #29410 (Santa Cruz Biotechnology)

2.19 Assessment of apoptosis by measurement of the sub-G1 peak

Analysis of apoptosis was performed by quantification of the sub-G1 peak by flow cytometry using a BD FACScalibur flow cytometer (BD Biosciences, Mississauga ON). Following treatment, the media and any detached cells were transferred to a 2 mL tube and 300 µl trypsin/EDTA was added to each well. Once cells were detached, the corresponding media was re-added to neutralize the trypsin. Samples were then centrifuged at 1500 rpm for 3 minutes at 4°C. Cells were washed twice with cold PBS and then resuspended in 500 µl hypotonic PI Lysis buffer (188 mL ddH_20, 10 mL 1.0 mg/mL PI, 0.2g sodium citrate, 0.2 mL triton-X-100, 100µg/mL RNase A) Cells were then incubated for 30 minutes in the dark at room temperature. Assessment of hypodiploid nuclei was performed on the FL3 channel in logarithmic data collection mode. Cellular debris was gated out by exclusion of minimally sized fragments in a plot of forward versus side scatter. Nuclei to the left of the G1 peak were considered apoptotic.

2.20 Assessment of Apoptosis - Annexin V / 7AAD staining

The accuracy of apoptosis assessment by measurement of the sub-G1 peak was confirmed using the additional method of Annexin V-FITC / 7AAD staining of cells. Following treatment, the media and any detached cells were transferred to a 2 mL tube and 300 µl trypsin/EDTA was added to each well. Once cells detached, the corresponding media was re-added to neutralize the trypsin. Cells were centrifuged at 1500 rpm for 5 minutes and washed twice with cold PBS. Cells were then resuspended at a concentration of 1 x 10^6 cells/mL in Annexin-V binding buffer (BD Biosciences). To a 100 µl cell suspension, 5 µl Annexin V-FITC (BD Biosciences) and 2 µl 7-AAD (BD Biosciences) were added and cell suspensions were mixed and incubated for 15 minutes in the dark at room temperature. Following incubation, 400 µl 1X Annexin V binding buffer was added and samples were analyzed by flow cytometry. Cells positive for Annexin V (FL1 channel) or Annexin V and 7AAD (FL3 channel) were considered apoptotic. Fluorescence intensity compensation was employed to eliminate signal contamination by spectral overlap.

2.21 Measurement of Cell Viability by the MTT Assay

Cell viability was measured using a colorimetric assay that uses the dye 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT). MTT is converted to insoluble purple formazan crystals by actively metabolizing cells. Cells were plated into 96-well plates. Following treatment, 10 µl of 5 mg/mL MTT was added to each well and plates were incubated for 3 hr at 37°C. Following incubation, plates were spun down at 90g for 10 minutes and the supernatant was removed by aspiration. The cells and

formazan crystals were dissolved in 200 µl DMSO and absorbance was measured at 570 nm.

2.22 Immunofluorescence of cells grown on coverslips

Immunofluorescence was performed on cells that had been grown on glass coverslips (Fisher Scientific). Sterile coverslips were placed on the bottom of a 6-well dish prior to plating of cells. Following treatment, coverslips were transferred to a 24-well dish and 300 µl PBS containing 3.7% formaldehyde was added. Coverslips were fixed for 10 minutes in the formaldehyde solution and were subsequently washed 2x 5 minutes with 500 µl PBS. Primary antibodies were diluted in PBS containing 0.1% NP40 and 10% FBS. 300 µl antibody solution was added to the appropriate well and cells were stained for 1 hr at room temperature. Coverslips were then washed 3x 5minutes in PBS containing 0.1% NP40 prior to the addition of secondary antibody. Fluorescently labelled secondary antibodies were diluted in PBS containing 0.1% NP40 and 10% FBS and added to the wells. Coverslips were incubated for 1 hr in the dark with the secondary antibody followed by 3 final washes in PBS-NP40. Coverslips were then mounted on superfrost microscope slides (Fisher Scientific) with Vectashield mounting medium containing the nuclear stain DAPI (Vector labs, Burlingame CA). Slides were visualized on a Olympus BX51 fluorescent microscope.

2.23 Immunofluorescence of paraffin embedded tissue sections

Tumour tissue sections were acquired from the Manitoba Breast Tumour Bank. To perform immunofluorescence, slides were baked at 60°C in an oven for 20 minutes. Slides were then immersed in Xylene for 10 minutes to remove paraffin. Sections were

subsequently immersed for 2 minutes in each of the following solutions: Xylene, 100% ethanol, 95% ethanol, 85% ethanol, 75% ethanol, 50% ethanol. Slides were then placed in ddH2O for 5 minutes. Antigen presentation was performed by placing slides in a pressure cooker with 10 mM citrate buffer pH 6.0 for 20 minutes. Following a subsequent 3 washes for 5 minutes in PBS, sections were blocked for 2 hours in PBS containing 5% goat serum, 0.2% Triton X-100, 0.02% sodium azide, 0.1% BSA. Primary antibodies were diluted in the same blocking buffer and sections were incubated with primary antibody in a humidified chamber at 4° C overnight. Slides were then washed 3x 5 minutes with PBS-T. Fluorescently conjugated secondary antibodies were diluted in blocking buffer and sections were incubated for 1 hour in a humidified chamber at room temperature with the secondary antibody. Following an additional 3 washes in PBS-T, coverslips were mounted with Vectashield mounting medium (Vector labs). Staining was scored by two separate observers using a 3 point system with an Olympus BX51 fluorescent microscope.

2.24 Immunohistochemistry of breast tumour tissue microarrays

Breast tumour tissue microarrays were sectioned, stained and developed at the Manitoba Breast Tumour Bank. Microarrays were scored by two blinded independent observers under the supervision of a pathologist. Her-2 scores and patient clinical data was provided by the tumour bank. All scores were performed using the following immunohistological scoring method: staining intensity was assessed on a scale of 0-3 and multiplied by the percentage of tumour cells demonstrating staining. All statistical analysis was performed using GraphPad Prism® software (GraphPad Inc. La Jolla, CA).

Chapter 3 Experimental Results

3.1 Breast cancer cell line survival is modulated both by stimulation with EGF and alteration of Mcl-1 protein expression and activity.

3.1.1 Rationale

We have previously determined that Mcl-1 protein expression correlates with the EGF receptor status of breast tumours (369). Over-expression/hyper-activation of the EGF receptors has been well documented to enhance the survival and proliferation of a number of tumour cell types including breast cancer (370). Activation of the EGF receptors stimulates multiple downstream pathways including the PI3K/AKT, JAK/STAT, Ras-MAPK and PLCγ signalling networks (36). All of these signalling pathways can activate cell survival and proliferation mechanisms through modulation of gene expression and target protein activity (49, 371-372). Mcl-1 expression has been undoubtedly linked to the regulation of the intrinsic apoptotic pathway and Mcl-1 has been identified as a downstream protein transcriptionally and post-translationally modified by all of the above-mentioned signalling pathways (269, 373). Mcl-1 has also been shown to be a negative prognostic indicator in breast cancer (249). Therefore, we set out to validate the known functional outcomes of EGF signalling and Mcl-1 expression in our breast cancer cell line models. These experiments were performed in order to confirm that our model system is a suitable means to study the relationship between EGF receptor activation and Mcl-1 regulation in breast cancer.

3.1.2 EGF prevents apoptosis induced by chemotherapeutic agents in the SK-BR-3 and MCF-7 breast cancer cell lines.

The EGF receptors, particularly ErbB2, are amplified in a large portion of breast tumours. ErbB2 alone is amplified in as many as 25% of total breast cancer cases (374). ErbB1 and ErbB2 have been definitively linked to poor prognosis and increased tumour aggressiveness in breast cancer as well as other tumour types (35, 54). Amplification of ErbB2 is associated with resistance to a number of therapeutic interventions including chemotherapy, radiotherapy and anti-estrogen treatments (53). To test whether stimulation with the EGF ligand conferred a survival advantage to our cell line models, SK-BR-3 and MCF-7 cells were treated with the chemotherapeutic drugs etoposide and doxorubicin either in the presence or absence of EGF. Tumour cells were plated in 12-well dishes in complete medium. Prior to addition of the drugs, cells were pre-treated for 1 hr with EGF or vehicle control. Cells were harvested 18 hr later for cell death measurements. We found that treatment with EGF significantly reduced the measured levels of apoptosis due to both drugs. As shown in Figure 3.1A, SK-BR-3 cells pre-treated with EGF demonstrated resistance to apoptosis across a broad range of etoposide concentrations. Similar results were obtained in Figure 3.1B upon treatment of SK-BR-3 cells with doxorubicin. The protective effect was more evident at lower drug concentrations where EGF showed a 2-fold reduction in etoposide induced apoptosis at 5 μM and a 3-fold reduction in doxorubicin induced apoptosis at 0.5 μM. While EGF maintained a highly significant protective effect at concentrations as high as 100 μM etoposide, the protective effect was lost at 4 μM doxorubicin. The data was confirmed with the second method of Annexin V-FITC/7AAD staining of SK-BR-3 cells following

etoposide treatment (Figure 3.1C). As doxorubicin is a highly fluorescent molecule and interferes with the assay it was excluded as a treatment condition. While MCF-7 cells were largely resistant to apoptosis induced by both drugs, possibly due to their inherent deficiency in caspase-3, a protective advantage was observed with EGF pre-treatment in etoposide treated cells. (Figure 3.1D).

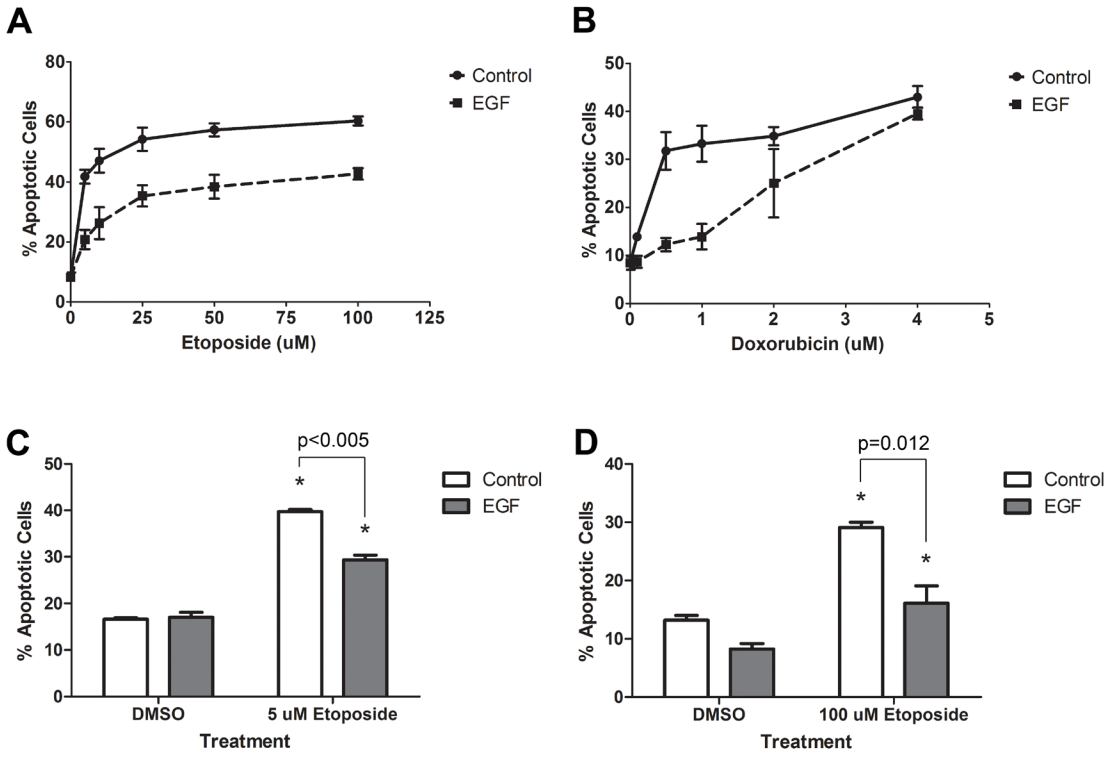

Figure 3.1 EGF prevents apoptosis induced by chemotherapeutic drugs.

(A) SK-BR-3 cells were seeded into 12-well plates and pre-treated with 1 µg/mL EGF or vehicle control for 1 hour. Following the 1 hr incubation, cells were treated with 5, 10, 25, 50 or 100 µM etoposide. Following an 18 hr incubation with etoposide, apoptosis was measured by quantification of the sub-G1 population (cells with hypodiploid DNA) by flow cytometry. The proportion of cells with hypodiploid DNA is indicated by the y-axis. Results represent three independent experiments +/- standard error. (B) Identical experiment as in (A) however SK-BR-3 cells were treated with 0.25, 0.5, 1, 2 or 4 µM doxorubicin. (C) To verify the data in (A) SK-BR-3 cells were pre-treated with EGF or control and then treated with 5 µM etoposide for 18 hr. Following etoposide treatment, apoptosis was assessed by staining with Annexin V-FITC/7AAD. The y-axis represents the proportion of cells staining positive for Annexin V or double positive for Annexin V and 7AAD. Data represents the mean of three experiments +/- standard error (D) Similar experiment as in (C) performed with MCF-7 cells treated with 100 µM etoposide following 1 hr pre-treatment with EGF or control. Data represents the mean of three experiments +/- standard error. p-values for (C) and (D) determined by paired, one-tail *t* test.

3.1.3 Mcl-1 promotes the survival of the SK-BR-3 breast cancer cell line.

Shortly after its discovery, Mcl-1 was confirmed as a regulator of the intrinsic apoptotic pathway (256). Mcl-1 expression has been linked to cell survival in differentiating hematopoietic cells and its over-expression prevents apoptosis in a variety of cell types (257, 327). To assess the contribution of Mcl-1 to the survival of breast cancer cells we performed a targeted knockdown experiment with Mcl-1 specific siRNA (Figure 3.2A). Twenty-four hours following siRNA transfection there was a significantly higher number of apoptotic cells (38%) when Mcl-1 was knocked down as compared to untreated cells (9%) or transfection with a scrambled control siRNA (12%). This implies that a minimal level of Mcl-1 expression is critical for tumour cell survival. Knock-down efficiency was confirmed by performing Western blot on lysates taken from the same experiment (Figure 3.2B). Over-expression of Mcl-1 by transfection of the Mcl-1 cDNA resulted in increased resistance to cell death. Figure 3.2C demonstrates that Mcl-1 over-expression reduced transfection associated toxicity (25% cell death) and etoposide induced cell death (45%) as compared to the empty vector (40% and 55% respectively). These results demonstrate the important role Mcl-1 plays in cell survival. SK-BR-3 cells were also sensitive to the pan-Bcl-2 family inhibitor GX15-070 (Figure 3.2E). Cells were plated into 96-well plates and treated with a concentration range of GX15-070 from 0-5 μM. Viability was measured by MTT assay relative to the viability of the DMSO treated control. At a concentration of 40nM a significant decrease in viability was observed and escalating drug concentration correlated with a continual decrease in viability.

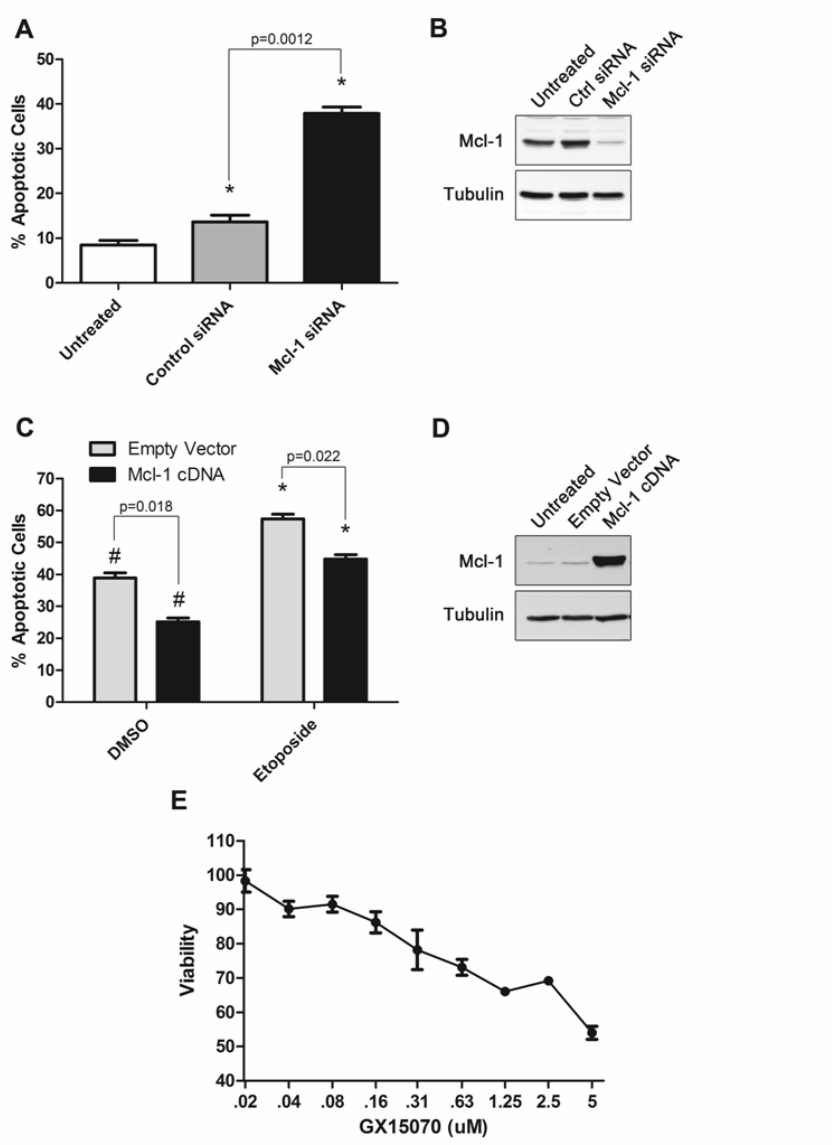

Figure 3.2 Mcl-1 modulates the survival of breast cancer cells

(A) SK-BR-3 cells were transfected with either a scrambled control siRNA or an siRNA targeting Mcl-1. 24 hr following transfection apoptosis was assessed flow cytometric analysis of the sub-G1 population. Data represents the mean of three experiments +/- standard error. p-value was determined by paired, one-tail *t* test. (B) A single well representing each treatment was lysed for protein analysis by SDS/PAGE and Western blotting to demonstrate effectiveness of the knock-down. (C) SK-BR-3 cells were transfected with either the empty pCDNA3 vector or pCDNA3-Mcl-1. The impact of Mcl-1 over-expression on apoptosis was measured in cells treated with DMSO alone (background and transfection induced apoptosis) or 5 μM etoposide. Data represents the mean of three experiments +/- standard error. p-value was determined by paired, one-tail *t* test. (D) A single well representing each treatment was lysed for protein analysis by SDS/PAGE and Western blotting to demonstrate effectiveness of Mcl-1 over-expression. (E) SK-BR-3 cells were treated with a dose-range of GX15-070 from 0-5 μM. Following 48hr, viability was assessed by the MTT assay relative to the vehicle treated control. Data represents the mean of four independent experiments.

3.1.4 Summary

The data in this section supports the following conclusions:

- Stimulation with EGF activates a survival program within breast cancer cells
- Activation of the EGF receptors confers resistance to drugs that activate the intrinsic apoptotic pathway
- A minimal threshold expression level of Mcl-1 is critical for breast cancer cell survival
- Increased expression of Mcl-1 is associated with a decrease in sensitivity to chemotherapeutic drugs
- Inhibition of the anti-apoptotic Bcl-2 family reduces the viability of breast tumour cells.

These results confirm the importance of both the EGF signalling network as well as Mcl-1 expression for survival in breast cancer. These experiments establish that the cell line models are responsive to EGF stimulation and are also suitable to study the contribution of Mcl-1 to cell survival and drug resistance.

3.2 Mcl-1 is a downstream target of EGF signalling in breast cancer but does not appear to be a target of estrogen mediated signalling pathways

3.2.1 Rationale

While the regulation of Mcl-1 has been studied in a variety of contexts, the control of expression in breast cancer is only poorly understood. Several studies have demonstrated correlations between Mcl-1 and activated signalling pathways or transcription factors in breast cancer (252, 307, 352); however, the regulatory mechanisms have yet to be explored in detail. We have previously demonstrated that Mcl-1 expression correlates with ErbB2 in breast tumours and that in cell line models over-expression of ErbB2 is associated with an increase in Mcl-1 protein levels (369). Several studies have suggested that EGF signalling regulates the transcription of the Mcl-1 gene (313, 375-376). Based on the correlative data demonstrating a strong association of elevated Mcl-1 protein with amplified ErbB2, we set out to determine if the Mcl-1 gene is transcriptionally activated by downstream signalling of the EGF receptors in breast cancer.

Another survival pathway that breast tumours are frequently reliant upon for survival is signalling mediated by estrogen receptor activation. Estrogen is well-known to support cell survival and proliferation in ERα-positive breast tumours (156). To determine if Mcl-1 is a downstream target of the estrogen receptor, we also sought to analyze the effects of estrogen stimulation on Mcl-1 transcription and translation. As cross-talk has been reported to occur between the EGF and estrogen mediated signalling pathways (157), and because estrogen can activate the EGF receptors through non-

classical signalling (154-155), we wished to investigate the possibility of a convergence of the two stimuli upon Mcl-1 up-regulation.

3.2.2 EGF up-regulates Mcl-1 protein and mRNA levels

To further assess whether Mcl-1 expression is modified by EGF receptor activation in breast cancer, we studied Mcl-1 protein and mRNA levels in MCF-7 and SK-BR-3 breast cancer cell lines following treatment with EGF. As shown in Figure 3.3A, both MCF-7 and SK-BR-3 cells demonstrated a marked elevation of Mcl-1 protein levels within 2 hours of treatment, which remained elevated greater than 8 hours following stimulation with EGF. Because Mcl-1 has a short half-life (~30 minutes), rapid fluctuations in protein levels can occur in the absence of a change in relative transcription of the Mcl-1 gene. Therefore it was necessary to determine whether the observed changes were a result of elevated transcription or modification of protein stability. Mcl-1 mRNA levels were detected by semi-quantitative real-time PCR over a 120 minute time-course following stimulation with EGF (Figure 3.3B). After 30 minutes of EGF treatment, the Mcl-1 mRNA level was increased four-fold in MCF-7 cells and at 60 minutes EGF treatment in SK-BR-3 cells the mRNA level had increased nearly three-fold. The mRNA levels peaked in both cell lines at 90 minutes post EGF stimulation at a 12 fold increase in MCF-7 cells and 4 fold increase in SK-BR-3 cells. Control treated cells failed to demonstrate an increase in Mcl-1 mRNA levels over the same time course (dashed line). This strongly suggests that signalling pathways activated by EGF stimulation elevate transcription of the Mcl-1 gene in breast cancer cells.

Measurement of Mcl-1 mRNA levels by RT-PCR is not a direct assessment of Mcl-1 gene transcription. Additional factors including modification of mRNA stability can result in fluctuations of total Mcl-1 mRNA. To obtain further evidence that the Mcl-1 promoter is directly activated by EGF treatment, luciferase assays were performed using an Mcl-1 promoter fragment generated by PCR from a BAC clone containing the appropriate region of human chromosome 1. A 4kb fragment upstream of the translation start site was cloned into the PGL3 luciferase reporter vector and the construct was transiently transfected into MCF-7 and SK-BR-3 cells. Cells were serum starved for 24 hr and then treated with EGF. Luciferase activity was measured five hours post-treatment and compared to vehicle control. As shown in Figure 3.3C, both cell lines demonstrated a 3-4 fold increase in luciferase activity when treated with EGF, confirming the Western blot and RT-PCR data.

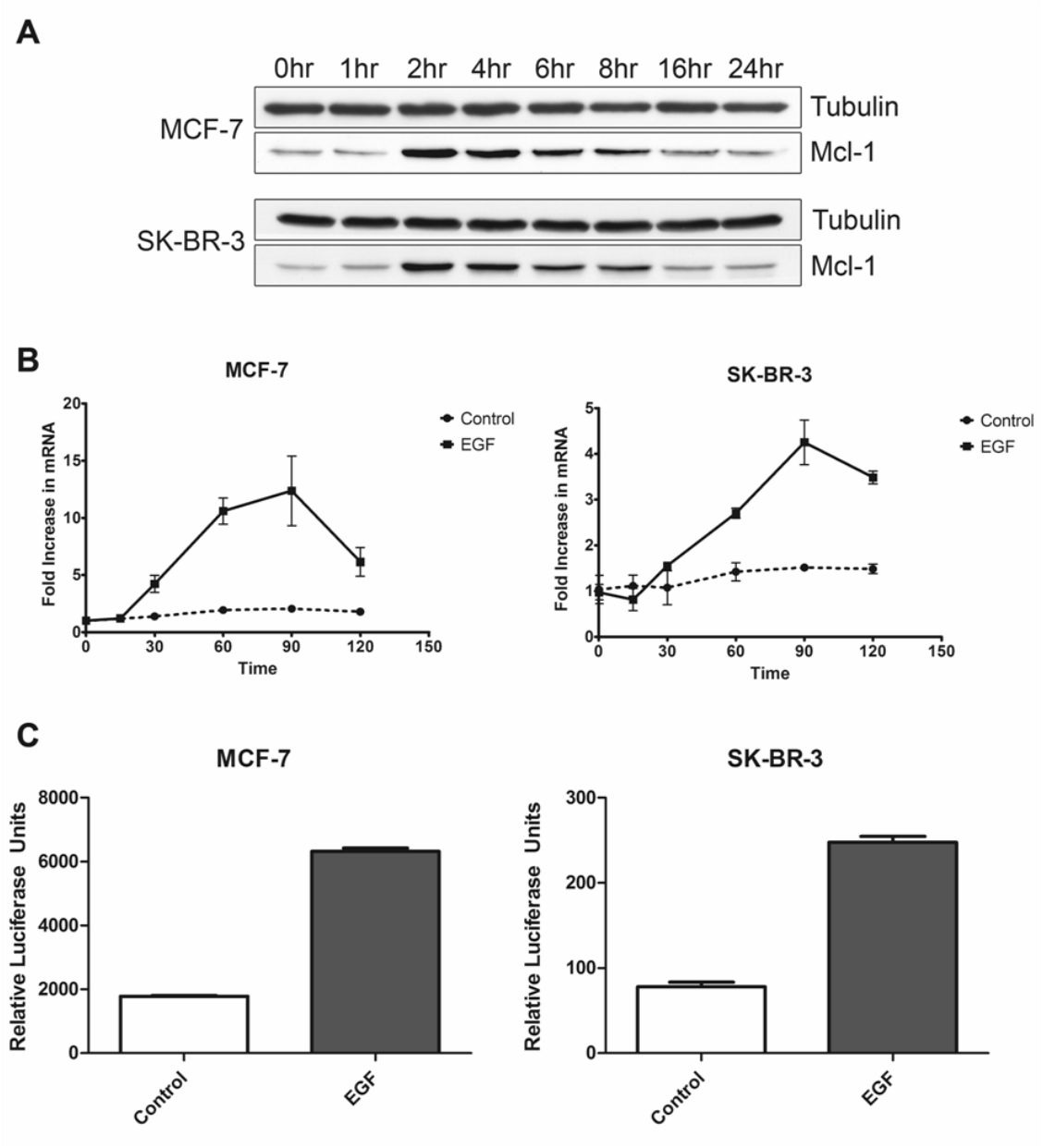

Figure 3.3 Mcl-1 mRNA and protein are up-regulated by EGF

(A) MCF-7 and SK-BR-3 cells were plated into 6-well plates. Cells were serum starved for 24hr and then treated with EGF. Lysates were collected at the indicated times and 30 µg protein was separated by SDS/PAGE. Mcl-1 protein levels were assessed by Western blot. Blots were re-probed for tubulin as a loading control. (B) MCF-7 and SK-BR-3 cells were plated and starved in the same manner as in (A). Cells were treated with EGF or vehicle control and total RNA was isolated over a 120 minute time-course. Mcl-1 mRNA levels were assessed by RT-PCR and the housekeeping cyclophilin was used to standardize results. Data represents the mean of three independent experiments +/- standard error. (C) MCF-7 and SK-BR-3 cells were transfected with the PGL-3 luciferase reporter vector under control of a 4kb fragment of the Mcl-1 promoter. 24hr following transfection, cells were serum starved for 24 hr and then treated with EGF or vehicle control. Lysates were collected after five hours and luciferase activity was measured with an LMax luminometer. Data represents the mean of three independent experiments +/- standard error.

3.2.3 Estrogen treatment does not impact Mcl-1 protein expression but does result in a significant change in the Mcl-1 mRNA level.

Similar to the EGF receptor ErbB2, the expression status of the estrogen receptor ERα is an important indicator for breast cancer prognosis and treatment (152). The estrogen receptor enhances proliferation and cell survival primarily through direct action at the promoters of target genes. Estrogen can also stimulate non-classical estrogen signalling mechanisms at the plasma membrane and in the cytosol and can activate additional receptors including GPR30 (155-156).

To determine if Mcl-1 is a target of estrogen mediated signalling pathways in breast cancer, a Western blot time-course was performed following addition of 10 nM estrogen to the cell culture medium of MCF-7 cells that had been maintained under estrogen depleted conditions for five days (Figure 3.4A). Control cells were treated with an equal volume of 100% ethanol. As is seen in Figure 3.4A, there was no discernable increase in Mcl-1 protein following estrogen stimulation over a 24 hr time-course.

In order to confirm the Western blot data, a real-time PCR experiment was performed to assess Mcl-1 mRNA levels following stimulation with estrogen. Cells were maintained in phenol-red free medium with charcoal stripped FBS for five days prior to the experiment. As a positive control, primers for the estrogen responsive gene PS2 were used to ensure the cells were responding to the estrogen treatment. Following addition of estrogen, the levels of the PS2 mRNA began to steadily increase (Figure 3.4B). Comparison of the ethanol and estrogen treated PS2 mRNA curves by Two-way ANOVA indicated a highly significant ($p<0.001$) change in PS2 mRNA between the control and estrogen treated cells over time. Bonferroni post-tests indicated that the change in PS2

mRNA levels became highly significant at 2 hours ($P<0.001$). PS2 mRNA levels continued to increase throughout the course of the experiment reaching an increase of 6-fold by 8 hours. In contrast to this, the maximal fold increase in Mcl-1 mRNA by estrogen stimulation was 1.801 at the 6 hour time point after which levels began to subside (1.35 fold at 8 hours). As measured by a Two-way ANOVA comparison of the control and estrogen treated curves, estrogen treatment did significantly change Mcl-1 mRNA over time ($p=0.0344$). The changes at 4 and 6 hr were statistically significant as measured by the Bonferroni post-tests ($p<0.05$ and $p<0.01$ respectively). As the mRNA increase is subtle and occurs several hours following stimulation and because the changes are not evident at the protein level, it is likely that it is not a direct effect of ERα at the Mcl-1 promoter. Supporting this conclusion, estrogen treatment did not demonstrate a significant change in activity of the Mcl-1 promoter as measured by luciferase assay in Figure 3.4C ($p=0.460$ as measured by one tailed t-test).

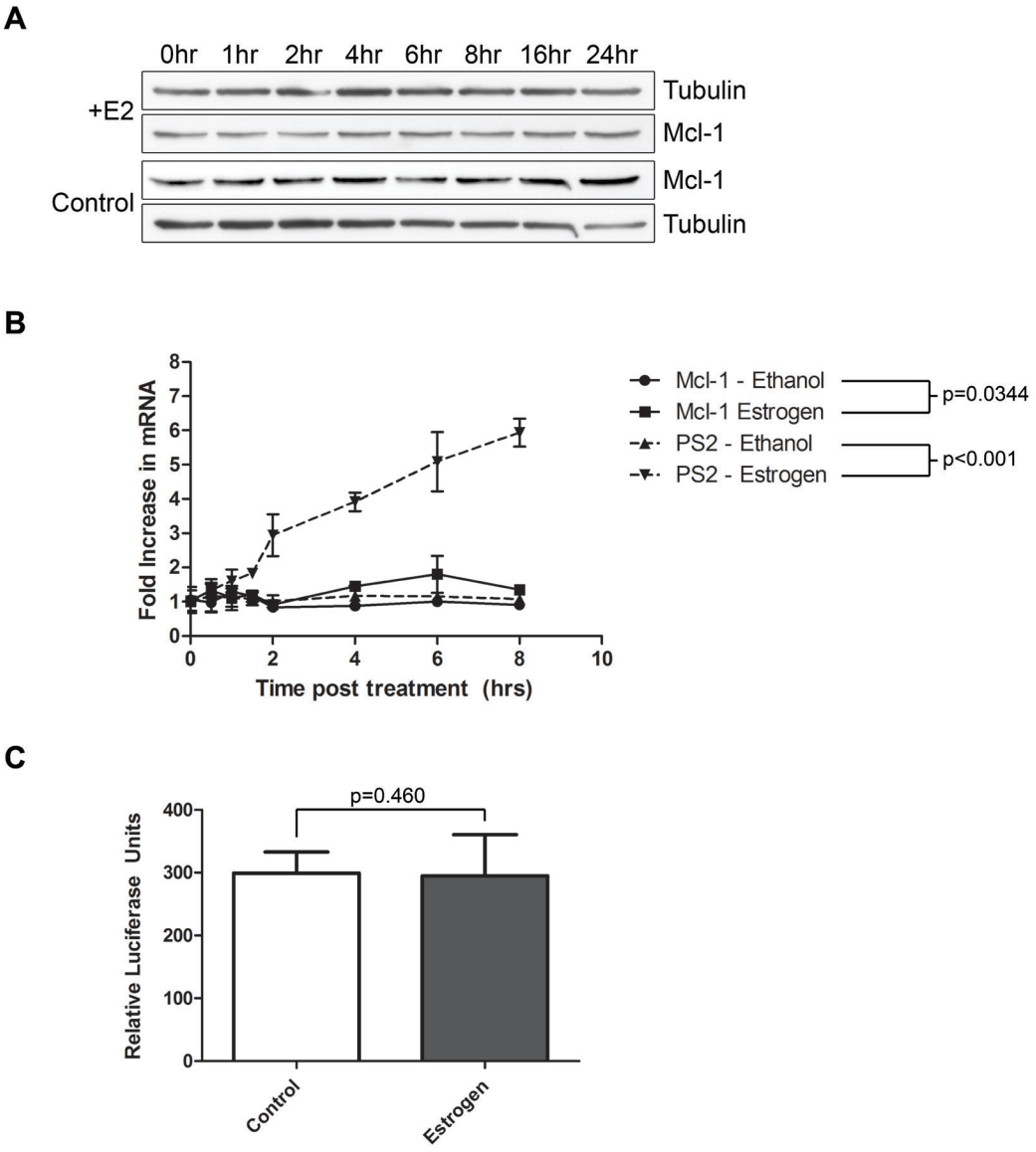

Figure 3.4 Estrogen does not change Mcl-1 protein but does increase Mcl-1 mRNA

(A) MCF-7 cells were grown in phenol red free medium with charcoal stripped FBS for 5 days prior to the experiment. Prior to treatment, cells were serum starved 24 hr. On the day of the experiment cells were treated with estrogen or an equal volume of ethanol for the indicated times. Cells were lysed and SDS/PAGE and Western blot performed to assess Mcl-1 protein levels. (B) Total RNA was isolated from cells treated with estrogen or vehicle control in a similar manner as (A). Mcl-1 mRNA was measured by RT-PCR and standardized to the housekeeping gene cyclophilin. As a positive control PS2 mRNA was also measured following estrogen stimulation. Data represents the mean of 3 independent experiments +/- standard error. (C) MCF-7 cells were transfected with the PGL3 luciferase vector under control of a 4kb fragment of the Mcl-1 promoter. Cells were grown in phenol red free medium with charcoal stripped FBS for 5 days prior to the experiment. Following 24 hr serum starvation, cells were treated with estrogen or ethanol control and luciferase activity was measured 6 hr following treatment. Data represents the mean of 3 independent experiments +/- standard error.

3.2.4 Summary

The data in this section supports the following conclusions:

- EGF stimulation results in a rapid increase in both Mcl-1 mRNA and protein levels
- Treatment with EGF results in enhanced transcriptional activity of the Mcl-1 promoter
- Estrogen treatment results in a measurable increase in Mcl-1 mRNA levels
- Estrogen does not alter Mcl-1 protein levels and fails to modulate the activity of the Mcl-1 promoter.

These results confirm that a direct relationship exists between activation of the EGF receptors and transcriptional activation of the Mcl-1 promoter. Mcl-1 mRNA levels are rapidly increased and the protein increases within 2 hours and the increase is sustained for ~8 hours. Treatment with estrogen resulted in a subtle increase in Mcl-1 mRNA following six hours exposure, but this change was not evident at the protein level. Estrogen also did not significantly change the activity of the Mcl-1 promoter as measured by luciferase assays. These data suggest that the estrogen does not play a major role in the regulation of the Mcl-1 gene and support previous studies that failed to find a correlation between the estrogen receptor status and Mcl-1 expression in breast tumours (252).

3.3 Stimulation of the EGF receptors activates the Ras/Raf/Mek/Erk signalling cascade resulting in Elk-1 dependent transcriptional activation of the Mcl-1 promoter.

3.3.1 Rationale

Both the EGF receptors and Mcl-1 are implicated in tumour development and progression (259, 377). The data in section 3.2 confirm a direct relationship between activation of the EGF receptors and dramatic increases in Mcl-1 transcription and translation. As Mcl-1 is a strongly anti-apoptotic Bcl-2 family member, it is probable that a significant portion of the drug resistance conferred by EGF receptor activation is dependent upon Mcl-1 up-regulation. For this reason we sought to investigate the mechanisms underlying EGF induced Mcl-1 expression. A complete understanding of these mechanisms provides further opportunities for therapeutic intervention. While targeted agents against the EGF receptors as well as Mcl-1 itself are in clinical use, the identification of the precise signalling pathways and critical transcription factors expands the number of potential therapeutic targets and provides opportunities to avoid selection for resistant tumour cells.

3.3.2 The promoter elements critical for basal and EGF induced transcriptional activity of the Mcl-1 gene reside within a ~300 bp fragment proximal to the transcription start site.

In order to gain further insight into the regulatory mechanisms governing EGF induced Mcl-1 expression, a series of deletion mutants were generated that sequentially removed large fragments from the 5' end of the Mcl-1 promoter. Primers were designed to generate seven deletions from the 5' end of the 3974 bp cloned promoter. Each of the constructs is shown cut with HindIII and MluI and separated by agarose gel

electrophoresis in Figure 3.5A. Upon transfection into MCF-7 cells, the originally cloned 3974 bp promoter demonstrated strong basal activity (~2000 relative luciferase units (RLU)) compared to the empty PGL3 vector (~4 RLU). Deletion of nearly 3650 bp from the 5' end yielded no reduction in both basal and EGF induced activity (Figure 3.5B). Minor variations observed in the constructs greater than 333 bp may be due to alterations of secondary structure and/or transfection efficiency of the various constructs. Highly similar results were obtained with the SK-BR-3 cell line; however the absolute values obtained were lower due to reduced transfection efficiency compared to MCF-7 (Figure 3.5C). The deletion of all but 204 bp upstream of the translation start site reduced both the basal and EGF induced promoter activity approximately 10-fold. Despite the overall reduction, EGF treatment still enhanced the activity of this small fragment approximately 3-fold. Deletion of another ~50 bp to yield a promoter insert of only 143 bp resulted in near complete abrogation of both basal and EGF induced activity. These results narrowed the region of interest to approximately 300 bp upstream of the translation start site in both the MCF-7 and SK-BR-3 cells.

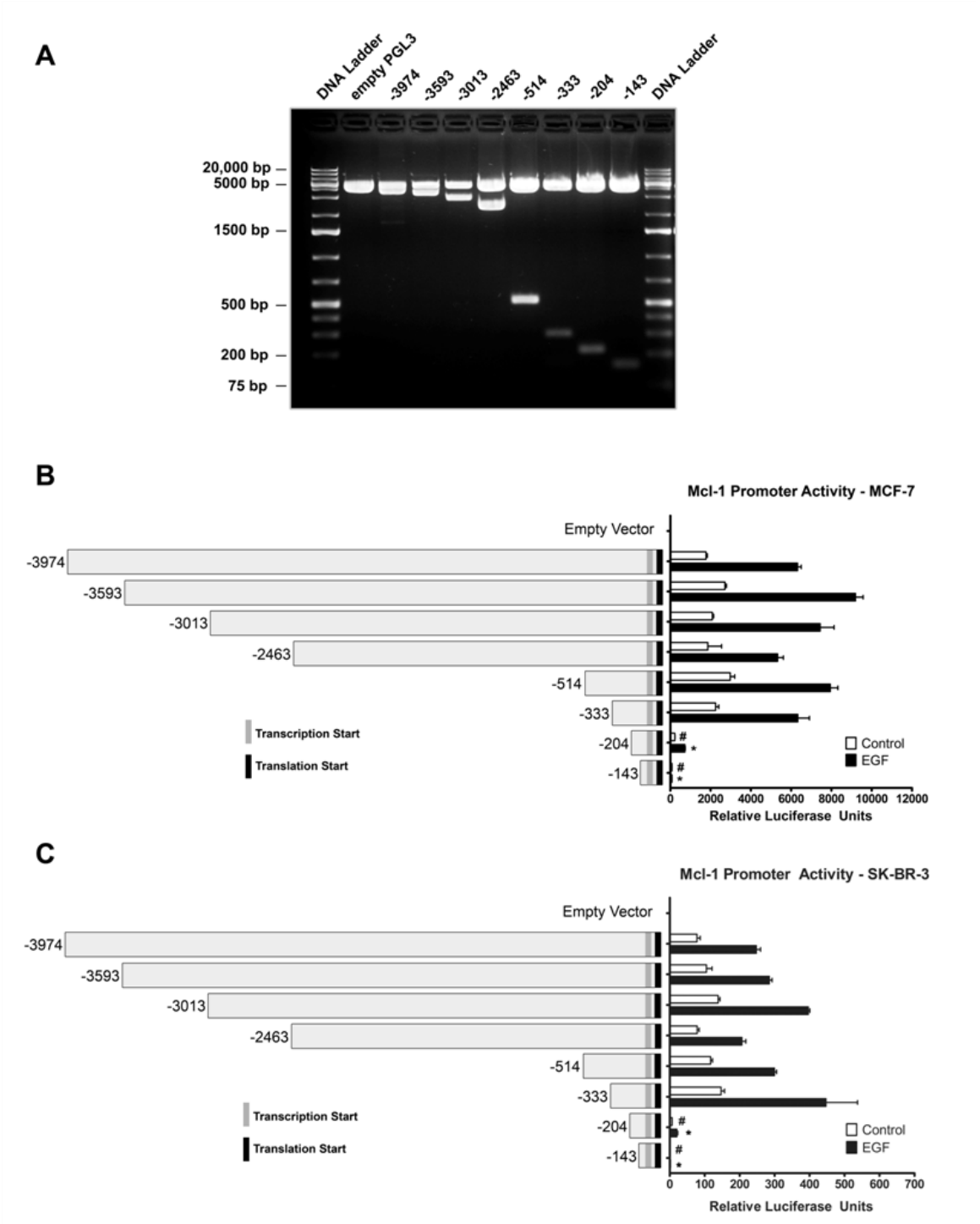

Figure 3.5 A small region of the Mcl-1 promoter proximal to the transcription start site is sufficient for EGF induced transcription

(A) A series of deletion mutants from the 5' end of the 3974 bp cloned Mcl-1 promoter were generated by PCR. PCR products were cloned into the PGL3 luciferase reporter vector. Promoter constructs were cut with HindIII and MluI restriction enzymes and run on a 1.5% agarose gel to verify insert size. All

constructs were further confirmed by sequencing. (B) MCF-7 cells were transfected with each of the Mcl-1 promoter constructs along with 0.2μg pCDNA3-β-Gal using the GenePorter transfection reagent. Cells were changed to serum free media 24 hours following transfection and serum-starved for a subsequent 24 hours. Cells were then stimulated with either EGF or vehicle control for five hours and harvested for luciferase assay. Luciferase activity was measured on an LMax luminometer and normalized to total β-Galactosidase activity. Promoter inserts cloned into the PGL3 vector are shown to scale to the left of the axis. Sequence length upstream from translation start site is indicated for each construct. Basal and EGF induced promoter activity is shown as relative luciferase units. Data represents the mean of 3 independent experiments +/- the standard deviation. # indicates p=<0.005 compared to control -3974, and * indicates p=<0.005 compared to EGF treated -3974. (C) Identical experiment as in (B) with SK-BR-3 cells.

3.3.3 A region of high identity between the human and mouse Mcl-1 promoters containing multiple transcription factor binding sites is critical for basal and EGF induced activity of the Mcl-1 promoter.

In order to distinguish critical DNA elements within the ~300bp region of interest, potential transcription factor binding sites were identified using the TFSEARCH software (368). High scoring transcription factor binding sites were singled out for site directed deletion. In addition to the results from the sequence submission to TFSEARCH, a comparative analysis of the human and mouse promoters was performed by aligning the sequences 2kb upstream of the human and mouse promoters with the ClustalW software (378). This analysis identified a region of high identity between the human and mouse promoter that also contained several high scoring transcription factor binding sites (Figure 3.6A). A total of 7 site directed mutations were made in the Mcl-1 promoter to identify transcription factor binding sites of interest by luciferase assay. In both MCF-7 and SK-BR-3 cells, deletion of a consensus ATF2 site had no effect on basal or induced promoter activity (Figure 3.6 B,C). In contrast, deletions made within the region of high identity between the human and mouse promoter yielded two regions that, when deleted, reduced both basal and EGF induced activity. These regions overlapped several potential transcription factor binding sites including an Ets binding site, CarG box Stat binding site and NF-κB site.

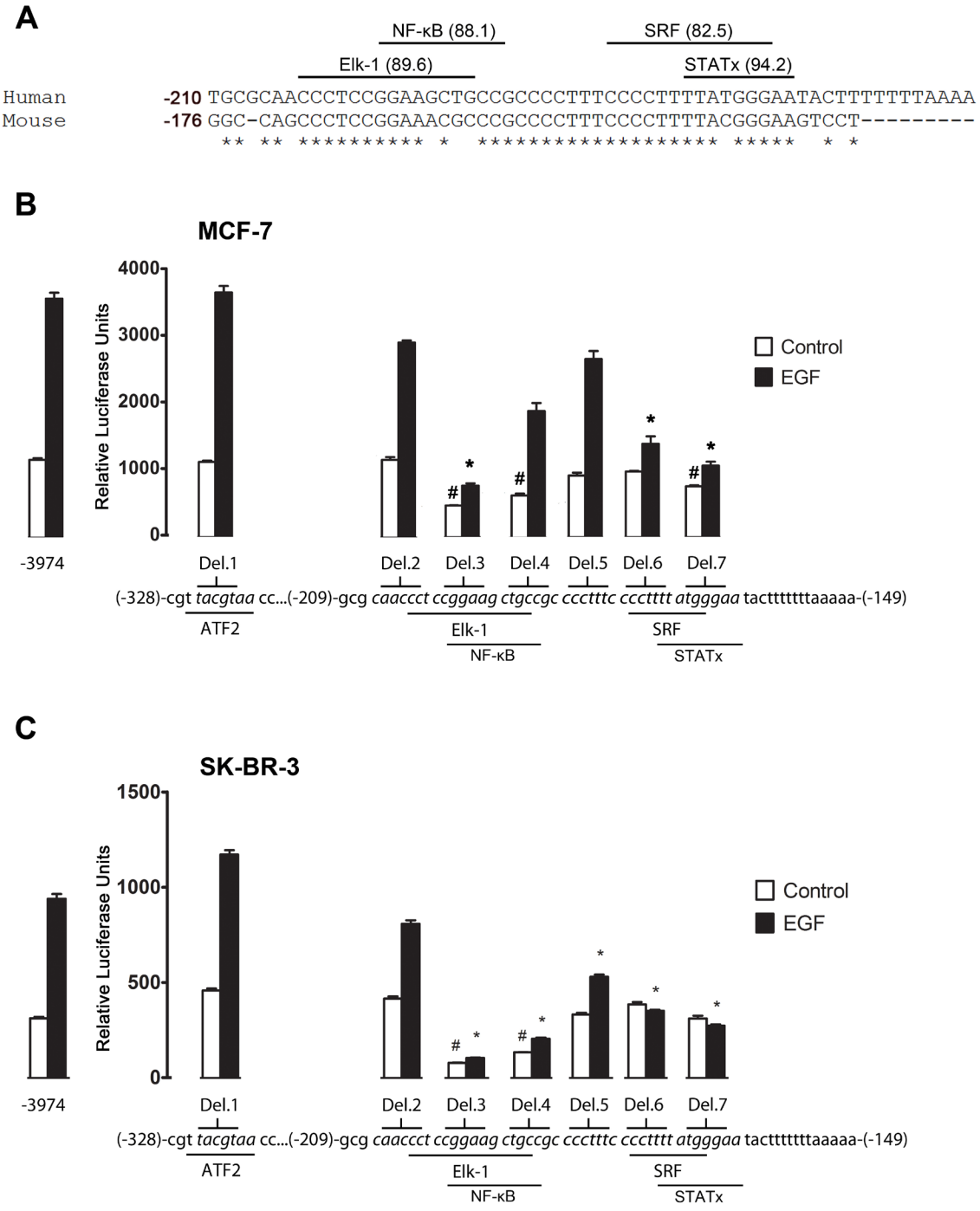

Figure 3.6 Identification of critical transcription factor binding sites in the Mcl-1 promoter

(A) 2kb of the DNA sequence upstream of the human and mouse Mcl-1 promoters was aligned using ClustalW (378). A short DNA sequence within the region of interest demonstrated a stretch of high identity. Identical nucleotides are marked with an asterisk. Transcription factor binding sites identified by the TFSEARCH software are indicated above the sequence with their score in parenthesis. (B) Site directed

deletions were made in the 3974 bp Mcl-1 promoter construct to pinpoint critical DNA elements for transcriptional regulation. 7 bp deletions were made within the region of high identity between the human and mouse promoter as well as within a perfect scoring ATF2 binding site upstream of this region. Luciferase assays were performed with the 7 bp deletion constructs alongside the unmutated 3974 bp promoter. The impact on promoter activity is shown compared to the unmutated 3974 bp fragment (bars to left of axis) in both control and EGF stimulated conditions. Deletions 3 and 7 demonstrated the strongest reduction in basal and EGF induced activity. Data is shown in the context of the Mcl-1 promoter sequence with predicted transcription factor binding sites indicated below the sequence. Deletions are separated by spaces and bases deleted are indicated by italics. * indicates a statistically significant reduction in activity compared to the EGF treated unmutated construct and # indicates a statistically significant reduction in activity compared to the control treated unmutated construct. (C) Identical experiment as in (B) performed with SK-BR-3 cells.

3.3.4 Stat3 is not required for basal or EGF-induced Mcl-1 protein expression.

As the Stat binding site was the highest scoring transcription factor binding site within the region of interest and because prior studies established a link between Stat3 and Mcl-1 in breast cancer (307), the role of Stat3 on Mcl-1 expression was investigated. To assess whether Stat3 is activated by EGF in breast cancer cells, a Western blot was performed over a 60-minute time-course of treatment with EGF (Figure 3.7A). Within 5 minutes of EGF stimulation, activation of the EGF receptors was detected with an antibody specific for tyrosine-phosphorylated ErbB1. This coincided with phosphorylation of Stat3 at Ser727 and Tyr705 indicating that Stat3 is activated in MCF-7 cells following EGF stimulation.

In order to determine whether Stat3 activation is necessary for transcription of the Mcl-1 promoter, a luciferase assay was performed with the 3974 bp Mcl-1 promoter in the presence of the Stat3 inhibitor JSI-124 (Cucurbitacin I) (Figure 3.7B). Compared to the DMSO treated control or untreated cells JSI-124 caused a statistically significant reduction in the EGF induced Mcl-1 promoter activity (3200 RLU as compared to 5000 RLU). The effect of the inhibitor on basal and EGF induced Mcl-1 protein levels was also assessed by Western blot. As is observed in Figure 3.7C, pre-treatment with 0.5 μM JSI-

124 reduced basal Mcl-1 expression and also shifted the kinetics of Mcl-1 induction by EGF. Increased concentration of JSI-124 at 5.0 μM demonstrated a discernible reduction in the induced expression level of Mcl-1.

As the mechanism of action of JSI-124 is not fully understood, and is believed to be acting indirectly through the activation of a phosphatase that de-activates Stat3 (379), an siRNA-mediated knock-down experiment was performed to obtain a more direct assessment of the impact of Stat3 on Mcl-1 expression. Stat3 expression was knocked down by transfection of Stat3 targeting siRNA. At time-points 24 and 48 hours post transfection cells were treated with EGF or control for three hours and the expression of Mcl-1 was assessed by Western blot. Neither basal nor EGF-induced Mcl-1 expression showed discernible variation despite a high efficiency of Stat3 knock-down. The siRNA data points strongly to the possibility that JSI-124 is modulating Mcl-1 expression through indirect non-specific interactions that are independent of Stat3.

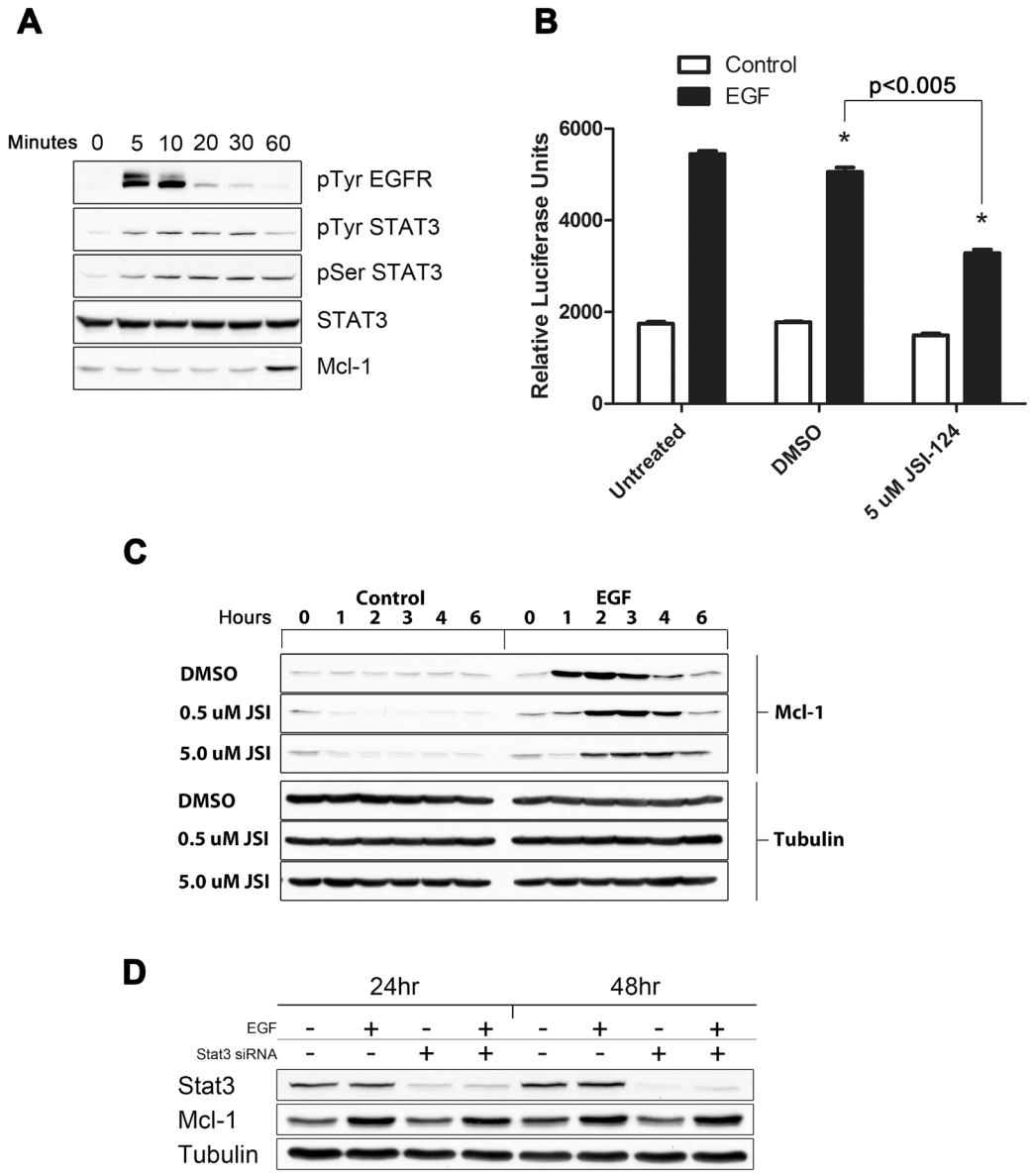

Figure 3.7 Evaluation of the role of Stat3 on Mcl-1 protein expression

(A) To determine if Stat3 is activated in breast cancer cells following stimulation with EGF, MCF-7 cells were treated with EGF and protein lysates collected over a 60 minute time-course. The levels of phospho-EGFR, phospho-Tyrosine Stat3, phospho-Serine Stat3, total Stat3 and Mcl-1 were assessed by Western blot. (B) A luciferase assay was performed using the 3974 bp Mcl-1 promoter with untreated cells, control treated cells or cells that had been pre-treated for 1 hour with the Stat3 inhibitor JSI-124 (cucurbitacin I). Data represents the mean of 3 experiments + standard error. (C) A six-hour Western blot time-course was performed to assess the impact of the Stat3 inhibitor JSI-124 on basal and EGF-induced Mcl-1 protein expression. Cells were pre-treated for one hour with the inhibitor or DMSO as a control. Cell lysates were taken at the indicated time-points following stimulation with EGF. (D) To assess the impact of Stat3 on Mcl-1 protein expression, Stat3 expression was knocked down by transfection with siRNA. Cell lysates were taken 24 or 48 hours following transfection after a three hour treatment with EGF or control. The effect of Stat3 siRNA on basal and EGF-induced Mcl-1 protein levels was assessed by Western blot.

3.3.5 Knock-down or inhibition of the transcription factor Elk-1 reduces both basal and EGF induced Mcl-1 expression.

As the results in Figure 3.7 cast doubt upon the role of Stat3 in the regulation of Mcl-1, additional small molecule inhibitors were employed to narrow down the transcription factor of interest. A luciferase assay was performed with the 3974 bp promoter in the presence or absence of small molecule inhibitors targeting Stat3, Elk-1 and NF-κB. Following transfection with the luciferase reporter vector, cells were starved for 24 hours. After 24 hours starvation cells were pre-treated for 60 min with the specific inhibitor and then stimulated with EGF. Basal and EGF induced luciferase activity was measured five hours later. As is shown in Figure 3.8A, both the Stat3 inhibitor S3I-201 and the NF-κB inhibitor Bay11-7082 had no effect on both basal and EGF induced promoter activity. This supports the hypothesis that the observed effect of JSI-124 is through a non-specific mechanism independent of Stat3. As inhibitors targeting Elk-1 were not commercially available, the Erk inhibitor 3-(2-Aminoethyl)-5-((4-ethoxyphenyl)methylene)-2,4-thiazolidinedione was used to prevent its upstream activation by Erk1/2. Inhibition of Erk1/2 demonstrated a near complete reduction in both basal and EGF induced activity.

To confirm these results, the transcription factors Elk-1, SRF, Stat3 and NF-κB were knocked down with small interfering RNA. siRNA was transfected by electroporation 48 hr prior to stimulation with EGF. Following 3 hr stimulation with EGF or vehicle control, cells were lysed and protein levels measured by Western blot. As is shown in Figure 3.8B, only knock-down of Elk-1 had a measurable impact on both basal and EGF induced Mcl-1 levels. As the region of interest contained a serum response

element (An Elk-1 binding site in close proximity to a CarG box) it was suspected that Elk-1 and SRF were binding the serum response element as a ternary complex. Unexpectedly, SRF knock-down did not reduce either basal or EGF induced Mcl-1 levels. This observation raised the possibility that Elk-1 is not acting in a ternary complex with SRF but binding to the Ets site autonomously.

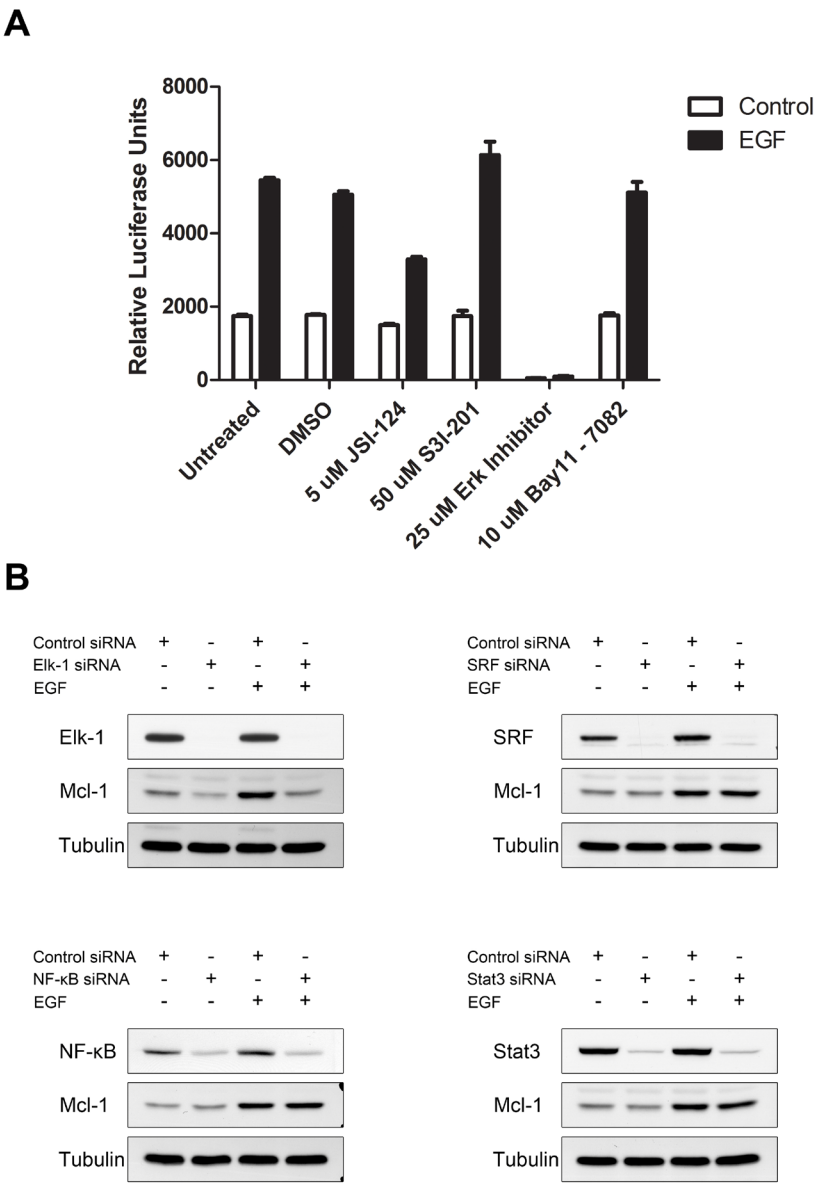

Figure 3.8 Elk-1 inhibition and knock-down reduces Mcl-1 expression

(A) A luciferase assay was performed in the presence of various inhibitors to assess the impact on the activity of the Mcl-1 promoter. MCF-7 cells were transfected with the 3974 bp Mcl-1 promoter construct and serum starved for 24 hours. On the day of treatment, cells were pre-treated with the inhibitors at the indicated concentrations for 1 hour prior to EGF stimulation. Luciferase assay was performed 5 hours post EGF stimulation. Results represent the mean of three independent experiments +/- standard error. (B) To gauge the impact of the transcription factors identified in Figure 3.6, each transcription factor was knocked down by transfection of a specific siRNA. 24 hours after transfection cells were serum starved for 24 hours and then treated with EGF or control for 3 hours. Mcl-1 protein levels were assessed by Western blot. The knock-down efficiency of each siRNA was also assessed by re-probing the same blot with the appropriate antibody. Tubulin was used as a loading control.

As previous studies indicated that TPA stimulated increases in Mcl-1 were dependent upon both Elk-1 and SRF (314), and as the data in Figure 3.8 implicated the involvement of Elk-1 in Mcl-1 regulation in the context of breast cancer, it was initially suspected that Elk-1 and SRF were acting in concert in a ternary complex. Therefore, the lack of effect of SRF knock-down on Mcl-1 protein levels in Figure 3.8B was unexpected. To confirm that SRF knock-down does not impact Mcl-1 levels, SRF was also knocked down in SK-BR-3 cells. As is shown in Figure 3.9A, knock-down of SRF failed to reduce either basal or EGF induced levels of Mcl-1 protein. This is in contrast to Elk-1 knock-down in the SK-BR-3 cells where a reduction in Mcl-1 protein is readily apparent. It was then hypothesized that SRF may be contributing to the residual basal and EGF-induced Mcl-1 levels observed in the absence of Elk-1 (Figure 3.8B and Figure 3.9A). To determine if a combined knock-down of Elk-1 and SRF could potentiate the effect observed with Elk-1 knock-down alone, SK-BR-3 cells were transfected with each siRNA separately and both siRNAs together. Figure 3.9B shows that the combined knock-down of Elk-1 and SRF demonstrates no greater reduction in Mcl-1 protein levels than when Elk-1 is knocked-down alone under all tested conditions. The added condition of cells cultured in 10% serum following siRNA transfection was included to determine if SRF knock-down would have an impact on the steady state levels of Mcl-1 in proliferating cells. Under all tested conditions SRF knock-down failed to demonstrate a discernible effect on Mcl-1 protein expression.

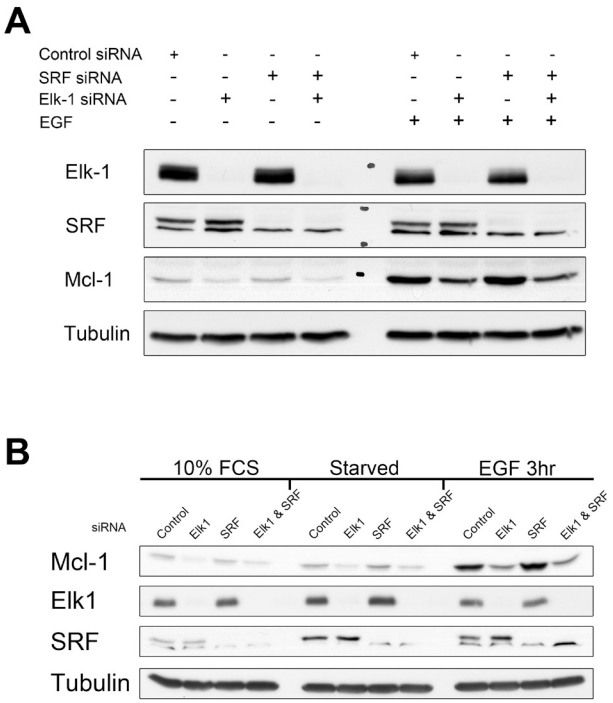

Figure 3.9 SRF knock-down does not measurably effect Mcl-1 protein levels
(A) To confirm the data acquired in Figure 3.8, Elk-1 and SRF were knocked down with siRNA in SK-BR-3 cells. SK-BR-3 cells were transfected with either scrambled, Elk-1 or SRF siRNAs for 24 hours and then serum starved for a subsequent 24 hours. Following starvation, cells were treated with EGF or vehicle control for 3 hours and the levels of Elk-1, SRF, and Mcl-1 were detected by Western blot. Tubulin was used as a loading control. (B) To determine if the combined knock-down of SRF and Elk-1 has a greater impact on Mcl-1 expression than knock-down of either alone, SK-BR-3 cells were transfected with each siRNA separately and both together. 24 hours following transfection one set of samples was left in 10% serum while the remaining two sets were serum starved for an additional 24 hours. The following day one serum starved set was treated with EGF for 3 hours. Mcl-1, Elk-1 and SRF were detected by Western blot. Tubulin was used as a loading control.

The results of Figure 3.8 and Figure 3.9 were further validated using a more quantitative approach. Mcl-1 mRNA levels were measured by real-time PCR following EGF stimulation over a 120 minute time course to assess the impact of both Elk-1 and SRF knock-down. In agreement with the Western blot data, Elk-1 knock-down reduced Mcl-1 mRNA levels significantly (Figure 3.10A). Basal levels of Mcl-1 mRNA in the Elk-1 transfected samples were nearly half that observed in control (0.54 vs 1.0). 30 minutes following stimulation Mcl-1 mRNA had increased nearly four-fold (3.8) in the

control siRNA samples, whereas samples with Elk-1 knock-down had only just surpassed the normal basal level (1.2). The maximal induction was also only half that of control (3.5 fold vs 6.5 fold). Unlike Elk-1, knockdown of SRF did not demonstrate a discernable decrease in either MCF-7 or SK-BR-3 cells (Figure 3.10B,C). On the contrary, in MCF-7 cells the maximal Mcl-1 mRNA in the SRF knock-down samples peaked higher than the control siRNA (15.0 fold increase as compared to a 10.2 fold increase). In SK-BR-3 cells it appears as though the Mcl-1 mRNA decreased more rapidly (2.74 fold vs. 4.94 fold at 120 minutes) following a nearly identical peak induction as compared to control (5.98 vs. 5.66). Regardless of the more subtle and inconsistent variations observed in the context of SRF knock-down, Elk-1 knock-down demonstrated an unmistakable reduction across all time-points. Thus, the sum of the data in Figures 3.8-3.10 suggests a critical role for Elk-1 in the regulation of Mcl-1 that is not dependent upon participation in a ternary complex with SRF. Whether the changes in Mcl-1 protein and mRNA that still occur when Elk-1 is knocked down are due to incomplete knock-down of Elk-1 or compensation by additional transcription factors is yet to be determined.

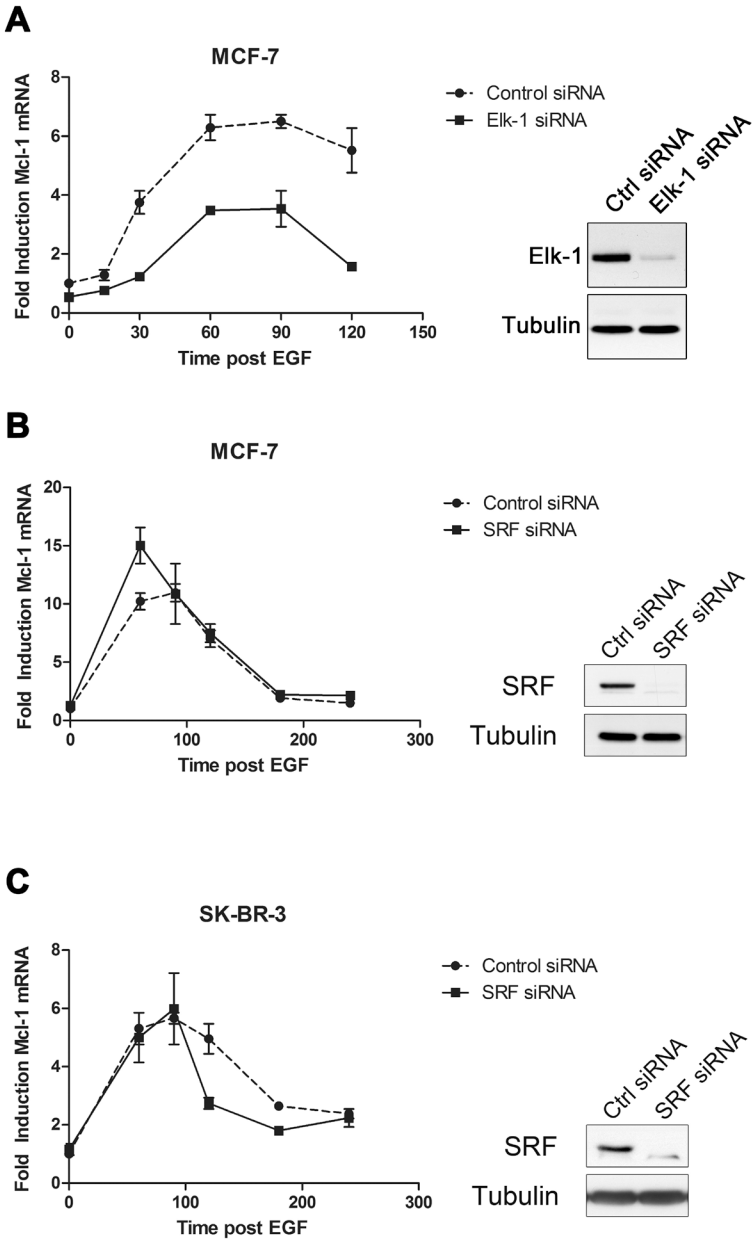

Figure 3.10 Elk-1 knock-down reduces Mcl-1 mRNA

(A) To determine the impact of Elk-1 knock-down on the transcriptional regulation of Mcl-1, MCF-7 cells were transfected with either scrambled or Elk-1 siRNA for 24 hours and then serum starved a subsequent 24 hours. Total RNA was isolated following EGF treatment over a 120 minute time-course. Mcl-1 mRNA was measured by RT-PCR and standardized to the housekeeping gene cyclophilin. Data represents the fold-change relative to the untreated control cells and is the mean of 3 independent experiments +/- standard error. A set of wells was reserved for protein extraction and Western blot to confirm knock-down efficiency (shown to right of graph). (B) To determine the impact of SRF knock-down on the transcriptional regulation of Mcl-1, an identical experiment as in (A) was performed with the exception that siRNA targeting SRF was used in place of Elk-1 siRNA. (C) To confirm the data in (B), an identical experiment was carried out with the SK-BR-3 cell line.

3.3.6 EGF stimulation activates Elk-1 via the Ras/Raf/Mek/Erk signalling pathway

To confirm the importance of Elk-1 in the EGF mediated up-regulation of Mcl-1, a 60-minute Western blot time-course was performed to assess whether Elk-1 was being activated following stimulation with EGF. Phosphorylation of Elk-1 at Ser383 is a critical post-translational modification that enhances both the DNA binding and transcriptional activity of the protein (126). As is shown in Figure 3.11A/B, prior to stimulation with EGF the phosphorylated forms of both Erk1/2 and Elk-1 are undetectable in MCF-7 and only faintly detectable in SK-BR-3 cells. Within 5 minutes of addition of EGF, Erk1/2 is phosphorylated and this phosphorylation persists through until the 60 minute time-point. Following similar kinetics, phosphorylation of Elk-1 at Ser383 was detected at 5 minutes following EGF treatment and decreased by 60 minutes. After 60 minutes, an increase in Mcl-1 protein was detected in MCF-7 cells (Figure 3.11A). Control treatment failed to induce phosphorylation of these proteins over the same time course.

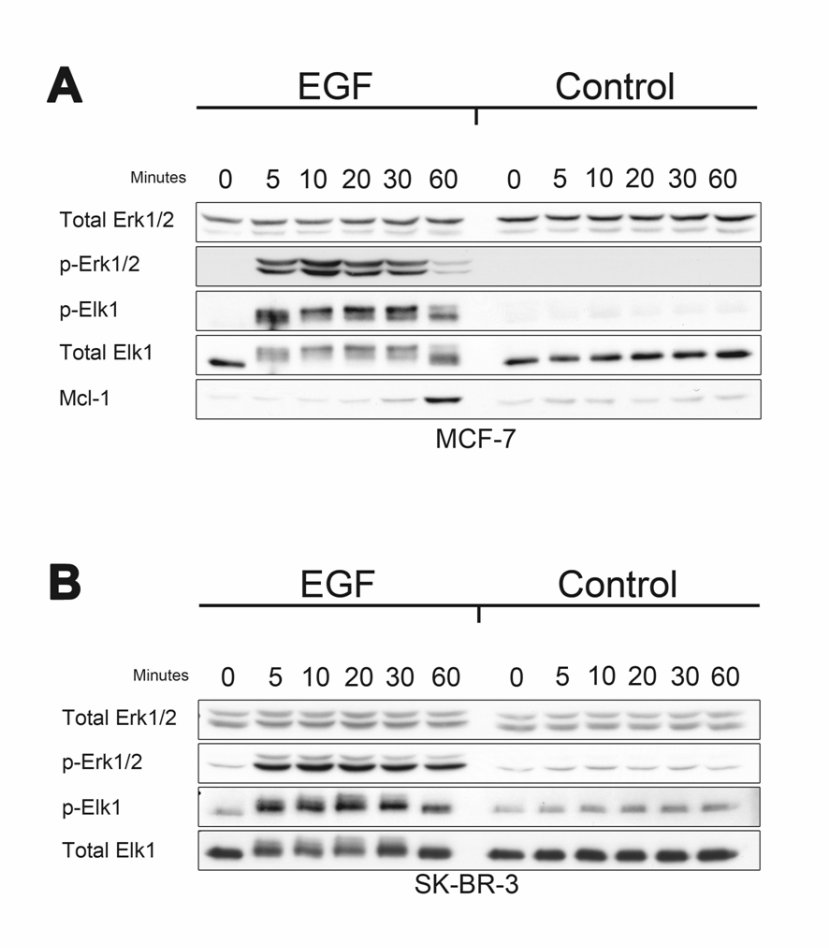

Figure 3.11 EGF stimulation activates Erk1/2 and Elk-1

(A) MCF-7 cells were seeded into a 6-well dish and allowed to grow until 75% confluence. Cells were serum starved for 48 hours and then treated with EGF for the indicated time periods. Cell lysates were taken and the activation of Erk1/2 and Elk-1 were assessed with antibodies specific to Erk1/2 when phosphorylated at Thr202/Tyr204 (p-Erk1/2) and specific to Elk-1 when phosphorylated at Ser383 (p-Elk1). As a loading control, blots were re-probed for total Erk1/2 and total Elk1 (non phospho-specific antibodies). Blots were also re-probed for total Mcl-1 protein levels. (B) Similar experiment as in (A) performed with SK-BR-3 cells. Mcl-1 protein was not measured for the SK-BR-3 cells.

In order to determine if the Ras/Raf/Mek/Erk signalling cascade is critical for the activation of Elk-1 and the transcriptional up-regulation of Mcl-1, two small molecule inhibitors were used that prevent the action of Mek and Erk. Pre-treatment with U0126, a highly specific inhibitor of Mek, prevented both Erk1/2 and Elk-1 phosphorylation in MCF-7 and SK-BR-3 cells (Figure 3.12A/B). This inhibition also prevented the elevation

of Mcl-1 protein levels following EGF treatment. The Erk inhibitor 3-(2-Aminoethyl)-5-((4-ethoxyphenyl)methylene)-2,4-thiazolidinedione, previously published to prevent Elk-1 phosphorylation at Ser383 (113), also completely prevented the EGF mediated induction of Mcl-1 protein observed in the control (Figure 3.12C). These results confirm the importance of Mek/Erk signalling in the transcriptional regulation of Mcl-1 in breast cancer cells.

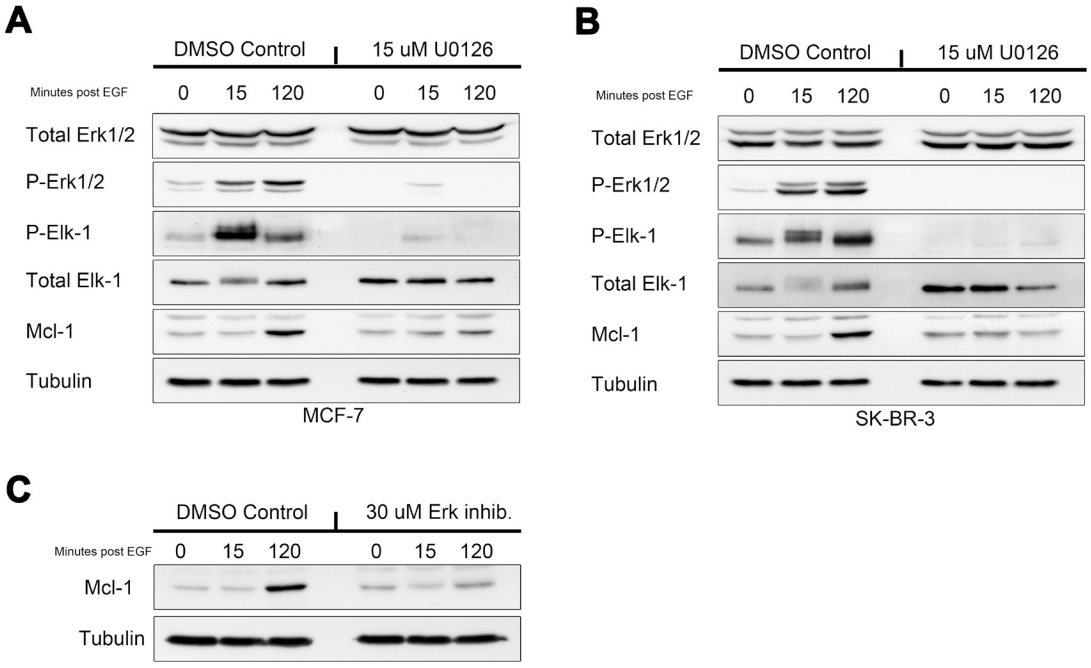

Figure 3.12 Mek and Erk inhibition prevents phosphorylation of Elk-1 and up-regulation of Mcl-1

(A) To establish the importance of Mek/Erk signalling on the regulation of Mcl-1 by EGF MCF-7 cells were pre-treated with Mek inhibitor U0126 for 30 minutes prior to stimulation with EGF. Cells were then stimulated with EGF and lysates were taken at 0, 15 and 120 minutes. Western blots were performed to detect phosphorylated levels of Erk1/2(Thr202/Tyr204) and Elk-1 (Ser383). Blots were re-probed for total Erk1/2, total Elk-1, Mcl-1 and tubulin. (B) Identical experiment as in (A) performed with SK-BR-3 cells. (C) Similar experiment as in (A) using the Erk inhibitor 3-(2-Aminoethyl)-5-((4-ethoxyphenyl)methylene)-2,4-thiazolidinedione in place of the Mek inhibitor U0126.

3.3.7 The transcription factors Elk-1 and SRF bind to the Mcl-1 promoter

To determine whether the transcription factors Elk-1 and SRF bind to the Mcl-1 promoter, a ChIP experiment was performed with MCF-7 cells. Primers were designed to amplify a 163 bp region containing the transcription factor binding sites identified as important in Figure 3.6. ChIP was performed with Elk-1 and SRF antibodies along with antibodies to Stat3 (Ctrl 1) and NF-κB (Ctrl 2) and a no antibody control (beads alone). Figure 3.13A clearly demonstrates that Elk-1 is detectable on the Mcl-1 promoter by ChIP prior to stimulation with EGF. Under unstimulated conditions, SRF was only marginally detectable on the Mcl-1 promoter; however, within 10 minutes of adding EGF there was a significant elevation (85 fold enrichment) of SRF at the promoter, which was maintained 30 minutes after stimulation (Figure 3.13A). The increase in SRF binding occurred alongside an elevation of Elk-1 at the 20 and 30 minute time-points. Isotype controls failed to immunoprecipitate Mcl-1 promoter fragments and demonstrated fold enrichment values close to zero. All of the antibodies failed to enrich a region of the third exon of the Mcl-1 gene (Figure 3.13B). The PCR products were separated by agarose gel electrophoresis following thermal cycling to confirm the presence of a single band of the appropriate sequence length (Figure 3.13A&B).

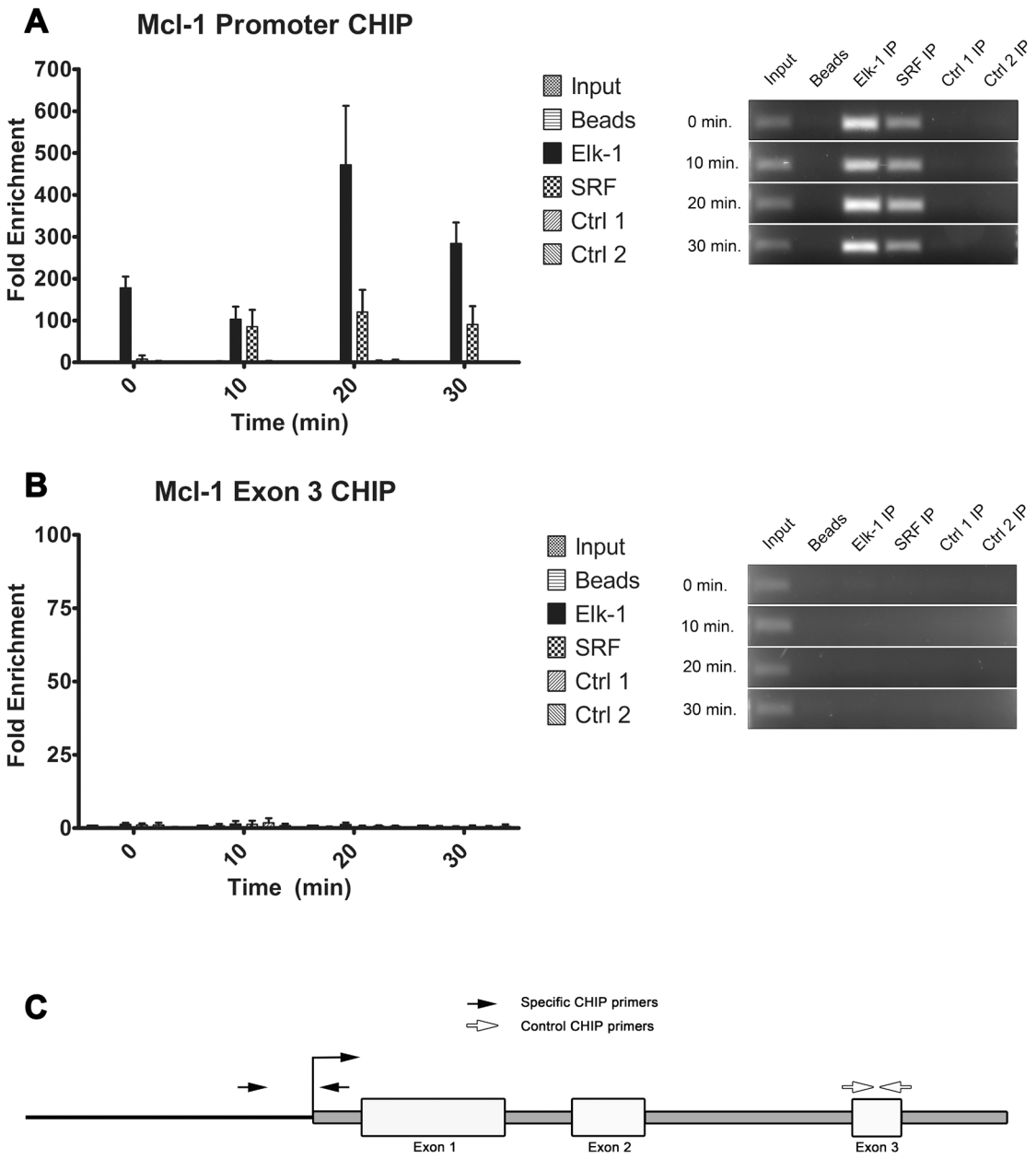

Figure 3.13 Elk-1 and SRF bind to the Mcl-1 promoter
(A) To determine if the transcription factors Elk-1 and SRF bind to the Mcl-1 promoter a ChIP assay was performed. MCF-7 cells were serum starved for 24 hours and then treated with EGF for 0, 10, 20 or 30 minutes and cross-linked with formaldehyde. Primers designed to amplify a 163 bp fragment of the Mcl-1 promoter were used to amplify the DNA fragments precipitated using antibodies to Elk-1, SRF, Stat3 (Ctrl 1) and NF-κB (Ctrl 2). RT-PCR was performed in triplicate on each ChIP sample and fold enrichment values were obtained by comparing the cT value of each ChIP sample to that of an equal amount of input DNA. Results represent 3 independent experiments performed in triplicate +/- standard deviation. Following RT-PCR, PCR products were run on a 2.0% agarose gel to confirm primer specificity. (B) To confirm specificity of the ChIP, primers were designed in the third exon of the Mcl-1 gene that amplified a 186 bp fragment. DNA from each ChIP sample was amplified using the control primers. (C) Schematic demonstrating the locations of both the specific and control ChIP primers.

Further validation that Elk-1 and SRF bind to the region of interest was obtained by performing a streptavidin pull-down assay using a biotin-labelled 50bp probe complementary to the region of interest (155-205 bp upstream of the translation start site (Figure 3.14B)). MCF-7 cells were serum starved for 24 hours after which nuclear lysates were taken at 0, 5, 10, 20 and 30 minutes post EGF stimulation. The Mcl-1 promoter specific probe was able to pull-down both Elk-1 and SRF from EGF treated nuclear lysates. The scrambled probe did not demonstrate observable binding and the presence of an excess of unlabelled probe successfully competed away the signal for both Elk-1 and SRF. Binding specificity was determined by using antibodies against two other transcription factors, NF-κB and Stat3. The decrease in SRF binding observed following stimulation in the pull-down assay may be due to increased recruitment of SRF to the chromatin and therefore reduced availability of the protein in the assay.

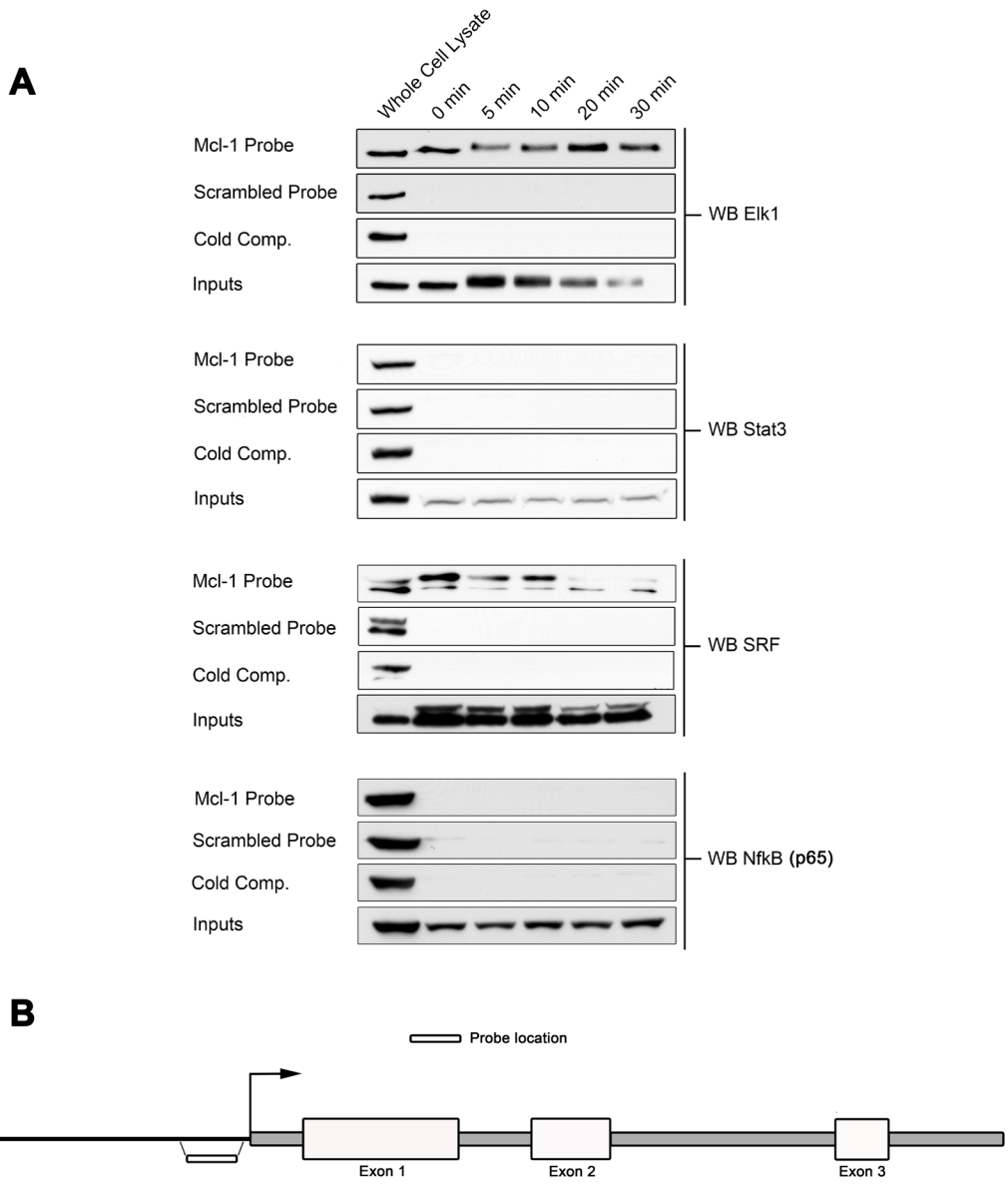

Figure 3.14 Elk-1 and SRF bind to an Mcl-1 promoter specific probe

(A) To determine whether Elk-1 and SRF are binding to the expected DNA elements identified in Figure 3.6, a 50 bp double stranded biotinylated oligonucleotide probe was designed matching the sequence containing the expected binding sites. MCF-7 cells were treated with EGF and nuclear lysates were taken at 0, 5, 10, 20 and 30 minutes post-stimulation. Nuclear extracts were combined in a binding reaction with the Mcl-1 specific probe. Probe-transcription factor complexes were pulled down using streptavidin-coated beads and complexes were separated by SDS/PAGE. Proteins bound to the probe were identified by performing Western blot on the samples following electrophoresis. Membranes were probed with antibodies directed against Elk-1 and SRF as well as Stat3 and NF-κB as specificity controls. As a further control, the Mcl-1 probe with the sequence randomized was used in a separate pull-down assay. In addition, binding of the transcription factors to the specific probe was competed away with a 100-fold molar excess of unlabeled probe (Cold Comp.) (B) Schematic demonstrating the location of the Mcl-1 specific probe in the context of the Mcl-1 gene.

3.3.8 Summary

The data in this section support the following conclusions:

- A small region of the Mcl-1 promoter containing a serum response element as well as potential binding sites for Stat3 and NF-κB is sufficient for basal and EGF induced transcription of the Mcl-1 promoter.
- Knock-down or inhibition of the transcription factors Stat3, NF-κB and SRF does not affect basal or EGF induced transcription of the Mcl-1 gene.
- The Stat3 inhibitor JSI-124 modulates Mcl-1 expression in a manner independent of Stat3.
- Knock-down of the transcription factor Elk-1 reduces basal and EGF induced Mcl-1 mRNA and protein levels
- Inhibition of Mek/Erk signalling prevents Elk-1 phosphorylation and EGF induced Mcl-1 up-regulation
- Elk-1 and SRF bind to the region containing the predicted transcription factor binding sites both in-vivo (ChIP) and in-vitro (Streptavidin pull-down assay)

The results of this section identify the critical DNA elements necessary for efficient transcription from the Mcl-1 promoter. Four transcription factor binding sites were identified within the promoter fragment necessary for transcriptional activity. The region containing these sites also exhibited high identify between the human and mouse Mcl-1 promoters. All four transcription factors have been implicated in Mcl-1 regulation. Stat3 and NF-κB have both been suspected to play a role in regulation of the Mcl-1 gene in breast cancer (307, 369); however, this study casts doubt on the importance of these

factors in the context of signalling from the EGF receptors. The results in this section lend support to the importance of Elk-1 in Mcl-1 transcriptional regulation.

3.4 EGF protects breast cancer cells from apoptosis through a mechanism that relies on signalling via the Mek/Erk pathway.

3.4.1 Rationale

We have confirmed that EGF treatment increases resistance to chemotherapy induced apoptosis in breast cancer cell lines. We have also demonstrated that EGF up-regulates Mcl-1 mRNA and protein levels dramatically in breast cancer cells. Furthermore, knock-down of Mcl-1 causes cells to undergo apoptosis whereas over-expression of Mcl-1 confers a protective effect. In sum, these data suggest that Mcl-1 can be attributed to part of the survival benefit that occurs when EGF signalling is amplified. This study finds that the Ras-MAPK signalling pathway is critical for Mcl-1 induction and we have shown that the prevention of Mek/Erk activation of Elk-1 prevents Mcl-1 protein expression by EGF. The goal of the following experiments therefore is to determine the importance of Mek/Erk signalling and Mcl-1 up-regulation for the prevention of apoptosis by EGF.

3.4.2 Mek/Erk inhibitors prevent Mcl-1 up-regulation and reverse the protection against apoptosis conferred by EGF.

Since Mcl-1 knockdown alone was sufficient to induce apoptosis, we determined whether prevention of Mcl-1 up-regulation could reverse the protective effects conferred by EGF pre-treatment. SK-BR-3 cells were treated with etoposide in the presence or absence of EGF and in the presence or absence of Mek and Erk inhibitors. The Mek inhibitor U0126 completely prevented EGF induced up-regulation of Mcl-1 protein levels

(Figure 3.15B) and also resulted in a significant reduction of the protective effect of EGF (Figure 3.15A). In both the control and U0126 treated cells etoposide induced nearly identical levels of apoptosis (39.4% in control and 41.4% in the U0126 pre-treated cells). Pre-treatment with EGF resulted in a 50% reduction in the levels of apoptosis induced by etoposide in the control cells with levels falling from an average of 39.4% to 19.12%. When the cells were pre-treated with U0126 the EGF treatment only resulted in a reduction from 41.4% to 31.4% (25% reduction). This result confirms the importance of Mek activation in EGF mediated survival signalling and supports the possibility that Mek inhibitors would be highly effective in tumours over-expressing EGF receptors or harbouring activating receptor mutations.

To verify the result with U0126, the experiment was repeated using an Erk1/2 inhibitor (Figure 3.15C/D). The Erk1/2 inhibitor alone displayed slight toxicity that appeared to be enhanced in the presence of EGF. Despite the toxicity of the inhibitor, levels of apoptosis induced by etoposide were only slightly higher with the inhibitor as compared to that of the control (28.7% and 33.6%). While EGF protection in the control samples was similar to that observed in Figure 3.15A (reduction from 28.7% to 12.8%), Erk inhibition completely reversed the protective effect conferred by EGF pre-treatment. In stark contrast to the control samples, co-treatment of etoposide with EGF in the presence of the Erk inhibitor demonstrated a mean level of apoptosis that was higher than that of etoposide alone. These results confirm that Mek/Erk signalling leads to both increased Mcl-1 expression and EGF survival response in breast cancer cells.

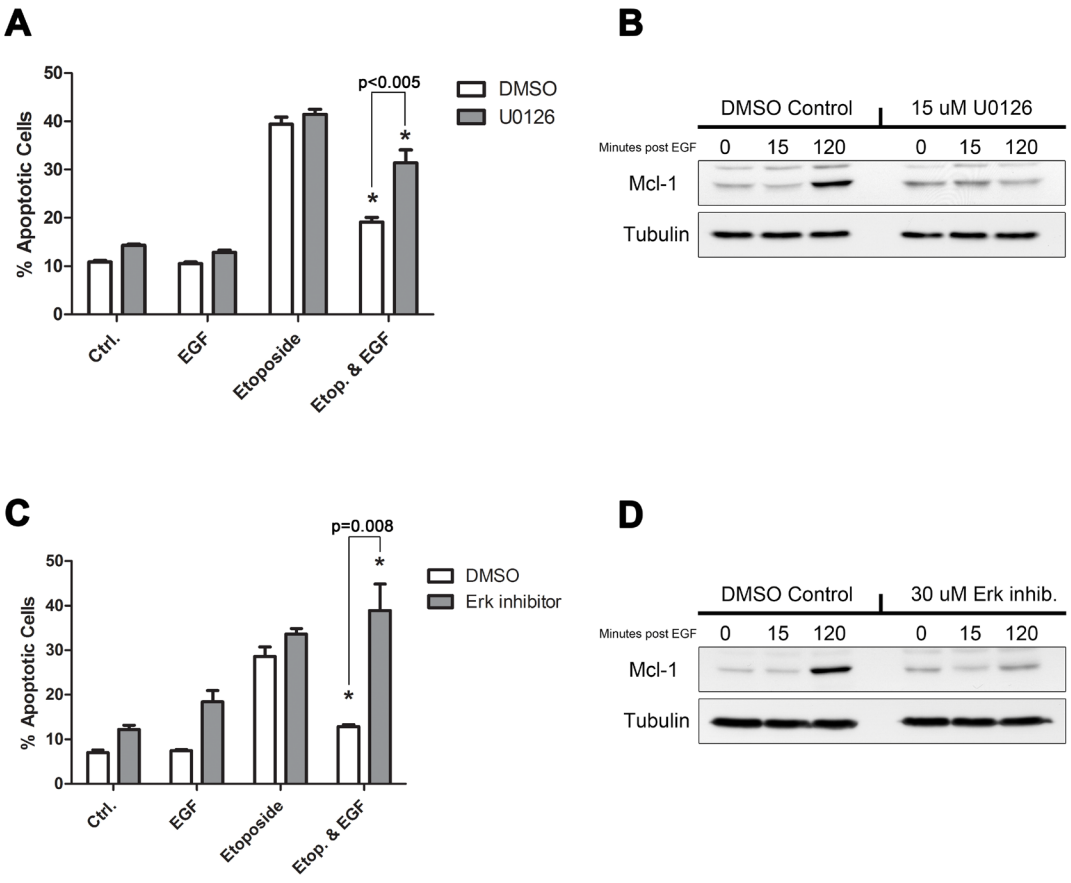

Figure 3.15 Protection conferred by EGF is reliant on Mek/Erk signalling

(A) To determine the impact of Mek inhibition on the protective effect conferred by EGF a cell death assay was performed using the Mek inhibitor U0126. SK-BR-3 cells were seeded into 12-well plates and treated with 15 µM U0126 or an equal volume of DMSO. Following a 30 minute incubation, the cells were then treated with EGF or vehicle control for 1 hour. Following 1 hour, cells were treated with 5 µM etoposide for 18 hours. Apoptosis was assessed by measurement of the sub-G1 population by flow cytometry. Results represent the mean of three independent experiments +/- standard error. (B) Western blot demonstrating the inhibition of Mcl-1 up-regulation by U0126. (C) Similar experiment as in (A) using the Erk inhibitor 3-(2-Aminoethyl)-5-((4-ethoxyphenyl)methylene)-2,4-thiazolidinedione in place of the Mek inhibitor U0126. (D) Western blot demonstrating the inhibition of Mcl-1 up-regulation by 3-(2-Aminoethyl)-5-((4-ethoxyphenyl)methylene)-2,4-thiazolidinedione.

3.4.3 Summary

The data in this section support the following conclusions:

- Inhibition of Mek prevents the induction of Mcl-1 by EGF
- Mek inhibition reverses the protective effect conferred by EGF
- Inhibition of Erk prevents the induction of Mcl-1 by EGF
- Erk inhibition reverses the protective effect conferred by EGF

These data demonstrate the importance of the Ras-MAPK signalling cascade for the survival advantage conferred through activation of the EGF receptors. Mek and Erk inhibition were sufficient to completely prevent a reduction in apoptosis in the presence of EGF. We have also determined that Mek and Erk inhibition completely prevent the up-regulation of Mcl-1 by EGF. While these data do not conclusively establish the relative contribution of Mcl-1 to the EGF induced survival advantage, they do strongly support the conclusion that downstream signals from Erk1/2 are critical in the prevention of apoptosis by EGF.

3.5 Activated Elk-1 correlates with increased levels of Mcl-1 in breast tumour samples.

3.5.1 Rationale

We have established that Mcl-1 expression is regulated by stimulation of the EGF receptors in two cell line models. Furthermore, we have identified the signalling pathway, transcription factors and promoter elements critical for Mcl-1 regulation. This knowledge, combined with previous studies that correlate Mcl-1 with poor prognosis in breast cancer (249), provides both the rationale and approach for targeting Mcl-1 in breast cancer. Knowledge of the molecular pathways governing Mcl-1 expression opens the door to developing rationale therapeutic approaches; however, for these results to be

clinically relevant they must hold true beyond cell line models of breast cancer. To that end, we sought to determine if the molecular pathways discovered in the MCF-7 and SK-BR-3 cell lines can be used to predict significant correlations in breast tumour samples. Additionally, we assessed the predictive capacity of Mcl-1 with regards to patient survival and prognosis.

3.5.2 Antibody validation and pilot study

Prior to proceeding to a large scale analysis, a pilot study was performed with 26 samples from the Manitoba Breast Tumour Bank. Tumour sections were stained for immunofluorescence with antibodies directed against Mcl-1 and phosphorylated Elk-1. Antibodies were pre-validated for immunofluorescence on tumour sections by over-expression and knock-down experiments in MCF-7 cells. Figure 3.16A demonstrates that staining for phospho-Elk-1 is primarily nuclear and overlaps with the nuclear stain DAPI. Treatment of the cells with EGF for 15 minutes resulted in increased signal intensity and cells that have Elk-1 knocked down with siRNA demonstrate reduced staining in the presence of EGF. Similar validation experiments were performed for the detection of Mcl-1. In Figure 3.16B, anti-Mcl-1 staining is primarily cytoplasmic and, upon transfection of the cells with Mcl-1 specific siRNA, the fluorescent signal is visibly diminished. Furthermore, over-expression of Mcl-1 cDNA greatly increases signal strength. These data support the specificity of the antibodies and increases confidence that they are suitable for analysis of Mcl-1 and phosphorylated Elk-1 in tumour sections.

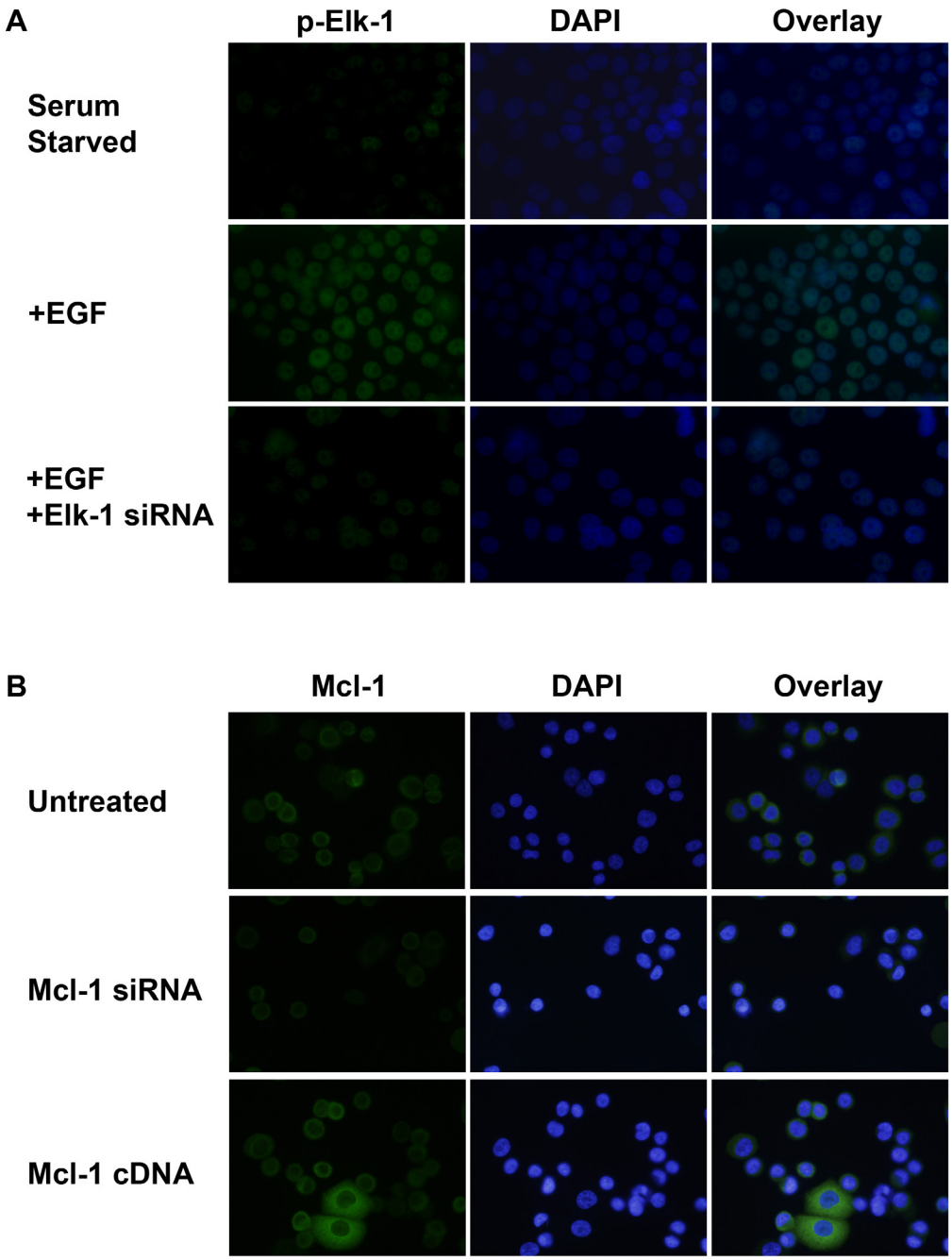

Figure 3.16 Validation of phospho-Elk-1 and Mcl-1 antibodies for Immunofluorescence

(A) MCF-7 cells were plated onto coverslips in 6-well dishes. Cells were serum starved or treated with EGF for 15 minutes. One set of EGF treated cells was transfected with Elk-1 siRNA 24 hr prior to the experiment. Cells were fixed and stained with antibodies against phosphorylated Elk-1 as well as the nuclear stain DAPI. Images were taken at 40x magnification using the DP Controller and DP Manager software (Olympus). (B) MCF-7 cells were plated onto coverslips in 6-well dishes. Cells were left untreated or transfected for 24 hr with Mcl-1 siRNA or a plasmid containing the Mcl-1 cDNA. Cells were fixed and stained with antibodies directed against Mcl-1 as well as the nuclear stain DAPI. Images were taken at 40x magnification.

Following antibody validation, a panel of 26 breast tumour sections were incubated separately with each primary antibody and subsequently stained with a fluorescently conjugated secondary antibody. Sections were examined on an Olympus BX51 fluorescent microscope and scored using a 0-3 point scoring system. In Figure 3.17A, data were analyzed by plotting the scores as an XY scatter and performing a Spearman correlation test. We found a statistically significant positive correlation between phosphorylation of Elk-1 and Mcl-1 expression (r_S=0.4303, p=0.0282) in the tested breast tumours. Representative images from the sampled tumours shown in Figure 3.17B demonstrate that regions within an individual tissue section having high phospho-Elk-1 (green signal), also demonstrate strong staining for Mcl-1 (red signal). Conversely, regions with low phospho-Elk-1 levels tended to have corresponding low levels of Mcl-1.

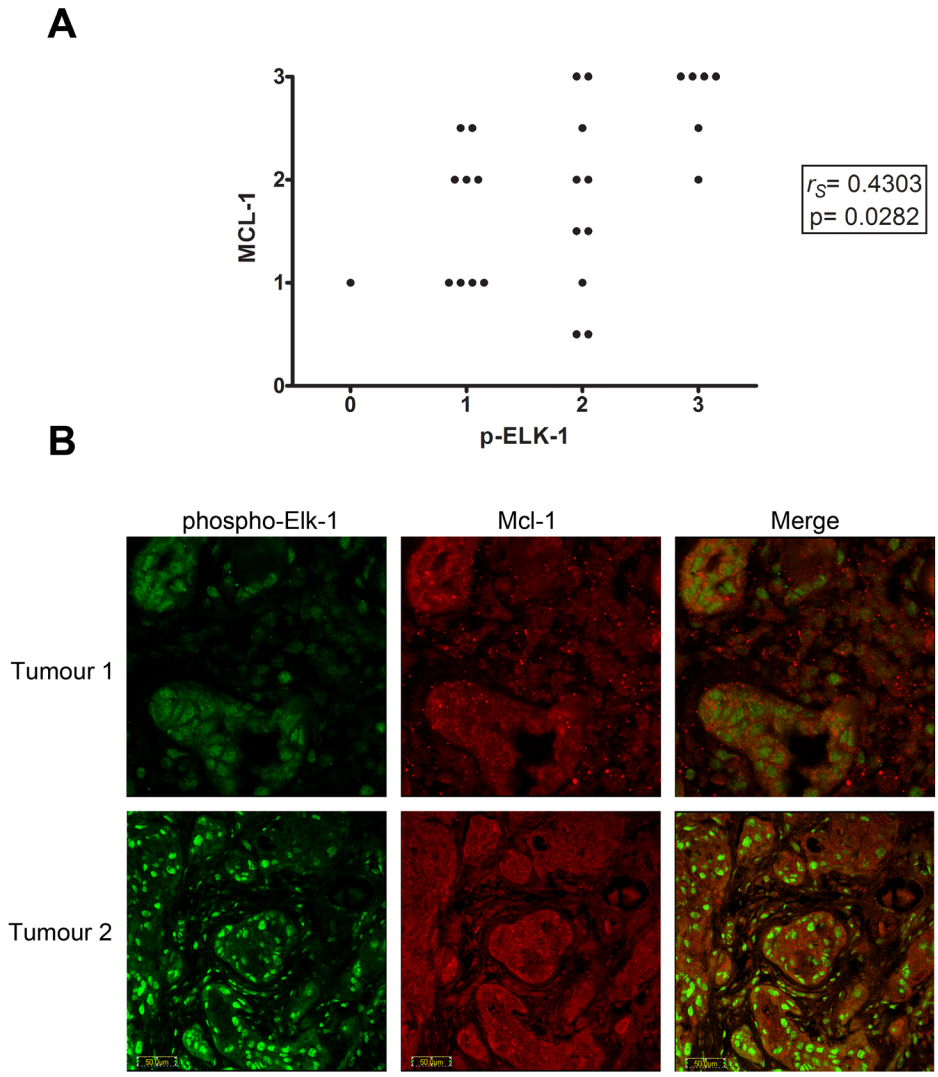

Figure 3.17 Mcl-1 correlates with p-Elk-1 in a pilot study of 26 breast tumours

(A) Paraffin imbedded tumour sections were provided by the Manitoba Breast Tumour Bank on glass slides. Slides were immunostained with antibodies against Mcl-1 and phospho-Elk-1 Ser383. Tumours were scored independently for Mcl-1 and p-Elk-1 stain intensity on a scale of 0-3. Scores were plotted as an XY scatter and data were jittered to prevent overlap of identical scores. Spearman correlation and p-value are indicated to the right of the graph. (B) Representative tumour sections double-stained for phospho-Elk-1 (green) and Mcl-1 (red) as well as both stains overlapped (Merge).

3.5.3 Mcl-1 correlates with phosphorylated Elk-1 and Erk1/2 in an array of 255 ERα negative breast tumours

As the pilot study suggested a positive association, we further investigated the relationship between activated Elk-1 and the expression level of Mcl-1 in a tissue microarray provided by the Manitoba Breast Tumour Bank. The TMA consisted of 255 tumours that were previously assessed as ERα negative by a ligand binding assay. Each tumour was represented by two separate spots on the TMA. Sectioning, slide preparation, antibody testing and IHC staining was performed by staff at the Manitoba Breast Tumour Bank. TMAs were immunostained with the Mcl-1 and phospho-Elk-1 antibodies previously demonstrated to be effective for immunofluorescence as well as antibodies directed against phospho-Erk1/2, ErbB1, ErbB2 and ErbB3 (Table 2.3). TMAs were assessed using the H-score method by two independent observers and data were analyzed using GraphPad Prism® 5 (GraphPad Software Inc. La Jolla, CA). In Figure 3.18 relative Mcl-1 expression levels for each tumour were compared to the staining intensity for phospho-Elk-1 and phospho-Erk1/2. As the cell line data have previously demonstrated regulation of Mcl-1 through Erk1/2 activation of Elk-1, it was hypothesized that both phospho-Elk-1 and phospho-Erk1/2 would positively correlate with each other and Mcl-1 expression.

In Figure 3.18A the tumours were separated into two groups based on the median Mcl-1 H-score of 120. Tumours with an Mcl-1 score of 120 or less were classified as "Low Mcl-1" and those with a score above the median were classified as "High Mcl-1". A Mann-Whitney test was performed to determine if the median phospho-Elk-1 score (indicated by the solid horizontal line) was significantly different between the two

groups. The median phospho-Elk-1 score for the Low-Mcl-1 category was 75.0 and the median score for the High Mcl-1 score was 140, a difference that demonstrated significance ($p<0.005$). In a similar manner, the phospho-Erk1/2 scores were assessed based on separation into two groups by the Mcl-1 median score. The median phospho-Erk1/2 scores were also significantly different with a score of 50.0 in the Low-Mcl-1 subset and a score of 100.0 in the high subset. The phospho-Erk1/2 scores were also separated into two groups based on the median phospho-Elk-1 score. As Elk-1 is a substrate for Erk1/2 it was hypothesized that elevated levels of phospho-Erk1/2 would correlate with elevated levels of phospho-Elk-1. A significantly higher phospho-Erk1/2 score (150.0) was observed in tumours that demonstrated high levels of phospho-Elk-1 as compared to those that did not (50.0) (Figure 3.18C).

The same data-set was also plotted as an XY scatter and analyzed by Spearman correlation in Figure 3.18D, E and F. The Spearman correlation test supported the associations identified in Figure 3.18A, B and C and also demonstrated that the association between phospho-Elk-1 and Mcl-1 ($r_S = 0.3724$) was stronger than that between phospho-Erk1/2 and Mcl-1 ($r_S = 0.2279$). Furthermore the association between phospho-Elk-1 and phospho-Erk1/2 was stronger than either relationship with Mcl-1 having an r_S value of 0.4728. The solid lines on each of the scatter plots represents the linear regression of the data. These data suggest that the molecular pathways delineated in cell line models translates into accurate predictions of protein expression/activity in breast cancer.

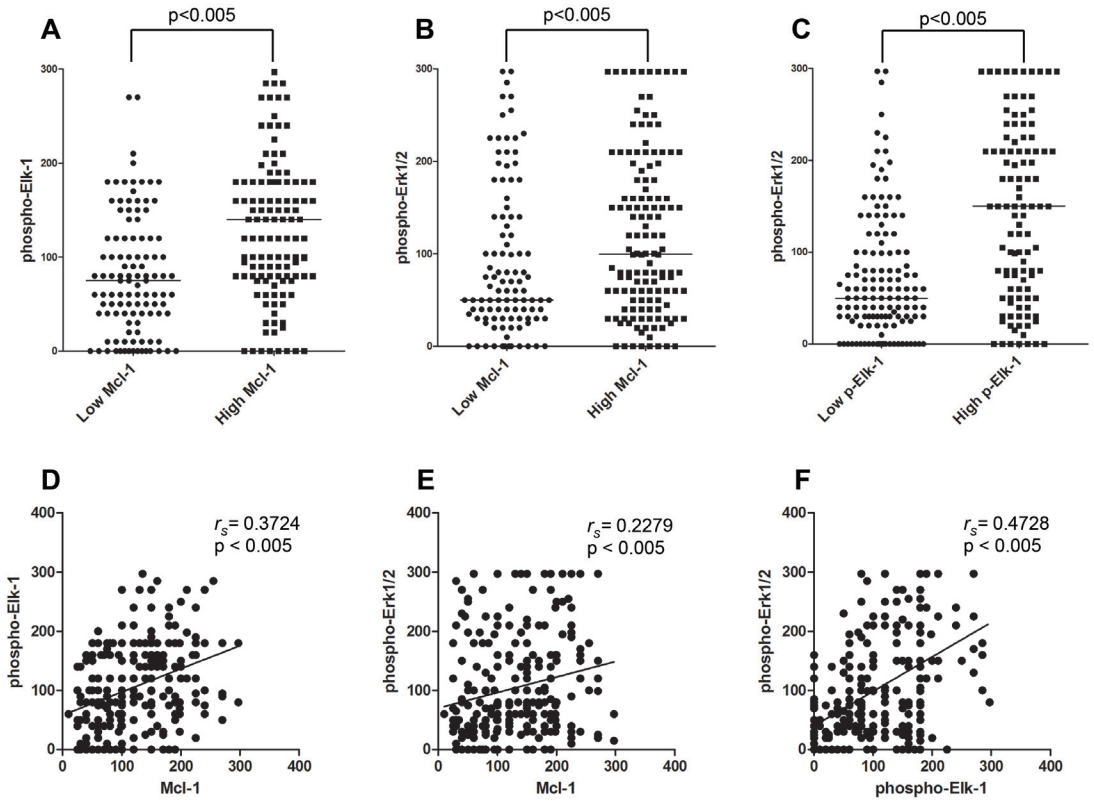

Figure 3.18 Mcl-1 correlates with elevated phospho-Elk-1 and phospho-Erk1/2

A TMA containing samples from 255 ERα negative breast tumours was stained by immunohistochemistry with antibodies specific for Elk-1 when phosphorylated at Ser383, Erk1/2 when phosphorylated at Thr202/Tyr204 and Mcl-1. TMAs were scored by two blinded independent observers. Tumours were assessed using the H-score method. An intensity score of 0-3 was multiplied by the percentage of tumour cells stained. (A) H-scores for phospho-Elk-1 were separated into two groups based on the median Mcl-1 H-score of 120. (B) H-scores for phospho-Erk1/2 were separated into two groups based on the median Mcl-1 H-score of 120. (C) H-scores for phospho-Erk1/2 were separated into two groups based on the median phospho-Elk-1 H-score of 100. Bars for A, B and C represent median scores for each category. p-values were calculated by Mann-Whitney test. (D) XY scatter plot of the H-score for Mcl-1 against the H-score of phospho-Elk-1. (E) XY scatter plot of the H-score for Mcl-1 against the H-score of phospho-Erk1/2. (F) XY scatter plot of the H-score for phospho-Elk-1 against the H-score of phospho-Erk1/2. Lines for D, E and F indicate linear regression. Spearman correlation coefficient (r_S) and p-value are indicated above each graph.

3.5.4 Mcl-1 correlates with the expression level of the EGF receptor family

The initial experiments performed in this study demonstrated that Mcl-1 transcription and translation are strongly activated by stimulation with the EGF ligand. We also have previously reported an association between ErbB2/Her-2 status and Mcl-1

expression in a panel of 29 ERα negative breast tumours (369). To confirm and expand upon these data, the tumour arrays were stained with antibodies specific to ErbB1 and ErbB3. Staining and scoring for ErbB2 was previously performed on this array by two independent observers in the lab of Dr. Leigh Murphy at the Manitoba Institute of Cell Biology and these scores were used in our analysis. Data were analyzed in a similar manner as in Section 3.5.3. In Figure 3.19A, B and C the Mcl-1 H-score was separated into two groups based upon the median H-score of the indicated receptor. The median Mcl-1 score for the low ErbB1 subset was 100.0 where as in the high subset the score was 130.0. This difference bordered upon significance with a Mann-Whitney p-value of 0.048. In the case of ErbB2, the median Mcl-1 score in the low subset was 120.0 where as in the high subset it was 150.0. This difference was statistically significant with a p-value of 0.0366. While ErbB1 and ErbB2 scores failed to strongly stratify the Mcl-1 H-scores, ErbB3 status showed a more striking difference. In the low ErbB3 subset the median Mcl-1 score was 80.0 whereas in the high subset the median score was 150.0. This generated a highly significant p-value of <0.005. The data were also plotted in an XY scatter to assess associations between the variables. Spearman correlations performed in Figure 3.19D, E and F generally reflected the data obtained in Figure 3.19A, B and C; however, in this case the correlation between Mcl-1 and ErbB1 was not significant. In summary, Mcl-1 correlates with the expression level of ErbB1 and ErbB2 and demonstrates strong association with the expression level of ErbB3. These data confirm already published studies that have found a relationship between EGF receptor status and Mcl-1 protein expression.

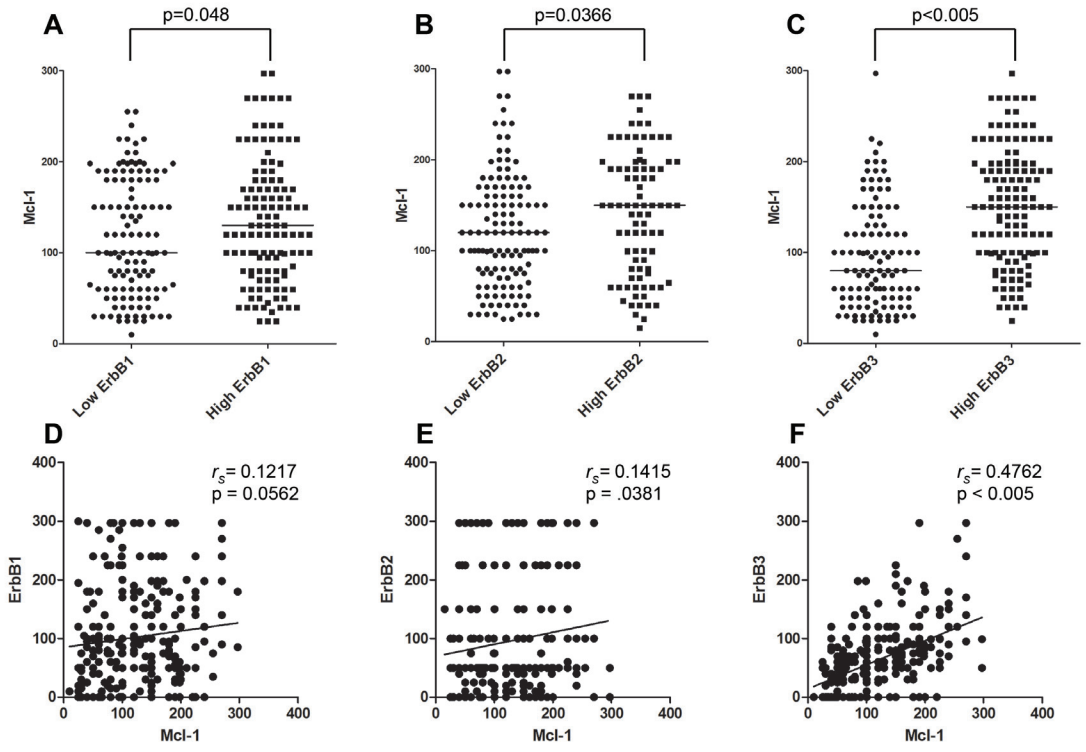

Figure 3.19 Mcl-1 correlates with EGF receptor expression

The same TMA as in Figure 3.18 was stained by immunohistochemistry with antibodies specific for ErbB1 ErbB2 and ErbB3. TMAs were scored by two blinded independent observers. Tumours were assessed using the H-score method. An intensity score of 0-3 was multiplied by the percentage of tumour cells stained. (A) H-scores for Mcl-1 were separated into two groups based on the median ErbB1 H-score of 85 and plotted in a vertical scatter (B) H-scores for Mcl-1 were separated into two groups based on the median ErbB2 H-score of 50 and plotted in a vertical scatter. (C) H-scores for Mcl-1 were separated into two groups based on the median ErbB3 H-score of 60 and plotted in a vertical scatter. Bars for A, B and C represent median scores for each category. p-values were calculated by Mann-Whitney test. (D) XY scatter plot of the H-score for Mcl-1 against the H-score of ErbB1. (E) XY scatter plot of the H-score for Mcl-1 against the H-score of ErbB2. (F) XY scatter plot of the H-score for Mcl-1 against the H-score of ErbB3. Lines for D,E and F indicate linear regression. Spearman correlation coefficient (r_S) and p=value are indicated above each graph.

3.5.5 Mcl-1 does not correlate with patient survival

As Mcl-1 is a pro-survival Bcl-2 family member, it was hypothesized that Mcl-1 may contribute to poor treatment response and ultimately poor patient prognosis. While one previous study has demonstrated a correlation between Mcl-1 and poor prognosis in breast cancer (249), other studies have not supported the association (252). In order to determine if Mcl-1 is able to predict patient survival in our patient cohort, Kaplan Meier

curves were generated to assess overall survival of patients with tumours expressing low or high levels of Mcl-1. The median Mcl-1 H-score was used to separate patients into two categories. Further analysis performed with varying cut-off points yielded no changes in the data trend (data not shown). As is demonstrated in Figure 3.20A, Mcl-1 expression did not predict any change in patient survival as the survival curves demonstrated a high degree of overlap. Kaplan Meier curves were also generated using the IHC data for phospho-Elk-1, phospho-Erk1/2, ErbB1, ErbB2 and ErbB3. The results of these analyses are shown in Figure 3.20B-F; however, none of the measured proteins demonstrated a statistically significant change in overall survival. ErbB2 expression did demonstrate divergence in the survival curves with high ErbB2 expression exhibiting reduced survival; however, the difference only bordered upon statistical significance (Figure 3.20E).

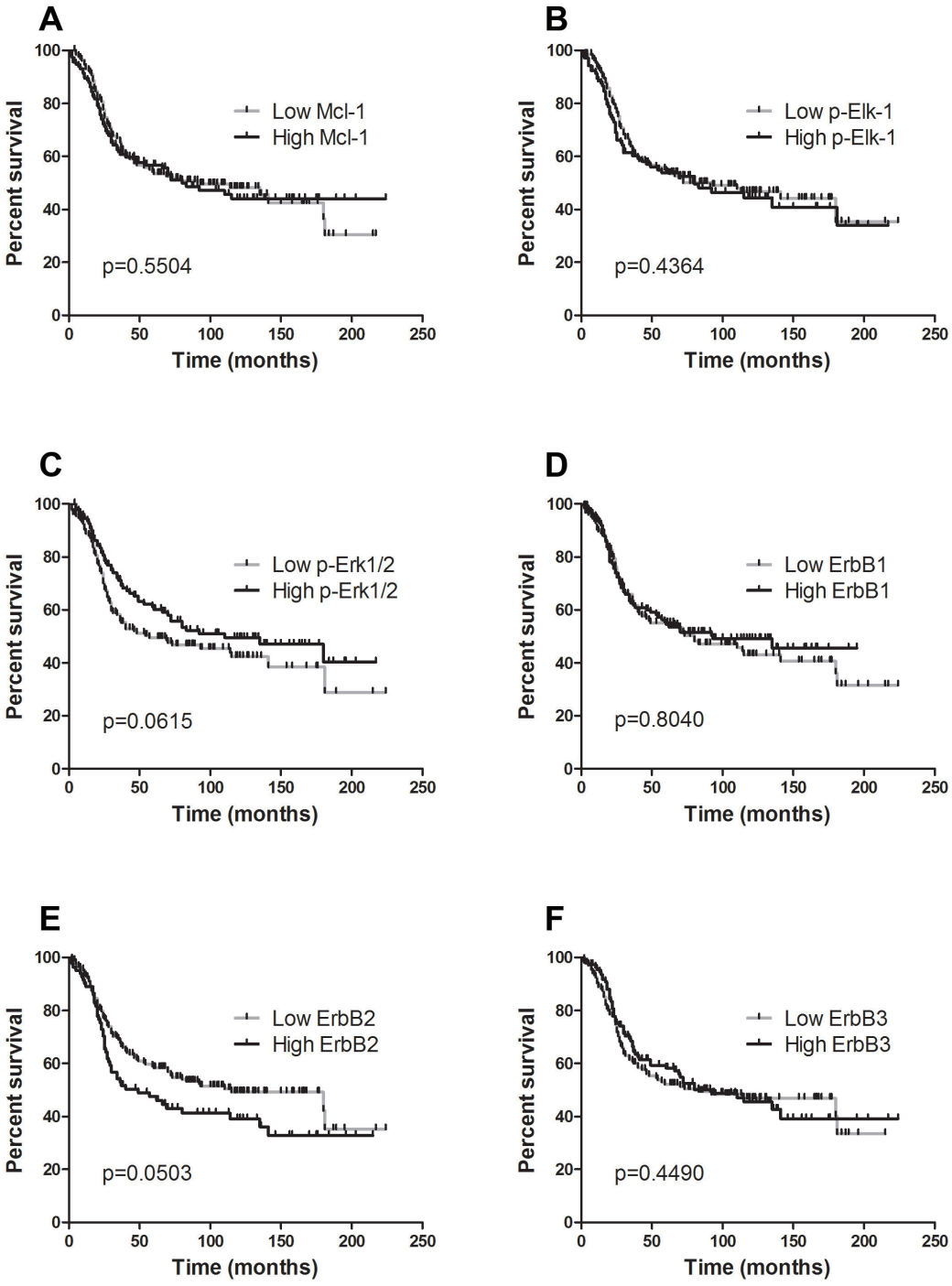

Figure 3.20 Mcl-1 does not correlate with overall breast cancer survival
Patients were separated into Low or High categories based on the median H-score. Kaplan Meier curves were generated to assess whether any parameters associated with a significant change in patient survival. p-values were calculated by the Gehan-Breslow-Wilcoxon test. (A) Survival analysis of low and high Mcl-1 expressing tumours. (B) Survival analysis of low and high p-Elk-1 expressing tumours. (C) Survival analysis of low and high p-Erk1/2 expressing tumours. (D) Survival analysis of low and high ErbB1 expressing tumours. (E) Survival analysis of low and high ErbB2 expressing tumours. (F) Survival analysis of low and high ErbB3 expressing tumours.

3.5.6 Mcl-1 does not correlate with disease progression

In order to determine if Mcl-1 or any of the other proteins measured by IHC could predict trends in progression free survival the data were further analyzed. Patients were separated based on median H-scores and progression free survival was plotted as a Kaplan-Meier curve. Progression was defined as disease recurrence, tumour metastasis or death due to the disease. The earliest progression time-point was used for each patient. Progression free survival demonstrated similar trends as overall survival with little differences observed in all variables with the exception of ErbB2 (Figure 3.21A-D, F). While ErbB2 did not statistically correlate with overall survival, in the case of progression free survival, ErbB2 expression did predict a poorer outcome (Figure 3.21E). The data in Sections 3.5.5 and 3.5.6 demonstrate that Mcl-1 expression alone was not able to be a predictive marker for patient prognosis. Furthermore, all variables, with the exception of ErbB2, expression did not associate with any statistically significant changes in overall survival or progression free survival. This indicates that in the panel of tumours assessed, Mcl-1 is not a valuable prognostic indicator.

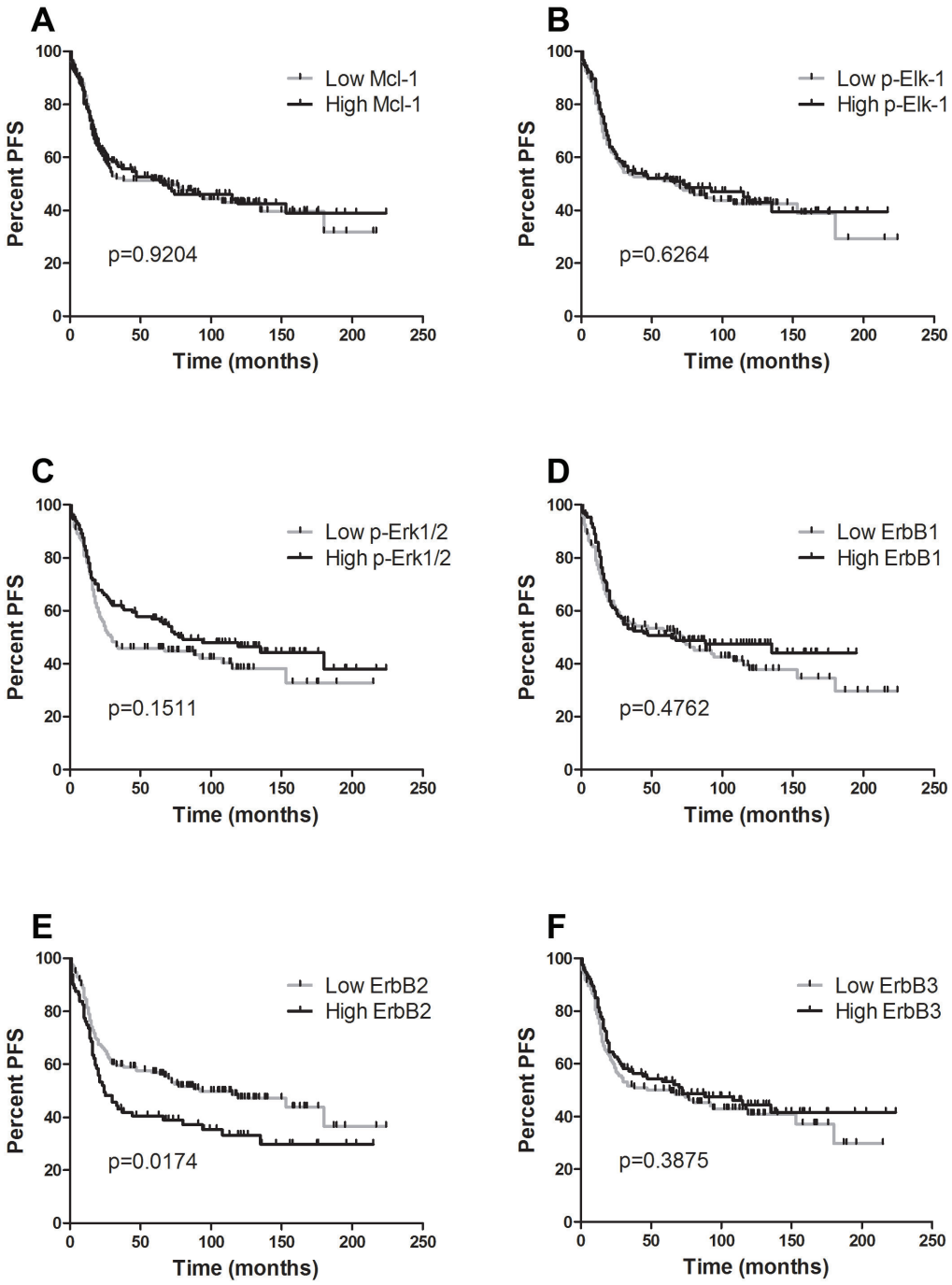

Figure 3.21 Mcl-1 does not correlate with disease progression
Patients were separated into Low or High categories based on the median H-score for each antibody. Kaplan Meier curves were generated to assess whether any parameters associated with a significant change in progression-free survival (PFS). p-values were calculated by the Gehan-Breslow-Wilcoxon test. (A) Comparison of PFS between low and high Mcl-1 expressing tumours. (B) Comparison of PFS between low and high p-Elk-1 expressing tumours. (C Comparison of PFS between low and high p-Erk1/2 expressing tumours. (D) Comparison of PFS between low and high ErbB1 expressing tumours. (E) Comparison of PFS between low and high ErbB2 expressing tumours. (F) Comparison of PFS between low and high ErbB3 expressing tumours.

3.5.7 Summary

The data in this section support the following conclusions:

- Mcl-1 expression positively correlates with the activation level of Elk-1 and Erk1/2 in ERα negative breast tumours
- Mcl-1 correlates with ErbB1 and ErbB2 expression and strongly correlates with the expression of ErbB3
- Mcl-1 is not a prognostic factor in terms of overall survival or progression free survival in the assessed patient cohort

These data support the concepts identified in cell line models in terms of the regulation of Mcl-1 protein expression. These results increase the relevance of the *in-vitro* data as the identified pathways have predictive value on protein expression levels within breast tumours. Further studies will be needed to determine the predictive value of Mcl-1 over-expression on patient prognosis. The TMA data from this study do not demonstrate a significant association of Mcl-1 with poor patient prognosis or reduced survival. The TMA data do, however, confirm the predictive value of ErbB2 with regard to patient survival.

Chapter 4 Discussion

The EGF receptors, particularly ErbB1 and ErbB2 are over expressed or mutated in many tumour types (380). While ErbB2 has been most extensively studied in the context of breast cancer, where it is amplified in as many as 25% of breast cancers, ErbB2 expression also contributes to the pathogenesis of many other tumour types including ovarian, bladder, gastric, colon, lung and head and neck tumours (381). ErbB2 over-expression is observed in several breast cancer subtypes including the Her2+ subtype as well as Luminal B and basal-like tumours. In the Luminal B subtype up to 50% of tumours are ErbB2+ (25). ErbB1 over-expression is even more widespread than ErbB2 and is observed at high levels in a significant proportion of nearly every solid tumour type. ErbB1 expression is frequently observed in both the basal subtype and triple-negative breast cancer, two similar and highly aggressive categories of breast cancer that have poor clinical outcome. In triple negative breast cancer ErbB1 over-expression is seen in more than 50% of cases. Under both classifications, ErbB1 expression is a poor prognostic indicator (382-383) . ErbB1 is also mutated in many cancers, with the most common mutation being the EGFR vIII mutation that causes deletion of a large portion of the extracellular domain of the receptor. This mutation is frequently observed in gliomas as well as breast, lung and ovarian cancer (384). While less studied, expression of the ErbB3 and ErbB4 receptors have also been assessed and found to be similar to that of ErbB1 (381). In breast, pancreatic and bladder tumours ErbB3 is associated with poorer prognosis while ErbB4 generally correlated with a favourable outcome in breast cancer (381, 385). While combined ErbB1 and ErbB2 over-

expression is not common in breast cancer (386), ErbB3 and ErbB2 co-expression is frequent (387) and some studies suggest that ErbB3 is critical for ErbB2 mediated tumour cell proliferation (388). Assessment of receptor expression in breast cancer shows that over-expression of the EGF receptors occurs in the breast cancer subtypes that are generally high grade and have the highest risk of recurrence (18-19, 24-25). As such, these receptors and there downstream signalling pathways are critical for the targeted treatment of breast cancer survival mechanisms.

Through this study we wished to examine the downstream signals that allow receptors of the EGF family to promote cell survival and confer resistance to apoptosis. We had previously reported an association of increased Mcl-1 expression with Her-2 amplification in breast tumours (369). We have demonstrated that stimulation of our cell line models with EGF results in resistance to etoposide and doxorubicin induced apoptosis and have also shown that knock-down of Mcl-1 with siRNA alone is sufficient to induce high levels of apoptosis. These results establish Mcl-1 as a critical survival factor in breast cancer. As knock-down alone was sufficient to induce levels of apoptosis comparable to treatment with doxorubicin or etoposide, it suggests a minimum threshold expression level of Mcl-1 is necessary for the cells to remain viable. These data alone identify Mcl-1 as a potential therapeutic target in breast cancer. Furthermore, transfection of the Mcl-1 cDNA protected the cells both against apoptosis induced by transfection toxicity as well as by etoposide treatment. This suggests that Mcl-1 expression levels may be predictive of a response to treatment. To expand the clinical relevance of these results, the pan-Bcl-2 family inhibitor GX15-070 demonstrated a reduction in cell viability when treated as a single agent. GX15-070 is currently undergoing clinical trials in several

tumour types including CLL (347) and lung cancer (349), but has only been tested in the context of breast cancer using cell line models (345-346). In these studies GX15-070 demonstrated synergistic activity when combined with the tyrosine kinase inhibitor lapatinib in sensitive cells and was able to overcome lapatinib resistance in tyrosine kinase inhibitor resistant cells. This agrees with prior studies implicating Mcl-1 over-expression in Lapatinib resistance (389) and lends support to the future use of Mcl-1 inhibitors in the treatment of breast cancer.

The connection between stimulation of the EGF receptors and increased expression of Mcl-1 has not been extensively studied to date. Increases in Mcl-1 in response to stimulation with EGF ligand or receptor over expression has been identified in non-small cell lung cancer (327), colorectal cancer (390) and esophageal cancer cells (313). In breast cancer we, and others, have demonstrated a correlation between ErbB2 amplification and increased expression of Mcl-1 (250, 369); however, a comprehensive study has not been performed in this context until now. The results of Figure 3.3 confirm the previous correlative studies and strongly support a causative link between activation of the EGF receptors and increased Mcl-1 protein expression. In both MCF-7 and SK-BR-3 cells, two cell lines of a differing molecular profile, EGF induced an increase in Mcl-1 transcription and translation with very similar kinetics. In addition to this, the cloned Mcl-1 promoter demonstrated a 3-4 fold increase in activity in cells that had been treated with EGF. These results provide strong evidence that downstream signalling from the EGF receptors is changing the presence or activation status of transcription factors at the Mcl-1 promoter.

While the measurement of total Mcl-1 mRNA by semi-quantitative real-time PCR was not a direct assessment of gene transcription these experiments did indicate that EGF induced significant increases in Mcl-1 mRNA within 30 minutes of stimulation. Reporter gene assays with the Mcl-1 promoter strengthen the argument that the change in Mcl-1 mRNA is primarily due to increases in Mcl-1 gene transcription. These experiments could have been further validated by assessing changes in Mcl-1 mRNA and protein levels in the presence of Actinomycin D, a known blocking agent of transcription (391). This experiment would have provided evidence as to the role of alterations in protein and mRNA stability in the observed induction by EGF. Furthermore, nuclear run-on assays could have been employed to directly measure the production of new Mcl-1 transcripts and would have been a strong confirmation of the importance in regulation of transcription by EGF. Nonetheless, the observed increases in Mcl-1 mRNA, coupled with measurements of promoter activity by luciferase assay and transcription factor knock-down experiments present convincing evidence that modulation of gene transcription is an important factor in Mcl-1 protein regulation by the EGF receptors. While alterations in both protein and mRNA stability are likely to play a contributing role to the total Mcl-1 protein expression level they were not a subject of focus for this study.

As signalling from the estrogen receptor ERα is highly important in a subset of breast tumours for cell proliferation and cell survival (152), we wished to determine if increased transcription of Mcl-1 was a point of convergence of both EGF and estrogen mediated signalling pathways. In addition to this, cross-talk between the two pathways is frequently reported in the literature. Estrogen has been demonstrated to activate growth factor receptors and downstream signalling pathways through signalling via GPR30 and

pathways downstream of EGF receptor activation can result in ligand independent activity of the estrogen receptor through post-translational modifications (156, 392). Estrogen has also been shown to activate the serum response element within the c-fos promoter in MCF-7 cells through Erk activation of Elk-1 (393). Therefore we hypothesized that Mcl-1 may mediate part of the survival response of both pathways. While correlative studies had previously not demonstrated an association between Mcl-1 and the estrogen receptor status of breast tumours (252, 394), it is quite possible that a molecule regulated by multiple survival signalling pathways would still be elevated in the ERα negative subset of breast tumours. Our data, however, demonstrated no discernible changes in Mcl-1 protein expression in response to stimulation of an ERα positive cell line with estrogen. Although assessment of the Mcl-1 mRNA did show a statistically significant increase 6 hours after estrogen stimulation, the change in messenger RNA over time followed very different kinetics than the known estrogen responsive gene PS2. In addition, because the Mcl-1 promoter did not demonstrate a change in activity following estrogen stimulation, it seems likely from this study that any changes observed at the mRNA level are secondary effects that can be attributed to global changes in gene expression and pathway activation following reintroduction of estrogen to estrogen and serum starved cells. Nonetheless the subtle changes observed suggest that Mcl-1 does not play as significant a role in the survival benefit elicited by activation of the estrogen receptor.

Cloning and large scale deletions made in the Mcl-1 promoter narrowed down the necessary promoter elements to a small sequence proximal to the transcription start site of the Mcl-1 gene. Reduction of the promoter down to a 200 bp fragment reduced the

total promoter activity by approximately 10-fold; however the fragment was still responsive to EGF and induced promoter activity by a similar fold-change as observed in the larger promoter clones. This small fragment contained a region of high identity discovered between the human and mouse Mcl-1 promoters and also the critical transcription factor binding sites. *In silico* promoter analysis identified four transcription factor binding sites contained in a region that spanned -202 to -163 bp upstream of the translation start site. Site-directed deletions made in this region greatly reduced but did not entirely eliminate both basal and EGF induced activity of the promoter. This same promoter region was identified by others as critical for TPA responsiveness of the Mcl-1 gene (314-315).

While in this study key promoter elements were detected by performing site-directed deletion of 7 base pair stretches within the Mcl-1 promoter, base substitutions rather than deletions could have been an alternative approach. While both deletions and substitutions have the ability to remove key DNA recognition elements and disrupt the secondary structure of the promoter, deletions have the added possibility of changing the relative proximity of DNA bound transcriptional regulators and the transcriptional machinery at the core promoter, which may have an impact on transcription that is difficult to predict. The advantage of performing deletions instead of substitutions when searching for unknown transcriptional regulatory sequences is that there is some difficulty in determining which bases to substitute when disrupting an unknown sequence element. Furthermore, the GC rich nature and highly repetitive sequences found within the Mcl-1 promoter impeded PCR. An increase in the denaturation temperature and length was necessary to successfully perform the site directed deletions, which employ

primers containing only complementary DNA. In all likelihood these challenges would have been exacerbated in attempts to perform substitution mutagenesis. Regardless, targeted substitutions to disrupt predicted transcription factor binding sites within the regions defined as critical by the site-directed deletion experiments would have increased the strength of the mutagenesis experiments; however, the subsequent knock-down and inhibitor studies combined with both the chromatin immunoprecipitation and streptavidin pull-down assays clearly identified the transcription factors binding to and controlling the Mcl-1 promoter.

As a Stat binding site was identified as the highest scoring transcription factor binding site in the region of interest and because previous studies had linked Stat3 to Mcl-1 expression in breast cancer, we investigated the role of Stat3 in the EGF mediated up-regulation of Mcl-1. Stat3 has been implicated in the regulation of Mcl-1 in response to varied stimuli in several other cell types including macrophages (305), cholangiocarcinoma cells (303) and lymphoma cells (306). Additionally, because the Jak/Stat pathway is activated by the EGF receptors it seemed a reasonable starting point. Our data, however, did not support the involvement of Stat3 in the regulation of Mcl-1. Despite decreases in Mcl-1 expression observed when cells were treated with the Stat3 inhibitor JSI-124 (cucurbitacin I), Stat3 knock-down did not alter the ability of EGF to induce Mcl-1 and did not reduce basal expression levels. JSI-124 has previously been described to have a negative impact on Mcl-1 expression (395). Our data suggest a non-specific mechanism by which JSI-124 negatively impacts Mcl-1 gene transcription and therefore implies caution in the interpretation of the impact of this natural compound on signalling pathways in the cell. The previously published study by Hsieh et al. that

identified a correlation between Mcl-1 and activated Stat3 in breast cancer was based on correlation of immunohistochemistry stains on breast tissue microarrays (307). Thus, our data does not conflict with the observation that Mcl-1 expression correlates with phosphorylated Stat3; however, based upon our analysis we would conclude that the correlation found in the previous study is not due to direct action of Stat3 on the Mcl-1 promoter.

The remaining potential transcription factor binding sites within the region of interest included an NF-κB binding site, an Elk-1 binding site and an SRF binding site (CarG box). A previous study demonstrated that NF-κB regulated Mcl-1 expression in response to EGF receptor activation in HEK293 and NIH3T3 cells (375), and work in unrelated cell lines had implicated both Elk-1 and SRF (314). Therefore, further analysis of the Mcl-1 promoter was performed using small molecule inhibitors and siRNA mediated knock-down. The results of Figure 3.8 clearly demonstrate involvement of Erk signalling and the transcription factor Elk-1 in the regulation of Mcl-1. In the context of breast cancer, EGF mediated regulation of the Mcl-1 gene appears to be independent of the transcription factors NF-κB and Stat3. The Ras-MAPK pathway and Elk-1 are also demonstrated to be activated efficiently in both MCF-7 cells and SK-BR-3 cells following stimulation with EGF. The previously mentioned study demonstrating NF-κB involvement in EGF regulated expression of Mcl-1 used two unrelated cell lines one of which has an ambiguous origin (HEK293) (396) and the other of which is a murine cell line (NIH3T3). Furthermore, the evidence for NF-κB involvement is indirect with demonstration that ΔIκB transfection or over-expression of a kinase-dead version of

AKT reduced Mcl-1 expression as measured by Western blot (375). Thus, it is possible that cell line specific variations contribute to the contrary findings or that the previous study was not rigorous enough to conclude the involvement of NF-κB.

The involvement of Elk-1 agreed well with previous studies that demonstrated that Elk-1 and SRF bind to a serum response element in the Mcl-1 promoter in response to TPA stimulation (314-315). Based upon these studies, it was expected that knock-down of SRF would impact Mcl-1 gene expression; however, SRF knock-down in both MCF-7 and SK-BR-3 cells failed to demonstrate any significant reduction in basal or induced levels of both the Mcl-1 protein and mRNA. This result was unexpected as site directed deletions within the CarG box negatively impacted the EGF induced activity of the Mcl-1 promoter luciferase reporter construct. The impact of these deletions was not as great as was seen with deletion of the Elk-1 binding site and there was only a slight reduction of basal promoter activity. It is possible that deletions in close proximity to the Elk-1 binding site altered the DNA secondary structure and therefore negatively impacted the affinity of Elk-1 for its DNA binding element. Whether the non-requirement of SRF is specific to breast cancer cell lines remains to be determined. The results have nevertheless demonstrated that activation of Elk-1 via the Ras/Raf/Mek/Erk signalling cascade is of critical importance in the up-regulation of Mcl-1 by the EGF receptors. These data suggest that inhibition of this pathway to prevent Mcl-1 up-regulation may block the survival benefit conferred by EGF through Mcl-1. Blockage of Mek/Erk signalling or treatment with small molecule inhibitors of Mcl-1 in breast tumours where Mcl-1 levels are highly expressed may be sufficient to trigger cells to undergo apoptosis or may be beneficial in sensitizing cells to chemotherapy induced apoptosis.

Further confirmation of the importance of Elk-1 was obtained by chromatin immunoprecipitation assays. Elk-1 bound to the Mcl-1 promoter under unstimulated, serum starved conditions. This indicates that Elk-1 is capable of binding to its site within the Mcl-1 promoter in the unphosphorylated state. Additionally, SRF was not detected on the Mcl-1 promoter under unstimulated conditions. This suggests that Elk-1 is not binding to the serum response element through a classical mechanism as is observed on the c-Fos promoter where Elk-1 is recruited by an activated SRF (121). The presence of Elk-1 in the absence of EGF stimulation and SRF suggests that Elk-1 is able to bind autonomously to the Mcl-1 promoter. Stimulation with EGF did not result in an increase of Elk-1 at the promoter within 10 minutes; however within 10 minutes SRF was detected by ChIP. It is possible therefore that activated Elk-1 recruits SRF to the Mcl-1 promoter; however SRF may not be required for recruitment of histone acetyl transferases and the basal transcriptional machinery. The recruitment of SRF by Elk-1 has been demonstrated by others at the promoters of other genes including nurr77 and pip92 (397).

The ChIP data were supported by streptavidin pull-down assays performed with a 50 bp probe designed to match the sequence of the region of interest. Both Elk-1 and SRF bound to this probe. The kinetics of the pull-down following EGF stimulation matched the ChIP data quite well, but the changes over time did not correlate between the two assays for SRF. This is likely due to the *in vitro* nature of the assay, the decreasing trend of SRF binding may be due to the decreased availability of the protein for the assay following stimulation if the bulk of the SRF becomes bound to chromatin and insoluble.

These results raise the possibility that another transcription factor is binding in concert with Elk-1 to this region or is compensating for SRF when it is knocked down. The other possibility, which is less likely based on the complete loss of the SRF specific band in Western blots, is that the knock-down efficiency was not great enough to significantly impact Mcl-1 expression. A third possibility is that Elk-1 binds autonomously on the Mcl-1 promoter and the close proximity deletions within the CarG box impacted the binding efficiency of Elk-1 to its DNA element or altered the conformation of the promoter and inhibited formation of the pre-initiation complex. Regardless of the ambiguous role of SRF, the results clearly identify Elk-1 as a critical transcription factor in the regulation of the Mcl-1 gene. While only loss of function experiments were performed to assess the impact of Elk-1 and SRF on Mcl-1 promoter activity (siRNA mediated knock-down and inhibitor studies) further information could have been gleaned by performing the converse gain of function assays with over-expression of both transcription factors individually or together. The difficulty with both gain and loss of function experiments in this context is that it is difficult to assess the specificity of the result. Over-expression or knock-down of Elk-1 potentially alters the expression of a large number of genes that may themselves impact Mcl-1 transcription as well as protein and mRNA stability. The direct binding of these transcription factors to the Mcl-1 promoter as determined by chromatin immunoprecipitation and streptavidin pull-down assays offer the strongest evidence of their direct involvement in Mcl-1 gene transcription.

The Ras-MAPK pathway is frequently deregulated in human tumours. Activating mutations in Ras, Raf and Mek are all means by which tumour cells exhibit sustained

activation of the pathway. Signalling mediated by Mek/Erk plays an important role in breast cancer progression (398-399). Although Ras mutations are infrequently observed in breast cancer, increased activation of the protein is detectable in a substantial proportion of tumours (398, 400). The basal-like subset of breast tumours has been identified as particularly susceptible to Mek inhibition (401). Furthermore, the Mek inhibitor PD0325901 is effective as a single agent as well as in combination with inhibitors of PI3K in murine xenograft models of basal-like breast cancer (402). Recently, the use of Mek inhibitors has demonstrated success in overcoming resistance to the tyrosine kinase inhibitor lapatinib (403-404).

We have demonstrated that the Mek inhibitor U0126 as well as an Erk inhibitor efficiently prevent downstream signalling from this pathway. Inhibitors of Mek/Erk block the activating phosphorylation of Elk-1 and completely prevent up-regulation of Mcl-1 by EGF. Our results point to the possibility that inhibitors of the Mek/Erk signalling cascade can be effective at blocking the expression of Mcl-1 in breast cancer. Furthermore, we have demonstrated that prevention of Mek/Erk signalling is sufficient to completely block EGF mediated resistance to chemotherapeutic drug induced apoptosis. This in good agreement with other studies that implicate Mek/Erk signalling rather than PI3K/Akt signalling as critical for the survival response mediated by EGF (313). Mek and Erk activation is frequently seen in tumours that have developed resistance to EGF receptor targeted therapies. Several studies have demonstrated effectiveness of Mek inhibition in tumours that rely on EGFR or Her-2 signalling (405). Our data suggest that downstream inhibition of Mek/Erk may be as effective as direct targeting of the EGF receptors and furthermore may be able to overcome resistance to monoclonal antibody

and tyrosine kinase inhibitor based therapies. This concept has already been demonstrated in the context of breast cancer where resistance to Lapatinib was reversed through Mek inhibition (406). As Mcl-1 is an anti-apoptotic protein downstream of this pathway, direct targeting of Mcl-1 with small molecule inhibitors may prove to be effective in the context of hyper-activated Ras-MAPK signalling.

Our work is in good agreement with prior studies that demonstrate the importance of Mek/Erk signalling and Mcl-1 in EGF receptor mediated survival responses and in resistance to EGF targeted therapies (313, 346, 389, 404, 407). In addition to this, a large body of literature exists that strongly implicates the PI3K/AKT signalling pathway as the primary mediator of resistance to targeted therapies such as Lapatinib and Trastuzumab (8, 408). In cell line models of lung cancer, resistance to Lapatinib was mediated through upregulation of the insulin-like growth factor receptor 1 (IGF1R), which activated the PI3K/Akt pathway through transphosphorylation of ErbB3 (409). Combined inhibition of EGFR and IGF1R prevented the outgrowth of resistant cells (408). Furthermore, other studies demonstrated that IGF1R signalling through both the PI3K and MAPK pathways was sufficient to completely compensate for loss of Her2 (410). Despite this convincing data from cell line models, IGF1R expression did not show any value as an indicator of a clinical response to trastuzumab or Lapatinib (411-412). Increased expression of the Met receptor is another mechanism of resistance to tyrosine kinase inhibitors that has been identified in cell line models. The Met receptor mediated resistance through activation of ErbB3 and subsequent downstream PI3K/Akt signalling. Met expression was also observed in 25% of tumours resistant to the TKI Gefitinib demonstrated Met gene amplification (413). Additional modes of resistance to EGF receptor targeted therapies

includes loss of PTEN expression as well as activating mutations in the p110 catalytic subunit of PI3K; however, both of these biomarkers served as poor prognostic indicators of trastuzumab resistance in clinical studies (8). While conflicting data exists as to the exact mechanisms by which resistance to these targeted therapies develops, response to EGF receptor targeting agents has been shown to correlate with their ability to reduce Her2, Raf, Akt and Erk phosphorylation (414). It is almost certain that resistance mechanisms involve activation of multiple survival signalling pathways including both the Mek/Erk pathway as well as the PI3K/Akt pathway. As this work has shown that in breast cancer, Mcl-1 transcription is regulated by the Mek/Erk pathway and as others have shown that Mcl-1 translation and mRNA/proteins stability can be regulated by the PI3K/Akt pathway (316) it is quite likely that increased expression of Mcl-1 contributes to resistance under a variety of mechanistic scenarios.

In order to expand the relevance of this study, the data acquired in cell line models were used as the basis for correlative studies in breast tumour samples. An initial pilot study of 26 breast tumours confirmed a positive association between high levels of nuclear phosphorylated Elk-1 and increased Mcl-1 expression. This trend was the basis for the broader tissue microarray study of 255 breast tumour samples. The primary aim of this study was to determine if the identification of Elk-1 as a critical regulator of Mcl-1 expression in cell line models would translate into a positive association between activated levels of Elk-1 and increased Mcl-1 expression. While Elk-1 expression and prognostic significance has not been extensively studied in breast cancer, expression of related Ets family members has been assessed and found to correlate with disease progression (415) and to be indicative of poor prognosis (416). Our data demonstrated a

significant correlation between p-Elk-1 and Mcl-1 expression, supporting the *in-vitro* assays that implicated Elk-1 in the regulation of Mcl-1.

In addition to Mcl-1 and phospho-Elk-1, the TMAs were scored for expression of phospho-Erk1/2, ErbB1, ErbB2 and ErbB3. Over-expression of Erk1/2 and increased phosphorylation status has previously been studied in breast cancer. Increased levels of Mek and Erk have been observed in breast tumours compared to normal tissues and expression found to be higher in the ERα negative subset (398, 417). Erk1/2 expression alone was not found to have any prognostic significance. In contrast, expression of phospho-Erk1/2 was found by one group to associate with low frequency of recurrence, early stage, negative nodal status and improved survival (418). We also found a correlation, albeit weaker, between phospho-Erk1/2 and Mcl-1. It is not unexpected that the correlation was weaker, as Erk1/2 are one step removed from Mcl-1 transcriptional regulation. High levels of phospho-Erk1/2 in the absence of strong Elk-1 expression would therefore not result in increased Mcl-1 transcription and may explain some of the outliers. We also demonstrated a strong correlation between phospho-Erk1/2 and phospho-Elk-1, which was expected as Elk-1 is a target substrate of activated Erk1/2.

Associations between expression of the EGF receptors and Mcl-1 were also assessed. Mcl-1 was found to correlate with ErbB1 and ErbB2; however, the data for ErbB1 were not significant by Spearman correlation. This is in general agreement with previous reports demonstrating a relationship between receptor status and Mcl-1 expression. In the case of ErbB3 the association was much stronger as measured by both a Mann-Whitney test and Spearman correlation. This association is a novel discovery, as

to our knowledge no previous studies have investigated the relationship between ErbB3 expression and Mcl-1. ErbB3 lacks intrinsic kinase activity but is a dimerization partner and phosphorylation substrate for ErbB2. ErbB3 is emerging as an increasingly important protein in terms of cancer initiation and progression (419). ErbB3 expression is also associated with resistance to ErbB1 and ErbB2 targeted therapies in both lung and breast cancer (55, 388). While ErbB3 is reported to preferentially signal through the PI3K/Akt pathway (420), it remains yet to be determined if ErbB3 is responsible for the up-regulation of Mcl-1 via Mek/Erk phosphorylation of Elk-1.

Moving beyond correlative studies of IHC scores, the expression level of each protein studied was examined in the context of clinical data provided by the Manitoba Breast Tumour Bank. The clinical data did not show any significant impact of Mcl-1, phospho-Elk-1 or phospho-Erk1/2 on survival or progression free survival. The survival curve for phospho-Erk1/2 did suggest that increased phospho-Erk1/2 correlated with improved survival; however, these data did not make the criteria to be statistically significant ($p=.0615$). This trend is in agreement with a previous study that examined Erk1/2 phosphorylation by Western blot and IHC in a panel of breast tumours and found that high levels of phospho-Erk1/2 associated with a favourable prognosis (418). The survival curves for ErbB1 and ErbB3 did not demonstrate any statistically significant deviations between patients with tumours expressing low or high levels of each receptor. That expression of ErbB2, on the other hand, was a stronger prognostic indicator. Patients with high levels of ErbB2 demonstrated poorer overall survival, a result that bordered upon significance with a p-value of 0.0503. In the case of progression free survival the

data were more clear with elevated expression correlating with poorer prognosis that was statistically significant.

While several measured parameters did not have any prognostic value for the patients represented by the TMA this result does not exclude the possibility that a more structured analysis would yield significant data. The patients whose samples made up the TMA were not standardized according to treatment type or tumour stage or grade at the time of biopsy. Additionally, the tumours analyzed were only segregated based on the ERα expression status as measured by ligand binding assay. Further studies with a more uniform sample set may uncover a prognostic significance of Mcl-1 that was not readily identifiable within our patient cohort.

Our data suggest that up-regulation of Mcl-1 via the Ras/Raf/Mek/Erk signalling cascade may be an important factor in the survival benefit conferred by over-expression of the EGF receptors. This pathway has been targeted in a number of disease contexts with inhibitors of the upstream signalling molecules including both Ras and Mek inhibitors (83, 96). Furthermore Mcl-1 is currently being targeted through the use of small molecule Bcl-2 family inhibitors such as GX15070 (341, 347, 349). Over-expression of Bcl-2 family members is a key feature distinguishing normal and cancer cells (421). Correlation of both Bcl-2 and Bcl-X_L with favourable prognosis suggests that other anti-apoptotic Bcl-2 family members may be necessary to provide apoptosis resistance in breast cancer (246, 248). While Mcl-1 may be an attractive therapeutic target, it's wide ranging expression in normal cells and critical roles in the development of both the nervous and immune system suggest that efficient targeting of Mcl-1 may

result in side effects (296). Current studies with GX15070 have demonstrated a high degree of tolerance and therefore bode well for the potential use of other Mcl-1 targeting approaches including modulators of Mcl-1 splicing (347, 349). As Mcl-1 is often expressed at low levels and expression only increased as specific stages of a cells' development, the side effects may be restricted to certain cell types and only under certain stages of differentiation (296). It is possible that factors that shift the splicing preference of Mcl-1 may be ineffective in the context of normal Mcl-1 expression levels. In this case the increase in the pro-apoptotic splice variant expression level may not be high enough to induce apoptosis. This raises the possibility that these types of treatments may be restricted to cells expressing aberrantly high levels of full length Mcl-1 and raises hope that these treatments may have a good degree of specificity. Furthermore the concept that tumour cells by their very nature elicit apoptotic signals that must be neutralized (421) suggests that Bcl-2 family inhibition may result in tumour cell specific induction of apoptosis. As these types of approaches are in their infancy have only be studied in cell line models it remains to be seen if the desired effects will pan out in animal models or future clinical trials.

Chapter 5 Final Conclusions

This study establishes that Mcl-1 is a direct target of the Ras/Raf/Mek/Erk signalling pathway following activation of the EGF receptors by stimulation with EGF ligand. Mcl-1 is transcriptionally up-regulated through phosphorylation of the transcription factor Elk-1 by active Erk1/2. Both Mcl-1 and EGF receptor activation offer a protective advantage to tumour cells and increase resistance to chemotherapeutic drug induced cell death. Prevention of Mcl-1 up-regulation by blocking Mek/Erk activation of Elk-1 reverses the protective effect of EGF ligand (Figure 5.1). This study establishes the relationship between the EGF receptor family and the pro-survival Bcl-2 family member Mcl-1, outlines the molecular mechanisms by which this relationship is mediated, and confirms Mcl-1 as a prospective therapeutic target in breast cancer.

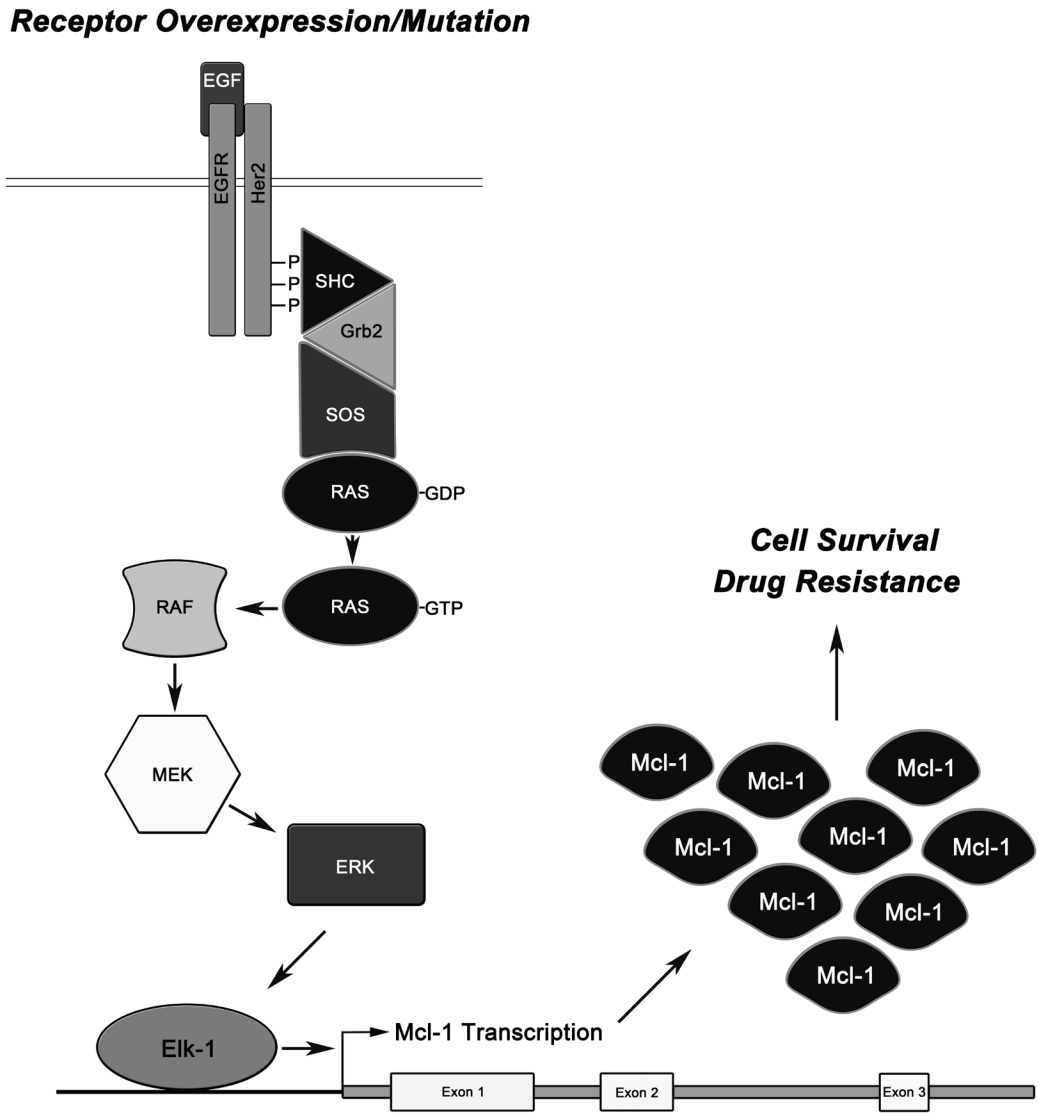

Figure 5.1 Summary of Mcl-1 regulation in breast cancer

This study establishes that the Ras-Raf-Mek-Erk signalling cascade activated downstream from the EGF receptors increases transcription of the Mcl-1 gene through phosphorylation of the transcription factor Elk-1. Increased expression of Mcl-1 leads to enhanced cell survival and resistance to chemotherapeutic drugs.

Chapter 6 Future Directions

Data from this study identifies several areas of future research. While all experiments were performed by stimulation of cultured cells with EGF ligand, the role of each member of the EGF receptor family was not directly assessed. IHC data from the TMA demonstrated a strong association between Mcl-1 and the EGF receptor ErbB3. ErbB3 has been reported to play an important role in breast cancer as well as other tumour types. Further analysis of the degree to which each receptor of the EGF receptor family contributes to the up-regulation of Mcl-1 would expand upon this study and narrow down a subset of tumours where Mcl-1 may be transcriptionally increased. These experiments could be performed by knocking down ErbB3 expression and assessing the impact on EGF stimulated Mcl-1 protein levels in both MCF-7 and SK-BR-3 cells.

This study raised questions as to the involvement of the transcription factor SRF in the regulation of Mcl-1. The data indicated that although SRF bound to the Mcl-1 promoter as determined by ChIP and pull-down assays, knock-down of SRF failed to effect Mcl-1 transcription. Further study could be aimed at delineating the role, if any, SRF plays in the transcriptional regulation of the Mcl-1 gene. Furthermore, if other transcription factors are compensating in the absence of SRF the nature of these interactions could be investigated. Additional transcription factors binding to the region of interest could be identified by performing streptavidin pull-down assays with the biotin labelled Mcl-1 promoter probe under stringent conditions and identifying the bound proteins by proteomics approaches.

The tumour samples from the microarray study demonstrated correlations that supported the in-vitro cell line experiments. The clinical data from these patient samples, however, did not demonstrate any prognostic value with regards to Mcl-1. Additionally, as the literature is ambiguous as to the prognostic significance of Mcl-1, future studies using a more stringently defined patient cohort may aid in the assessment of the prognostic significant of Mcl-1 over-expression. A TMA with patients receiving standardized treatment would shed further light in this regard. RNA or protein could also be measured from samples from patients enrolled in a clinical trial where treatment is uniform. Assessment of Mcl-1 expression by measurement of mRNA levels or protein expression by Western blot would also allow for differentiation between Mcl-1 splice variants providing greater understanding than can be assessed by immunohistochemistry. The significance of Mcl-1 may also need to be assessed in the context of a complete understanding of the expression status of all anti-apoptotic Bcl-2 family members within the patient samples.

Chapter 7 References

1. Canadian Cancer Society's Steering Committee: Canadian Cancer Statistics 2009. Toronto: Canadian Cancer Society, 2009. April 2009, ISSN 0835-2976.
2. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000; 100: 57-70.
3. Lazebnik Y. What are the hallmarks of cancer? Nat Rev Cancer; 10: 232-3.
4. The Canadian Cancer Encyclopedia. 2010 [cited 2010; Available from: http://info.cancer.ca/cce-ecc/
5. Romond EH, Perez EA, Bryant J, et al. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. N Engl J Med 2005; 353: 1673-84.
6. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. N Engl J Med 2005; 353: 1659-72.
7. Ward HW. Anti-oestrogen therapy for breast cancer: a trial of tamoxifen at two dose levels. Br Med J 1973; 1: 13-4.
8. Browne BC, O'Brien N, Duffy MJ, Crown J, O'Donovan N. HER-2 signaling and inhibition in breast cancer. Curr Cancer Drug Targets 2009; 9: 419-38.
9. Honrado E, Benitez J, Palacios J. The molecular pathology of hereditary breast cancer: genetic testing and therapeutic implications. Mod Pathol 2005; 18: 1305-20.
10. Gudmundsdottir K, Ashworth A. The roles of BRCA1 and BRCA2 and associated proteins in the maintenance of genomic stability. Oncogene 2006; 25: 5864-74.
11. Stratton MR, Rahman N. The emerging landscape of breast cancer susceptibility. Nat Genet 2008; 40: 17-22.
12. Tan DS, Marchio C, Reis-Filho JS. Hereditary breast cancer: from molecular pathology to tailored therapies. J Clin Pathol 2008; 61: 1073-82.
13. Weyandt J, Ellsworth RE, Hooke JA, Shriver CD, Ellsworth DL. Environmental chemicals and breast cancer risk--a structural chemistry perspective. Curr Med Chem 2008; 15: 2680-701.
14. Salehi F, Turner MC, Phillips KP, Wigle DT, Krewski D, Aronson KJ. Review of the etiology of breast cancer with special attention to organochlorines as potential endocrine disruptors. J Toxicol Environ Health B Crit Rev 2008; 11: 276-300.
15. Brody JG, Rudel RA, Michels KB, et al. Environmental pollutants, diet, physical activity, body size, and breast cancer: where do we stand in research to identify opportunities for prevention? Cancer 2007; 109: 2627-34.
16. Narod SA. Modifiers of risk of hereditary breast cancer. Oncogene 2006; 25: 5832-6.
17. Key TJ, Verkasalo PK, Banks E. Epidemiology of breast cancer. Lancet Oncol 2001; 2: 133-40.
18. Perou CM, Sorlie T, Eisen MB, et al. Molecular portraits of human breast tumours. Nature 2000; 406: 747-52.
19. Sorlie T, Perou CM, Tibshirani R, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 2001; 98: 10869-74.
20. Ellis IO, Galea M, Broughton N, Locker A, Blamey RW, Elston CW. Pathological prognostic factors in breast cancer. II. Histological type. Relationship with survival in a large study with long-term follow-up. Histopathology 1992; 20: 479-89.

21. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 1991; 19: 403-10.
22. Weigelt B, Reis-Filho JS. Histological and molecular types of breast cancer: is there a unifying taxonomy? Nat Rev Clin Oncol 2009; 6: 718-30.
23. Tavassoeli FA, Devilee, P. Pathology and Genetics Tumours of the Breast and Female Genital Organs. Lyon: Lyon Press; 2003.
24. Peppercorn J, Perou CM, Carey LA. Molecular subtypes in breast cancer evaluation and management: divide and conquer. Cancer Invest 2008; 26: 1-10.
25. Carey LA, Perou CM, Livasy CA, et al. Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. JAMA 2006; 295: 2492-502.
26. Dowsett M, Dunbier AK. Emerging biomarkers and new understanding of traditional markers in personalized therapy for breast cancer. Clin Cancer Res 2008; 14: 8019-26.
27. Maughan KL, Lutterbie MA, Ham PS. Treatment of breast cancer. Am Fam Physician 2010; 81: 1339-46.
28. Aubel-Sadron G, Londos-Gagliardi D. Daunorubicin and doxorubicin, anthracycline antibiotics, a physicochemical and biological review. Biochimie 1984; 66: 333-52.
29. Keizer HG, Pinedo HM, Schuurhuis GJ, Joenje H. Doxorubicin (adriamycin): a critical review of free radical-dependent mechanisms of cytotoxicity. Pharmacol Ther 1990; 47: 219-31.
30. Nitiss JL. DNA topoisomerase II and its growing repertoire of biological functions. Nat Rev Cancer 2009; 9: 327-37.
31. Nitiss JL. Targeting DNA topoisomerase II in cancer chemotherapy. Nat Rev Cancer 2009; 9: 338-50.
32. Murphy CG, Seidman AD. Evolving approaches to metastatic breast cancer previously treated with anthracyclines and taxanes. Clin Breast Cancer 2009; 9 Suppl 2: S58-65.
33. Montecucco A, Biamonti G. Cellular response to etoposide treatment. Cancer Lett 2007; 252: 9-18.
34. Rowinsky EK. The development and clinical utility of the taxane class of antimicrotubule chemotherapy agents. Annu Rev Med 1997; 48: 353-74.
35. Herbst RS. Review of epidermal growth factor receptor biology. Int J Radiat Oncol Biol Phys 2004; 59: 21-6.
36. Bazley LA, Gullick WJ. The epidermal growth factor receptor family. Endocr Relat Cancer 2005; 12 Suppl 1: S17-27.
37. Stein RA, Staros JV. Evolutionary analysis of the ErbB receptor and ligand families. J Mol Evol 2000; 50: 397-412.
38. Burgess AW, Cho HS, Eigenbrot C, et al. An open-and-shut case? Recent insights into the activation of EGF/ErbB receptors. Mol Cell 2003; 12: 541-52.
39. Hynes NE, Horsch K, Olayioye MA, Badache A. The ErbB receptor tyrosine family as signal integrators. Endocr Relat Cancer 2001; 8: 151-9.
40. Heldin CH. Dimerization of cell surface receptors in signal transduction. Cell 1995; 80: 213-23.
41. Lemmon MA, Schlessinger J. Regulation of signal transduction and signal diversity by receptor oligomerization. Trends Biochem Sci 1994; 19: 459-63.
42. Gan HK, Kaye AH, Luwor RB. The EGFRvIII variant in glioblastoma multiforme. J Clin Neurosci 2009; 16: 748-54.
43. Gazdar AF. Activating and resistance mutations of EGFR in non-small-cell lung cancer: role in clinical response to EGFR tyrosine kinase inhibitors. Oncogene 2009; 28 Suppl 1: S24-31.

44. Hubbard SR, Till JH. Protein tyrosine kinase structure and function. Annu Rev Biochem 2000; 69: 373-98.
45. Kuriyan J, Cowburn D. Modular peptide recognition domains in eukaryotic signaling. Annu Rev Biophys Biomol Struct 1997; 26: 259-88.
46. Scaltriti M, Baselga J. The epidermal growth factor receptor pathway: a model for targeted therapy. Clin Cancer Res 2006; 12: 5268-72.
47. Carpenter G, Ji Q. Phospholipase C-gamma as a signal-transducing element. Exp Cell Res 1999; 253: 15-24.
48. Lin SY, Makino K, Xia W, et al. Nuclear localization of EGF receptor and its potential new role as a transcription factor. Nat Cell Biol 2001; 3: 802-8.
49. Lo HW, Hsu SC, Hung MC. EGFR signaling pathway in breast cancers: from traditional signal transduction to direct nuclear translocalization. Breast Cancer Res Treat 2006; 95: 211-8.
50. Lo HW, Xia W, Wei Y, Ali-Seyed M, Huang SF, Hung MC. Novel prognostic value of nuclear epidermal growth factor receptor in breast cancer. Cancer Res 2005; 65: 338-48.
51. Lo HW, Hsu SC, Ali-Seyed M, et al. Nuclear interaction of EGFR and STAT3 in the activation of the iNOS/NO pathway. Cancer Cell 2005; 7: 575-89.
52. Wang SC, Lien HC, Xia W, et al. Binding at and transactivation of the COX-2 promoter by nuclear tyrosine kinase receptor ErbB-2. Cancer Cell 2004; 6: 251-61.
53. Nanda R. Targeting the human epidermal growth factor receptor 2 (HER2) in the treatment of breast cancer: recent advances and future directions. Rev Recent Clin Trials 2007; 2: 111-6.
54. Chrysogelos SA, Dickson RB. EGF receptor expression, regulation, and function in breast cancer. Breast Cancer Res Treat 1994; 29: 29-40.
55. Sithanandam G, Anderson LM. The ERBB3 receptor in cancer and cancer gene therapy. Cancer Gene Ther 2008; 15: 413-48.
56. Sundvall M, Iljin K, Kilpinen S, Sara H, Kallioniemi OP, Elenius K. Role of ErbB4 in breast cancer. J Mammary Gland Biol Neoplasia 2008; 13: 259-68.
57. Johnston JB, Navaratnam S, Pitz MW, et al. Targeting the EGFR pathway for cancer therapy. Curr Med Chem 2006; 13: 3483-92.
58. Booy EP, Johar D, Maddika S, et al. Monoclonal and bispecific antibodies as novel therapeutics. Arch Immunol Ther Exp (Warsz) 2006; 54: 85-101.
59. Garnock-Jones KP, Keating GM, Scott LJ. Trastuzumab: A review of its use as adjuvant treatment in human epidermal growth factor receptor 2 (HER2)-positive early breast cancer. Drugs; 70: 215-39.
60. Lurje G, Lenz HJ. EGFR signaling and drug discovery. Oncology 2009; 77: 400-10.
61. Tevaarwerk AJ, Kolesar JM. Lapatinib: a small-molecule inhibitor of epidermal growth factor receptor and human epidermal growth factor receptor-2 tyrosine kinases used in the treatment of breast cancer. Clin Ther 2009; 31 Pt 2: 2332-48.
62. Nahta R, Esteva FJ. HER2 therapy: molecular mechanisms of trastuzumab resistance. Breast Cancer Res 2006; 8: 215.
63. Lievre A, Bachet JB, Le Corre D, et al. KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. Cancer Res 2006; 66: 3992-5.
64. Di Nicolantonio F, Martini M, Molinari F, et al. Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer. J Clin Oncol 2008; 26: 5705-12.
65. Sartore-Bianchi A, Martini M, Molinari F, et al. PIK3CA mutations in colorectal cancer are associated with clinical resistance to EGFR-targeted monoclonal antibodies. Cancer Res 2009; 69: 1851-7.

66. Viloria-Petit A, Crombet T, Jothy S, et al. Acquired resistance to the antitumor effect of epidermal growth factor receptor-blocking antibodies in vivo: a role for altered tumor angiogenesis. Cancer Res 2001; 61: 5090-101.
67. Nagata Y, Lan KH, Zhou X, et al. PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. Cancer Cell 2004; 6: 117-27.
68. Valabrega G, Montemurro F, Sarotto I, et al. TGFalpha expression impairs Trastuzumab-induced HER2 downregulation. Oncogene 2005; 24: 3002-10.
69. Kruser TJ, Wheeler DL. Mechanisms of resistance to HER family targeting antibodies. Exp Cell Res; 316: 1083-100.
70. Shattuck DL, Miller JK, Carraway KL, 3rd, Sweeney C. Met receptor contributes to trastuzumab resistance of Her2-overexpressing breast cancer cells. Cancer Res 2008; 68: 1471-7.
71. Sergina NV, Rausch M, Wang D, et al. Escape from HER-family tyrosine kinase inhibitor therapy by the kinase-inactive HER3. Nature 2007; 445: 437-41.
72. Yoon S, Seger R. The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. Growth Factors 2006; 24: 21-44.
73. Ramos JW. The regulation of extracellular signal-regulated kinase (ERK) in mammalian cells. Int J Biochem Cell Biol 2008; 40: 2707-19.
74. Lawrence MC, Jivan A, Shao C, et al. The roles of MAPKs in disease. Cell Res 2008; 18: 436-42.
75. Uhlik MT, Abell AN, Cuevas BD, Nakamura K, Johnson GL. Wiring diagrams of MAPK regulation by MEKK1, 2, and 3. Biochem Cell Biol 2004; 82: 658-63.
76. Kolch W. Coordinating ERK/MAPK signalling through scaffolds and inhibitors. Nat Rev Mol Cell Biol 2005; 6: 827-37.
77. Zebisch A, Czernilofsky AP, Keri G, Smigelskaite J, Sill H, Troppmair J. Signaling through RAS-RAF-MEK-ERK: from basics to bedside. Curr Med Chem 2007; 14: 601-23.
78. Hancock JF. Ras proteins: different signals from different locations. Nat Rev Mol Cell Biol 2003; 4: 373-84.
79. Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell 2000; 103: 211-25.
80. Gamblin SJ, Smerdon SJ. GTPase-activating proteins and their complexes. Curr Opin Struct Biol 1998; 8: 195-201.
81. Dhillon AS, Hagan S, Rath O, Kolch W. MAP kinase signalling pathways in cancer. Oncogene 2007; 26: 3279-90.
82. Schubbert S, Shannon K, Bollag G. Hyperactive Ras in developmental disorders and cancer. Nat Rev Cancer 2007; 7: 295-308.
83. Downward J. Targeting RAS signalling pathways in cancer therapy. Nat Rev Cancer 2003; 3: 11-22.
84. Lobell RB, Omer CA, Abrams MT, et al. Evaluation of farnesyl:protein transferase and geranylgeranyl:protein transferase inhibitor combinations in preclinical models. Cancer Res 2001; 61: 8758-68.
85. Zebisch A, Troppmair J. Back to the roots: the remarkable RAF oncogene story. Cell Mol Life Sci 2006; 63: 1314-30.
86. Wellbrock C, Karasarides M, Marais R. The RAF proteins take centre stage. Nat Rev Mol Cell Biol 2004; 5: 875-85.
87. Daum G, Eisenmann-Tappe I, Fries HW, Troppmair J, Rapp UR. The ins and outs of Raf kinases. Trends Biochem Sci 1994; 19: 474-80.

88. Chong H, Vikis HG, Guan KL. Mechanisms of regulating the Raf kinase family. Cell Signal 2003; 15: 463-9.
89. Kolch W. Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. Biochem J 2000; 351 Pt 2: 289-305.
90. Stancato LF, Chow YH, Hutchison KA, Perdew GH, Jove R, Pratt WB. Raf exists in a native heterocomplex with hsp90 and p50 that can be reconstituted in a cell-free system. J Biol Chem 1993; 268: 21711-6.
91. Fantl WJ, Muslin AJ, Kikuchi A, et al. Activation of Raf-1 by 14-3-3 proteins. Nature 1994; 371: 612-4.
92. Avruch J, Khokhlatchev A, Kyriakis JM, et al. Ras activation of the Raf kinase: tyrosine kinase recruitment of the MAP kinase cascade. Recent Prog Horm Res 2001; 56: 127-55.
93. Rushworth LK, Hindley AD, O'Neill E, Kolch W. Regulation and role of Raf-1/B-Raf heterodimerization. Mol Cell Biol 2006; 26: 2262-72.
94. Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. Nature 2002; 417: 949-54.
95. Garnett MJ, Rana S, Paterson H, Barford D, Marais R. Wild-type and mutant B-RAF activate C-RAF through distinct mechanisms involving heterodimerization. Mol Cell 2005; 20: 963-9.
96. Beeram M, Patnaik A, Rowinsky EK. Raf: a strategic target for therapeutic development against cancer. J Clin Oncol 2005; 23: 6771-90.
97. Pearson G, Robinson F, Beers Gibson T, et al. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev 2001; 22: 153-83.
98. Zheng CF, Guan KL. Activation of MEK family kinases requires phosphorylation of two conserved Ser/Thr residues. EMBO J 1994; 13: 1123-31.
99. Fremin C, Meloche S. From basic research to clinical development of MEK1/2 inhibitors for cancer therapy. J Hematol Oncol; 3: 8.
100. Seger R, Ahn NG, Posada J, et al. Purification and characterization of mitogen-activated protein kinase activator(s) from epidermal growth factor-stimulated A431 cells. J Biol Chem 1992; 267: 14373-81.
101. Wu X, Noh SJ, Zhou G, Dixon JE, Guan KL. Selective activation of MEK1 but not MEK2 by A-Raf from epidermal growth factor-stimulated Hela cells. J Biol Chem 1996; 271: 3265-71.
102. Xu S, Khoo S, Dang A, et al. Differential regulation of mitogen-activated protein/ERK kinase (MEK)1 and MEK2 and activation by a Ras-independent mechanism. Mol Endocrinol 1997; 11: 1618-25.
103. Marks JL, Gong Y, Chitale D, et al. Novel MEK1 mutation identified by mutational analysis of epidermal growth factor receptor signaling pathway genes in lung adenocarcinoma. Cancer Res 2008; 68: 5524-8.
104. Murugan AK, Dong J, Xie J, Xing M. MEK1 mutations, but not ERK2 mutations, occur in melanomas and colon carcinomas, but none in thyroid carcinomas. Cell Cycle 2009; 8: 2122-4.
105. Estep AL, Palmer C, McCormick F, Rauen KA. Mutation analysis of BRAF, MEK1 and MEK2 in 15 ovarian cancer cell lines: implications for therapy. PLoS One 2007; 2: e1279.
106. LoRusso PM, Krishnamurthi SS, Rinehart JJ, et al. Phase I pharmacokinetic and pharmacodynamic study of the oral MAPK/ERK kinase inhibitor PD-0325901 in patients with advanced cancers. Clin Cancer Res; 16: 1924-37.
107. Boulton TG, Nye SH, Robbins DJ, et al. ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell 1991; 65: 663-75.

108. Pages G, Guerin S, Grall D, et al. Defective thymocyte maturation in p44 MAP kinase (Erk 1) knockout mice. Science 1999; 286: 1374-7.
109. Saba-El-Leil MK, Vella FD, Vernay B, et al. An essential function of the mitogen-activated protein kinase Erk2 in mouse trophoblast development. EMBO Rep 2003; 4: 964-8.
110. Lloyd AC. Distinct functions for ERKs? J Biol 2006; 5: 13.
111. Gonzalez FA, Raden DL, Davis RJ. Identification of substrate recognition determinants for human ERK1 and ERK2 protein kinases. J Biol Chem 1991; 266: 22159-63.
112. Gille H, Sharrocks AD, Shaw PE. Phosphorylation of transcription factor p62TCF by MAP kinase stimulates ternary complex formation at c-fos promoter. Nature 1992; 358: 414-7.
113. Ohori M, Kinoshita T, Okubo M, et al. Identification of a selective ERK inhibitor and structural determination of the inhibitor-ERK2 complex. Biochem Biophys Res Commun 2005; 336: 357-63.
114. Burkhard K, Smith S, Deshmukh R, MacKerell AD, Jr., Shapiro P. Development of extracellular signal-regulated kinase inhibitors. Curr Top Med Chem 2009; 9: 678-89.
115. Chen F, Hancock CN, Macias AT, et al. Characterization of ATP-independent ERK inhibitors identified through in silico analysis of the active ERK2 structure. Bioorg Med Chem Lett 2006; 16: 6281-7.
116. Leprince D, Gegonne A, Coll J, et al. A putative second cell-derived oncogene of the avian leukaemia retrovirus E26. Nature 1983; 306: 395-7.
117. Sharrocks AD. The ETS-domain transcription factor family. Nat Rev Mol Cell Biol 2001; 2: 827-37.
118. Dittmer J, Nordheim A. Ets transcription factors and human disease. Biochim Biophys Acta 1998; 1377: F1-11.
119. Buchwalter G, Gross C, Wasylyk B. Ets ternary complex transcription factors. Gene 2004; 324: 1-14.
120. Treisman R. The serum response element. Trends Biochem Sci 1992; 17: 423-6.
121. Shaw PE, Schroter H, Nordheim A. The ability of a ternary complex to form over the serum response element correlates with serum inducibility of the human c-fos promoter. Cell 1989; 56: 563-72.
122. Sharrocks AD. Complexities in ETS-domain transcription factor function and regulation: lessons from the TCF (ternary complex factor) subfamily. The Colworth Medal Lecture. Biochem Soc Trans 2002; 30: 1-9.
123. Shore P, Sharrocks AD. The transcription factors Elk-1 and serum response factor interact by direct protein-protein contacts mediated by a short region of Elk-1. Mol Cell Biol 1994; 14: 3283-91.
124. Cruzalegui FH, Cano E, Treisman R. ERK activation induces phosphorylation of Elk-1 at multiple S/T-P motifs to high stoichiometry. Oncogene 1999; 18: 7948-57.
125. Jacobs D, Glossip D, Xing H, Muslin AJ, Kornfeld K. Multiple docking sites on substrate proteins form a modular system that mediates recognition by ERK MAP kinase. Genes Dev 1999; 13: 163-75.
126. Janknecht R, Ernst WH, Pingoud V, Nordheim A. Activation of ternary complex factor Elk-1 by MAP kinases. EMBO J 1993; 12: 5097-104.
127. Sharrocks AD. ERK2/p42 MAP kinase stimulates both autonomous and SRF-dependent DNA binding by Elk-1. FEBS Lett 1995; 368: 77-80.
128. Yang SH, Vickers E, Brehm A, Kouzarides T, Sharrocks AD. Temporal recruitment of the mSin3A-histone deacetylase corepressor complex to the ETS domain transcription factor Elk-1. Mol Cell Biol 2001; 21: 2802-14.

129. Boros J, O'Donnell A, Donaldson IJ, Kasza A, Zeef L, Sharrocks AD. Overlapping promoter targeting by Elk-1 and other divergent ETS-domain transcription factor family members. Nucleic Acids Res 2009; 37: 7368-80.
130. Fitzsimmons D, Hodsdon W, Wheat W, Maira SM, Wasylyk B, Hagman J. Pax-5 (BSAP) recruits Ets proto-oncogene family proteins to form functional ternary complexes on a B-cell-specific promoter. Genes Dev 1996; 10: 2198-211.
131. Janknecht R, Nordheim A. MAP kinase-dependent transcriptional coactivation by Elk-1 and its cofactor CBP. Biochem Biophys Res Commun 1996; 228: 831-7.
132. Li QJ, Yang SH, Maeda Y, Sladek FM, Sharrocks AD, Martins-Green M. MAP kinase phosphorylation-dependent activation of Elk-1 leads to activation of the co-activator p300. EMBO J 2003; 22: 281-91.
133. Boyer TG, Martin ME, Lees E, Ricciardi RP, Berk AJ. Mammalian Srb/Mediator complex is targeted by adenovirus E1A protein. Nature 1999; 399: 276-9.
134. Zhang HM, Li L, Papadopoulou N, et al. Mitogen-induced recruitment of ERK and MSK to SRE promoter complexes by ternary complex factor Elk-1. Nucleic Acids Res 2008; 36: 2594-607.
135. Vickers ER, Kasza A, Kurnaz IA, et al. Ternary complex factor-serum response factor complex-regulated gene activity is required for cellular proliferation and inhibition of apoptotic cell death. Mol Cell Biol 2004; 24: 10340-51.
136. Shore P, Sharrocks AD. The MADS-box family of transcription factors. Eur J Biochem 1995; 229: 1-13.
137. Treisman R. Identification and purification of a polypeptide that binds to the c-fos serum response element. EMBO J 1987; 6: 2711-7.
138. Chai J, Tarnawski AS. Serum response factor: discovery, biochemistry, biological roles and implications for tissue injury healing. J Physiol Pharmacol 2002; 53: 147-57.
139. Norman C, Runswick M, Pollock R, Treisman R. Isolation and properties of cDNA clones encoding SRF, a transcription factor that binds to the c-fos serum response element. Cell 1988; 55: 989-1003.
140. Johansen FE, Prywes R. Identification of transcriptional activation and inhibitory domains in serum response factor (SRF) by using GAL4-SRF constructs. Mol Cell Biol 1993; 13: 4640-7.
141. Kemp PR, Metcalfe JC. Four isoforms of serum response factor that increase or inhibit smooth-muscle-specific promoter activity. Biochem J 2000; 345 Pt 3: 445-51.
142. Belaguli NS, Zhou W, Trinh TH, Majesky MW, Schwartz RJ. Dominant negative murine serum response factor: alternative splicing within the activation domain inhibits transactivation of serum response factor binding targets. Mol Cell Biol 1999; 19: 4582-91.
143. Patten LC, Belaguli NS, Baek MJ, Fagan SP, Awad SS, Berger DH. Serum response factor is alternatively spliced in human colon cancer. J Surg Res 2004; 121: 92-100.
144. Zhang X, Azhar G, Huang C, et al. Alternative splicing and nonsense-mediated mRNA decay regulate gene expression of serum response factor. Gene 2007; 400: 131-9.
145. Misra RP, Rivera VM, Wang JM, Fan PD, Greenberg ME. The serum response factor is extensively modified by phosphorylation following its synthesis in serum-stimulated fibroblasts. Mol Cell Biol 1991; 11: 4545-54.
146. Posern G, Treisman R. Actin' together: serum response factor, its cofactors and the link to signal transduction. Trends Cell Biol 2006; 16: 588-96.
147. Arsenian S, Weinhold B, Oelgeschlager M, Ruther U, Nordheim A. Serum response factor is essential for mesoderm formation during mouse embryogenesis. EMBO J 1998; 17: 6289-99.

148. Brandt DT, Xu J, Steinbeisser H, Grosse R. Regulation of myocardin-related transcriptional coactivators through cofactor interactions in differentiation and cancer. Cell Cycle 2009; 8: 2523-7.
149. Park MY, Kim KR, Park HS, et al. Expression of the serum response factor in hepatocellular carcinoma: implications for epithelial-mesenchymal transition. Int J Oncol 2007; 31: 1309-15.
150. Kim HJ, Kim KR, Park HS, et al. The expression and role of serum response factor in papillary carcinoma of the thyroid. Int J Oncol 2009; 35: 49-55.
151. Farra R, Dapas B, Pozzato G, et al. Serum response factor depletion affects the proliferation of the hepatocellular carcinoma cells HepG2 and JHH6. Biochimie; 92: 455-63.
152. Chen GG, Zeng Q, Tse GM. Estrogen and its receptors in cancer. Med Res Rev 2008; 28: 954-74.
153. Safe S, Kim K. Non-classical genomic estrogen receptor (ER)/specificity protein and ER/activating protein-1 signaling pathways. J Mol Endocrinol 2008; 41: 263-75.
154. Filardo EJ. Epidermal growth factor receptor (EGFR) transactivation by estrogen via the G-protein-coupled receptor, GPR30: a novel signaling pathway with potential significance for breast cancer. J Steroid Biochem Mol Biol 2002; 80: 231-8.
155. Filardo E, Quinn J, Pang Y, et al. Activation of the novel estrogen receptor G protein-coupled receptor 30 (GPR30) at the plasma membrane. Endocrinology 2007; 148: 3236-45.
156. Schiff R, Massarweh SA, Shou J, et al. Advanced concepts in estrogen receptor biology and breast cancer endocrine resistance: implicated role of growth factor signaling and estrogen receptor coregulators. Cancer Chemother Pharmacol 2005; 56 Suppl 1: 10-20.
157. Schiff R, Massarweh SA, Shou J, Bharwani L, Mohsin SK, Osborne CK. Cross-talk between estrogen receptor and growth factor pathways as a molecular target for overcoming endocrine resistance. Clin Cancer Res 2004; 10: 331S-6S.
158. Newman SP, Bates NP, Vernimmen D, Parker MG, Hurst HC. Cofactor competition between the ligand-bound oestrogen receptor and an intron 1 enhancer leads to oestrogen repression of ERBB2 expression in breast cancer. Oncogene 2000; 19: 490-7.
159. Campbell RA, Bhat-Nakshatri P, Patel NM, Constantinidou D, Ali S, Nakshatri H. Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for anti-estrogen resistance. J Biol Chem 2001; 276: 9817-24.
160. Robertson JF. Selective oestrogen receptor modulators/new antioestrogens: a clinical perspective. Cancer Treat Rev 2004; 30: 695-706.
161. Abrial C, Mouret-Reynier MA, Cure H, et al. Neoadjuvant endocrine therapy in breast cancer. Breast 2006; 15: 9-19.
162. Keating GM. Letrozole: a review of its use in the treatment of postmenopausal women with hormone-responsive early breast cancer. Drugs 2009; 69: 1681-705.
163. Jordan VC. Antiestrogenic action of raloxifene and tamoxifen: today and tomorrow. J Natl Cancer Inst 1998; 90: 967-71.
164. Prasad S, Ravindran J, Aggarwal BB. NF-kappaB and cancer: how intimate is this relationship. Mol Cell Biochem 2010; 336: 25-37.
165. Wu JT, Kral JG. The NF-kappaB/IkappaB signaling system: a molecular target in breast cancer therapy. J Surg Res 2005; 123: 158-69.
166. Dejardin E, Bonizzi G, Bellahcene A, Castronovo V, Merville MP, Bours V. Highly-expressed p100/p52 (NFKB2) sequesters other NF-kappa B-related proteins in the cytoplasm of human breast cancer cells. Oncogene 1995; 11: 1835-41.

167. Nakshatri H, Bhat-Nakshatri P, Martin DA, Goulet RJ, Jr., Sledge GW, Jr. Constitutive activation of NF-kappaB during progression of breast cancer to hormone-independent growth. Mol Cell Biol 1997; 17: 3629-39.
168. Biswas DK, Iglehart JD. Linkage between EGFR family receptors and nuclear factor kappaB (NF-kappaB) signaling in breast cancer. J Cell Physiol 2006; 209: 645-52.
169. Biswas DK, Shi Q, Baily S, et al. NF-kappa B activation in human breast cancer specimens and its role in cell proliferation and apoptosis. Proc Natl Acad Sci U S A 2004; 101: 10137-42.
170. Jing N, Tweardy DJ. Targeting Stat3 in cancer therapy. Anticancer Drugs 2005; 16: 601-7.
171. Bowman T, Garcia R, Turkson J, Jove R. STATs in oncogenesis. Oncogene 2000; 19: 2474-88.
172. Bromberg JF, Wrzeszczynska MH, Devgan G, et al. Stat3 as an oncogene. Cell 1999; 98: 295-303.
173. Devarajan E, Huang S. STAT3 as a central regulator of tumor metastases. Curr Mol Med 2009; 9: 626-33.
174. Diaz N, Minton S, Cox C, et al. Activation of stat3 in primary tumors from high-risk breast cancer patients is associated with elevated levels of activated SRC and survivin expression. Clin Cancer Res 2006; 12: 20-8.
175. Lin L, Amin R, Gallicano GI, et al. The STAT3 inhibitor NSC 74859 is effective in hepatocellular cancers with disrupted TGF-beta signaling. Oncogene 2009; 28: 961-72.
176. Yue P, Turkson J. Targeting STAT3 in cancer: how successful are we? Expert Opin Investig Drugs 2009; 18: 45-56.
177. Lin L, Hutzen B, Zuo M, et al. Novel STAT3 phosphorylation inhibitors exhibit potent growth-suppressive activity in pancreatic and breast cancer cells. Cancer Res 2010; 70: 2445-54.
178. Fiers W, Beyaert R, Declercq W, Vandenabeele P. More than one way to die: apoptosis, necrosis and reactive oxygen damage. Oncogene 1999; 18: 7719-30.
179. Sun Y, Peng ZL. Programmed cell death and cancer. Postgrad Med J 2009; 85: 134-40.
180. Degterev A, Yuan J. Expansion and evolution of cell death programmes. Nat Rev Mol Cell Biol 2008; 9: 378-90.
181. Leist M, Single B, Castoldi AF, Kuhnle S, Nicotera P. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. J Exp Med 1997; 185: 1481-6.
182. Bonfoco E, Krainc D, Ankarcrona M, Nicotera P, Lipton SA. Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. Proc Natl Acad Sci U S A 1995; 92: 7162-6.
183. Klionsky DJ, Emr SD. Autophagy as a regulated pathway of cellular degradation. Science 2000; 290: 1717-21.
184. Codogno P, Meijer AJ. Autophagy and signaling: their role in cell survival and cell death. Cell Death Differ 2005; 12 Suppl 2: 1509-18.
185. Christofferson DE, Yuan J. Necroptosis as an alternative form of programmed cell death. Curr Opin Cell Biol; 22: 263-8.
186. Galluzzi L, Kroemer G. Necroptosis: a specialized pathway of programmed necrosis. Cell 2008; 135: 1161-3.
187. Cho YS, Park SY, Shin HS, Chan FK. Physiological consequences of programmed necrosis, an alternative form of cell demise. Mol Cells; 29: 327-32.
188. Fadeel B, Orrenius S. Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. J Intern Med 2005; 258: 479-517.

189. Fadok VA, Bratton DL, Frasch SC, Warner ML, Henson PM. The role of phosphatidylserine in recognition of apoptotic cells by phagocytes. Cell Death Differ 1998; 5: 551-62.
190. Kerr JF, Winterford CM, Harmon BV. Apoptosis. Its significance in cancer and cancer therapy. Cancer 1994; 73: 2013-26.
191. Thornberry NA, Lazebnik Y. Caspases: enemies within. Science 1998; 281: 1312-6.
192. Salvesen GS, Riedl SJ. Caspase mechanisms. Adv Exp Med Biol 2008; 615: 13-23.
193. Riedl SJ, Shi Y. Molecular mechanisms of caspase regulation during apoptosis. Nat Rev Mol Cell Biol 2004; 5: 897-907.
194. Fan TJ, Han LH, Cong RS, Liang J. Caspase family proteases and apoptosis. Acta Biochim Biophys Sin (Shanghai) 2005; 37: 719-27.
195. Kischkel FC, Hellbardt S, Behrmann I, et al. Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. EMBO J 1995; 14: 5579-88.
196. Zou H, Li Y, Liu X, Wang X. An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. J Biol Chem 1999; 274: 11549-56.
197. Tinel A, Tschopp J. The PIDDosome, a protein complex implicated in activation of caspase-2 in response to genotoxic stress. Science 2004; 304: 843-6.
198. Bao Q, Shi Y. Apoptosome: a platform for the activation of initiator caspases. Cell Death Differ 2007; 14: 56-65.
199. Shi Y. Caspase activation: revisiting the induced proximity model. Cell 2004; 117: 855-8.
200. Riedl SJ, Fuentes-Prior P, Renatus M, et al. Structural basis for the activation of human procaspase-7. Proc Natl Acad Sci U S A 2001; 98: 14790-5.
201. Fischer U, Janicke RU, Schulze-Osthoff K. Many cuts to ruin: a comprehensive update of caspase substrates. Cell Death Differ 2003; 10: 76-100.
202. Chipuk JE, Moldoveanu T, Llambi F, Parsons MJ, Green DR. The BCL-2 family reunion. Mol Cell; 37: 299-310.
203. Brunelle JK, Letai A. Control of mitochondrial apoptosis by the Bcl-2 family. J Cell Sci 2009; 122: 437-41.
204. Sorenson CM. Bcl-2 family members and disease. Biochim Biophys Acta 2004; 1644: 169-77.
205. Yip KW, Reed JC. Bcl-2 family proteins and cancer. Oncogene 2008; 27: 6398-406.
206. Danial NN. BCL-2 family proteins: critical checkpoints of apoptotic cell death. Clin Cancer Res 2007; 13: 7254-63.
207. van Delft MF, Huang DC. How the Bcl-2 family of proteins interact to regulate apoptosis. Cell Res 2006; 16: 203-13.
208. Tsujimoto Y, Cossman J, Jaffe E, Croce CM. Involvement of the bcl-2 gene in human follicular lymphoma. Science 1985; 228: 1440-3.
209. Kroemer G, Reed JC. Mitochondrial control of cell death. Nat Med 2000; 6: 513-9.
210. Lomonosova E, Chinnadurai G. BH3-only proteins in apoptosis and beyond: an overview. Oncogene 2008; 27 Suppl 1: S2-19.
211. Kutuk O, Letai A. Regulation of Bcl-2 family proteins by posttranslational modifications. Curr Mol Med 2008; 8: 102-18.
212. Wajant H. Death receptors. Essays Biochem 2003; 39: 53-71.
213. Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. Science 1998; 281: 1305-8.
214. Guicciardi ME, Gores GJ. Life and death by death receptors. FASEB J 2009; 23: 1625-37.

215. Mahmood Z, Shukla Y. Death receptors: targets for cancer therapy. Exp Cell Res; 316: 887-99.
216. Debatin KM, Krammer PH. Death receptors in chemotherapy and cancer. Oncogene 2004; 23: 2950-66.
217. Igney FH, Krammer PH. Tumor counterattack: fact or fiction? Cancer Immunol Immunother 2005; 54: 1127-36.
218. Igney FH, Krammer PH. Immune escape of tumors: apoptosis resistance and tumor counterattack. J Leukoc Biol 2002; 71: 907-20.
219. Chen L, Park SM, Tumanov AV, et al. CD95 promotes tumour growth. Nature 2010; 465: 492-6.
220. Chan FK, Chun HJ, Zheng L, Siegel RM, Bui KL, Lenardo MJ. A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. Science 2000; 288: 2351-4.
221. Tartaglia LA, Ayres TM, Wong GH, Goeddel DV. A novel domain within the 55 kd TNF receptor signals cell death. Cell 1993; 74: 845-53.
222. Schneider P, Tschopp J. Apoptosis induced by death receptors. Pharm Acta Helv 2000; 74: 281-6.
223. Sartorius U, Schmitz I, Krammer PH. Molecular mechanisms of death-receptor-mediated apoptosis. Chembiochem 2001; 2: 20-9.
224. Yin XM. Bid, a BH3-only multi-functional molecule, is at the cross road of life and death. Gene 2006; 369: 7-19.
225. Wang S, El-Deiry WS. TRAIL and apoptosis induction by TNF-family death receptors. Oncogene 2003; 22: 8628-33.
226. Papenfuss K, Cordier SM, Walczak H. Death receptors as targets for anti-cancer therapy. J Cell Mol Med 2008; 12: 2566-85.
227. French LE, Tschopp J. The TRAIL to selective tumor death. Nat Med 1999; 5: 146-7.
228. Bonavida B, Ng CP, Jazirehi A, Schiller G, Mizutani Y. Selectivity of TRAIL-mediated apoptosis of cancer cells and synergy with drugs: the trail to non-toxic cancer therapeutics (review). Int J Oncol 1999; 15: 793-802.
229. Kruyt FA. TRAIL and cancer therapy. Cancer Lett 2008; 263: 14-25.
230. Saelens X, Festjens N, Vande Walle L, van Gurp M, van Loo G, Vandenabeele P. Toxic proteins released from mitochondria in cell death. Oncogene 2004; 23: 2861-74.
231. Hengartner MO. The biochemistry of apoptosis. Nature 2000; 407: 770-6.
232. Shimizu S, Narita M, Tsujimoto Y. Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature 1999; 399: 483-7.
233. Shore GC. Apoptosis: it's BAK to VDAC. EMBO Rep 2009; 10: 1311-3.
234. Galluzzi L, Kroemer G. Mitochondrial apoptosis without VDAC. Nat Cell Biol 2007; 9: 487-9.
235. Cain K, Bratton SB, Langlais C, et al. Apaf-1 oligomerizes into biologically active approximately 700-kDa and inactive approximately 1.4-MDa apoptosome complexes. J Biol Chem 2000; 275: 6067-70.
236. Susin SA, Lorenzo HK, Zamzami N, et al. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 1999; 397: 441-6.
237. Li LY, Luo X, Wang X. Endonuclease G is an apoptotic DNase when released from mitochondria. Nature 2001; 412: 95-9.
238. Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell 2000; 102: 33-42.

239. Suzuki Y, Imai Y, Nakayama H, Takahashi K, Takio K, Takahashi R. A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. Mol Cell 2001; 8: 613-21.
240. Kang MH, Reynolds CP. Bcl-2 inhibitors: targeting mitochondrial apoptotic pathways in cancer therapy. Clin Cancer Res 2009; 15: 1126-32.
241. Tse C, Shoemaker AR, Adickes J, et al. ABT-263: a potent and orally bioavailable Bcl-2 family inhibitor. Cancer Res 2008; 68: 3421-8.
242. Vogler M, Dinsdale D, Dyer MJ, Cohen GM. Bcl-2 inhibitors: small molecules with a big impact on cancer therapy. Cell Death Differ 2009; 16: 360-7.
243. Krajewski S, Krajewska M, Turner BC, et al. Prognostic significance of apoptosis regulators in breast cancer. Endocr Relat Cancer 1999; 6: 29-40.
244. Johnston SR, MacLennan KA, Sacks NP, Salter J, Smith IE, Dowsett M. Modulation of Bcl-2 and Ki-67 expression in oestrogen receptor-positive human breast cancer by tamoxifen. Eur J Cancer 1994; 30A: 1663-9.
245. Teixeira C, Reed JC, Pratt MA. Estrogen promotes chemotherapeutic drug resistance by a mechanism involving Bcl-2 proto-oncogene expression in human breast cancer cells. Cancer Res 1995; 55: 3902-7.
246. Joensuu H, Pylkkanen L, Toikkanen S. Bcl-2 protein expression and long-term survival in breast cancer. Am J Pathol 1994; 145: 1191-8.
247. Callagy GM, Webber MJ, Pharoah PD, Caldas C. Meta-analysis confirms BCL2 is an independent prognostic marker in breast cancer. BMC Cancer 2008; 8: 153.
248. Krajewski S, Krajewska M, Shabaik A, et al. Immunohistochemical analysis of in vivo patterns of Bcl-X expression. Cancer Res 1994; 54: 5501-7.
249. Ding Q, He X, Xia W, et al. Myeloid cell leukemia-1 inversely correlates with glycogen synthase kinase-3beta activity and associates with poor prognosis in human breast cancer. Cancer Res 2007; 67: 4564-71.
250. Petry IB, Fieber E, Schmidt M, et al. ERBB2 induces an antiapoptotic expression pattern of Bcl-2 family members in node-negative breast cancer. Clin Cancer Res 2010; 16: 451-60.
251. Krajewski S, Bodrug S, Krajewska M, et al. Immunohistochemical analysis of Mcl-1 protein in human tissues. Differential regulation of Mcl-1 and Bcl-2 protein production suggests a unique role for Mcl-1 in control of programmed cell death in vivo. Am J Pathol 1995; 146: 1309-19.
252. O'Driscoll L, Cronin D, Kennedy SM, et al. Expression and prognostic relevance of Mcl-1 in breast cancer. Anticancer Res 2004; 24: 473-82.
253. Gil J, Yamamoto H, Zapata JM, Reed JC, Perucho M. Impairment of the proapoptotic activity of Bax by missense mutations found in gastrointestinal cancers. Cancer Res 1999; 59: 2034-7.
254. Krajewski S, Blomqvist C, Franssila K, et al. Reduced expression of proapoptotic gene BAX is associated with poor response rates to combination chemotherapy and shorter survival in women with metastatic breast adenocarcinoma. Cancer Res 1995; 55: 4471-8.
255. Kozopas KM, Yang T, Buchan HL, Zhou P, Craig RW. MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. Proc Natl Acad Sci U S A 1993; 90: 3516-20.
256. Reynolds JE, Yang T, Qian L, et al. Mcl-1, a member of the Bcl-2 family, delays apoptosis induced by c-Myc overexpression in Chinese hamster ovary cells. Cancer Res 1994; 54: 6348-52.
257. Zhou P, Qian L, Kozopas KM, Craig RW. Mcl-1, a Bcl-2 family member, delays the death of hematopoietic cells under a variety of apoptosis-inducing conditions. Blood 1997; 89: 630-43.

258. Zhou P, Qian L, Bieszczad CK, et al. Mcl-1 in transgenic mice promotes survival in a spectrum of hematopoietic cell types and immortalization in the myeloid lineage. Blood 1998; 92: 3226-39.
259. Akgul C. Mcl-1 is a potential therapeutic target in multiple types of cancer. Cell Mol Life Sci 2009; 66: 1326-36.
260. Warr MR, Shore GC. Unique biology of Mcl-1: therapeutic opportunities in cancer. Curr Mol Med 2008; 8: 138-47.
261. Rinkenberger JL, Horning S, Klocke B, Roth K, Korsmeyer SJ. Mcl-1 deficiency results in peri-implantation embryonic lethality. Genes Dev 2000; 14: 23-7.
262. Opferman JT, Iwasaki H, Ong CC, et al. Obligate role of anti-apoptotic MCL-1 in the survival of hematopoietic stem cells. Science 2005; 307: 1101-4.
263. Opferman JT, Letai A, Beard C, Sorcinelli MD, Ong CC, Korsmeyer SJ. Development and maintenance of B and T lymphocytes requires antiapoptotic MCL-1. Nature 2003; 426: 671-6.
264. Craig RW, Jabs EW, Zhou P, et al. Human and mouse chromosomal mapping of the myeloid cell leukemia-1 gene: MCL1 maps to human chromosome 1q21, a region that is frequently altered in preneoplastic and neoplastic disease. Genomics 1994; 23: 457-63.
265. Atkin NB. Chromosome 1 aberrations in cancer. Cancer Genet Cytogenet 1986; 21: 279-85.
266. Gendler SJ, Cohen EP, Craston A, Duhig T, Johnstone G, Barnes D. The locus of the polymorphic epithelial mucin (PEM) tumour antigen on chromosome 1q21 shows a high frequency of alteration in primary human breast tumours. Int J Cancer 1990; 45: 431-5.
267. Akgul C, Turner PC, White MR, Edwards SW. Functional analysis of the human MCL-1 gene. Cell Mol Life Sci 2000; 57: 684-91.
268. Pietrzak M, Puzianowska-Kuznicka M. p53-dependent repression of the human MCL-1 gene encoding an anti-apoptotic member of the BCL-2 family: the role of Sp1 and of basic transcription factor binding sites in the MCL-1 promoter. Biol Chem 2008; 389: 383-93.
269. Michels J, Johnson PW, Packham G. Mcl-1. Int J Biochem Cell Biol 2005; 37: 267-71.
270. Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. Genes Dev 1999; 13: 1899-911.
271. Petros AM, Olejniczak ET, Fesik SW. Structural biology of the Bcl-2 family of proteins. Biochim Biophys Acta 2004; 1644: 83-94.
272. Yang T, Kozopas KM, Craig RW. The intracellular distribution and pattern of expression of Mcl-1 overlap with, but are not identical to, those of Bcl-2. J Cell Biol 1995; 128: 1173-84.
273. Rogers S, Wells R, Rechsteiner M. Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. Science 1986; 234: 364-8.
274. Bingle CD, Craig RW, Swales BM, Singleton V, Zhou P, Whyte MK. Exon skipping in Mcl-1 results in a bcl-2 homology domain 3 only gene product that promotes cell death. J Biol Chem 2000; 275: 22136-46.
275. Bae J, Leo CP, Hsu SY, Hsueh AJ. MCL-1S, a splicing variant of the antiapoptotic BCL-2 family member MCL-1, encodes a proapoptotic protein possessing only the BH3 domain. J Biol Chem 2000; 275: 25255-61.
276. Kim JH, Sim SH, Ha HJ, Ko JJ, Lee K, Bae J. MCL-1ES, a novel variant of MCL-1, associates with MCL-1L and induces mitochondrial cell death. FEBS Lett 2009; 583: 2758-64.
277. Kim MH. Protein phosphatase 1 activation and alternative splicing of Bcl-X and Mcl-1 by EGCG + ibuprofen. J Cell Biochem 2008; 104: 1491-9.

278. Shieh JJ, Liu KT, Huang SW, Chen YJ, Hsieh TY. Modification of alternative splicing of Mcl-1 pre-mRNA using antisense morpholino oligonucleotides induces apoptosis in basal cell carcinoma cells. J Invest Dermatol 2009; 129: 2497-506.
279. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 2005; 120: 15-20.
280. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009; 136: 215-33.
281. Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet 2008; 9: 102-14.
282. Mott JL, Kobayashi S, Bronk SF, Gores GJ. mir-29 regulates Mcl-1 protein expression and apoptosis. Oncogene 2007; 26: 6133-40.
283. Crawford M, Batte K, Yu L, et al. MicroRNA 133B targets pro-survival molecules MCL-1 and BCL2L2 in lung cancer. Biochem Biophys Res Commun 2009; 388: 483-9.
284. Subramaniam D, Natarajan G, Ramalingam S, et al. Translation inhibition during cell cycle arrest and apoptosis: Mcl-1 is a novel target for RNA binding protein CUGBP2. Am J Physiol Gastrointest Liver Physiol 2008; 294: G1025-32.
285. Abdelmohsen K, Lal A, Kim HH, Gorospe M. Posttranscriptional orchestration of an anti-apoptotic program by HuR. Cell Cycle 2007; 6: 1288-92.
286. Fritsch RM, Schneider G, Saur D, Scheibel M, Schmid RM. Translational repression of MCL-1 couples stress-induced eIF2 alpha phosphorylation to mitochondrial apoptosis initiation. J Biol Chem 2007; 282: 22551-62.
287. Herrant M, Jacquel A, Marchetti S, et al. Cleavage of Mcl-1 by caspases impaired its ability to counteract Bim-induced apoptosis. Oncogene 2004; 23: 7863-73.
288. Le Gouill S, Podar K, Harousseau JL, Anderson KC. Mcl-1 regulation and its role in multiple myeloma. Cell Cycle 2004; 3: 1259-62.
289. Snowden RT, Sun XM, Dyer MJ, Cohen GM. Bisindolylmaleimide IX is a potent inducer of apoptosis in chronic lymphocytic leukaemic cells and activates cleavage of Mcl-1. Leukemia 2003; 17: 1981-9.
290. Michels J, O'Neill JW, Dallman CL, et al. Mcl-1 is required for Akata6 B-lymphoma cell survival and is converted to a cell death molecule by efficient caspase-mediated cleavage. Oncogene 2004; 23: 4818-27.
291. Domina AM, Smith JH, Craig RW. Myeloid cell leukemia 1 is phosphorylated through two distinct pathways, one associated with extracellular signal-regulated kinase activation and the other with G2/M accumulation or protein phosphatase 1/2A inhibition. J Biol Chem 2000; 275: 21688-94.
292. Domina AM, Vrana JA, Gregory MA, Hann SR, Craig RW. MCL1 is phosphorylated in the PEST region and stabilized upon ERK activation in viable cells, and at additional sites with cytotoxic okadaic acid or taxol. Oncogene 2004; 23: 5301-15.
293. Maurer U, Charvet C, Wagman AS, Dejardin E, Green DR. Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1. Mol Cell 2006; 21: 749-60.
294. Inoshita S, Takeda K, Hatai T, et al. Phosphorylation and inactivation of myeloid cell leukemia 1 by JNK in response to oxidative stress. J Biol Chem 2002; 277: 43730-4.
295. Kobayashi S, Lee SH, Meng XW, et al. Serine 64 phosphorylation enhances the antiapoptotic function of Mcl-1. J Biol Chem 2007; 282: 18407-17.
296. Thomas LW, Lam C, Edwards SW. Mcl-1; the molecular regulation of protein function. FEBS Lett 2010; 584: 2981-9.

297. Nijhawan D, Fang M, Traer E, et al. Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation. Genes Dev 2003; 17: 1475-86.
298. Opferman JT. Unraveling MCL-1 degradation. Cell Death Differ 2006; 13: 1260-2.
299. Zhong Q, Gao W, Du F, Wang X. Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis. Cell 2005; 121: 1085-95.
300. Ding Q, He X, Hsu JM, et al. Degradation of Mcl-1 by beta-TrCP mediates glycogen synthase kinase 3-induced tumor suppression and chemosensitization. Mol Cell Biol 2007; 27: 4006-17.
301. Stewart DP, Koss B, Bathina M, Perciavalle RM, Bisanz K, Opferman JT. Ubiquitin-Independent Degradation of Antiapoptotic MCL-1. Mol Cell Biol; 30: 3099-110.
302. Valentino L, Pierre J. JAK/STAT signal transduction: regulators and implication in hematological malignancies. Biochem Pharmacol 2006; 71: 713-21.
303. Isomoto H, Kobayashi S, Werneburg NW, et al. Interleukin 6 upregulates myeloid cell leukemia-1 expression through a STAT3 pathway in cholangiocarcinoma cells. Hepatology 2005; 42: 1329-38.
304. Rahaman SO, Harbor PC, Chernova O, Barnett GH, Vogelbaum MA, Haque SJ. Inhibition of constitutively active Stat3 suppresses proliferation and induces apoptosis in glioblastoma multiforme cells. Oncogene 2002; 21: 8404-13.
305. Liu H, Ma Y, Cole SM, et al. Serine phosphorylation of STAT3 is essential for Mcl-1 expression and macrophage survival. Blood 2003; 102: 344-52.
306. Tsutsui M, Yasuda H, Suto H, et al. Frequent STAT3 activation is associated with Mcl-1 expression in nasal NK-cell lymphoma. Int J Lab Hematol 2009.
307. Hsieh FC, Cheng G, Lin J. Evaluation of potential Stat3-regulated genes in human breast cancer. Biochem Biophys Res Commun 2005; 335: 292-9.
308. Kuo ML, Chuang SE, Lin MT, Yang SY. The involvement of PI 3-K/Akt-dependent up-regulation of Mcl-1 in the prevention of apoptosis of Hep3B cells by interleukin-6. Oncogene 2001; 20: 677-85.
309. Wang JM, Chao JR, Chen W, Kuo ML, Yen JJ, Yang-Yen HF. The antiapoptotic gene mcl-1 is up-regulated by the phosphatidylinositol 3-kinase/Akt signaling pathway through a transcription factor complex containing CREB. Mol Cell Biol 1999; 19: 6195-206.
310. Wang JM, Lai MZ, Yang-Yen HF. Interleukin-3 stimulation of mcl-1 gene transcription involves activation of the PU.1 transcription factor through a p38 mitogen-activated protein kinase-dependent pathway. Mol Cell Biol 2003; 23: 1896-909.
311. Longo PG, Laurenti L, Gobessi S, Sica S, Leone G, Efremov DG. The Akt/Mcl-1 pathway plays a prominent role in mediating antiapoptotic signals downstream of the B-cell receptor in chronic lymphocytic leukemia B cells. Blood 2008; 111: 846-55.
312. Boucher MJ, Morisset J, Vachon PH, Reed JC, Laine J, Rivard N. MEK/ERK signaling pathway regulates the expression of Bcl-2, Bcl-X(L), and Mcl-1 and promotes survival of human pancreatic cancer cells. J Cell Biochem 2000; 79: 355-69.
313. Leu CM, Chang C, Hu C. Epidermal growth factor (EGF) suppresses staurosporine-induced apoptosis by inducing mcl-1 via the mitogen-activated protein kinase pathway. Oncogene 2000; 19: 1665-75.
314. Townsend KJ, Zhou P, Qian L, et al. Regulation of MCL1 through a serum response factor/Elk-1-mediated mechanism links expression of a viability-promoting member of the BCL2 family to the induction of hematopoietic cell differentiation. J Biol Chem 1999; 274: 1801-13.
315. Townsend KJ, Trusty JL, Traupman MA, Eastman A, Craig RW. Expression of the antiapoptotic MCL1 gene product is regulated by a mitogen activated protein kinase-mediated

pathway triggered through microtubule disruption and protein kinase C. Oncogene 1998; 17: 1223-34.
316. Schubert KM, Duronio V. Distinct roles for extracellular-signal-regulated protein kinase (ERK) mitogen-activated protein kinases and phosphatidylinositol 3-kinase in the regulation of Mcl-1 synthesis. Biochem J 2001; 356: 473-80.
317. Zhai D, Jin C, Huang Z, Satterthwait AC, Reed JC. Differential regulation of Bax and Bak by anti-apoptotic Bcl-2 family proteins Bcl-B and Mcl-1. J Biol Chem 2008; 283: 9580-6.
318. Willis SN, Chen L, Dewson G, et al. Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. Genes Dev 2005; 19: 1294-305.
319. Fujise K, Zhang D, Liu J, Yeh ET. Regulation of apoptosis and cell cycle progression by MCL1. Differential role of proliferating cell nuclear antigen. J Biol Chem 2000; 275: 39458-65.
320. Liu H, Peng HW, Cheng YS, Yuan HS, Yang-Yen HF. Stabilization and enhancement of the antiapoptotic activity of mcl-1 by TCTP. Mol Cell Biol 2005; 25: 3117-26.
321. Bae J, Donigian JR, Hsueh AJ. Tankyrase 1 interacts with Mcl-1 proteins and inhibits their regulation of apoptosis. J Biol Chem 2003; 278: 5195-204.
322. Jamil S, Sobouti R, Hojabrpour P, Raj M, Kast J, Duronio V. A proteolytic fragment of Mcl-1 exhibits nuclear localization and regulates cell growth by interaction with Cdk1. Biochem J 2005; 387: 659-67.
323. Zhou P, Levy NB, Xie H, et al. MCL1 transgenic mice exhibit a high incidence of B-cell lymphoma manifested as a spectrum of histologic subtypes. Blood 2001; 97: 3902-9.
324. Miyamoto Y, Hosotani R, Wada M, et al. Immunohistochemical analysis of Bcl-2, Bax, Bcl-X, and Mcl-1 expression in pancreatic cancers. Oncology 1999; 56: 73-82.
325. Chung TK, Cheung TH, Lo WK, et al. Expression of apoptotic regulators and their significance in cervical cancer. Cancer Lett 2002; 180: 63-8.
326. Sano M, Nakanishi Y, Yagasaki H, et al. Overexpression of anti-apoptotic Mcl-1 in testicular germ cell tumours. Histopathology 2005; 46: 532-9.
327. Song L, Coppola D, Livingston S, Cress D, Haura EB. Mcl-1 regulates survival and sensitivity to diverse apoptotic stimuli in human non-small cell lung cancer cells. Cancer Biol Ther 2005; 4: 267-76.
328. Sieghart W, Losert D, Strommer S, et al. Mcl-1 overexpression in hepatocellular carcinoma: a potential target for antisense therapy. J Hepatol 2006; 44: 151-7.
329. Hussein MR, Haemel AK, Wood GS. Apoptosis and melanoma: molecular mechanisms. J Pathol 2003; 199: 275-88.
330. Zhuang L, Lee CS, Scolyer RA, et al. Mcl-1, Bcl-XL and Stat3 expression are associated with progression of melanoma whereas Bcl-2, AP-2 and MITF levels decrease during progression of melanoma. Mod Pathol 2007; 20: 416-26.
331. Thallinger C, Wolschek MF, Maierhofer H, et al. Mcl-1 is a novel therapeutic target for human sarcoma: synergistic inhibition of human sarcoma xenotransplants by a combination of mcl-1 antisense oligonucleotides with low-dose cyclophosphamide. Clin Cancer Res 2004; 10: 4185-91.
332. Derenne S, Monia B, Dean NM, et al. Antisense strategy shows that Mcl-1 rather than Bcl-2 or Bcl-x(L) is an essential survival protein of human myeloma cells. Blood 2002; 100: 194-9.
333. Cho-Vega JH, Rassidakis GZ, Admirand JH, et al. MCL-1 expression in B-cell non-Hodgkin's lymphomas. Hum Pathol 2004; 35: 1095-100.
334. Wuilleme-Toumi S, Robillard N, Gomez P, et al. Mcl-1 is overexpressed in multiple myeloma and associated with relapse and shorter survival. Leukemia 2005; 19: 1248-52.

335. Kitada S, Andersen J, Akar S, et al. Expression of apoptosis-regulating proteins in chronic lymphocytic leukemia: correlations with In vitro and In vivo chemoresponses. Blood 1998; 91: 3379-89.
336. Khoury JD, Medeiros LJ, Rassidakis GZ, McDonnell TJ, Abruzzo LV, Lai R. Expression of Mcl-1 in mantle cell lymphoma is associated with high-grade morphology, a high proliferative state, and p53 overexpression. J Pathol 2003; 199: 90-7.
337. Mandelin AM, 2nd, Pope RM. Myeloid cell leukemia-1 as a therapeutic target. Expert Opin Ther Targets 2007; 11: 363-73.
338. Oltersdorf T, Elmore SW, Shoemaker AR, et al. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature 2005; 435: 677-81.
339. van Delft MF, Wei AH, Mason KD, et al. The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. Cancer Cell 2006; 10: 389-99.
340. Lin X, Morgan-Lappe S, Huang X, et al. 'Seed' analysis of off-target siRNAs reveals an essential role of Mcl-1 in resistance to the small-molecule Bcl-2/Bcl-XL inhibitor ABT-737. Oncogene 2007; 26: 3972-9.
341. Nguyen M, Marcellus RC, Roulston A, et al. Small molecule obatoclax (GX15-070) antagonizes MCL-1 and overcomes MCL-1-mediated resistance to apoptosis. Proc Natl Acad Sci U S A 2007; 104: 19512-7.
342. Pan J, Cheng C, Verstovsek S, Chen Q, Jin Y, Cao Q. The BH3-mimetic GX15-070 induces autophagy, potentiates the cytotoxicity of carboplatin and 5-fluorouracil in esophageal carcinoma cells. Cancer Lett 2010; 293: 167-74.
343. Li J, Viallet J, Haura EB. A small molecule pan-Bcl-2 family inhibitor, GX15-070, induces apoptosis and enhances cisplatin-induced apoptosis in non-small cell lung cancer cells. Cancer Chemother Pharmacol 2008; 61: 525-34.
344. Smoot RL, Blechacz BR, Werneburg NW, et al. A Bax-mediated mechanism for obatoclax-induced apoptosis of cholangiocarcinoma cells. Cancer Res 2010; 70: 1960-9.
345. Witters LM, Witkoski A, Planas-Silva MD, Berger M, Viallet J, Lipton A. Synergistic inhibition of breast cancer cell lines with a dual inhibitor of EGFR-HER-2/neu and a Bcl-2 inhibitor. Oncol Rep 2007; 17: 465-9.
346. Martin AP, Mitchell C, Rahmani M, Nephew KP, Grant S, Dent P. Inhibition of MCL-1 enhances lapatinib toxicity and overcomes lapatinib resistance via BAK-dependent autophagy. Cancer Biol Ther 2009; 8: 2084-96.
347. O'Brien SM, Claxton DF, Crump M, et al. Phase I study of obatoclax mesylate (GX15-070), a small molecule pan-Bcl-2 family antagonist, in patients with advanced chronic lymphocytic leukemia. Blood 2009; 113: 299-305.
348. Schimmer AD, O'Brien S, Kantarjian H, et al. A phase I study of the pan bcl-2 family inhibitor obatoclax mesylate in patients with advanced hematologic malignancies. Clin Cancer Res 2008; 14: 8295-301.
349. Paik PK, Rudin CM, Brown A, et al. A phase I study of obatoclax mesylate, a Bcl-2 antagonist, plus topotecan in solid tumor malignancies. Cancer Chemother Pharmacol 2010.
350. Wilhelm SM, Carter C, Tang L, et al. BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. Cancer Res 2004; 64: 7099-109.
351. Rahmani M, Davis EM, Bauer C, Dent P, Grant S. Apoptosis induced by the kinase inhibitor BAY 43-9006 in human leukemia cells involves down-regulation of Mcl-1 through inhibition of translation. J Biol Chem 2005; 280: 35217-27.

352. Ding Q, Huo L, Yang JY, et al. Down-regulation of myeloid cell leukemia-1 through inhibiting Erk/Pin 1 pathway by sorafenib facilitates chemosensitization in breast cancer. Cancer Res 2008; 68: 6109-17.
353. Katz SI, Zhou L, Chao G, et al. Sorafenib inhibits ERK1/2 and MCL-1(L) phosphorylation levels resulting in caspase-independent cell death in malignant pleural mesothelioma. Cancer Biol Ther 2009; 8: 2406-16.
354. Guoan X, Hanning W, Kaiyun C, Hao L. Adenovirus-mediated siRNA targeting Mcl-1 gene increases radiosensitivity of pancreatic carcinoma cells in vitro and in vivo. Surgery 2010; 147: 553-61.
355. Schulze-Bergkamen H, Fleischer B, Schuchmann M, et al. Suppression of Mcl-1 via RNA interference sensitizes human hepatocellular carcinoma cells towards apoptosis induction. BMC Cancer 2006; 6: 232.
356. Ashihara E, Kawata E, Maekawa T. Future prospect of RNA interference for cancer therapies. Curr Drug Targets 2010; 11: 345-60.
357. Tiemann K, Rossi JJ. RNAi-based therapeutics-current status, challenges and prospects. EMBO Mol Med 2009; 1: 142-51.
358. Soule HD, Vazguez J, Long A, Albert S, Brennan M. A human cell line from a pleural effusion derived from a breast carcinoma. J Natl Cancer Inst 1973; 51: 1409-16.
359. Levenson AS, Jordan VC. MCF-7: the first hormone-responsive breast cancer cell line. Cancer Res 1997; 57: 3071-8.
360. Chen B, Mao R, Wang H, She J. Cell line and drug-dependent effect of ERBB3 on cancer cell proliferation, chemosensitivity, and multidrug actions. International Journal of High Throughput Screening 2010; 1: 49-55.
361. Zhan Q, Fan S, Bae I, et al. Induction of bax by genotoxic stress in human cells correlates with normal p53 status and apoptosis. Oncogene 1994; 9: 3743-51.
362. Fan S, Smith ML, Rivet DJ, 2nd, et al. Disruption of p53 function sensitizes breast cancer MCF-7 cells to cisplatin and pentoxifylline. Cancer Res 1995; 55: 1649-54.
363. Weng LP, Smith WM, Dahia PL, et al. PTEN suppresses breast cancer cell growth by phosphatase activity-dependent G1 arrest followed by cell death. Cancer Res 1999; 59: 5808-14.
364. Brockhoff G, Heiss P, Schlegel J, Hofstaedter F, Knuechel R. Epidermal growth factor receptor, c-erbB2 and c-erbB3 receptor interaction, and related cell cycle kinetics of SK-BR-3 and BT474 breast carcinoma cells. Cytometry 2001; 44: 338-48.
365. Brodowicz T, Kandioler D, Tomek S, et al. Anti-Her-2/neu antibody induces apoptosis in Her-2/neu overexpressing breast cancer cells independently from p53 status. Br J Cancer 2001; 85: 1764-70.
366. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248-54.
367. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 2008; 3: 1101-8.
368. Heinemeyer T, Wingender E, Reuter I, et al. Databases on transcriptional regulation: TRANSFAC, TRRD and COMPEL. Nucleic Acids Res 1998; 26: 362-7.
369. Henson ES, Hu X, Gibson SB. Herceptin sensitizes ErbB2-overexpressing cells to apoptosis by reducing antiapoptotic Mcl-1 expression. Clin Cancer Res 2006; 12: 845-53.
370. Klapper LN, Kirschbaum MH, Sela M, Yarden Y. Biochemical and clinical implications of the ErbB/HER signaling network of growth factor receptors. Adv Cancer Res 2000; 77: 25-79.
371. Carnero A. The PKB/AKT pathway in cancer. Curr Pharm Des 2010; 16: 34-44.

372. Klampfer L. Signal transducers and activators of transcription (STATs): Novel targets of chemopreventive and chemotherapeutic drugs. Curr Cancer Drug Targets 2006; 6: 107-21.
373. Iglesias-Serret D, Pique M, Gil J, Pons G, Lopez JM. Transcriptional and translational control of Mcl-1 during apoptosis. Arch Biochem Biophys 2003; 417: 141-52.
374. Singleton TP, Strickler JG. Clinical and pathologic significance of the c-erbB-2 (HER-2/neu) oncogene. Pathol Annu 1992; 27 Pt 1: 165-90.
375. Henson ES, Gibson EM, Villanueva J, Bristow NA, Haney N, Gibson SB. Increased expression of Mcl-1 is responsible for the blockage of TRAIL-induced apoptosis mediated by EGF/ErbB1 signaling pathway. J Cell Biochem 2003; 89: 1177-92.
376. Andersson Y, Juell S, Fodstad O. Downregulation of the antiapoptotic MCL-1 protein and apoptosis in MA-11 breast cancer cells induced by an anti-epidermal growth factor receptor-Pseudomonas exotoxin a immunotoxin. Int J Cancer 2004; 112: 475-83.
377. Mendelsohn J, Baselga J. The EGF receptor family as targets for cancer therapy. Oncogene 2000; 19: 6550-65.
378. Larkin MA, Blackshields G, Brown NP, et al. Clustal W and Clustal X version 2.0. Bioinformatics 2007; 23: 2947-8.
379. Blaskovich MA, Sun J, Cantor A, Turkson J, Jove R, Sebti SM. Discovery of JSI-124 (cucurbitacin I), a selective Janus kinase/signal transducer and activator of transcription 3 signaling pathway inhibitor with potent antitumor activity against human and murine cancer cells in mice. Cancer Res 2003; 63: 1270-9.
380. Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. Crit Rev Oncol Hematol 1995; 19: 183-232.
381. Normanno N, Bianco C, Strizzi L, et al. The ErbB receptors and their ligands in cancer: an overview. Curr Drug Targets 2005; 6: 243-57.
382. Burness ML, Grushko TA, Olopade OI. Epidermal growth factor receptor in triple-negative and basal-like breast cancer: promising clinical target or only a marker? Cancer J 2010; 16: 23-32.
383. Siziopikou KP, Cobleigh M. The basal subtype of breast carcinomas may represent the group of breast tumors that could benefit from EGFR-targeted therapies. Breast 2007; 16: 104-7.
384. Normanno N, De Luca A, Bianco C, et al. Epidermal growth factor receptor (EGFR) signaling in cancer. Gene 2006; 366: 2-16.
385. Koutras AK, Fountzilas G, Kalogeras KT, Starakis I, Iconomou G, Kalofonos HP. The upgraded role of HER3 and HER4 receptors in breast cancer. Crit Rev Oncol Hematol 2010; 74: 73-8.
386. Grupka NL, Lear-Kaul KC, Kleinschmidt-DeMasters BK, Singh M. Epidermal growth factor receptor status in breast cancer metastases to the central nervous system. Comparison with HER-2/neu status. Arch Pathol Lab Med 2004; 128: 974-9.
387. Lee-Hoeflich ST, Crocker L, Yao E, et al. A central role for HER3 in HER2-amplified breast cancer: implications for targeted therapy. Cancer Res 2008; 68: 5878-87.
388. Holbro T, Beerli RR, Maurer F, Koziczak M, Barbas CF, 3rd, Hynes NE. The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation. Proc Natl Acad Sci U S A 2003; 100: 8933-8.
389. Martin AP, Miller A, Emad L, et al. Lapatinib resistance in HCT116 cells is mediated by elevated MCL-1 expression and decreased BAK activation and not by ERBB receptor kinase mutation. Mol Pharmacol 2008; 74: 807-22.

390. Schulze-Bergkamen H, Ehrenberg R, Hickmann L, et al. Bcl-x(L) and Myeloid cell leukaemia-1 contribute to apoptosis resistance of colorectal cancer cells. World J Gastroenterol 2008; 14: 3829-40.
391. Sobell HM. Actinomycin and DNA transcription. Proc Natl Acad Sci U S A 1985; 82: 5328-31.
392. Nicholson RI, McClelland RA, Robertson JF, Gee JM. Involvement of steroid hormone and growth factor cross-talk in endocrine response in breast cancer. Endocr Relat Cancer 1999; 6: 373-87.
393. Duan R, Xie W, Burghardt RC, Safe S. Estrogen receptor-mediated activation of the serum response element in MCF-7 cells through MAPK-dependent phosphorylation of Elk-1. J Biol Chem 2001; 276: 11590-8.
394. Rochaix P, Krajewski S, Reed JC, Bonnet F, Voigt JJ, Brousset P. In vivo patterns of Bcl-2 family protein expression in breast carcinomas in relation to apoptosis. J Pathol 1999; 187: 410-5.
395. Lui VW, Yau DM, Wong EY, et al. Cucurbitacin I elicits anoikis sensitization, inhibits cellular invasion and in vivo tumor formation ability of nasopharyngeal carcinoma cells. Carcinogenesis 2009; 30: 2085-94.
396. Shaw G, Morse S, Ararat M, Graham FL. Preferential transformation of human neuronal cells by human adenoviruses and the origin of HEK 293 cells. FASEB J 2002; 16: 869-71.
397. Latinkic BV, Zeremski M, Lau LF. Elk-1 can recruit SRF to form a ternary complex upon the serum response element. Nucleic Acids Res 1996; 24: 1345-51.
398. Sivaraman VS, Wang H, Nuovo GJ, Malbon CC. Hyperexpression of mitogen-activated protein kinase in human breast cancer. J Clin Invest 1997; 99: 1478-83.
399. Mueller H, Flury N, Eppenberger-Castori S, Kueng W, David F, Eppenberger U. Potential prognostic value of mitogen-activated protein kinase activity for disease-free survival of primary breast cancer patients. Int J Cancer 2000; 89: 384-8.
400. von Lintig FC, Dreilinger AD, Varki NM, Wallace AM, Casteel DE, Boss GR. Ras activation in human breast cancer. Breast Cancer Res Treat 2000; 62: 51-62.
401. Mirzoeva OK, Das D, Heiser LM, et al. Basal subtype and MAPK/ERK kinase (MEK)-phosphoinositide 3-kinase feedback signaling determine susceptibility of breast cancer cells to MEK inhibition. Cancer Res 2009; 69: 565-72.
402. Hoeflich KP, O'Brien C, Boyd Z, et al. In vivo antitumor activity of MEK and phosphatidylinositol 3-kinase inhibitors in basal-like breast cancer models. Clin Cancer Res 2009; 15: 4649-64.
403. Zoppoli G, Moran E, Soncini D, et al. Ras-induced resistance to lapatinib is overcome by MEK inhibition. Curr Cancer Drug Targets; 10: 168-75.
404. Sambade MJ, Camp JT, Kimple RJ, Sartor CI, Shields JM. Mechanism of lapatinib-mediated radiosensitization of breast cancer cells is primarily by inhibition of the Raf>MEK>ERK mitogen-activated protein kinase cascade and radiosensitization of lapatinib-resistant cells restored by direct inhibition of MEK. Radiother Oncol 2009; 93: 639-44.
405. Faber AC, Li D, Song Y, et al. Differential induction of apoptosis in HER2 and EGFR addicted cancers following PI3K inhibition. Proc Natl Acad Sci U S A 2009; 106: 19503-8.
406. Zoppoli G, Moran E, Soncini D, et al. Ras-induced resistance to lapatinib is overcome by MEK inhibition. Curr Cancer Drug Targets 2010; 10: 168-75.
407. Mitchell C, Yacoub A, Hamed H, et al. Inhibition of MCL-1 in breast cancer cells promotes cell death in vitro and in vivo. Cancer Biol Ther 2010; 10.

408. Rexer BN, Engelman JA, Arteaga CL. Overcoming resistance to tyrosine kinase inhibitors: lessons learned from cancer cells treated with EGFR antagonists. Cell Cycle 2009; 8: 18-22.
409. Jin Q, Esteva FJ. Cross-talk between the ErbB/HER family and the type I insulin-like growth factor receptor signaling pathway in breast cancer. J Mammary Gland Biol Neoplasia 2008; 13: 485-98.
410. Jerome L, Alami N, Belanger S, et al. Recombinant human insulin-like growth factor binding protein 3 inhibits growth of human epidermal growth factor receptor-2-overexpressing breast tumors and potentiates herceptin activity in vivo. Cancer Res 2006; 66: 7245-52.
411. Kostler WJ, Hudelist G, Rabitsch W, et al. Insulin-like growth factor-1 receptor (IGF-1R) expression does not predict for resistance to trastuzumab-based treatment in patients with Her-2/neu overexpressing metastatic breast cancer. J Cancer Res Clin Oncol 2006; 132: 9-18.
412. Spector NL, Xia W, Burris H, 3rd, et al. Study of the biologic effects of lapatinib, a reversible inhibitor of ErbB1 and ErbB2 tyrosine kinases, on tumor growth and survival pathways in patients with advanced malignancies. J Clin Oncol 2005; 23: 2502-12.
413. Engelman JA, Zejnullahu K, Mitsudomi T, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science 2007; 316: 1039-43.
414. Konecny GE, Pegram MD, Venkatesan N, et al. Activity of the dual kinase inhibitor lapatinib (GW572016) against HER-2-overexpressing and trastuzumab-treated breast cancer cells. Cancer Res 2006; 66: 1630-9.
415. Seth A, Watson DK. ETS transcription factors and their emerging roles in human cancer. Eur J Cancer 2005; 41: 2462-78.
416. Lincoln DW, 2nd, Bove K. The transcription factor Ets-1 in breast cancer. Front Biosci 2005; 10: 506-11.
417. Wang S, Zhu X, Zhang J, et al. [Significance of MEK-ERK cascade in the development of human breast carcinoma]. Zhonghua Wai Ke Za Zhi 2002; 40: 171-4.
418. Milde-Langosch K, Bamberger AM, Rieck G, et al. Expression and prognostic relevance of activated extracellular-regulated kinases (ERK1/2) in breast cancer. Br J Cancer 2005; 92: 2206-15.
419. Baselga J, Swain SM. Novel anticancer targets: revisiting ERBB2 and discovering ERBB3. Nat Rev Cancer 2009; 9: 463-75.
420. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol 2001; 2: 127-37.
421. Leber B, Geng F, Kale J, Andrews DW. Drugs targeting Bcl-2 family members as an emerging strategy in cancer. Expert Rev Mol Med 2010; 12: e28.