UNIVERSITY OF LEICESTER

Transcriptional and Translational control of the expression of the EMT transcription factor ZEB2 in cancer cells

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By

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ABSTRACT

Epithelial-mesenchymal transition (EMT) is a crucial process involved in the metastasis and invasion of cancer cells. EMT promotes migration, invasion and separation of individual cells from the primary cancer enabling them to access the vascular system and promoting tumour dissemination. In different types of human cancer, invading cells recapitulate elements of embryonic EMT pathways to extravasate into the blood stream and form metastases at distant sites. Several pleiotropically activated transcription factors, categorised as master regulators of EMT (EMT-TFs), acting downstream of EMT pathways have a central role in cancer. EMT-TFs include Zn finger transcription factors of SNAIL (SNAIL1 and SNAIL2) and ZEB (ZEB1 and ZEB2) families, basic helix-loop-helix (bHLH) proteins E47, TWIST1, TWIST2, a forkhead transcription factor FOXC2, and a few other relatively less studied proteins. Though ZEB family members, ZEB1 and ZEB2, are both efficient executers of EMT programs in human cancer and their roles in tumourigensis might be different. In particular, ZEB2, but not ZEB1 exhibited tumour-suppressive features in malignant melanoma and, possibly, hepatocellular carcinoma. Regulation of ZEB proteins expression occurs at different levels. The activity of three putative transcriptional enhancer controlling ZEB2 gene expression were analysed, and found that RE-4 regulatory element known to control ZEB2 expression in human embryonic stem cells (hESC) is also active in malignant melanoma cells. However, in contrast with hESC, in melanoma cells ZEB2 expression is not regulated by core pluripotency factors. Instead, in these cells, the activity of RE-4 element is affected by ZEB1- or ZEB2- induced EMT programs. By studying post-transcriptional level of ZEB regulation, a novel mechanism that limits ZEB2 protein synthesis was described in this study. A protein motif adjacent to the smad-binding domain within ZEB2 protein induces ribosome stalling and compromises translation. The activity of this motif is dependent on triplets of rare codons, Leu(UUA)-Gly(GGU)-Val(GUA). Introducing these stretches in the homologous region of ZEB1 has no effect on protein expression. By using retroviral expression of pBABE-ZEB2-mut, it was shown that these stretches might contribute to EMT. The data suggest that rare codons play a regulatory role in the context of appropriate protein structures and we speculate that pools of tRNA available for protein translation influence configuration of EMT programs in cancer cells.

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LIST OF ABBREVIATIONS

Amp	Ampicillin
ANOVA	Analysis of variance
BCA	Bicinchoninic acid
bHLH	Basic helix-loop-helix
β-ME	βeta-mercaptoethanol
BMP	Bone morphogenetic protein
bp	Base pair
BRAG1	Braham-related protein 1
BSA	Bovine serum albumin
CBD	C-terminal binding domain or CtBP-binding domain
cDNA	Complementary deoxyribonucleic acid
CSCs	Cancer stem cells
CtBP	C-terminal binding protein
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle media
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DOX	Doxycycline
ECL	Enhanced chemiluminescence
EMT	Epithelial-mesenchymal transition
ERK1/2	Extracellular signal-regulated proteins 1 and 2
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FOXD3	Forkhead box D3
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
HD	Homeodomain
HEM	Human epidermal melanocytes
HMEC	human mammary epithelial cells
HRP	Horseradish peroxidase
IF	Immunofluorescence
kb	Kilobase
kDa	Kilodalton
KLF4	Krueppel-like factor 4
LB	Luria broth
MAPK	Mitogen-activated protein kinase
MCF-7	Michigan Cancer Foundation-7 (Human BC cell line)
MEK1/2	MAPK/ERK kinase
MET	Mesenchymal-epithelial transition
min	Minutes
miR	MicroRNA
MITF	Microphthalmia-associated transcription factor
MITF-M	Melanocyte-specific MITF
MR-EMT	Master regulators of epithelial-mesenchymal transitions

mRNA	Messenger ribonucleic acid
MW	Molecular weight
MYC	Myelocytomatosis viral oncogene
N/A	Not applicable
NaCl	Sodium chloride
NEAA	Non-essential amino acid
NHEM	Neonatal human epidermal melanocytes
NuRD	Nucleosome remodelling and deacetylase
OCT4	Octamer-binding transcription factor 4
p16INK4A	Cyclin-dependent kinase inhibitor 2A
PAX3	Paired box 3
PBS	Phosphate buffered saline
P/CAF	P300/CBP-associated factor
pCMV	CMV (cytomegalovirus) promoter
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinases
P/S	Penicillin-streptomycin
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene fluoride
Rb	Retinoblastoma protein
RGP	Radial growth phase
RLU	Relative fluorescent units
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
RPM	Rosewell Park Memorial Institute (medium)
RT-PCR	Reverse transcriptase polymerase chain reaction
SBD	Smad-binding domain
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec	Seconds
SEM	Standard error of the mean
SID	Smad-interacting domain
SIP1	Smad-interacting protein 1
siRNA	Short interfering ribonucleic acid
SOX2	SRY (sex determining region Y)-box 2
SOX10	SRY (sex determining region Y)-box 10
SNAIL1/2	Snail homolog 1/2
SWI/SNF	SWItch/Sucrose NonFermentable
SUMO	Small ubiquitin-like modifier
TAE	Tris acetic acid EDTA
Taq	Thermus aquaticus
TBE	Tris boric acid EDTA
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline-Tween
TE	Tris-EDTA

TEMED	Tetramethylethylenediamine
TGF-β	Transforming growth factor-beta
TWIST1/2	Twist-related protein 1/2
UK	United Kingdom
UTR	Untranslated region
UV	Ultraviolet
UVR	Ultraviolet radiation
VDR	Vitamin D receptor
VGP	Vertical growth phase xiii
v/v	Volume by volume
WB	Western blotting
w/v	Weight by volume
ZEB1	Zinc finger E-box-binding homeobox 1
ZEB2	Zinc finger E-box-binding homeobox 2
ZHX1	Zinc finger homeodomain 1

CHAPTER 1: INTRODUCTION

1.1 CANCER

The term cancer, medically known as a malignant tumour, indicates abnormal cell growth within the human body. Not all tumours are cancerous and invasive; when benign tumours are present they do not spread within the human body. The development of cancer, termed carcinogenesis, is a process involving multiple stages that require genetic and epigenetic modifications to transform normal human cells progressively into malignant cells. All types of malignant tumours share the same set of cancer traits; these common hallmarks of cancer growth include autocrine signalling, insensitivity to antigrowth signals, resistance to apoptosis, an infinite replicative potential, the ability to induce angiogenesis, and the propensity for invasion and metastasis. Although an inefficient process, direct or indirect genetic changes are required in most cancer cells Hanahan and Weinberg (2000). In addition to these changes, cancer progression requires sustained cellular energy metabolism and immune-system avoidance. Moreover, the enabling characteristics of genomic stability and inflammation promote tumour progression. It is important to understand the molecular complexity of the evolving tumour; it is also equally important to understand the function of the tumour microenvironment. Not only is the interaction between stromal cells and tumour cells important in cancer growth and progression, tumour interaction with inflammatory cells is an important determinant of invasiveness and metastasis. However, most of the understanding of the molecular mechanisms underlying the metastatic progression of human tumours remains incomplete (Hanahan and Weinberg, 2011).

The core cause for cancer-related deaths (accounting for 90% of deaths from solid tumours) are metastasis and resistance to therapy, meaning that malignant cells have spread from the primary tumour to different organs and are often resistant to current therapeutic regimes (Nguyen and Massague, 2007). A series of sequential stages, each of which can be rate-limiting, is required for a malignant tumour to be established and for metastasis to occur. Indeed, the entire process can fail due to the inherent properties of the tumour cells and due to the organism's immune response. Following primary transformation, tumour cells migrate to and invade the host stroma. Additionally, they acquire a capability to activate angiogenesis and establish new networks of blood vessels. Tumour cells then spread into the circulatory system, a process known as intravasation and can reach

lymphatic channels. During invasion and intravasation tumour cells must avoid the host immune response. At the end of the process, tumour cells reach and arrest in capillary beds, exit the circulatory system through a process known as extravasation, invade secondary organs, proliferate and subsequently successfully colonise to develop into a metastases (figure1-1) (Talmadge and Fidler, 2010). The 'seed and soil' hypothesis, proposed by Stephan Paget in 1889, suggests that metastasis is not a random process but that tumour cells from the primary tumour need to be compatible with the secondary organ to establish a metastasis. Indeed, this is a highly unstable process, with the importance of colonisation and the presence of a favourable microenvironment remaining highly challenging areas of research to explain how tumours control metastatic progression (Fidler, 2003).

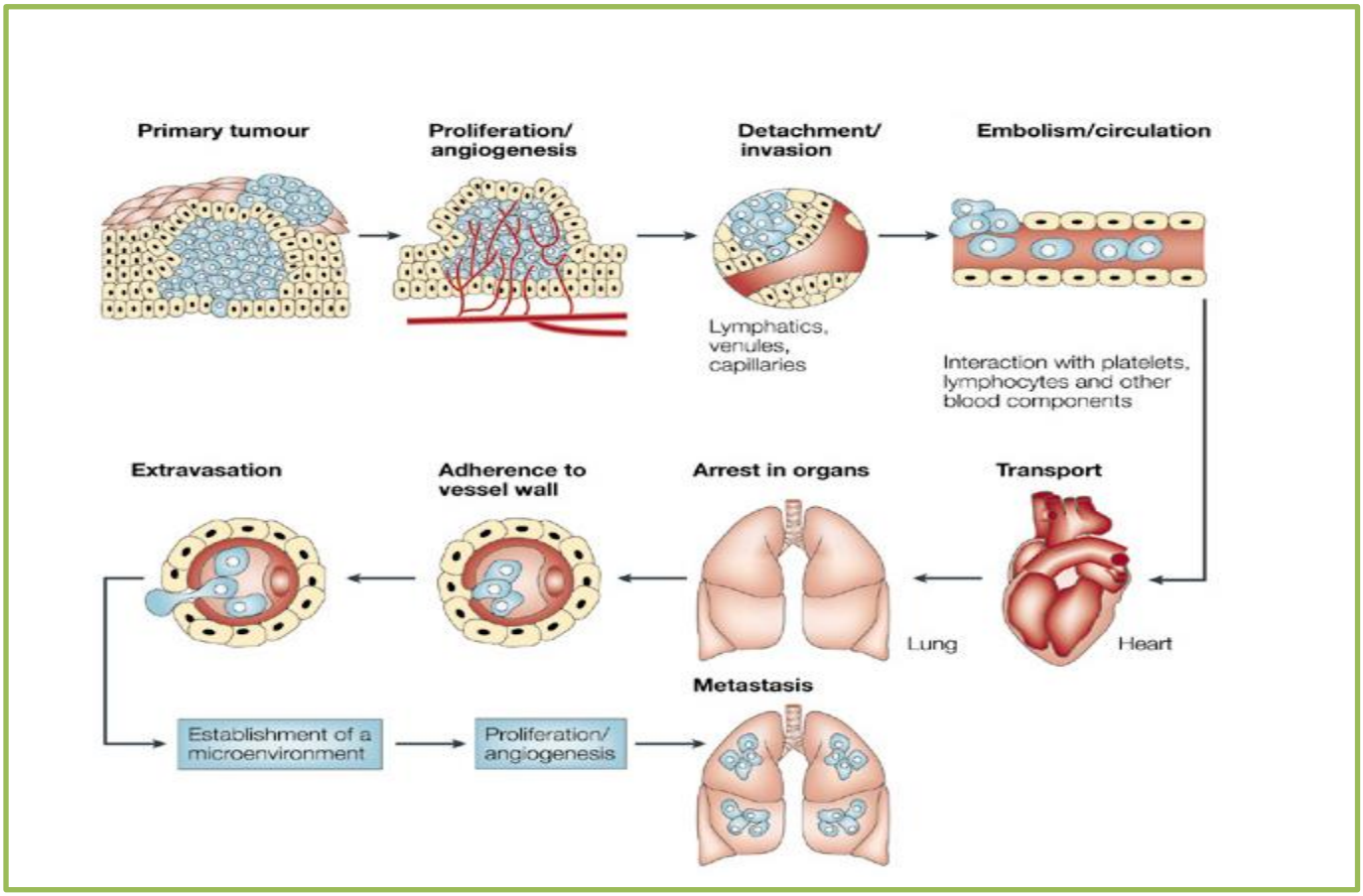


Figure 1-1: A schematic representation of metastatic growth.
Once initial transformation has occurred, angiogenesis establishes a new vascular network within the tumour. The invasion into the stroma is operated by lymphatic channels; the tumour cells enter the circulatory system and, if they survive, can become arrested in capillary beds, where they can adhere to the vessel wall. Secondary tumours may then be established within the new host environment where they must establish a vascular network and evade immune detection. Figure adapted from (Fidler, 2003)

1.1.1 MELANOMA

Skin melanoma was first described in the fifth century B.C as "black cancer" and "fatal black tumours with metastases" by the Greek physician Hippocrates. Unfortunately, in spite of recently developed treatments, melanoma remains a common and the deadliest skin cancer in European countries (Chin et al., 1998). Originating from the malignant transformation of melanocytes, melanoma has relatively high mortality and morbidity rates, a trend that has continued to increase worldwide. It has been shown that the incidence of melanoma has significantly grown in the young population being the second most common cancer type in the 15-29 year old age group (Bleyer et al., 2006). In the UK in 2011, 13,348 new cases of malignant melanoma were diagnosed; however, melanoma remains not common cancer in the UK. Nevertheless, malignant melanoma remains a less common cause of death in the UK compared to the other types of cancer (Figure 1.2) (*Skin cancer incidence statistics: Cancer Research UK*). Malignant melanoma can be classified into two categories: i) the radial growth phase (RGP), where melanocytes spread but are restricted within the epidermis, and ii) the vertical growth phase (VGP), where tumorigenic melanocytes infiltrate the dermis and metastasise. This is considered clinically dangerous as it the process resulting in the dissemination of tumour cells to distant locations in the body (Gaggioli and Sahai, 2007).

Despite the fact that the majority of melanocytes are found in the skin, malignant melanoma does not exclusively cause skin cancer, as melanocytes are present also within several internal organs. The range of locations includes ocular, mucosal, gastrointestinal, genitourinary, leptomeninges and lymphatic sites, all of which can be the origin of primary extracutaneous melanomas (Mihajlovic et al., 2012). Melanocytes are known to arise developmentally from the neural crest and mature in hair follicles with each mature melanocyte intimately adjacent to keratinocytes. Melanocytes create melanin and transfer it to the keratinocyte, via dendritic processes, within an organelle known as a melanosome. The main function of melanin is to shield the upper surface of the keratinocyte nucleus, so protecting its DNA from any damage that might occur through absorbance of ultraviolet radiation (UVR). Paradoxically, the DNA of melanocytes is damaged via exposure to UVR, with melanoma actually being more aggressive than the squamous cell and basal cell carcinoma that originate from the keratinocytes (Markovic et al., 2007).

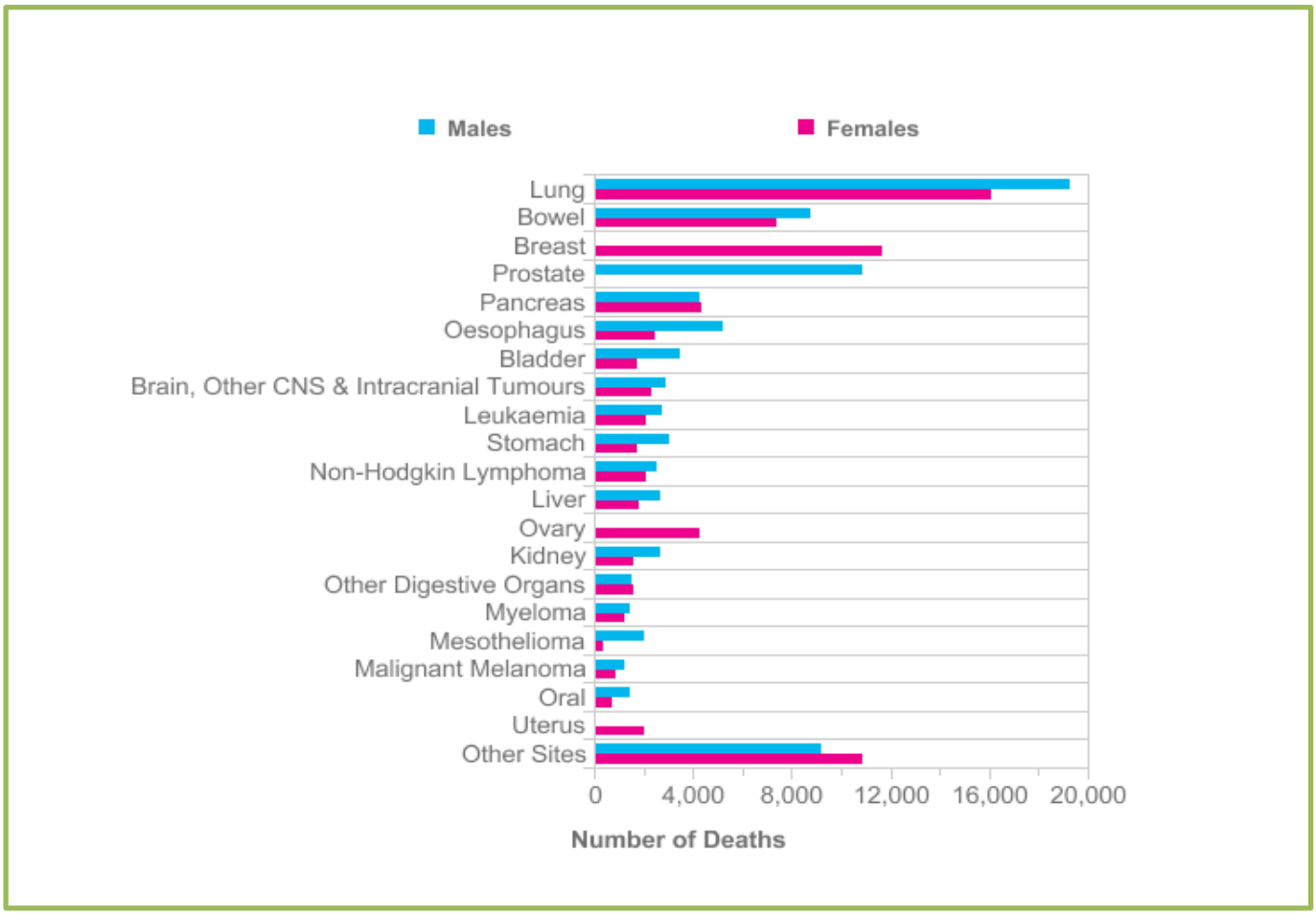


Figure 1-2: The 20 most common causes of death according to cancer type in the UK. MM is ranked eighteenth in the UK. Adapted from *Skin cancer incidence statistics: Cancer Research UK*.

Many factors increase the risk of melanoma such as male gender, a family history of melanoma, a higher number of nevi, a history of severe sunburn, a weakened immune system and a light hair colour (Cho et al., 2005). The two forms of melanin, eumelanin and pheomelanin, are important in determining skin and hair colour. The production of these two forms of melanin is controlled by the melanocortin receptor 1 (MCR1), which is present on melanocytes and is activated via linkage with the α-melanocyte-stimulating hormone (αMSH). Once activated, this triggers intracellular signalling to stimulate cells to produce eumelanin but failure to follow this process leads to pheomelanin production which results in a higher risk of skin cancer associated with sun exposure and levels of UV radiation (Scott et al., 2002).

Melanocytes originally differentiate from highly migratory, self-renewing and multipotent cells known as neural crest cells. Neural crest cells are formed from the dorsal neural tube during embryogenesis. At the stage of neural tube closure, they undergo an epithelial-mesenchymal transition (EMT) and migrate to specific locations throughout the body to complete their differentiation into components of bone, cartilage, melanocytes within the skin, and the peripheral nervous system (Thomas and Erickson, 2008). Several transcription factors, such as microphthalmia-associated transcription factor (MITF), paired box 3 (PAX3), sex-determining region Y-box 10 (SOX10), SNAIL homolog 2 (SNAIL2), endothelin receptor (EDNR), tyrosine kinase receptor (C-Kit), forkhead-box transcription factor D3 (FOXD3) (Nissan et al., 2011, Sommer, 2011), and ZEB1 and ZEB2, are known to be vital for the differentiation of melanocytes (Denecker et al., 2014). Specific genetic mutations during tumorigenesis including tumour suppressor genes and oncogenes, have been found to correlate with melanoma development (Rodolfo et al., 2004).

The major signalling pathway involved in melanoma formation is the mitogen-activated protein kinase (MAPK) pathway (Inamdar et al., 2010). Pathological activation of MAPK signalling switches cellular fate from differentiation to apoptosis or tumourigenicity depending on the cell background. The core MAPK pathway is a cascade of several enzymes consecutively phosphorylating a downstream protein kinase: a MAP3K that activates MAP2K, which in turn activates the phosphorylated MAPK (Qi and Elion, 2005). The MAPK family can be divided into three groups, including the extracellular signal-regulated kinase

(Spaderna et al.) 1/2, ERK3/4, ERK5, ERK7/8; Jun N-terminal kinase (JNK) 1/2/3 and the p38 isoforms α/β/γ (ERK6)/ δ. ERK is a primary signalling pathway in melanoma and has been well studied in many other cancer types as well. The downstream kinase in this pathway is ERK1/2 which is activated by MEK 1/2, with RAF (A-RAF, B-RAF, and C-RAF) directly activating MEK. In turn, RAF is activated at the plasma membrane via RAS GTPase, which is itself activated by different receptor tyrosine kinases. A correlation between melanoma and mutations within the MAPK signalling pathway has been identified in many studies. Strikingly, a very high proportion of all cutaneous melanoma samples harbour activating mutations either in N-RAS (15%) or B-RAF (66%). The most common mutation within B-RAF was identified at codon 600 in exon 15, with a substitution of valine to glutamic acid (Val600Glu; B-RAFV600E), which leads to a higher basal kinase activity. Interestingly, the activity of the kinase in the B-RAF mutants is approximately 500-fold higher than in the wild-type. Ultimately, when it cooperates with the microenvironment and other mutationally activated signalling pathways, B-RAF mutations promote melanocyte de-differentiation and enhanced invasion (Lin et al., 2010).

The high frequency of mutations in RAS or BRAF genes in melanomas and other tumour types highlighted are of vital importance to the designing of specific chemical inhibitors of these proteins for therapeutic purposes (Dhillon et al., 2007). Indeed, recently progress in the development of small chemical inhibitors of $BRAF^{V600E}$ has been achieved. The uses of these inhibitors clinically have shown promise by reducing the number of metastases and increasing survival of patients with metastatic cutaneous melanoma. However, in all cases resistance later developed, and new attempts are currently being made to use BRAF inhibitors in combination with other therapies as new treatment schemes (Siroy et al., 2015).

1.1.2 CARCINOMA

Carcinoma is a type of cancer that originates in the epithelial tissues of the body including the skin, lung, breast, prostate and colon to name but a few. Carcinoma has been identified as an independent entity which has the ability to invade tissues that line human organs and can metastasise (Springer, 1984). Epithelial tissues cover the outer and inner surfaces of the

human body, with distinct functions attributed to different epithelial cells within the same tissue. The absolute majority of human cancers (around 90%) are represented by carcinomas highlighting the importance of research on the molecular mechanisms of tumourigenesis in epithelial tissues. Epithelial cells are tightly connected with each other through different types of epithelial cell-cell adhesion structures, which in combination form the epithelial junctional complex. These structures including adherens junctions, tight junctions, gap junctions and desmosomes are key determinants of epithelial integrity. They play a key role in the maintenance of epithelial apical-basal polarity, and are responsible for communications between epithelial cells and have signalling functions. Loss of the epithelial junctional complex is often observed at later stages of carcinoma development and is associated with an increased metastatic propensity of carcinoma cells (Royer and Lu, 2011).

Basal-cell carcinoma, which is formed upon the transformation of epidermal keratinocytes and is not highly metastatic, and squamous cell carcinoma, which is an invasive tumour, are considered the most common subtypes of non-melanoma skin cancer (Nindl et al., 2007). Exposure to UV is the main cause of basal-cell carcinoma. Other risk factors, such as exposure to ionising radiation, arsenic and immunosuppression, have been linked to a predisposition to basal-cell carcinoma. Eighty per cent of basal-cell carcinoma cases have been observed in the head and neck, with less common occurrences at other sites, such as the arms and legs (Rubin et al., 2005). On the other hand, squamous cell carcinoma (SCC), which is the second most common skin cancer in the Caucasian population and is responsible for 1300-2300 non-melanoma skin cancer-related deaths a year in the United States, is caused by different factors such as ultraviolet radiation, chemical agents and chronically injured or inflamed skin. Invasive squamous cell carcinoma may originate from an actinic keratinocyte and can be observed as phantom intraepithelial neoplasia (Hawrot et al., 2003).

In relation to carcinomas of the breast, basal-like carcinoma, which account for 10-15% of all breast cancers, are most common in young African women. Histologically, they are high-grade, with high mitotic indices and are associated with prominent lymphoid invasion. Moreover, basal-like and triple-negative breast cancers share phenotypic similarities, in that 71% of triple-negative cancers are of the basal-like carcinoma phenotype, whereas 77% of

tumours expressing basal- like markers are triple-negative (Badve et al., 2011, Bosch et al., 2010). The basal subtype was associated with expression of genes related to proliferation, inhibition of apoptosis and tumour invasion (Sorlie et al., 2006) . Additionally, these basal-like cells express a group of protein markers, such as CK5 and CK17, P-cadherin, caveolin 1 and 2, nestin, CD44 and EGFR, which are present in normal breast myoepithelial cells (Eroles et al., 2012). In clinical trials, a panel of five markers (ER, HER1, HER2, and cytokeratin 5/6) was able to identify basal-like tumours with 76% sensitivity by IHC and 100% specificity using microarray (Nielsen et al., 2004).

As it originates within epithelial cells that line the terminal lobular duct unit, breast cancer remains non-invasive once they are sited at the basement membrane; however, when cells are disseminated outside the basement membrane they begin to invade the adjacent tissue (Sainsbury et al., 2000). The de-regulation of proliferation, differentiation, migration and apoptosis contributes to an invasive phenotype (Vargo-Gogola and Rosen, 2007). The Invasive lobular carcinoma is responsible for 5-15% of all invasive breast cancers (Yerushalmi et al., 2009). Special clinical and biological characteristics of invasive lobular carcinoma have been highlighted; for instance, it can be large in size, it is estrogen receptor (ER) and progesterone receptor (PgR) positive and has a low to absent expression of human epidermal growth factor receptor-2(HER2) (Jung et al., 2010).

1.1.3 CANCER STEM CELLS

The stem cell is the origin of all human life and all organisms in general. It is a single cell that can differentiate into more specialised cells with specific functions and also has the ability to renew itself. For instance, the bone marrow stem cell, which has no specialisation, is able to differentiate into red blood cells and white blood cells, and these new types of cells have special functions, such as being able to produce antibodies in the induction of an immune response. The stem cells remain uncommitted until they receive a signal to develop into specialised cells. Moreover, stem cells have the unique properties of being able to develop into various cell types of a particular tissue and of being able to divide asymmetrically (Bongos and Hin Lee, 2004).

The scarcity of stem cells in most tissues justifies the need for accurate identification and proper purification of the stem cell populations. Although it seems reasonable to propose that each tissue arises from a tissue-specific stem cell, the accurate identification and isolation of these somatic stem cells has been accomplished in only a few instances. For example, mice and humans have been used to isolate haematopoietic stem cells (HSCs), which are multipotent and have been considered responsible for the generation and regeneration of the blood and cells of the immune system (Taipale and Beachy, 2001). The ability to isolate HSCs has allowed recent progress in the understanding of the molecular control of their function, particularly regarding self-renewal and maintenance. The implication of Notch, Hedgehog and Wnt pathways in stem cell biology provide growing evidence that they control many developmental processes and are deregulated in cancer, and that they may be responsible for the regulation of self-renewal of haematopoietic progenitors and stem cells (Reya et al., 2003). The interplay between these pathways and signals from the stem cell niche provides a crucial regulatory balance between self-renewal and differentiation. In Wnt signalling, Wnt proteins such as Wnt3A and Wnt5A ligands are produced by HSCs, as well as by the micro-environment. The Wnt signalling pathways are important in the development and maintenance of stem cells and in disease, particularly the canonical Wnt signalling pathway (Reya and Clevers, 2005). Transcriptional activation of canonical Wnt occurs through the Frizzled family during tissue regeneration associated with chronic, persistent inflammation and the up-regulation of Wnt1, Wnt3 or Wnt10b can be due to the fact that mammary tumour virus integration leads to mammary carcinogenesis in mice (Taipale and Beachy, 2001).

Notch signalling is involved in normal embryonic stem cells and in the regulation of the self-renewal of stem cells. The Notch ligand, Notch 1, is known to play a crucial role in the regeneration of HSCs from hemogenic endothelium cells during early embryogenesis. It also represents an integral part in mediating signalling between adjacent cells. For example, Notch has the ability to inhibit the prevalence of cellular differentiation within tissue or to prompt adjacent cells to adopt the same cellular fate (Hadland et al., 2004)

An important focus of the study of stem cells is the cell division mechanism. Compared to progenitor cells, the division of stem cells is slower. Stem cells undergo asymmetric division, thereby generating two types of cells: one identical to the mother stem cell and the other a

specialised one. However, they also have the ability to generate two identical stem cells by undergoing symmetric division. Symmetric division is slower and through cell differentiation, the continuous regeneration of organs and tissues ensure the persistence of a pool of adult stem cells (Potten et al., 1997). Obtaining “lineage expansion” or “lineage extinction” depends on the type of cell division. Generating stem cells produces “lineage expansion”, whereas “lineage extinction” is achieved if differentiated cells are propagated. It is widely accepted that stem cells give rise to cancer, simply because their slow cycles of division and their longevity allow them to accumulate different mutations over time (Clarke, 2005). It has been proposed that neoplastic cells retrain the feature to divide asymmetrically and to produce cells with different tumorigenic potentials. Cancer cells with higher tumorigenic propensity have been categorised as “cancer stem cells” (CSC), which are capable of self-renewal and generation of the bulk of a tumour. Apparently, this feature is an important factor contributing to cancer heterogeneity (McDonald et al., 2006).

One of the essential downstream effectors, required for the regulation of the pluripotency in both mouse and human embryonic stem cells (ES), is the unique homeodomain transcriptional factor Nanog, which supports self-renewal and maintains the pluripotency of embryonic stem cells (Chambers et al., 2003, Pan and Thomson, 2007). It has been shown that Nanog is expressed in some cancers such as breast cancer (Ezeh et al., 2007), prostate cancer (Jeter et al., 2011), colorectal cancer (Zhang et al., 2013) and somatic Ewing’s sarcoma (Brown et al., 2013). Functionally, Nanog cooperates with other two stem cell transcriptional factors, OCT4 and SOX2, to maintain pluripotency (Kuroda et al., 2005, Rodda et al., 2005). Stable transfection of NANOG and OCT4 into the A375 melanoma cell line strongly enhances cell motility and transmigration of the cells (Borrull et al., 2012). Sox2 was also discovered to be expressed in 67% of human primary melanomas and 80% of metastatic melanomas. Functionally, SOX2 is associated with human melanoma dermal invasion. The high expression of these stem cell factors in different types of cancer and particularly in melanoma suggests that the invasive front of solid tumours might be enriched for cells with stem cell properties (Girouard et al., 2012).

1.2 EPITHELIAL-MESENCHYMAL TRANSITION (EMT)

Epithelial cells can be converted into mesenchymal cells by the EMT process. An epithelial mesenchymal transition (EMT) is a vital and fundamental embryonic epigenetics programme that often becomes active during tumour progression at the stage of invasion and metastasis (Gibbons et al., 2009). EMT plays a major role in the morphogenesis of organs; without it, tissues and organs would never be formed. Some characteristics of EMT are as follow:

- Loss of epithelial polarity.
- Loss of cell adhesion and disruption of intracellular contacts.
- Increased cell motility.
- Degradation of the basement membrane.
- Increased cell invasion and migration.
- Contributes to chemoresistance.
- Induce cell cycle arrest.
- Induce stem cell properties.

The transition from epithelial cells to characteristic mesenchymal cells encompasses inter- and intracellular processes. Epithelial and mesenchymal cells differ in these various properties. Epithelial cells are morphologically round and grow as compact clusters. These phenotypic characteristics allow for highly organised cellular adhesion with their neighbours, allowing for the formation of layered structures. These include tight junctions, adherens junctions, desmosomes and gap junctions (Figure1-3). Cells lose many of their epithelial characteristics and acquire properties that are typical of mesenchymal cells during this conversion. In contrast, mesenchymal cells have a spindle shape, fibroblast-like morphology, make focal contact only with neighbouring cells, and show a loss of organised cell junctions, which makes them highly migratory. Mesenchymal cells can produce epithelial cells through the reverse process (mesenchymal to epithelial transition MET) (Figure1-4). Molecular differences have been observed between mesenchymal and

epithelial cells; for example, mesenchymal cells do not express epithelial E-cadherin and Laminin-1, whereas epithelial cells do. Several molecular events have been observed during the EMT process. These involve changes in gene regulation, in cell adhesion cytoskeletal organisation and in the expression of specific microRNAs (Thiery and Sleeman, 2006).

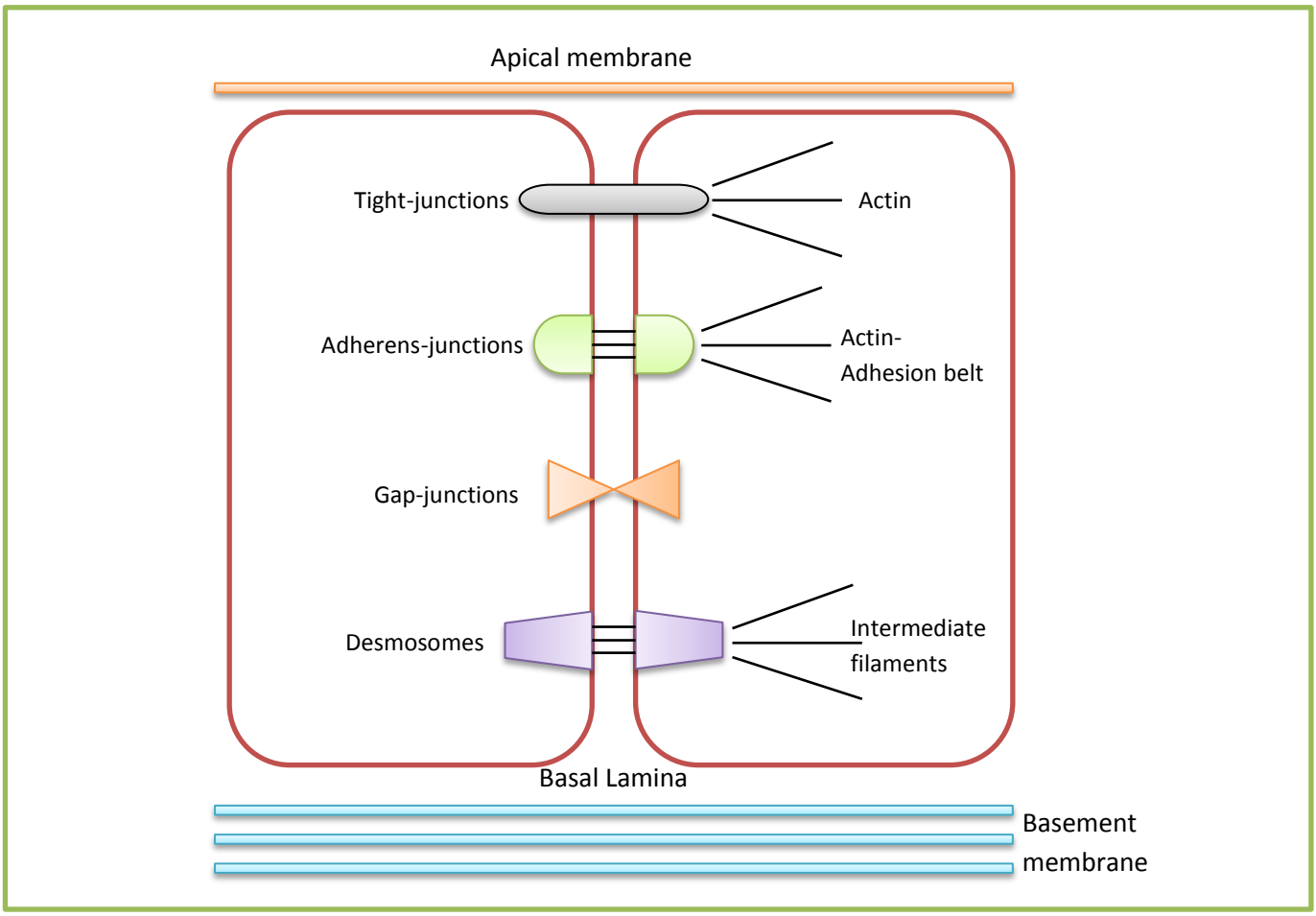


Figure 1-3: Epithelial junctional complex.
The junctional complexes that exist within epithelial cells provide adhesion contact and build the polarised structure of the cellular matrix. A tight junction is sited near to the apical surface controlling para-cellular permeability. Adherens junctions consist of E-cadherin which binds to the cytoskeletal component (Actin) and indirectly through the catenin family. Gap junctions are intercellular channels that allow the exchange of ions and small molecules between direct cell-cell transfers. Finally, Desmosomes are specialised for strong adhesion of cells via linking to intermediate filaments. Figure redrawn from (Rodriguez-Boulan and Macara, 2014).

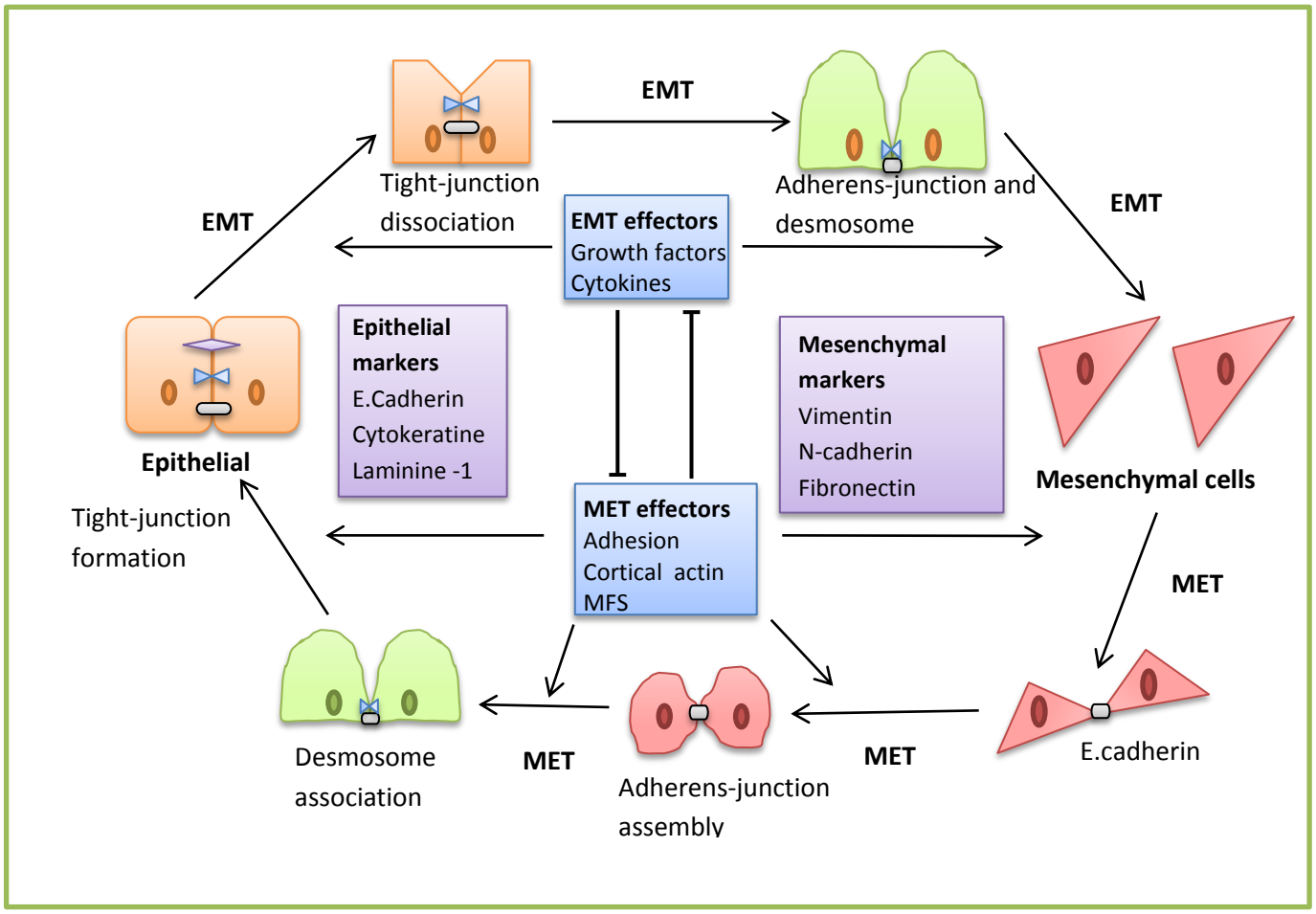


Figure 1-4: EMT process.
The process of transforming epithelial cells into mesenchymal cells is shown in this diagram. The differences in stages between EMT and MET are regulated by effectors of EMT and MET. An EMT involves a functional transition of epithelial cells into mesenchymal cells. The epithelial and mesenchymal cell markers commonly used by EMT researchers are shown. Adapted from (Thiery and Sleeman, 2006).

1.2.1 EMT AND DEVELOPMENT

EMT programmes, originally identified in the chicken primitive streak, are activated at early (gastrulation, neural crest delamination) or late (organ formation) stages of embryonic development in response to a number of extracellular signals. EMT is required during gastrulation to form three embryonic germ layers, ectoderm, mesoderm and endoderm - from the primitive streak. Neural crest cells are generated from epithelial cells by EMT, and migrate through the dorsal neural epithelium, after which they differentiate into a wide range of cell types like neurons of the peripheral nervous system, glial and satellite cells, pigment cells, odontoblasts, the craniofacial cartilage, as well as other cell types (Acloque et al., 2009, Thiery et al., 2009). The activation of signalling pathways including Wnt, transforming growth factor β (TGF-β) which is an essential suppressor of epithelial cell proliferation and thus primary tumorigenesis, fibroblast growth factor (FGF), and bone morphogenetic protein (BMP) results in the activation of transcription factors that act downstream of EMT pathways and are classified as master regulators of EMT (MR-EMT) (Figure 1-5). They include Zn finger transcriptional factors SNAIL (SNAIL1, SNAIL2) and ZEB (ZEB1, ZEB2) families, basic helix-loop-helix (bHLH) proteins E47, TWIST1, TWIST2, and a forkhead transcription factors, such as FOXC2 (Adhikary et al., 2014). These transcription factors have been found to confer malignant traits, such as motility, invasiveness and resistance to apoptosis (Huber et al., 2005, Mani et al., 2008, Oft et al., 2002, Savagner et al., 2005). Some of these transcription factors also appear to play a key role in wound healing (Savagner et al., 2005). EMT is classified into three types, each of which has different functions and pathological consequences. Type-1 EMT occurs in the normal process of organ formation and embryogenesis, allowing generation of mesenchymal cells that have the ability to undergo a mesenchymal to epithelial transition (MET) to generate epithelial cells. MET enhances cell proliferation and growth of epithelial tumour cells at a secondary tumour site. Type-2 EMT appears during tissue regeneration, fibrosis, inflammation and wound healing (Kalluri and Weinberg, 2009). In this case, EMT forms new active fibroblasts that stimulate tissue repair after tissue inflammation or injury (Okada et al., 1997). Type-3 EMT relates to neoplastic cells; it occurs in epithelial cells during cancer progression as they undergo genetic and epigenetic changes transforming them into invasive metastatic mesenchymal cells often with defects in oncogenes and tumour suppressor genes (Kalluri

and Weinberg, 2009). For example, a developmental type-2 EMT occurs during wound healing, tissue repair and fibrosis. A type-3 cancer-related EMT has been observed within a genetically unstable cell background which is able to change cellular properties such as stem cell-like features and latterly its phenotype. A variety of biomarkers are acquired to identify the three subtypes of EMT (Zeisberg and Neilson, 2009).

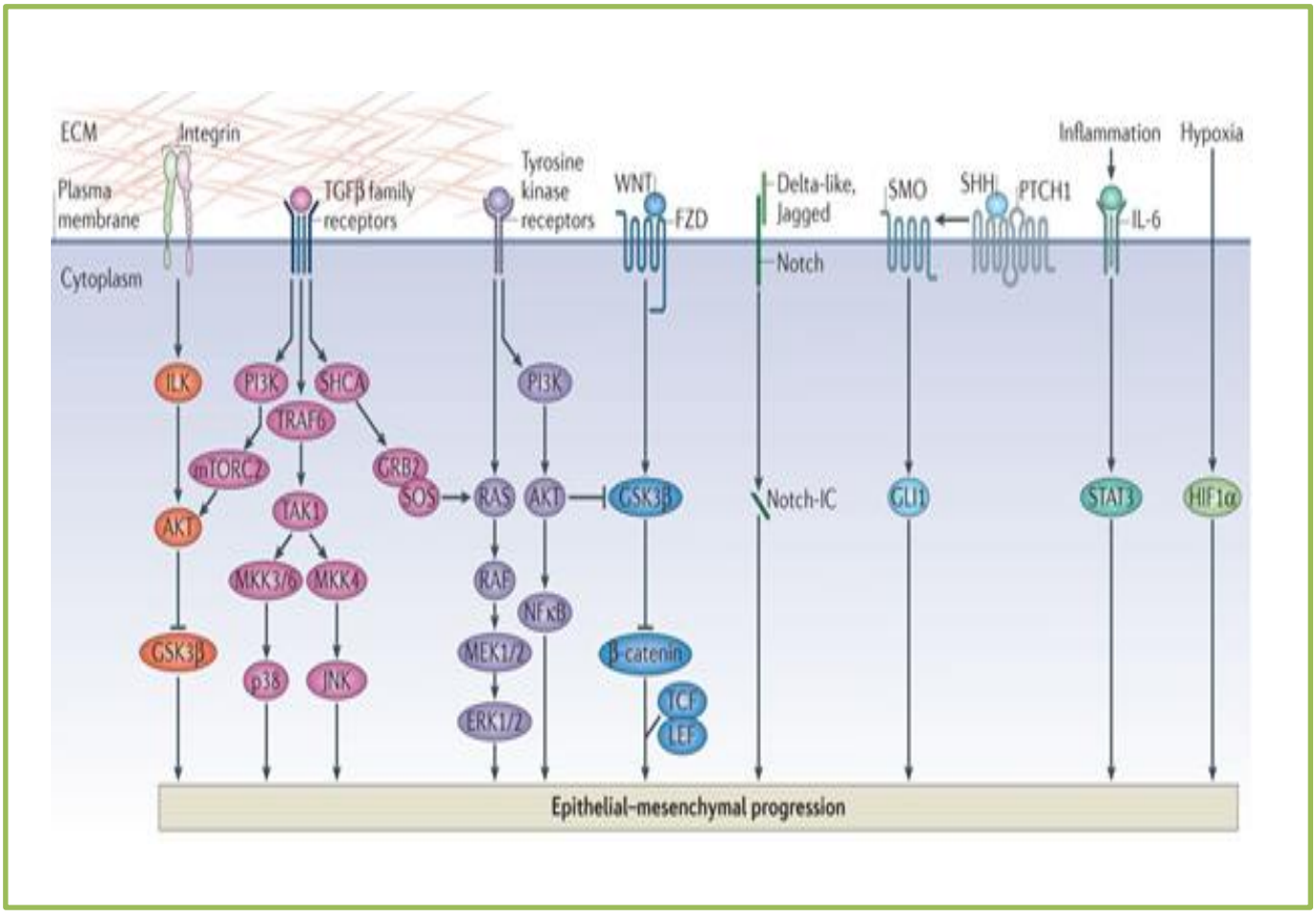


Figure 1-5: Overview of signaling pathways involved in EMT.
Progression of EMT is regulated through several signalling pathways including TGF-β, Wnt and Notch. For example, TGF-β promotes EMT via SMAD proteins and is able to activate the PI3K–AKT, ERK MAPK, p38 MAPK and JUN N-terminal kinase (JNK) pathways. These signalling pathways play a critical role in the reprogramming of gene expression during EMT. Adapted from (Lamouille et al., 2014)

1.2.2 EMT AND CANCER

EMT has been found in many types of carcinoma such as lung cancer, breast cancer and colon cancer (Voulgari and Pintzas, 2009). Cancer-related EMT not only leads to conversion of an epithelial cell into a mesenchymal cell but can also result in a reversible metastable EMT state and an epigenetically-fixed mesenchymal state can be achieved, while partial or complete reversion of EMT is achieved by removal of EMT-induced signals such as TGFβ (Thomson et al., 2011). In addition, the importance of mesenchymal to epithelial transition (MET) has been highlighted in the formation of clinically significant metastasis (Chaffer et al., 2006). This indicates that EMT has a strong effect on the development of the motility of tumour cells during dedifferentiation, while MET supports metastatic colonisation. The capability of tumour cells to undergo either EMT or MET is dependent upon signals from the microenvironment (Brabletz, 2012, Brabletz et al., 2001, Thiery et al., 2009). Several EMT-TFs have the capacity to enhance tumour formation (Thiery, 2002). For example, expression of SNAIL in breast cancer increases its malignancy, and its high expression relates to decreased survival rates (Moody et al., 2005). Twist and FOXC2 are two transcriptional factors that regulate development of EMT and are involved in enhancing metastasis. A high level of Twist expression has been shown to correlate with invasive lobular carcinoma via inducing EMT in breast cancer (Yang et al., 2004). However, FOXC2 up-regulates the mesenchymal transcriptional genes, rather than directly repressing the expression of E-cadherin (Kume, 2012). Thus, EMT has been considered as a fundamental process not only in the case of embryo development but also in different types of diseases, particularly in metastatic cancers in which tumour cells acquire invasive and motile features.

1.2.3 EMT AND CSC

Many studies have provided evidence to prove the link between cancer stem cells (CSC) and EMT. Theoretically, this link was first proposed by Brabletz's group using colorectal cancer as a model tumour (Brabletz et al., 2001) and subsequently it was experimentally studied by inducing EMT in non-tumorigenic, immortalised human mammary epithelial cells (HMLEs) (Mani et al., 2008). HMLE cells undergoing TWIST or SNAIL1- induced EMT acquire a $CD44^{high}/CD24^{low}$ stem cell phenotype. Cells that have undergone EMT followed by

tamoxifen treatment using modified estrogen receptor and activated ER-TWIST or ER-SNAIL formed at least 10-fold more tumour spheres than control cells that had not been exposed to tamoxifen. In a different set of experiments, the gene expression profile of $CD44^{high}/CD24^{low}$ cells isolated from various normal and neoplastic tissues have been established. Data showed high levels of the mRNAs encoding mesenchymal markers, specifically N-cadherin, Vimentin, Fibronectin, ZEB2, FOXC2, SNAIL1, SNAIL2, TWIST1 and TWIST2, and low level of E-cadherin were associated with the $CD44^{high}/CD24^{low}$ tumour cell populations (Mani et al., 2008). However, a subsequent study has reported that suppression of the expression of EMT transcription factors inhibits the pluripotency gene network of tumour cells. This study also suggests that in some cancers, acquisition of mesenchymal characteristics occur dependent on their self-renewal ability (Celia-Terrassa et al., 2012). Several attempts have been made to establish a functional link between EMT programs and embryonic stem cells. A study of non-small-cell lung cancer (NSCLC) has shown that high levels of SNAIL expression upregulates transcription factor NANOG and correlates with enhanced metastatic potential (Liu et al., 2014). Further studies in human embryonic stem cells (hES cells) showed a correlation between EMT and stem cell pathways and suggested that the EMT transcriptional factor ZEB2 has an effect on regulating stem cells. In hES cells, ZEB2 is modulated by Activin-Nodal signalling to regulate the cell-fate decision in favour of neuroectodermal differentiation and to repress pluripotency. In addition, ZEB2 decreased NANOG levels during differentiation, while ZEB2 transcription in turn is repressed by *NANOG, OCT4* and *SOX2* stem cell factors that are responsible for the maintenance of self-renewal. Binding to the regulatory element located at -4kb in the *ZEB2* promoter leads to repression. The expression of NANOG is repressed by ZEB2 and shows its ability to shift embryonic stem cells from pluripotency to differentiation (Chng et al., 2010).

1.3 TRANSCRIPTION FACTORS EXECUTING THE EMT PROGRAMME (EMT-TFs)

It has been proven that EMT is orchestrated by EMT Inducer transcription factors represented by different protein families. Some of these are reactivated during tumour progression, such as ZEB, TWIST and SNAIL. It also has been shown that ZEB and TWIST proteins are preventing cells from undergoing oncogene-induced senescence and apoptosis by functionally inhibiting p53 and RB-dependent pathways (Morel et al., 2012)

1.3.1 SNAIL PROTEINS AND miR-34

The family of SNAIL proteins includes three members, SNAIL1, SNAIL2 (known as Slug) and SNAIL3 that vertebrates needed for embryonic development. The family members of SNAIL are zinc-finger transcriptional factors which are needed during mesoderm formation and gastrulation. All three family members exhibit similar organisation with four to six highly conserved zinc-fingers within the C- terminus domains. These carboxy-terminal domains allow DNA binding and to recognise the E2-box element (CAGGTG). In the same context of DNA binding, SNAIL factors mediate transcriptional repression and their repressor capacity is dependent upon the presence of the SNAG domain located within the N-terminus (Peinado et al., 2007, Tania et al., 2014). SNAIL1 plays an essential role in repressing the epithelial marker E-cadherin which causes invasion and cell migration. Moreover, SNAIL1 and E-cadherin expression was shown to inversely correlate during early and late stages of mouse embryonic development within neural crest cells and mesodermal tissue. However, the function of SNAIL1 in repressing E-cadherin in melanoma cells remains contradictory (Tsutsumida et al., 2004). It has been reported in several epithelial cell lines, such as colon, ovarian, breast and gastric cancers, that the overexpression of SNAIL is correlated with deacetylation of histones (H3 and H4) within the E-cadherin promoter resulted in the repression of E-cadherin (Peinado et al., 2004). Developmentally, SNAIL and SLUG are indirectly increasing the activation of a self-renewal programme by loss-binding to specific stem cells gene promoters including NANOG, and through the induction of OCT4 (Tania et al., 2014).

SNAIL2 expression has been developed in melanocytes and has been detected in malignant melanoma and in benign nevi. SNAIL2 is required for metastatic melanoma because its depletion appears to attenuate growth of primary tumours and highly reduced metastatic potential (Gupta et al., 2005). However, a recent study by Caramel et al. has shown that SNAIL behaves as a tumour-suppressor during melanogenesis (Caramel et al., 2013). Interestingly, SNAIL2 expression has no association with E-cadherin but has a clear relationship with MITF in melanoma. Expression of SNAIL2 in the melanocyte leads to a minor effect on E-cadherin, induction of MITF and increased migration of melanoma cells, which suggests that SNAIL2 has an essential role in the transformation of melanocytes to melanoma cells (Shirley et al., 2012).

It has been found that SNAIL1 expression and activity is indirectly repressed by the loss of the tumour suppressor protein p53 or by mutation of the p53 gene. Two potential targeting sites for the p53-regulated miR-34a, miR-34b and miR-34c were identified within the 3' SNAIL1 UTR. Loss of p53 leads to decreased expression of miR-34 and resulted in up-regulation of SNAIL, which enhanced the cells ability to display EMT markers and become invasive and migratory. In the absence of p53, SNAIL1 is activated in several cancers such as colon, breast and lung. However, a role for the miR-34 family in EMT is broader than regulation of SNAIL1 expression. miR-34a has shown to down-regulate ZEB1 and also the stem cell factors CD44 and c-Myc. In turn, both SNAIL1 and ZEB1 suppress the miR-34 gene family expression by binding to the miR-34a/b/c promoters thereby forming a double negative loop (Kim et al., 2011, Siemens et al., 2011).

1.3.2 TWIST PROTEINS

TWIST1 and TWIST2, which share high structural homology, are basic helix-loop-helix (bHLH) transcription factors that have a vital role to in early embryogenesis and at the stage of gastrulation and mesoderm formation (Vernon and LaBonne, 2004). It also has been shown that TWIST1 is required for directing the migration of the neural crest cell, and at late stages for the proper differentiation of the first arch tissues into bone, muscle, and teeth (Soo et al., 2002), while TWIST2 knockout in mice resulted in postnatal atrophy of multiple tissues, apoptosis, and ultimately death (Sosic et al., 2003). In addition, TWIST1 has been involved in

the induction of cancer-EMT and invasive phenotypes (Vernon and LaBonne, 2004). Previous studies showed that TWIST1 is implicated in many types of human aggressive tumours, such as prostate cancer (Gajula et al., 2013) and breast cancer, as well as sarcomas (Martin et al., 2005). The common structure for all (bHLH) protein family members includes two parallel amphipathic α-helices joined by a loop, which is required for dimerisation. TWIST proteins are able to bind as either homodimers or heterodimers to DNA via the E-box (CANNTG) site (Ellenberger et al., 1994, Peinado et al., 2007). By using a tumourigenesis mouse cell line model, it was proved that TWIST1 works as a metastasis inducer, enhancing intravasation and inversely correlating with E-cadherin expression. Moreover, TWIST was identified as an inducer of metastatic spread in a mouse mammary cell line model of tumourigensis (Yang et al., 2004). EMT was induced during ectopic expression of TWIST in MDCK cells, with changes in cells morphology (fibroblast-like), induction of fibronectin, vimentin, N-cadherin, and down-regulation of E-cadherin and β-catenin (Yang et al., 2004). This has a direct corollary in human malignant prostate tissue where TWIST was expressed in 90% of prostate cancers but only expressed in 6% of benign prostate cases (Kwok et al., 2005).

1.3.3 ZEB PROTEINS AND miR-200 REGULATORY CIRCUIT

The ZEB protein family includes the two proteins ZEB1 (δ-EF1, Areb6, BZP, MEB1, Nil-2-a, TCF8, ZEB, ZEB-1, Zfhep1 or Zfhx1a) and ZEB2 (SIP1 or Zfhx1b), which are complex structures, containing two zinc-finger domains and as well as a homeodomain (Figure 1-6). Both human and murine ZEB1 and ZEB2 proteins contain a high degree of sequence homology within the N-terminal containing four zinc fingers and the C-terminal containing three zinc fingers, which suggests that both proteins have similarities in their DNA- binding sites to ZEB boxes (Sanchez-Tillo et al., 2011, Vandewalle et al., 2009). The central sites of ZEB proteins include the POU-like homeodomain which is not able to bind to DNA but might have the ability to interact with other proteins that are essential for transcriptional activation or even repression (Browne et al., 2010).

A high degree of homology was observed during the study of the zinc finger family in the chicken, mouse, hamster and human (Sekido et al., 1996). As these clusters show high-

sequence homology in ZEB1 and ZEB2, each of the zinc-fingers in both proteins share the same CA(C/G)(C/G)TG sequence located in the promoter region of the target genes. (Remacle et al., 1999). Despite ZEB1 and ZEB2 showing common structural and functional similarities, they may behave differently and act in opposing directions. In 1991, ZEB1 was named as δEF1 due to its correlation with the activity of the lens specific δ-crystallin enhancer (Funahashi et al., 1991). Cloning the *ZEB1* gene from the chicken indicates that ZEB1 consists of nine exons.

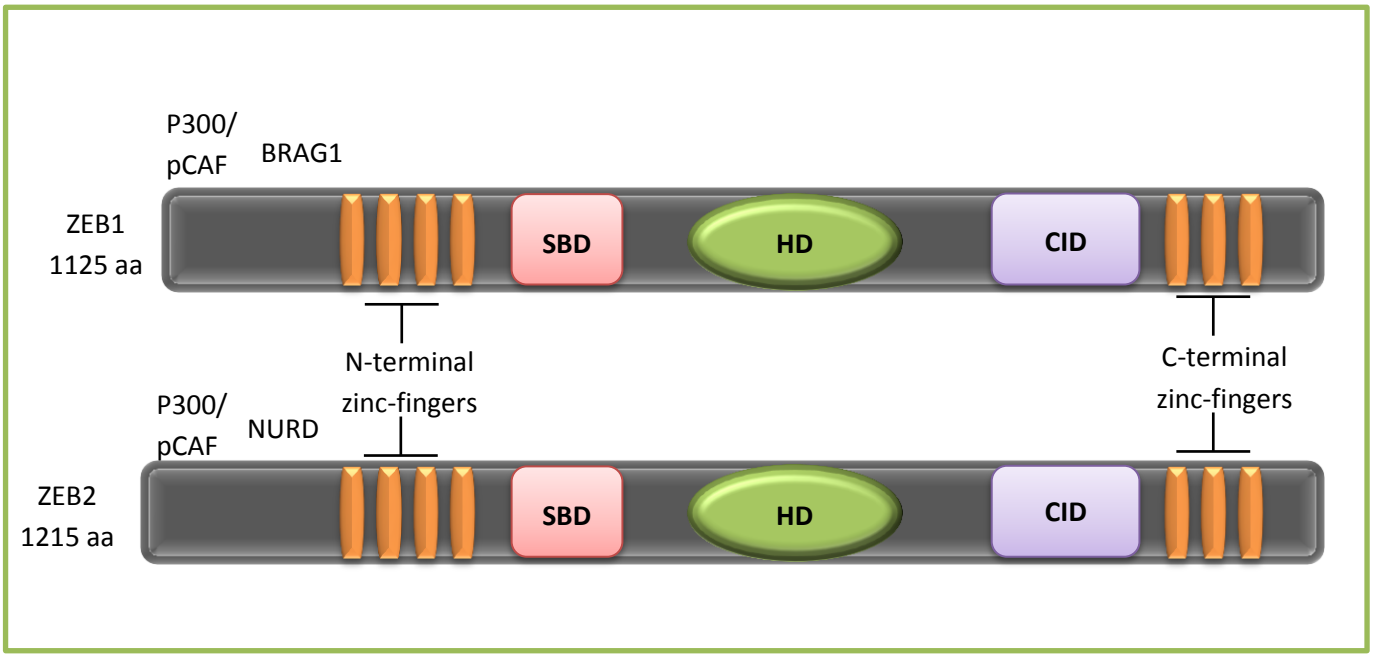


Figure 1-6: Schematics of ZEB1 and ZEB2 proteins structure.
Both proteins ZEB1 and ZEB2 contain main domains including N-terminals and C-terminals zinc finger clusters, the smad binding domain (SBD), the homeodomain located at the centre and the CtBP interacting domain (CID).

The N-terminal zinc fingers are encoded by exons 5 and 6, the C-terminal zinc fingers are encoded by exons 8 and 9, while the homeodomain and the central region is encoded by the large exon 7 (Sekido et al., 1996). ZEB2 was identified by using the yeast two-hybrid, which due to its interaction with the MH2 domain of the receptor-regulated Smad, resulted in the name of Smad-interacting protein 1 (SIP1) (Verschueren et al., 1999). ZEB2 is a complex protein with a size of 136 KDa which follows the widely separated evolution of ZEB1. Analysis of ZEB2 5'UTR in the mouse has identified nine untranslated exons (U1-U9) located up-stream of the first translated exon (exon1). All of these untranslated exons were variously spliced to exon one, but there were no upstream in-frame start codons observed. In addition, three potential cell-type dependent promoters were observed, promoter P1 sited upstream of exon U5, promoter P2 sited upstream of exon U1 and promoter P3 sited upstream of exon 1. It is noteworthy that an antisense transcript of ZEB2, which is highly conserved between human and mouse, was identified (Nelles et al., 2003).

ZEB1 and ZEB2 function as transcriptional repressors, due to their interaction with the presence of CtBP located at the centre of both proteins (Postigo and Dean, 1999, van Grunsven et al., 2003) . Both ZEB1 and ZEB2 contain the smad binding domain (SBD) which is located in the middle of the protein region between the N- and C-terminal zinc-finger clusters (Figure 1-5). They have been shown to interact with R-smad, with strong binding efficiency between ZEB2 and smad3 (Verschueren et al., 1999). The interaction between ZEB proteins and smad was induced by TGFβ and BMP signalling. Remarkably, the cooperation of ZEB1-smad signalling derives transcriptional activation and induces cell growth arrest, while the interaction of ZEB2-smad signalling represses gene transcription (Postigo, 2003). The opposing transcriptional function of ZEB1-smad and ZEB2-smad occurs via cooperation between smad, and the coactivator or corepressor. Functionally, the N-terminal region of ZEB1 binds to p300 and p/CAF which acetylates histones. ZEB2 has also been shown to bind to the corepressor p300 and P/CAF. However, ZEB1 and ZEB2 stabilise smad-p300 and smad-ZEB1 interactions, but not the smad-ZEB2 interaction. The function of P/CAF in binding to ZEB1 is to acetylate several lysine residues resulting in the prevention of the interaction between ZEB1 and CtBP which converts ZEB1 from a repressor to an activator (van Grunsven et al., 2006). Additionally, ZEB1 is able to recruit the Lys-specific

demethylation 1 (LSD1) by attaching it to the histone demethylation located in EMT (Wang et al., 2007).

ZEB1 and ZEB2 have been shown to work as E-cadherin transcriptional repressors during tumour progression (Bolos et al., 2003). The repression of E-cadherin by ZEB2 occurs via binding to E2-boxes sited within the E-cadherin promoter region. Introducing ZEB2 expression into E-cadherin-positive epithelial MDCK cells results in the loss of E-cadherin at both protein and mRNA levels, which disturbs cell-cell adhesion and results in invasive phenotypes showing the fundamental role of ZEB2 in the EMT process (Comijn et al., 2001). The association of ZEB1 and ZEB2 with E-cadherin expression has been reported in solid tumours including brain cancer, breast cancer, cervical cancer, colon cancer, pancreatic cancer, and bladder and renal cancers (Wong et al., 2014). Using the epithelial mouse mammary tumour model EpFosER to induce EMT resulted in the loss of apical-basal polarity and growth disruption in the multilayers (Eger et al., 2005). ZEB1 was up-regulated following EMT induction which in turn repressed E-cadherin expression. In comparison, using the same EMT model, ZEB2 and SNAIL expression was not altered indicating that the immediate repression of E-cadherin occurred by ZEB1. ZEB1 is implicated in repression of the tight junction ZO-1 and loss of desmosomes contact. Interestingly, ectopic expression of ZEB1 was remarkably, able to induce the mesenchymal marker proteins Vimentin and N-cadherin. Moreover, using the breast cancer model MDA-MB-231 which lacks E-cadherin, and ZR-75-1 which expresses high E-cadherin has confirmed the direct association of ZEB1 with E-cadherin (Eger et al., 2005). Additionally, ZEB1 showed a strong effect on regulating E-cadherin expression in early zebrafish development (Vannier et al., 2013). Previous studies using mouse NMuMG EMT model treated with TGF-β resulted in EMT induction enabling cells to acquire mesenchymal properties and suppress E-cadherin (Kondo et al., 2004). In another study, TGF-β treatment in NMuMG induces E-cadherin repression via E-box binding of ZEB1 and ZEB2 to the E-cadherin promoter, with no involvement of SNAIL1 and SNAIL2 in this repression. In addition, knockdown of ZEB1 and ZEB2 had no effect on the expression of mesenchymal markers including fibronectin, N-cadherin and vimentin. Moreover, the up-regulation of ZEB1 and ZEB2 by TGF-β appeared to have an indirect influence, probably via TGF-β-induced of Ets (Shirakihara et al., 2007). In several non-small cell lung cancer cell lines, overexpression of ZEB1 and ZEB2 using a dox-inducible system resulted in repression

of E-cadherin expression levels, while its expression increased during knockdown of ZEB1 and ZEB2 with strong reactivation following silencing of ZEB1 compared to ZEB2. However, the knockdown of ZEB1 and ZEB2 appeared synergistic in the re-expression of E-cadherin (Gemmill et al., 2011)

The roles of ZEB1 and ZEB2 transcription factors and several microRNA species (predominantly, miR-200 family members) are to regulate EMT and MET. A crosstalk between ZEB/miR-200 axis and a number of oncogenic and tumour suppressor pathways takes place at different stages of the metastatic cascade. MicroRNAs are small non-coding 20-22 nucleotide RNAs that function as gene expression inhibitor (He et al., 2005). A panel of 60 cell lines maintained by NCI were analysed to determine the correlation between epithelial phenotypes and miRNAs. Interestingly, the miRNA-200 family indirectly interacted with E-cadherin expression and negatively with the expression of vimentin. Induced miRNA-200 is inversely correlated with ZEB1 and ZEB2 expression (Figure 1-7). Transfected MDA-MB-231 with miR200a and miRNA-200c resulted in the up-regulation of E-cadherin, low levels of ZEB1 and ZEB2, and observed reduction in cell motility. Conversely, inhibition of miRNA-200 in HCT116 resulted in an increase in ZEB1 and ZEB2, reduction of E-cadherin and promotion of EMT. The highly efficient involvement of miRNA-200 in EMT regulation is confirmed due to its direct binding to the eight and nine sites within ZEB1 and ZEB2 3'UTRs mRNA (Christoffersen et al., 2007, Park et al., 2008). Likewise, TGF-β induced EMT in MDCK cells was regulated by the miR-200 family indicating involvement in cancer progression (Brabletz and Brabletz, 2010).

Several data have confirmed that a reverse relationship exists between miR-200 and ZEB proteins, not only in their different functions, but also in controlling each other's expression. These studies have also shown that all of the miR-200 members: miR-200a, miR-200b, miR-200c, miR-141 and miR-429 are transcriptional targets of ZEB proteins. Functionally, a ZEB/miR-200 double negative feedback loop has been identified, in which ZEB1 and ZEB2 transcriptionally repress miR-200, while miR-200 inhibits ZEB1 and ZEB2 expression at post-transcriptional level, consequently inhibiting EMT progression (Figure 1-8). In addition, the ZEB/miR-200 loop is able to control cellular processes such as differentiation, proliferation and apoptosis (Brabletz and Brabletz, 2010, Bracken et al., 2008, Hill et al., 2013, Lamouille

et al., 2014a). This mechanism was studied in MDCK-Pez cells through the overexpression of the protein tyrosine phosphatase Pez, which resulted in down-regulation of the miR-200 family due to the induction of EMT. Moreover, TGF-β-induced EMT in MDCK cells resulted in the down-regulation of the same miRNA-200 family, suggesting that this mechanism is directly involved in the EMT and MET processes (Gregory et al., 2008b). In an alternative study, overexpression of both miR-200c and miR-141 in the colorectal cancer cell line SW480 resulted in the reduced expression of TGF-β2 indicating that the EMT-induced pathway is the miR-200 target. Additionally, miR-141 showed the strongest repressor influence on TGF-β2 expression, whereas ZEB1 had the highest effect on expression via miR-200c (Burk et al., 2008).

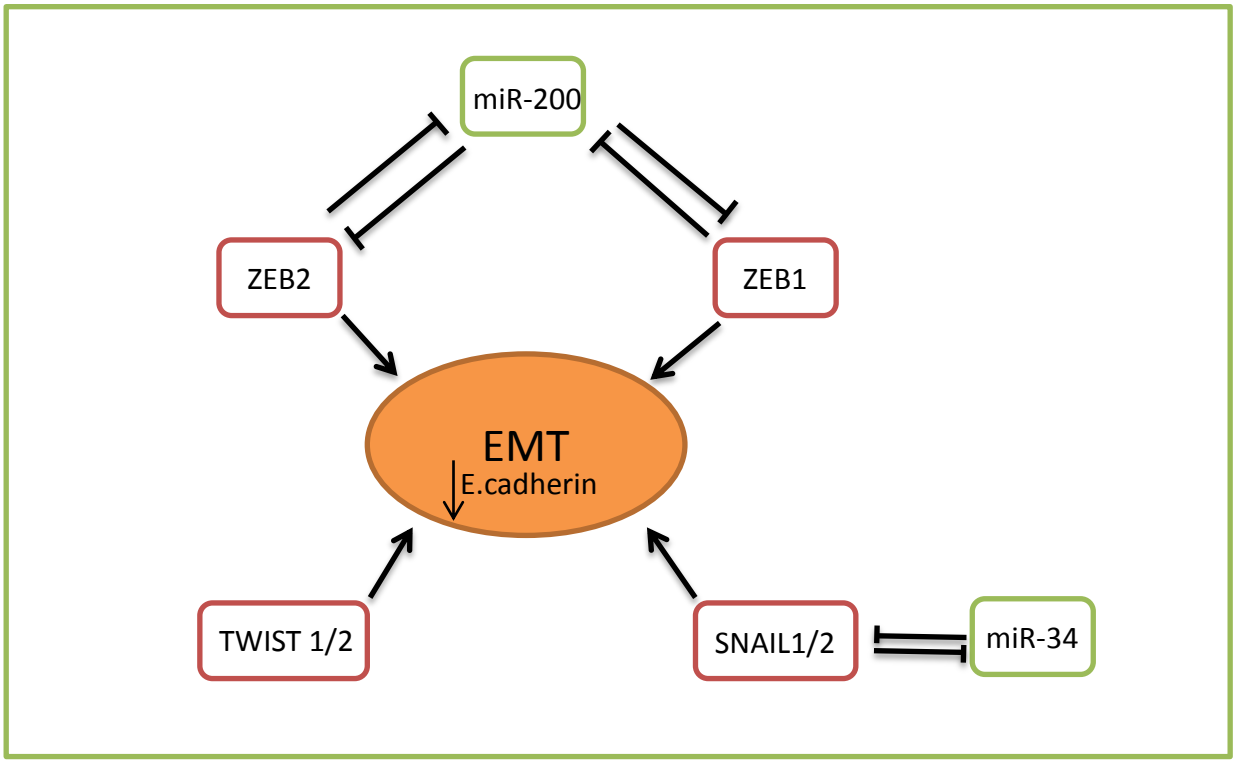


Figure 1-7: Regulation of EMT-TFs during carcinogenesis.
Expression of all the EMT-TFs increases during carcinogenesis, in a tumour specific manner all the EMT-TFs identified as transcriptional regulators of EMT and repressors of E-cadherin. A double negative feedback loop exists between the miR-200 family and the ZEB proteins, and between the miR-34 and SNAIL proteins.

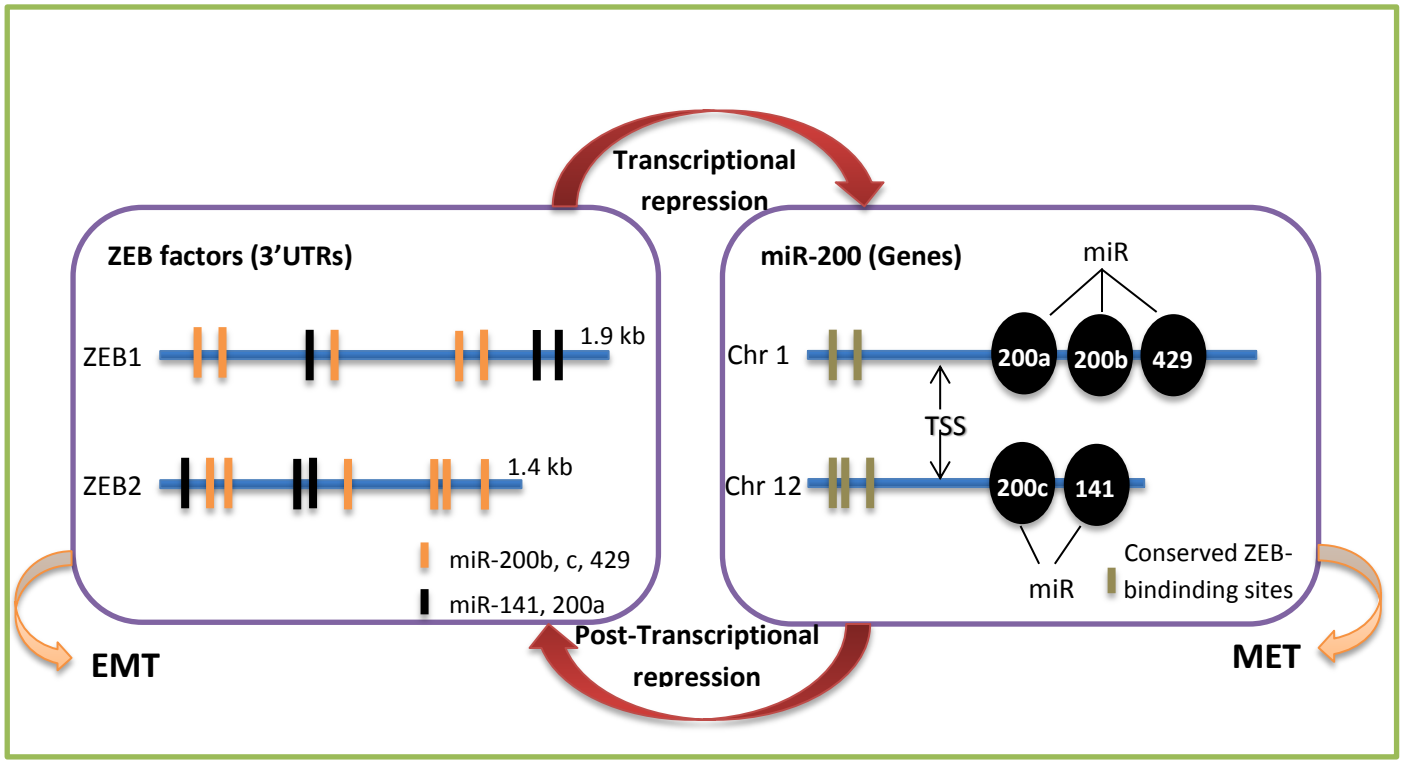


Figure 1-8: The ZEB/miR-200 double negative feedback loop.
Both ZEB factors are inhibited at the post-transcriptional level by the miR-200 family at multiple sites located within ZEB1 and ZEB2 3' UTRs promoting EMT. In return, both ZEB1 and ZEB2 transcriptionally repress the gene of the miR-200 by binding to the E-box recognition site in their promoter resulting in EMT.

1.4 POST-TRANSLATIONAL MODIFICATIONS

Post-translational modification is an important mechanism involved in eukaryotic cells to control their protein function by alerting its chemical structures and regulating complex signalling networks. Based on several studies, PTMs are known to play a vital role in regulating cellular events including gene expression, signal transduction, protein-protein interaction, cell-cell contacts and the intracellular and extracellular environment (Wang et al., 2014). The most common types of PTMs which are involved in cellular alteration are methylation, phosphorylation, glycosylation, acetylation and ubiquitination (Figure1-8) (Deribe et al., 2010).

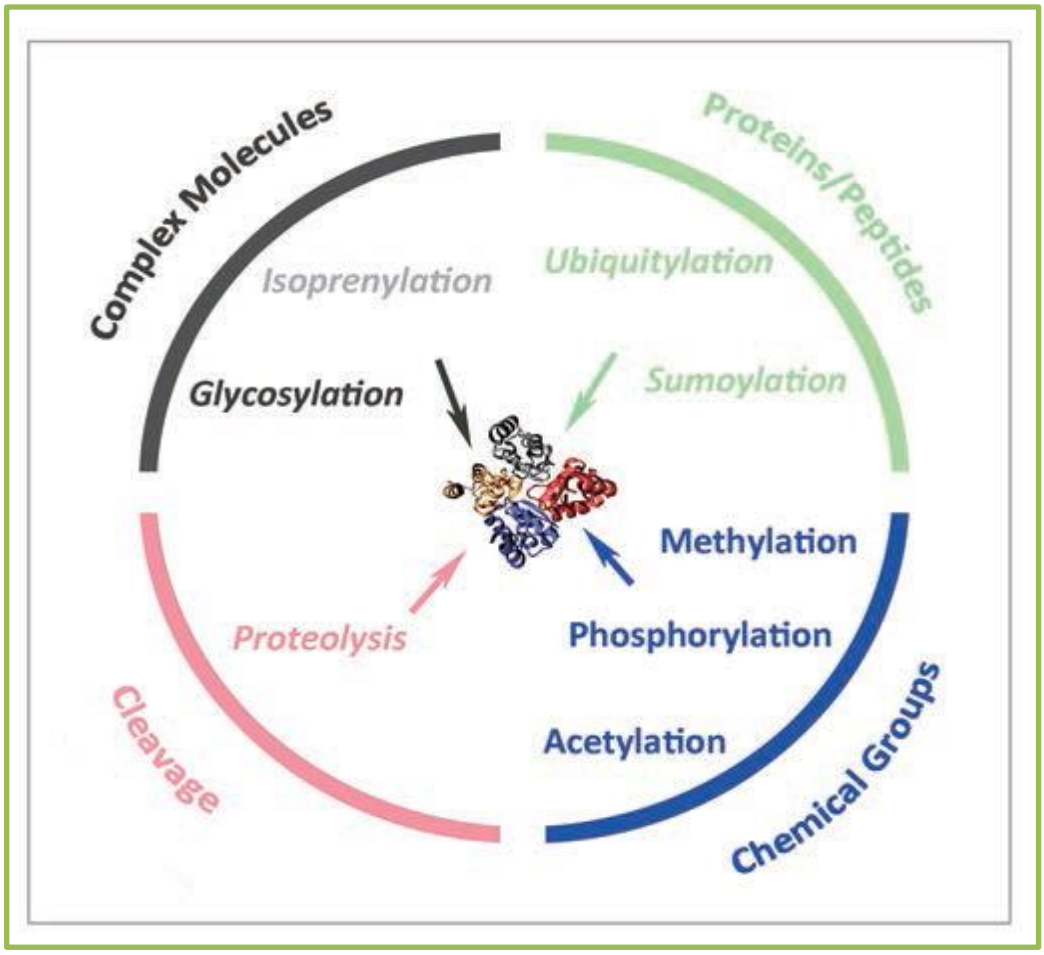


Figure 1-9: Characteristics of post-translational modifications.
Different types of reversible and irreversible post-translational mechanisms that edit the structure, stability and function of proteins after translation. Adapted from (Wang et al., 2014)

1.4.1 THE UBIQUITIN PROTEOSOME SYSTEM (UPS) AND EMT

Ubiquitin, an 8 kDa polypeptide comprising 76 amino acids, is a member of the conserved protein family which share common biochemical mechanisms and regulate intracellular processes in eukaryotic cells (Pickart and Eddins, 2004). Ubiquitin is linked to target proteins via the isopeptide ligament between the ubiquitin carboxyl-terminal glycine and an internal lysine substrate. The ubiquitination of targeted proteins consists of three steps requiring three enzymes E1, E2 and E3 and starts with activation of ubiquitin by the enzyme E1 followed by linking it to the E2 enzyme. In the last step, the ubiquitin is conjugated to the lysine residues of the target protein through the E3 enzyme (Figure 1-9) (Deribe et al., 2010, Wang and Maldonado, 2006). Ubiquitin chains are able to bind to the target proteins through seven lysine residues located within ubiquitin at positions 6, 11 27, 29, 33, 48 and 63 (Galan and Haguenauer-Tsapis, 1997). There are two types of E1 enzymes encoded by the human genome defined as Ubiquitin-activating enzyme 1 (UBE1) and Ubiquitin-like modifier activating enzyme 6 (UBA6) which activates the same members of the ubiquitin-like modifier (ULM) family such as SUMO, ISG15, or NEDD8 (Groettrup et al., 2008). The E2 enzymes include 40 active proteins and inactive E2 variants and all share a core ubiquitin conjugation (UBC) domain that contains 150-200 amino acids. In humans, 35 active E2 enzymes have been identified. Moreover, several E2s contain N- and/or C-terminal protein sequences which play a role in intracellular localisation and regulation properties. The classification of E2 members into three groups is based on the additional extension to the catalytic core, which differs between their functional roles. Class 1 E2 enzymes contain only the catalytic domain, while class2 and class3 contain additional N- or C-terminal extensions, respectively (Sheng et al., 2012, van Wijk and Timmers, 2010).

E3 ubiquitin-protein ligases, of which approximately 600 are coded for by the human genome, are considered to have a critical functional role in UPS as they control the transfer of ubiquitin from the E2 enzyme to the target protein and specify the substrate recognition (Fernandez-Saiz et al., 2013). Four types of E3 have been identified, HECT (homologous to E6-AP C-terminus), RING (really interesting new gene) finger, U-box domain, and the PHD-finger type (Nakayama and Nakayama, 2006). The HECT directly catalyses substrate proteins attached to ubiquitin and, through its interaction with target proteins via conjugation to E2,

results in transfer ubiquitin. The importance of HECT E3, also known as E6-AP, is demonstrated by the loss of expression leading to Angelman Syndrome. Although both RING-finger and U-box E3s have no catalytic role in the ubiquitination of the target protein, they do mediate between E2 and target proteins (Hatakeyama et al., 2001). RING-finger E3s are classified into two types, the N-recognin ligase which regulates the N-end rule pathway, and the multicomponent complex SCF (Skp1/Cullin/F-box/Rbx1/2) family (Ardley and Robinson, 2005).

On the other hand, the proteasome is multisubunit enzyme complex which has an essential role in transcriptional regulation and catalysing the degradation of poly-ubiquitinated proteins. The degradation of ubiquitin proteins is carried out by the 26S (2000 KDa) via an ATP-dependent process (Coux et al., 1996). The 26S proteasome is a multi-protein complex including the 20S core which contains proteolytic sites that break peptide bonds, and the 19S cap that binds to the ubiquitin-chain and transfers protein substrate into the 20S core for degradation (Lecker et al., 2006).

Importantly, development of regulatory EMT transcriptional factors including TWIST, SNAIL and ZEB family degradation are regulated by the ubiquitin proteasome system (UPS). It has been found that TWIST undergoes post-transcriptional regulation and is essential for cleavage by caspase during apoptosis which results in degradation via ubiquitination and the ubiquitin-proteasome system (Demontis et al., 2005, Lander et al., 2011). In contrast, the interaction of SNAIL with E3 ligase β-Trcp promotes the ubiquitination of SNAIL (Zhou et al., 2004). In addition, SNAIL stability is UPS-dependent through its regulation by the E3 ligases MDM2, while both ZEB1 and SLUG are regulated by the E3 ligase cullin7/FBXW8 complex which results in loss of E-cadherin and increased invasiveness (Voutsadakis, 2012). Interestingly, ZEB2 is degraded by the E3 ligase complex Skp1-Pam-Fbxo45, indicating that ZEB2 is the specific substrate of the E3 ligase complex SPF. Ubiquitination of ZEB2 was significantly decreased during Fbxo45 or Pam knockdown. Additionally, the SBD domain of ZEB2 is fundamental for its regulation by Fbxo45 ubiquitination. On the contrary, Fbxo45 expression is down-regulated by miR-27a which results in inhibiting degradation of EMT transcriptional factors (Xu et al., 2015a).

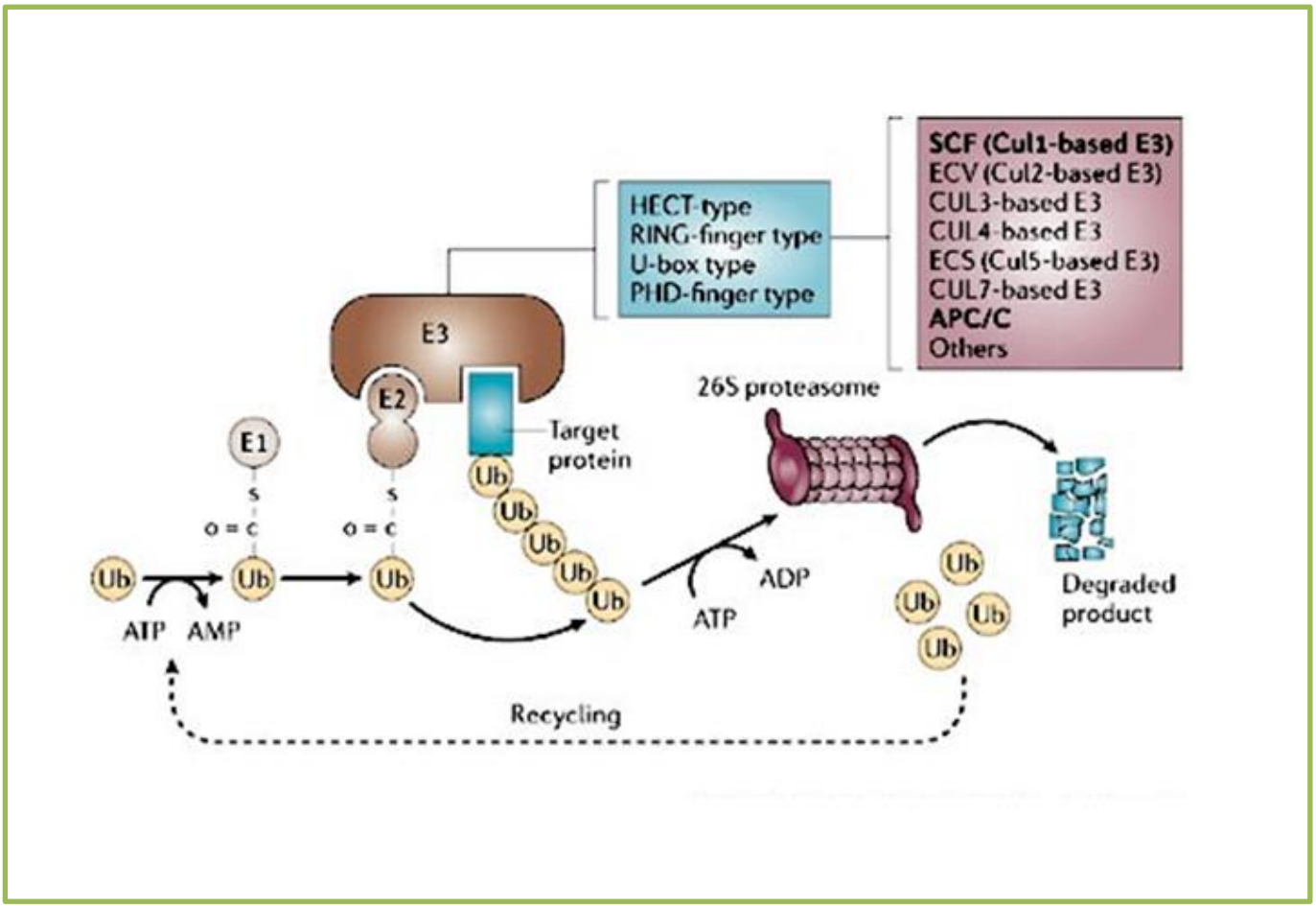


Figure 1-10: Schematics of the ubiquitin-proteasome pathway.
Ubiquitin protein binds to the ubiquitin-activating enzyme E1, and is transferred to the ubiquitin-conjugating enzyme E2. At the final stage, ubiquitin is attached to its targeted protein through E3 ligase followed by the poly-ubiquitin chain that interacts with 26s proteasome which lead to a degraded protein. E3 ligase is classified into four types: HECT-type, RING-finger-type, U-box-type and PHD-finger-type. Adapted from (Nakayama and Nakayama, 2006)

1.5 RAS PATHWAY IN HUMAN CANCER

1.5.1 OVERVIEW OF RAS PATHWAY

An intracellular process within normal cells is controlled by a complex signalling pathway network to ensure that cells are only proliferating when they are required to. Different signalling pathways such as the RAS pathway has been shown to play a major role in helping proliferative signals to pass through membrane receptors including K-RAS, N-RAS and H-RAS. The link between this pathway and malignancy has been demonstrated based on the fact that components of these pathways are present in a mutated form in cancer (Steelman et al., 2011). On the contrary, activation of this pathway by growth factor mutation may control cancer growth. It has been found that approximately 50% of metastatic cancers contain a RAS mutation including a point mutation in the RAS effector domain which allows RAS to interact with various targets (Ward et al., 2001).

The RAS proteins, which were identified as retroviral oncogenes, were found to control signal pathways that regulate normal cellular proliferation and cell growth in most human tumours. The RAS proteins are members of low molecular-weight GTP-binding proteins which are classified into different groups according to the degree of sequence conservation such as the RHO family which controls the actin cytoskeleton (Downward, 2003). RAS family members are very closely related and have 85% amino acid similarity and function in a very similar manner (Lowy and Willumsen, 1993). In addition to their role in regulating cell proliferation, a previous study by Johnson and his colleagues showed that K-RAS is essential for mouse development as it is expressed in all cell types, while knockout of N-RAS or/and H-RAS had no influence in mouse embryogenesis (Johnson et al., 1997). RAS protein activities were found to be regulated through binding to GTP which resulted in activated enzymes that allow RAS to control proliferation (Campbell et al., 1998).

RAS is a common molecule located upstream of multi-signalling pathways including RAF/MEK/ERK, PI3K/AKT and RALGDS. The main effector of RAS is the protein serine/threonine kinase (RAF) which has been extensively studied. RAF is classified into three types (A-RAF, B-RAF and C-RAF), and its activation is by interaction with RAS-GTP (Leevers et al., 1994). Mutations within the three members H-RAS, K-RAS and N-RAS have

been found to play a critical role in human tumorigenesis. These three closely related proteins promote cancer induction through their highly common mutations located at codons 12, 13 and 61. Although the three types share a common similarity, K-RAS mutations are more abundant in cancer types. Data collected from the COSMIC dataset have shown that K-RAS mutation occurs in 22% of all cancers, while 8% of N-RAS and 3% of H-RAS are present in all tumours analysed. Interestingly, 80% of K-RAS mutations at codon 12 and 60% of N-RAS mutated at codon 61 were found, while H-RAS was found to mutated by approximately 50% and 40% at codons 12 and 61, respectively (Prior et al., 2012).

As mentioned previously, RAS mutations are abundant in most cancer types with prevalence for K-RAS. For instance, RAS mutations in Pancreatic Ductal Adenocarcinoma range from 69-95%, with increasing K-RAS mutations appearing during progression of the pancreatic cancer (Fernandez-Medarde and Santos, 2011). In colorectal carcinoma, K-RAS was found in 40-45% of all samples that were analysed, with 80% of mutations occurring at codon 12, while 1-3% of mutations in N-RAS were observed in the same samples (Vaughn et al., 2011). In addition, non-small cell lung carcinomas (NSCLC) expressed a high number - 40% - of K-RAS mutations, with about 94% of all K-RAS mutations appearing at codon 12 (Garassino et al., 2011, Suzuki et al., 1990). It has been shown that RAS mutation is triggered in human NSCLC due to a long-term exposure to chemicals such as smoking tobacco (DeMarini et al., 2001). In contrast, malignant melanoma displays a high frequency of N-RAS mutations compared to K-RAS mutations. The mutation of N-RAS was found in 20-30% of samples analysed, with 86% of N-RAS mutations resulting at codon 61 via exposure to UV irradiation (Hocker and Tsao, 2007). The study of N-RAS mutations in melanoma at various stages of tumour progression has observed its mutation at the early stages and has not shown any increase in metastasis from the same patient suggesting that N-RAS mutation occurs during the early stages of melanoma progression (Omholt et al., 2002). It is worthy of note that RAS' downstream effector B-RAF is activated in human melanoma via RAS-GTP association with the RAS binding domain (RBD) which promote changes in RAF phosphorylation and stimulates its serine/threonine activity, suggesting that both N-RAS and B-RAF are initiating factors promoting malignant melanoma (Ugurel et al., 2007, Wan et al., 2004). Finally, RAS signalling has been found to contribute to other human illnesses such as diabetes and immunological and inflammatory disorders (Fernandez-Medarde and Santos, 2011).

RAS protein activity requires post-translational modification by farnesylation. However, inhibition of farnesyltransferase resulted in the rescue of the RAS isoform process via K-RAS and N-RAS catalysis geranylageranyltransferase (GGTase) (Downward, 2003). Furthermore, the modification of RAS includes the attachment of either farnesyl pyrophosphate or geranylgeranyl pyrophosphate. Modification by farnesylation occurs via attachment with any amino acid, not leucine and ending with a CAAX sequence, while geranylgernylation occurs when the leucine ends with CAAX sequence. Importantly, H-RAS can only be farnesylated, whereas K-RAS and N-RAS are able be farnesylated and geranylgeranylated (Konstantinopoulos et al., 2007).

1.5.2 MAPK PATHWAY IN CANCER

Activated RAF leads to the activation of mitogen-activated protein kinases (MEK1 and MEK2) resulting in the ultimate activation of extracellular signal-regulated kinases (ERK1 and ERK2) (Figure 1-10) (Downward, 2003). It has been demonstrated that K-RAS is the most common activator of C-RAF (Rajalingam et al., 2007) and the activation of RAF has been found to occur through:

i. Recruitment to the plasma membrane mediated by an interaction with RAS

ii. Dimerisation of RAF proteins

iii. Phosphorylation/ dephosphorylating on different domains

However, activation of both C-RAF and A-RAF requires the combined activity of RAS and Src, while B-RAF is only Src-dependent. The role of RAS in activating RAF/MEK/ERK and PI3K/AKT vary as K-RAS has been shown to associate with RAF/MEK/ERK, whereas H-RAS associates with PI3K/AKT (Chang et al., 2003). The interaction of down-stream RAS pathway, the RAF/MEK/ERK and PI3K/PTEN/Akt pathways were found to play an essential role in regulating cell growth and tumorigenesis (McCubrey et al., 2007). In addition, the interaction between RAS and the tumour suppressor p53 has been largely studied. Various mutations on p53 were found to regulate RAS pathway and induced metastasis (Solomon et al., 2012). The inactivation of p53 has been found to cooperate with activated RAS during the development of melanomagenesis (Bardeesy et al., 2001). Moreover, the interaction of RAF/MEK/ERK with the p53 pathway in prostate cancer has been found to promote cell cycle arrest which leads to sensitivity of cells to chemotherapeutic drugs. It also has been shown that the RAF/MEK/ERK pathway regulates apoptosis via phosphorylation of apoptotic regulatory elements. The induction of phosphorylation can be performed by RAF through downstream MEK and ERK, or through independent MEK and ERK. Additionally, inhibition of the RAF/MEK/ERK expression in hematopoietic cells induces proliferation and drug resistance. Therefore, the RAF/MEK/ERK pathway has different implication for cell growth or inhibition of apoptosis (McCubrey et al., 2007).

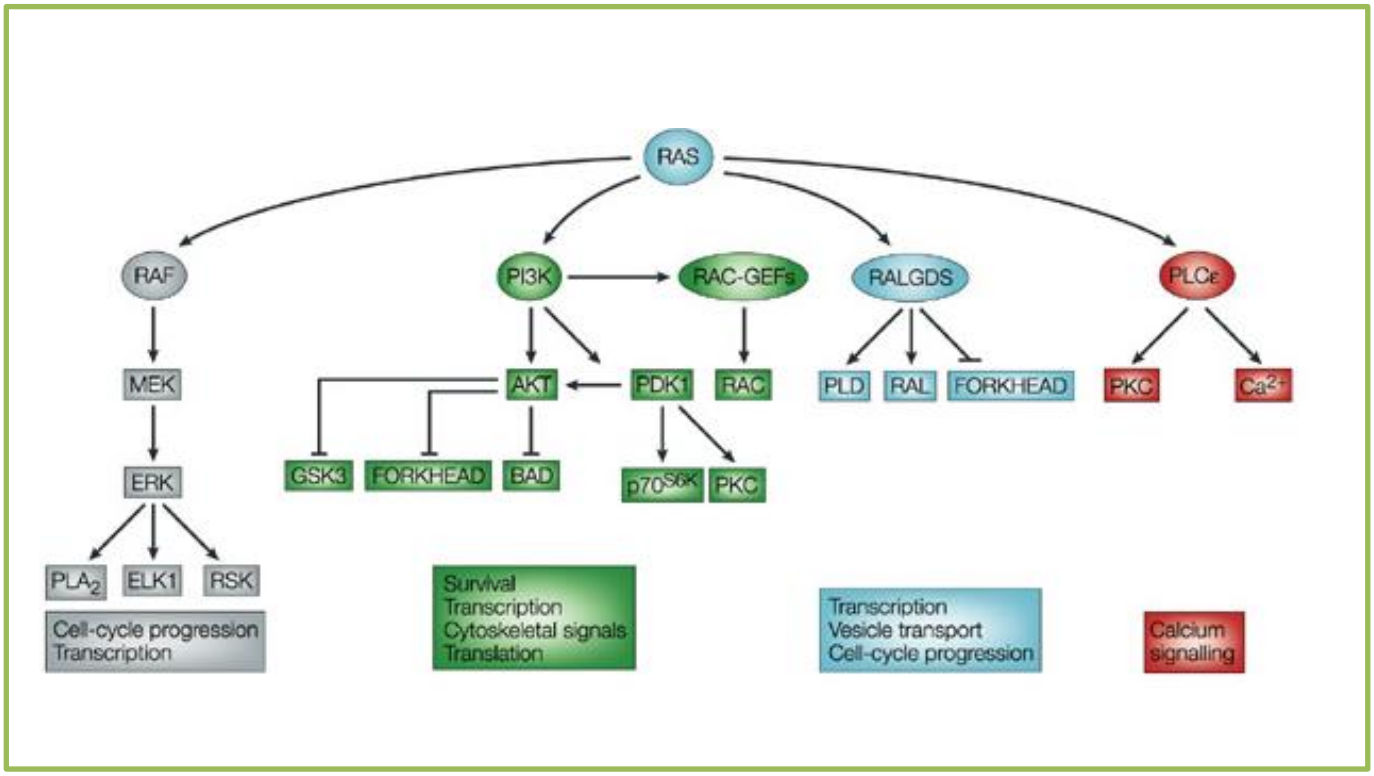


Figure 1-11: Overview of RAS downstream signaling pathways.
The activity of RAS is controlled by its binding to GTP. Once it is activated it will stimulate catalytic activity of several effectors. RAF is considered as the main effector that promotes MAPK which leads to ERK activation. ERK interferes with various substrates in the cytoplasm and the nucleus including EMT transcriptional factors. Downstream of RAS includes PI3Ks, RALGDS and PLCε. Adaptad from (Downward, 2003).

1.5.3 RAS PATHWAY IN CELL INVASION AND EMT

Several oncogenic pathways including RAS and its downstream effectors have been found to induce EMT and are involved in cancer invasiveness as well as metastatic properties. As previously mentioned, the down-regulation of the cell adhesive factor, E-cadherin, is the critical molecular feature during the EMT process. Signalling of RAS to EMT transcriptional factors such as SNAIL and SLUG occurs through RAF and MEK which are inducers of EMT. Furthermore, the activation of the PI3K/AKT pathway, which is located downstream of RAS, regulates E-cadherin during EMT (Larue and Bellacosa, 2005). It has been shown that the phosphorylation of PI3K/AKT is required for TGF-β-induced EMT and invasion (Bakin et al., 2000), while hyperactive RAF/MAPK regulates EMT, suggesting that the interaction between RAS and TGF-β is essential during EMT and cell migration (Janda et al., 2002). The mitogen-activated protein kinase (MAPK) pathway and its subfamily (ERKs, JUNKs and p38 MAPK) interact with TGF-β signalling pathway and promote EMT. The activation of ERK was found to play a crucial role during EMT which led to the acquisition of mesenchymal properties. Furthermore, ERK activation via TGF-β was found to repress E-cadherin expression in various models of EMT (Gui et al., 2012). MAPK has been shown to be involved in EMT due to its role in repressing TGF-β-induced SLUG via MEK (Choi et al., 2007).

In pancreatic cancer, the interaction between K-RAS and CDK8 regulates SNAIL and ZEB1 expression via the Wnt/ β-catenin signalling pathway which leads to EMT (Xu et al., 2015b). Additionally, inducing pancreatitis in adult K-RAS mutant mice resulted in increased EMT and circulating pancreatic cell numbers (Rhim et al., 2012). On the other hand, metastatic melanomas contained a B-RAF mutation in 70% of diagnosed melanoma cases (Tsao et al., 2012). Interestingly, B-RAF mutations are responsible for the activation of several anti-apoptotic pathways including RAS downstream effectors such as MAPK which in turn regulates the expression of EMT transcriptional factors. Actually, B-RAF activates NF- κB through IKKbeta and induces EMT via up-regulation of SNAIL; it activated by NFkB, and suppresses expression of PTEN. On the contrary, suppression of PTEN expression results in the activation of PI3K/AKT (Lin et al., 2010). Ultimately, it has been shown that activation of the B-RAF pathway in melanoma cells resulted in up-regulation of ZEB1 and TWIST1 the down-regulation of ZEB2 and SNAIL2 (SLUG). In contrast, chemical inhibition of both B-RAF

and MEK led to down-regulation of ZEB1 and TWIST1, but up-regulation of ZEB2 and SNAIL2 (Caramel et al., 2013). Therefore, the RAS pathway and its downstream effectors, particularly the MEK-ERK pathway, are essential in regulating EMT.

1.6 HYPOTHESIS

A protein motif adjacent to the smad-binding domain within the ZEB2 protein induces ribosome stalling and compromises translation. This regulatory mechanism is important for the ZEB1/ZEB2 balance which may determine a configuration of EMT programs in normal and pathological conditions.

1.7 AIM AND OBJECTIVES OF THE STUDY

The overall aim of this study was to study both the **transcription and translation** of ZEB2 in different cancer cells, to better understand the role of ZEB2 during the EMT process.

Objectives:

- Analysis of the activity of the ZEB2 U5 and E1 promoters in melanoma cell lines by the measurement of luciferase activity.
- To examine whether mutations in the RAS pathway modify the network of transcription factors and regulate stem cell properties and EMT, in cancer cells via ZEB2.
- To examine the different levels of ZEB2 expression and whether it is controlled at the transcriptional or translational level.
- To examine the regulation of ZEB2 expression by describing a novel mechanism that controls its protein synthesis.

CHAPTER 2: MATERIALS AND METHODS

2.1 CHEMICALS AND REAGENTS

Table 2-1: Reagents

Reagent	Company	Catalogue Number
α-Select Chemically Competent Cells Bronze and Gold efficiency	Bioline (London,UK)	BIO-85025 BIO-85027
ApaI restriction enzyme	New England Biolabs (Hertfordshire, UK)	R0114S
BamHI HF® restriction enzyme	New England Biolabs (Hertfordshire, UK)	R3136S
Bovine serum albumin powder protease free,fraction	Fisher Scientific	BPE9701-100
BglII restriction enzyme	New England Biolabs (Hertfordshire, UK)	R144S
Cycloheximide	Calbiochem (EMD Millipore) (Watford, UK)	239764
Dulbecco's modified eagle media (DMEM) high glucose without L-Glutamine	PAA (Pasching, Austria)	E15-009
Dulbecco's modified eagle media (DMEM) (4.5g/l) liquid with Sodium Pyruvate with L-Glutamine	Lonza (Switzerland)	BE12-604F/12
Heat inactivated fetal bovine serum (FBS)	Sera-Lab	EU-000-F
HighRanger 1kb DNA Ladder	Geneflow (Staffordshire, UK)	L3-0020-S
HindIII restriction enzyme	New England Biolabs (Hertfordshire, UK)	R0104S
Ingenio® electroporation solution	Geneflow	E7-0516
Kod hot start master mix	Millipore (U.K.) Limited	
QuikChange Lightning Multi Site-Directed Mutagenesis Kit – Academic	Agilent Technology	210515
LB Agar, powder (Lennox L Agar)	Invitrogen (Paisley, UK)	22700-025
Lipofectamine™ 2000	Invitrogen	11668-019
Lipofectamine™3000	Invitrogen	L3000008
Luciferase Assay System	Promega (Southampton, UK)	E4030

Marvel Dried Skimmed Milk Powder	Morrison brans	N/A
Miller's LB Broth Base® (Luria Broth Base)	Invitrogen	12795-027
MluI restriction enzyme	New England Biolabs (Hertfordshire, UK)	R0198S
NheI-HF® restriction enzyme	New England Biolabs (Hertfordshire, UK)	R3131S
Penicillin-Streptomycin (PS) (100X)	Life Technologies	15070-063
Phusion™ Flash High-Fidelity PCR Master Mix	Fisher Scientific	F-548S
Puromycin	Life Technologies	A11138-03
Roswell Park Memorial Institute (RPMI) 1640 with glutamine	Lonza	BE12-702f/12
SalI restriction enzyme	New England Biolabs (Hertfordshire, UK)	R0138S
SacI restriction enzyme	New England Biolabs (Hertfordshire, UK)	R0156S
Sodium Dodecyl Sulphate (SDS) (20%)	Fisher Scientific	10607443
Shrimp Alkaline Phosphatase	New England Biolabs (Hertfordshire, UK)	M0371S
Trypsin-EDTA (10X), no Phenol Red	LIFE TECHNOLOGIES	15400-054
Tris-acetic acid ethylenediaminetetraacetic acid (EDTA) buffer (van Grunsven et al.)	Thermo scientific	B49
Tris-borate EDTA buffer (TBE)	Thermo scientific	B52
Tet System Approved FBS, USDA-Approved	Clontech	631106
T4 DNA Ligase	New England Biolabs (Hertfordshire, UK)	M0202S
U0126	Calbiochem (EMD Millipore) (Watford, UK)	662005
Wizard SV Gel and PCR Clean-Up System	Promega (Southampton, UK)	A9281
Wizard® SV Genomic DNA Purification System	Promega (Southampton, UK)	A2360
XbaI restriction enzyme	New England Biolabs (Hertfordshire, UK)	R0145S
XcmI restriction enzyme	New England Biolabs (Hertfordshire, UK)	R0533S

Table 2-2: Reagents made in-house

Reagents Name	Component
β-galactosidase assay master mix	• 66 µL 4 mg/ml ONPG (ortho nitrophenyl β-galactosidase) • in 0.1 M NaPO4, pH 7.5; • 201µL of 0.1 M NaPO4, pH 7.5; • 3µL of 4.5M β-Me in 0.1M MgCl2
Laemmli lysis buffer (cell lysis buffer) (4X)	• 200 mM Tris-HCl (pH 6.8) • 8% SDS • 40% (v/v) glycerol
Phosphate buffered saline (PBS) (g/L:- 8.0 sodium chloride; 0.2 potassium chloride; 1.15 di-odium hydrogen phosphate; 0.2 potassium dihydrogen phosphate; pH 7.3± 0.2 at 25 oC)	Dissolve 10 PBS tablets/1 litre
Ponceau S staining solution	• 0.1% (w/v) Ponceau S • 5% (v/v) acetic acid
Protein loading buffer	• 0.2% (w/v) bromophenol blue • 1% (v/v) 2-mercaptoethanol
Protein running buffer	• 25 mM Tris Base • 190 mM Glycine • 0.1% SDS
Protein transfer buffer	• 25 mM Tris • 190 mM Glycine • 20% Methanol
Tris-buffered saline with Tween (TBS-T)	• 50mM Tris-HCl (pH 8.0) • 150 mM NaCl • 0.1% (v/v) Tween-20
TBS-T with bovine serum albumin (BSA)	• TBS-T • 5% BSA
TBS-T with milk	• TBS-T • 5% (w/v) Marvel dried milk powder

Table 2-3: Primary Antibodies Table

Antibody	Clone no.	Isotype	Migration in SDS/PAGE (KDa)	Dilution	Catalogue no.	Supplier	Reference
α-tubulin	B-5-1-2	Monoclonal mouse IgG1	55	1:20000 WB	T5168	Sigma-Aldrich (Dorset, UK)	-
Cyclin D1	EPR224(IHC)-32)	Monoclonal rabbit IgG	31	1:1000 WB	04-1151	Merck Millipore, UK	-
E-cadherin	36/E-Cadherin	Mouse monoclonal IgG	120	1:2000 WB	610181	BD Transduction Laboratories™ (Oxford, UK)	-
FBXO45	-	Polyclonal rabbit IgG	31	1:100 WB	Ab126521	Abcam (Cambridge, UK)	
NANOG	-	Polyclonal rabbit IgG	34	1:1000 WB	2929.00.02	Novus Biological (Cambridge ,UK)	-
OCT4	-	Polyclonal rabbit IgG	44	1:500 WB	NB100-2379	Novus Biological (Cambridge, UK)	-
P-cadherin	56/P-Cadherin	Monoclonal mouse IgG1	120	1:500 WB	610228	BD Transduction Laboratories™(Oxford, UK)	-
RB	Ab-5	Monoclonal mouse IgG1	105	1:100 WB	OP66-100UG	Merck Millipore, UK	-
Sox2	D6D9	Monoclonal rabbit IgG	35	1:1000 WB	3579	Cell signalling Technology	-
TWIST	Twist2C1a	Monoclonal mouse IgG1	21	1:50 WB	ab50887	Abcam (Cambridge, UK)	-
ZEB1	H-102	Polyclonal rabbit IgG	250	1:2000 WB	sc-25388	Santa Cruz Biotechnology, INC	-
ZEB2 (CUK2)	-	Polyclonal rabbit IgG	250	1:5000 WB	-	In house	Sayan *et al.*, 2009; Oztas *et al.*, 2010)

Table 2- 4: Secondary Antibodies

Antibody	Type	Dilution	Catalogue no.	Supplier
Anti-Mouse Immunoglobulins/HRP	Polyclonal goat	1:2500 WB	00063800	DAKO
Anti-Rabbit Immunoglobulins/HRP	Polyclonal goat	1:2500 WB	00072118	DAKO
Alexa Fluor® 488 Anti–Mouse IgG Antibodies	Polyclonal donkey	1:500 IF	A21202	Invitrogen
Alexa Fluor® 594 Anti–Rabbit IgG Antibodies	Polyclonal donkey	1:500 IF	A21207	Invitrogen

2.2 CELL CULTURE

2.2.1 ROUTINE CELL PASSAGING

All cell culture procedures were performed in a class II laminar flow cabinet, with cell lines maintained in an incubator at 37^{o}C, 5% CO_2 and 100% humidity. The various cell lines used can be found in (table 2-5) including guidance on the type of cell culture media used. All media was stored at 4^{o}C and warmed to 37^{o}C prior to use. Tissue culture flasks, T25, T75 and T175, were supplied from Thermofisher Scientific. Cells were culture and to approximately 70% confluence before passage. Cells were not passaged more than 20 times. Adherent cells were washed with two washes of phosphate-buffered saline (PBS), prior to trypsinisation with 1X trypsin EDTA. Cells were returned to the incubator at 37^{o}C for 5 to 10 minutes, after which the flask was gently tapped to detach cells. The 1X trypsin EDTA was neutralised with the addition of cell culture media. Cells were transferred to a 50 ml centrifuge tube (CFT-900-031F) and pelleted via centrifugation at 1000 rpm for 5 minutes and the pellet re-suspended in 1 ml of media. The cell concentration was calculated using the following formula:

(Total cell number = ((counted cell/4) x 10^4) x volume medium re-suspended in)

The cells were seeded at the required cellular density in the correct sized flask and media added. Cells were immediately returned to the incubator.

2.2.2 FREEZING AND DEFROSTING STORED CELLS

Cells were washed with PBS, trypsinised and pelleted. Therefore, cells were counted, re-pelleted with 2 × 10^6 cells and re-suspended in 1 ml of freezing media (80% complete cell culture media; 10 % FBS; 10 % DMSO) and aliquoted into Cryo.s™ Freezing Tubes from Greiner Bio-One (122263). These tubes were placed in a CoolCell® alcohol-free cell freezing containers, and stored at -80^{o}C overnight. Cells were then transferred into the liquid nitrogen container for long-term storage. In case of thawing, cells taken from liquid nitrogen were immediately placed in a 37^{o}C water bath to thaw. Cells were subsequently mixed with 10 ml of cell culture media and pelleted by centrifugation. The pellet was re-suspended in 5 ml of complete media, transferred to a T25 tissue culture flask and placed in the incubator.

Table 2-5: Cell lines

Cell line	Culture media	Cellular origin	Reference
A375 M	RPMI 1640; 10% FBS; 1% P/S	54 year old female; malignant melanoma; derived from the A375P cell line that were injected into the tail vein of nude mice and lung metastases removed	(Kozlowski *et al.*, 1984)
A375 P	RPMI 1640; 10% FBS; 1% P/S	54 year old female; malignant melanoma	(Kozlowski *et al.*, 1984)
A431	DMEM with L-glutamine; 10% FBS; 1% P/S	85 year old female; human epidermoid carcinoma	(Giard et al., 1973)
DLD-ZEB2	RPMI 1640; 10% FBS; 1% P/S	An adult male; human colorectal adenocarcinoma cell line	-
Human epidermal melanocytes neonatal (HEMN)	Melanocyte Medium(MelM); 1% melanocyte growth supplement; 0.5% FBS; 1% P/S	Human neonatal epidermal melanocytes supplied by TCS Cell works	-
H1299	DMEM with L-glutamine; 10% FBS; 1% P/S	Human non-small cell lung carcinoma cell line derived from metastatic site of lymph node of 43-years old Caucasian male	(Giaccone et al., 1992)
IPC-298	RPMI 1640; 10% FBS; 1% P/S	64 year old female; human cutaneous melanoma established from the primary tumour	-
J82	DMEM with L-glutamine; 10% FBS; 1% P/S	Human bladder carcinoma cell line derived from a poorly differentiated and invasive transitional cell bladder carcinoma (stage 3) of 58-year old Caucasian male.	Marshall et al., 1977, O'Toole et al., 1978)
MCF-7 Tet-on	DMEM with L-glutamine; 10% Tet free FBS; 1% P/S	Human breast adenocarcinoma cell line derived from a 69-year old Caucasian woman in 1970 with a malignant pleural effusion secondary to breast carcinoma	Clontech (631153)
MCF7-ZEB1-EGFP	DMEM with L-glutamine; 10% Tet free FBS; 1% P/S	Human breast adenocarcinoma cell line derived from a 69-year old Caucasian woman in 1970 with a malignant pleural effusion secondary to breast carcinoma; transformed to express ZEB1EGFP by addition of 2 µg/µl of doxycycline (DOX) to the culture medium	Cell model was established by Youssef Alghamdi.

MCF7-ZEB2-EGFP	DMEM with glutamine; 10% Tet free FBS; 1% P/S	Human breast adenocarcinoma cell line derived from a 69-year old Caucasian woman in 1970 with a malignant pleural effusion secondary to breast carcinoma; transformed to express ZEB2-EGFP by addition of 2 µg/µl of doxycycline (DOX) to the culture medium	Cell models was stablished by Youssef Alghamdi.
MDA-231	DMEM with L-glutamine; 10% FBS; 1% P/S	Human breast adenocarcinoma cell line derived in 1973 from 51-year old Caucasian woman with pleural effusion.	(Brinkley et al., 1980, Liu and Feng, 2010)
MDA-468	DMEM with L-glutamine; 10% FBS; 1% P/S	Human breast adenocarcinoma cell line obtained in 1977 from a 51-year old black woman with a pleural effusion	(Cailleau, Olive & Cruciger 1978)
RPMI-7951	EMEM (MG4655), 1% sodium pyruvate; 1% NEAA; 10% FBS; 1%P/S	18 year old Caucasian female; human malignant melanoma established from lymph node metastasis	-
RT112	DMEM with L-glutamine; 10% FBS; 1% P/S	The poorly invasive human bladder carcinoma cell line derived in 1973 from Caucasian female.	Benham et al., 1977, Kawanishi et al., 2008)
SK-MEL-2	EMEM; 10% FBS; 1% P/S	60 year old Caucasian male; human malignant melanoma obtained from the metastatic site	-
SaOs-2	DMEM with L-glutamine; 10% FBS; 1% P/S	Human osteosarcoma cell line derived in 1973 from an 11-years old Caucasian female.	(Fogh and Trempe, 1975)
SK-MEL-5	DMEM with L-glutamine; 1% NEAA; 10% FBS; 1% P/S	24 year old Caucasian female; human cutaneous melanoma	-
SK-MEL-28	DMEM with L-glutamine; 1% NEAA; 10% FBS; 1% P/S	51 year old male; human melanoma	-
SK-MEL-30	RPMI 1640; 10% FBS; 1% P/S	67 year old Caucasian male; human malignant melanoma established from subcutaneous tumour tissue	-
T24	DMEM with L-glutamine; 10% FBS; 1% P/S	The highly invasive human bladder transitional cell carcinoma derived in 1970 from an 81-year old Caucasian woman.	(O'Toole et al., 1972, Ware et al., 2007, Kawanishi et al., 2008)

T-47D	DMEM with L-glutamine; 10% FBS; 1% P/S	Human breast adenocarcinoma cell line isolated from a 54-year old woman with pleural effusion and infiltrating ductal carcinoma.	(Keydar et al., 1979, Ware et al., 2007)
UACC-257	RPMI 1640; 10% FBS; 1% P/S	Human melanoma	-
UMU-C3	DMEM with L-glutamine; 10% FBS; 1% P/S	Bladder carcinoma cell line derived from a urinary bladder transitional cell carcinoma of human male.	(Grossman et al., 1986, Bellet et al., 1997)
U2Os	DMEM with L-glutamine; 10% FBS; 1% P/S	Human bone osteosarcoma cell line, originally known as the 2T line derived in 1964 from a moderately differentiated sarcoma on the tibia of a 15 year old Caucasian female.	(Ponten and Saksela, 1967, Raile et al., 1994)
WM-266-4	DMEM high glucose with L-glutamine ; 1% NEAA; 10%FBS; 1%P/S	58 year old female; human malignant melanoma derived from the metastatic site	(Wan et al., 2004)
ZR-75-1	DMEM with L-glutamine; 10% FBS; 1% P/S	Human breast adenocarcinoma cell line derived from a malignant ascetic effusion of a 63-year old Caucasian woman.	(Engel et al., 1978)

2.2.3 TRANSIENT TRANSFECTIONS

Plasmids (Table 2-6) and siRNA (Table 2-7) were transiently transfected into cell lines using either the Ingenio® electroporation solution in combination with the GenePulser Xcell electroporator from Bio-Rad (165-2660), which was set at 250 V and 250 µF, or Lipofectamine® 2000 Transfection Reagent. Lipofectamine RNAimax was used for siRNA transfection.

2.2.3.1 ELECTROPORATION TRANSFECTIONS

Cells were trypsinised, pelleted and counted and two million cells aliquoted into a 1.5 ml microcentrifuge safe-lock tube from Fisher Scientific (CFA-112-020P). Cells were again pelleted and re-suspended in 60 µl of the Ingenio® electroporation solution and subsequently mixed with the appropriate plasmid DNA or siRNA at the required concentration. The sample was transferred to a 4 mm electroporation cuvette from Geneflow (E6-0076) and placed in the electroporator and the cells electroporated. Cells were immediately transferred to a 6 cm^2 cell culture dish from Greiner Bio-One (628960), containing pre-warmed media and returned to the incubator. For each set of transfections, a positive control for transfection efficiency was included, which consisted of transfection with the pEGFP-C1 plasmid (Table 2-5). Transfection efficiency was determined the following day and cell culture media replaced.

2.2.3.2 LIPOFECTAMINE® 2000 TRANSFECTION

Cells were seeded into 6 well plates to be 70% confluent at the time of transfection. Plasmid DNA was diluted in Opti-MEM® to a concentration of 0.5-5 µg/µl and mixed briefly. 5 µl of Lipofectamine 2000 transfection reagent was diluted in 250 µl Opti-MEM which then added into diluted plasmid DNA. This was gently mixed by flicking the tube and incubated at room temperature for 5 minutes. The transfection complex was then added to cells and incubated at 37°C / 5% CO_2. 4 hours post-transfection, Opti-MEM medium was replaced by cell culture medium and incubated at 37°C / 5% CO_2 for 24-72 hours and transfected cells were analysed.

Table 2-6: Plasmid constructs and vectors

Plasmid name	Plasmid	Insert	Provided by
pGL3_p1047_RE-4	pGL3-Basic vector, promega E1751	Contains ZEB2 U5 promoter and the regulatory element RE/-4	In Lab
pGL3_RE -395	pGL3-Basic vector, promega E1751	Contains -395 promoter cloned from ZEB2.	In Lab
pGL3_p1047_RE/E1	pGL3-Basic vector, promega E1751	Contains ZEB2 U5 promoter and the regulatory element RE/E1	In Lab
pGL3_p1047_RE/Vista	pGL3-Basic vector, promega E1751	Contains ZEB2 U5 promoter and the regulatory element RE/vista	In Lab
PBABE-PURO-HA-ZEB2-double Mutant.	pBABE_PURO	Contains the mutated mouse ZEB2 codons.	In Lab
pEGFP_C1_ZEB2	pEGFP_C1 Clontech	Full length mouse ZEB2.	In Lab
pBI-HA-ZEB2-EGFP	pBI Tet Vector Clontech 631006	Mouse ZEB1 with N-terminal HA-tag and EGFP.	In Lab
pBI-ZEB1-HA	pBI Tet Vector Clontech 631006	Mouse ZEB1 with N-terminal HA-tag.	In Lab
pGL3 CMVluv	pGL3 basic, Promega E1751	CMV promoter	In Lab
pCMV β-gal	pCMV-SPORT1, Invitrogen 10586-014	β-galactosidase (β-gal)	In Lab
pEGFP-C1	pEGFP-C1 Clontech 6084-1	Green fluorescent protein (GFP)- red shifted variant of wild-type GFP	In Lab

Table 2-7: siRNA sequences

Oligo Name	Sense sequence 5'-3'	Anti-sense sequence 5'-3'	Reference
OCT4	AGCAGCUUGGGCUCGAGAA	UUCUCGAGCCCAAGCUGCU	-
SOX2	AGUGGAAACUUUUGUCGGA	UCCGACAAAAGUUUCCACU	(Fang*et al., 2011)*
FBXO45 no.1	GGCUUUACUUUACAUCGAATT	UUCGAUGUAAAGUAAAGCCTT	(Xu et al., 2015a)
FBXO45 no.2	GGACAAUAAUCUACUACAUTT	AUGUAGUAGAUUAUUGUCCTT	(Xu et al., 2015)
Control-siRNA	AUGAACGUGAAUUGCUCAA	UUGAGCAAUUCACGUUCAU	-

2.2.4 CHEMICAL TREATMENT

A375M and A375P (B-RAF mutant) cell lines were treated with B-RAF and MEK inhibitors for 24 hours. Stock solutions at 10 mM were produced in DMSO for the MEK inhibitor U0126. The inhibitor were used at a final concentration of 10 µM dissolved in the appropriate tissue culture media. Additionally, for induction of ZEB1/EGFP and ZEB2/EGFP expression in the MCF7-ZEB1-EGFP and MCF7-ZEB2-EGFP cell lines, doxycycline at 2µg/ml was added to the tissue culture media, from a stock solution of 2 mg/ml in water. The MCF7-ZEB2-EGFP cell line was treated for 8 hours with the protein synthesis inhibitor Cycloheximide at a final concentration of 20µg/ml and presence of doxycycline.

2.3 NUCLEIC ACIDS ISOLATION AND MANIPULATION

2.3.1 EXTRACTING GENOMIC DNA

Cultured cells at the required density were pelleted, as previously described; the genomic DNA was extracted using Wizard® SV Genomic DNA Purification System (Cat. no.A2360 Promega), and stored at -20^{o}C for future use.

2.3.2 TOTAL RNA EXTRACTION

Cells were grown in T25 flasks, washed twice with PBS, followed by the addition of 1 ml of TRI Reagent® (Invitrogen, Cat. no. 15596-026). TRI Reagent® contains a mix of guanidine thiocyanate and phenol to dissolve DNA, RNA and protein. Cells were lysed by pipetting up and down a few times, and transferred to separate 1.5ml Eppendorf tubes and then incubated at room temperature for 5 minutes. Samples could be stored at -80^{o}C.

To every 1ml that had been transferred, 200µl of chloroform was added and shaken vigorously for 15 seconds, followed by 5 minutes incubation at room temperature. Samples were then centrifuged at 12,000 rpm for 15 minutes at 4°C. This generated three phases; the upper phase containing the RNA, the interface containing the DNA and the lower phase containing the proteins. The colourless upper aqueous phase was transferred to a clean Eppendorf tube. 0.5ml of 100% isopropanol was added to this upper aqueous phase to

precipitate the RNA. The tube was incubated for 10 minutes at room temperature and then centrifuged at 12,000 rpm for 30 minutes at 4°C. The pellet was collected after the supernatant had been discarded. The RNA was washed with 1ml of 75% ethanol and centrifuged at 10,000 rpm for 5 minutes at 4°C. The supernatant was discarded, and then the pellet was dried on the bench. Next, the pellet was re-suspended in 50µl RNase-free water and incubated at 55–60°C for 15 minutes. To remove any DNA contamination, 2µl of DNase (RNase-free) was added to sample and incubated for 10 minutes at 35^{o}C and then incubated for an additional 10 minutes at 75^{o}C and finally stored at -80^{o}C.

2.3.3 DNA/RNA QUANTIFICATION

Nucleic acid concentration and total yield were determined using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA). At the beginning and in between samples the pedestal and lid were cleaned with optical instrument cleaner. The program was initialised with ultra-pure water and blanked with the buffer used to dissolve the nucleic acid. Samples were applied onto the pedestal in 1.2µl volumes. Samples were measured in duplicate. Sample purity was determined by monitoring the absorbance readings at 260 and 280 nm, with DNA and RNA having an optimal $A_{260/280}$ ratio of between 1.8 and 2.1 respectively.

2.3.4 VISUALISING DNA

PCR products and DNA plasmids were separated by horizontal agarose gel electrophoresis with equipment obtained from Bio-Rad (170-4485EDU). DNA in 10µl volumes was mixed with 2 µl of 6X DNA loading dye. Varying percentages of agarose gels were produced, ranging from 0.8-1.5%, depending on DNA size. Agarose was dissolved in either TAE or TBE buffers and ethidium bromide added to a final concentration of 2 µg/ml. Samples were loaded, a DNA ladder included, and gels run at 100 volts for 30-45 minutes. DNA was visualised using an UVP BioDoc-H System UV transilluminator and gel images taken.

2.3.5 GENERATION OF cDNA BY REVERSE TRANSCRIPTION (RT)

First Strand cDNA Synthesis Kit cDNA synthesis Kit (Thermo Scientific, Cat. No.K1611) was used to synthesis complementary DNA (cDNA) from total RNA. To a sterile PCR tube, 1µg of total RNA and 1µl of random hexamer primers were made up to 11µl with treated ddH2O water. This combination was mixed gently by pipetting and then heated to 65°C for 5 minutes, followed by a brief chill on ice.

The components (table2-8) were added in the indicated order. This mixture was incubated for 5 min at 25^{o}C, followed by 60 minutes at 37^{o}C. Finally, the reaction was terminated by heating at 70^{o}C for 5 minutes. Samples containing cDNA were stored at -80^{o}C for further work.

Table 2-8: Summary of RT-PCR first Strand cDNA Synthesis

Solution	Volume (µl)
Total RNA	1 µl
Random hexamer primers	1 µl
5X Reaction buffer	4 µl
RiboLock RNase inhibitor (20 u/ µl)	1 µl
10 mM dNTP Mix	2 µl
m-MuLV Reverse transcriptase (20 u/ µl)	2 µl
H_2O	9 µl
Total volume	**20 µl**

2.3.6 DNA CLONING

2.3.6.1 DNA OLIGONUCLETIDES

Pelleted DNA primers (table 2-9) were re-suspended in water to produce a stock solution of 100 µM. A 10 µM working solution was subsequently produced. Additionally, dried siRNA oligonucleotides (table 2-7) were re-suspended in water to a concentration of 100 µM. 2 µl of siRNA were used for each transient transfection.

Table 2-9: PCR Primers

Primers	Sequence	Oligo length	Annealing temp(C^o)
5-ZEB2 RE-4	caccAGATCTGCGCGGAAAATTGGGGACAC	30	61°C
5-ZEB2 RE-4	caccGGATCCGCGCGGAAAATTGGGGACAAC	31	61°C
3-ZEB2 RE-4	catGTCGACAATGGGGATCGTGGGATCAGG	30	61°C
5-Vista-MLU	caACGCGTGCACTCCCTGAGTCCTTCTC	28	68°C
3- Vista-NHE	caccGCTAGCACATCCCTTTTGCTTCAAAGGAT	33	68°C
5-E1-MLU	caACGCGTAGGAAGTTTATAGACAAGTTCC	30	72°C
3- E1-NHE	caccGCTAGCTACCTTATACAGGAAAGGGG	30	72°C
5-promMLUshort	caACGCGTATGCCAAAGGCTTGACGGGCG	29	72°C
3-promNHEnew	caACGCGTGGGAGTGGCAGAGCGCGCGGA	29	72°C
LV- MUTANT	CACCAGCCCTCTGGGTGTGCACCATCTG	28	65°C
G- MUTANT	CACCAGCCCTTTAGGCGTACACCCATCTG	29	65°C
K1,2 MUTANT	AGGCTTACTTAGGATTAGAACAGAACCAC	29	65°C
K3 MUTANT	CTTCAATGACTATAGAGTTCTTATGGCAAC	30	65°C
LGV_MUT1	CACCAGCCCTCTGGGCGTGCACCCATCTG	29	65°C
LGV_MUT2	CAATGCAGCACCTGGGCGTGGGGATGGAAGC	31	65°C

2.3.6.2 POLYMERASE CHAIN REACTION (PCR)

PCR was carried out using 2µl of forward and reverse primers (10pmol/µl) designed to include the targeted DNA fragment (Table 2-8), 20 ng/µl of the genomic DNA, and 25µl of 2 x Phusion™ Flash PCR Master Mix (Cat. No. E0553L, Biolabs), and 20 µl of RNase-free water in a 50µl PCR reaction. The programme was set for pre-heating at 94^{o}C for 30 sec, then 40 cycles of [denaturation at 94^{o}C for 20 sec; annealing at 61^{o}C for 20 sec; and extension at 65^{o}C for 3 minutes]. The final extension was at 72^{o}C for 1 minute. The PCR products were purified using Wizard® SV Gel and the PCR Clean-up kit (Promega, cat. No. A2360). The final 30µl volume of purified PCR products was stored at 4^{o}C.

2.3.6.3 RESTRICTION ENZYME DIGEST OF AMPLIFYING DNA AND PLASMID DNA

Restriction digests were performed to check the quality of the plasmids and to confirm the size of the plasmid insert. Restriction digests were performed as 20 µl reactions, using 1 µg of plasmid, 1µl of restriction enzyme, 2µl of the corresponding 10X buffer, with the remaining volume composed of nuclease-free water. The reaction was incubated on a heat block for 1 hour at 37^{o}C. When a second sequential digestion was required, 1µl of the first digest was retained to check restriction enzyme activity, with the remaining 19µl used in the second reaction. The additional components of the second digestion include 1µl of restriction enzyme. The reaction was incubated at 37^{o}C for 2 hours. A 1µl sample of the resulting double digest was visualised using a 0.8% TAE agarose gel, along with the uncut plasmid and single digest. Digested plasmids were purified using the Wizard® SV Gel and PCR Clean-Up System (section 2.3.6.4).

2.3.6.4 LIGATION OF PLASMID DNA WITH INSERT DNA

The ligation mixture was set up with 1µl of digested vector, 1µl of T4 DNA ligase, and 2µl of T4 DNA ligase buffer. Varying amounts of DNA digest were added to produce a final concentration of a 1:3 molar ratio of vector: insert (DNA digest). Distilled water was added to produce a reaction of 10µl. The ligation mix was incubated overnight at 16^{o}C.

2.3.6.5 DNA PURIFICATION

DNA was purified using the Wizard® SV Gel and PCR Clean-Up System. This product removed contaminants, such as excess nucleotides and primers. The DNA was either purified after excision from an agarose gel or directly from solution. DNA was mixed with a Membrane Binding Solution (4.5M guanidine isothiocyanate, 0.5M potassium acetate (pH 5.0)), which contained guanidine isothiocyanate to denature proteins, so preventing degradation of the DNA. Through a series of centrifugations, the DNA became bound to the silica membrane and was washed with a Membrane Wash Solution (10mM potassium acetate (pH 5.0), 80% ethanol, 16.7µM EDTA (pH 8.0)). The DNA was finally eluted in nuclease-free water.

2.3.6.6 CHEMICAL TRANSFORMATION OF *E.Coli*

Plasmid DNA was chemically transformed into α-Select Chemically Competent Cells, of either bronze or gold efficiency. Prior to use, cells were stored at -80^{o}C and thawed on ice. Cells were mixed by gentle flicking of the tube and 50 µl of thawed competent cells were aliquoted into pre-chilled 1.5 ml micro-centrifuge tubes. 2.5 µl of plasmid DNA was added to the competent cells, gently flicked and incubated on ice for 30 minutes. Cells were then heat shocked in a water bath at 42^{o}C for 30 seconds. Samples were returned to ice for 2 minutes. 400 µl of SOC medium (2% (w/v) tryptone; 0.5% (w/v) yeast extract, 8.6 mM NaCl, 2.5 mM KCl, 20 mM MgSO4, 20 mM glucose) was added to the transformation reaction and shaken for 1 hour at 37^{o}C. As a positive control for transformation, pUC19 DNA was provided with the competent cells and used in a separate reaction. Additionally, a negative control for transformation was included, which lacked plasmid DNA and so was antibiotic sensitive.

Meanwhile, previously dissolved and autoclaved LB agar (10g SELECT peptone 140; 5 g SELECT yeast extract; 5 g sodium chloride; 12 g SELECT agar per litre) was melted by heating and allowed to cool prior to the addition of either ampicillin or kanamycin to a final concentration of 100 µg/ml or 50 µg/ml, respectively. This was then poured into culture dishes and allowed to set. 100 µl of the transformation reaction was then spread onto the culture dish and incubated overnight at 37^{o}C. Single colonies were then picked from the plate using a sterile pipette tip and the bacterial population expanded following the steps for small scale production of plasmid DNA.

2.3.6.7 ISOLATION OF PLASMID DNA

2.3.6.7.1 SMALL SCALE ISOLATION OF PLASMID DNA

Colonies were directly picked from the agar plate and added to 5 ml of LB broth (10 g SELECT peptone 140; 5 g SELECT yeast extract; 10 g sodium chloride per litre) containing antibiotic at the previously mentioned concentrations and incubated at 37^{o}C for approximately 8 hours or overnight with orbital shaking. This was classed as the starter culture. The plasmid DNA was then extracted and purified using the QIAprep Spin Miniprep Kit, following the protocol for isolation of high-copy plasmid DNA from *E.*coli, which can isolate up to 30 µg of plasmid DNA. The saturated LB broth was initially transferred into a 1.5 ml micro-centrifuge tube and the cells pelleted by centrifugation at 8000 rpm for 3 minutes. The pellet was re-suspended in Buffer P1 containing RNase and LyseBlue, followed by the addition of Buffer P2, which contains SDS, resulting in cellular lysis and release of plasmid DNA. Buffer N3 was then added and the tube inverted, ensuring neutralisation of the lysate to allow optimal binding of plasmid DNA to the silica membrane of the spin columns. Samples were then centrifuged at 13,000 rpm for 10 minutes to pellet cell debris, protein and genomic DNA and the resulting supernatant passed through a spin column by centrifugation at 13,000 rpm for 1 minute, whereby plasmid DNA became bound to the membrane. The membrane was washed by centrifugation in Buffer PE, which contains ethanol to remove contaminants. The DNA was finally eluted in Buffer EB (10 mM Tris/HCl, pH 8.5). Subsequently, the concentration and quantity of plasmid DNA was determined (Section 2.3.3). Additionally, the integrity of the plasmid DNA was visualised on a 0.8% agarose gel (Section 2.3.4).

In certain situations, the resulting plasmid preparation was further analysed to ensure the presence of the correct insert. This was either performed via a restriction digest (Section 2.3.6.3); using restriction enzymes that specifically cut the plasmid DNA at known sites to generate identifiable fragments. Alternatively, the plasmid DNA was sent for DNA sequencing (Section 2.3.7).

2.3.6.7.2 LARGE SCALE ISOLATION OF PLASMID DNA

In order to generate high quantities of purified plasmid DNA, the NucleoBond® Xtra Maxi Plus kit was used, following the protocol for maxi high-copy plasmid purification. This advised recovery of up to 1000 µg of plasmid DNA. A large overnight culture was prepared using 300 ml of LB Broth with antibiotic and diluting the starter culture 1:1000. This culture was grown for 16 hours at 37^{o}C with orbital shaking. The culture was then pelleted by centrifugation at 6,000 *x g* for 20 minutes at 4^{o}C. The pellet was re-suspended in Buffer RES, which contained RNase A. Cells were lysed in Buffer LYS, containing sodium hydroxide and SDS. Samples were mixed by inversion and incubated for 5 minutes. Meanwhile, the NucleoBond® Xtra Column and Filter were equilibrated with Buffer EQU by gravity flow. The cell lysate was then neutralised with Buffer NEU, which contained potassium acetate. The precipitated lysate was then applied to the NucleoBond® Xtra Column and Filter and contaminating protein, chromosomal DNA and cell debris were cleared from the lysate and the plasmid DNA became bound to the silica resin. The column and filter were then washed with Buffer EQU, after which the column was discarded. The silica resin was washed again and the plasmid DNA eluted under high salt conditions. The protocol was then transferred to the Concentration of NucleoBond® Xtra eluates with the NucleoBond® Finalizers. The eluted DNA was first precipitated by the addition of isopropanol. The sample was then loaded into a syringe with attached NucleoBond® Finalizer, containing another silica membrane, and the sample passed through. The membrane was then washed with the addition of 70% ethanol to the syringe. Excess ethanol was removed from the syringe and the membrane dried. The purified plasmid DNA was subsequently eluted in Tris buffer (5 mM Tris/HCl, pH 8.5).

The quantity and quality of the purified plasmid DNA was checked and visualised on an agarose gel (Sections 2.3.3 and 2.3.4). The plasmid DNA was then ready for use in downstream process, such as in transient transfections.

2.3.7 DNA SEQUENCING

Prior to DNA sequencing, PCR products and plasmids were cleaned using the Wizard® SV Gel and PCR Clean-Up System (Section 2.3.9). DNA sequencing was undertaken by GATC Biotech Ltd (London, UK). Plasmids at 30-100 ng/µl and the corresponding sequencing primers at 10pmol/µl, were sent to the GATC Biotech laboratories. Sequencing data was returned with Phred20 base calling quality and data provided in ABI, SEQ and FAS file formats.

2.3.8 SITE-DIRECTED MUTAGENESIS CLONING

QuikChange Lightning Multi Site-Directed Mutagenesis kit (Agilent Technology, cat. No. 210515) was used to introduce single or up to five different sites concurrently. The three-step method was firstly performed using mutagenic primers to set up mutant strand synthesis PCR reaction (table 2-10). The programme was set for pre-heating at 95^{o}C for 2 minutes, then 30 cycles of [denaturation at 95^{o}C for 20 sec; annealing at 55^{o}C for 30 sec; and extension at 65^{o}C for 3 minutes]. The final extension was at 65^{o}C for 5 minutes. Subsequently, 1 µl of *Dpn* I restriction enzyme was added directly to the sample, gently mixed, spin down for 1 minuet and incubated at 37^{o}C for 5 minutes. Finally, transformation was carried out using 45 µl of the XL10-Gold ultracompetent cells thawed on ice. 2 µl of the β-ME was added to the cells, mixed gently and incubated on ice for 10 minutes, flicked gently every 2 minutes. 1.5 µl of the *Dpn* I-treated DNA from mutagenesis reaction was transferred to the ultracompetent cells containing β-ME and followed steps in (section 2.3.6.6).

Table 2-10: Summary of PCR component reaction

Reaction Component	Templates >5 kb
10x QuickChange Lightening Multi reaction buffer	2.5 µl
H_2O	17.5 µl
QuickSolution	1 µl
ds-DNA Template	1 µl
Mutagenic primers	1 µl
dNTP Mix	1 µl
QuickChange Lightening Multi enzyme blend	1 µl

2.3.9 RT-qPCR REACTION

RT-qPCR reactions were prepared under a highly sterile environment. Ultrapure DNAse/RNase-free distilled water and RT-qPCR Fast SYBR® Green Master Mix (Applied Biosystems, cat. No. 4385612) was prepared with cDNA and primers (Table 2-7) in 20µl total reaction volume as follows:

Table 2-11: RT-qPCR Reaction components

Component	Volume (µl)
Fast SYBR® Green Master Mix	10 µl
Forward primers	0.5 µl
Reverse primers	0.5 µl
cDNA	4 µl
DNAse/RNase-free distilled water	5 µl
Total	**20 µl**

Expression of each gene was analysed in triplicate in 96 well PCR plates. After adding the appropriate volume of RT-qPCR Master Mix, cDNA Sample, primers and dH_2O in each well, with each plate containing a non-template control, the plate was sealed and centrifuged for 10 seconds.

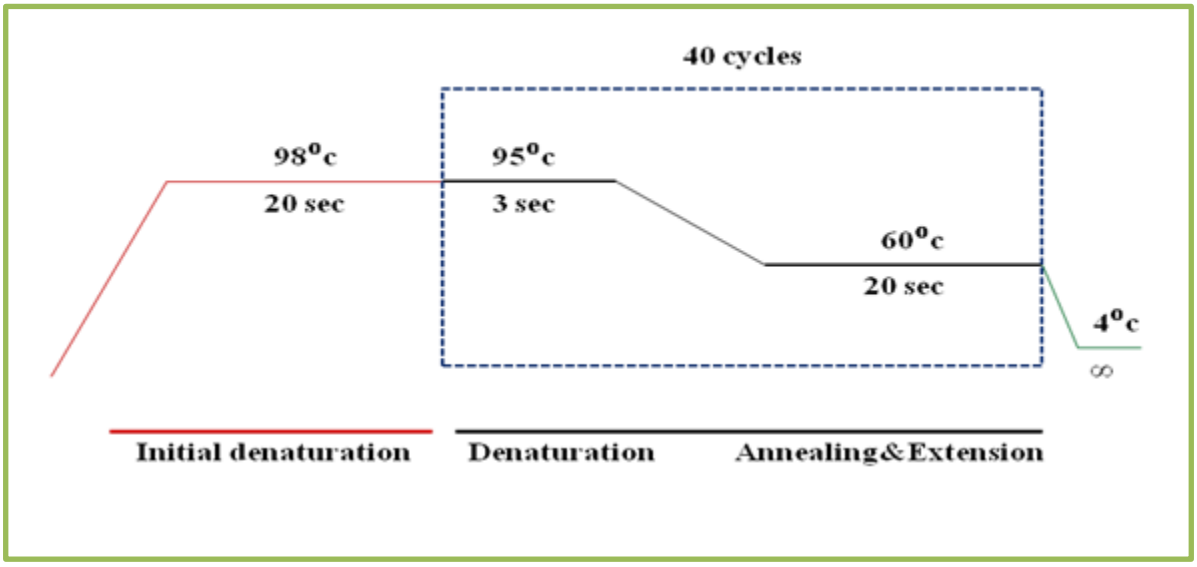


Figure 2-1: RT-qPCR cycling condition.

2.4 GENERATION OF A STABLE CELL LINE

The MCF-7 Tet-on breast cancer cell line was used to establish a stable cell line. Expression plasmids were transfected as shown in (section 2.2.3) using 4 µg of plasmid with addition of 1 µg of pIRES_Puro as a selection marker into MCF-7 Tet-on. Cell suspension was added to the appropriate culture medium. 100 µl of medium containing transfected cell suspension were transferred into all wells in the 96 well plates and incubated for 24 hours. The next day, medium was aspirated and cells were fed with fresh medium containing 1 µg/ml Puromycin (Life technology, cat.no. A11138-03).

2.5 WESTERN BLOTTING

2.5.1 SAMPLE PREPARATION

2.5.1.1 PROTEIN ISOLATION

Cells were obtained from the cell culture incubator and washed with PBS. Dependent upon the number of cells present within the flask, an appropriate volume of Laemmli buffer, diluted to 1X, was added. The cells were scraped to promote detachment from the base of the flask. Lysate containing detached cells was transferred into a 1.5 ml microcentrifuge tube. Samples were then boiled on a heat block at 94^{o}C for 10 minutes. The samples were centrifuged and sonicated on a Soniprep 150 from MSE for 10 seconds to fragment DNA. Protein concentration was then determined and samples were stored at -20^{o}C for future use.

2.5.1.2 DETERMINATION OF PROTEIN CONCENTRATION

Protein quantification was performed using the BCA Protein Assay Kit, which is compatible with the SDS detergent present within Laemmli buffer. This was a colorimetric based detection method for total protein quantification, using bicinchoninic acid (BCA). Quantification occurred in a 96-well format (Greiner, 655180), with 200 µl of BCA reagent required for each sample, consisting of 50 parts BCA Reagent A to 1 part BCA Reagent B. Additionally, a series of protein standards was included, ranging from 2 µg/µl to 25 ng/µl of bovine serum albumin (BSA). The colorimetric reaction commenced with the addition of 5 µl of pre-boiled protein sample or protein standard. A blank sample was also included, whereby the protein sample was replaced with Laemmli buffer. The 96-well plate was placed on a shaker for 30 seconds, then at 37^{o}C for 30 minutes. The plate was allowed to cool to room temperature, and an absorbance reading at 562 nm was taken on a BioTek ELx808 Absorbance Microplate Reader (BTELX808). The absorbance value obtained for the blank sample was subtracted from the absorbance readings for the protein samples and standards. A standard curve was generated for the protein standards, allowing the concentration of the unknown protein samples to be determined. Protein samples were diluted with the addition of 1X Laemmli buffer to a concentration of 1 µg/µl.

2.5.1.3 GEL PREPARATION

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed for the separation of proteins using various percentages of resolving gel and 5% stacking gel (table 2-11). The Mini-PROTEAN Tetra Electrophoresis System from Bio-Rad (165-8006) was used to cast 1.5 mm thick vertical gels. Acrylamide solution, Tris-HCl, SDS and water were mixed before polymerisation was initiated by addition of 10% ammonium persulphate (APS) and *N, N, N', N'*-tetramethylethylenediamine (TEMED). The resolving gel was pipetted into the cassette and then water saturated isobutanol applied to the top to remove air bubbles. The gel was allowed to set for 20 – 30 minutes. The isobutanol was removed and the top of the gel rinsed with water and dried. The stacking gel was loaded between the glass plate and a 15 well comb inserted and left for 20 minutes at room temperature.

Table 2-12: Summary of gel preparation

Solution component	8% resolving gel (ml) (10ml)	12% resolving gel (ml) (10ml)	5% stacking gel (ml) (10ml)
Sterile H_2O	5.3	3.3	6.8
30% acrylamide mix	2.0	4.0	1.7
1.5 M Tris (pH8.8)	2.5	2.5	-
1.0 M Tris (pH 6.8)	-	-	1.25
10% SDS	0.1	0.1	0.1
10% APS	0.1	0.1	0.1
TEMED	0.008	0.004	0.01

2.5.1.4 LOADING PROTEIN SAMPLES

Proteins samples at 1 µg/µl were combined with protein loading buffer and samples heated to 96^{o}C for 10 minutes and centrifuged. 20 µl of each sample were loaded into the cast wells, including a protein standard, and electrophoresis performed in protein running buffer at 170 volts for approximately 60 minutes. The bromophenol blue dye front was monitored to check for efficient electrophoresis.

2.5.1.5 PROTEIN TRANSFER

After electrophoresis, the gel was washed in transfer buffer for 5 minutes. Additionally, the Immobilon-P PVDF transfer membrane from Merck Millipore (IPVH00010) (Watford, UK) was immersed in 100% methanol for 5 minutes. Wet electrophoresis transfer was performed using the Trans-Blot system from Bio-Rad. The transfer cassette was immersed in transfer buffer and the gel and transfer membrane were sandwiched between filter paper from GE Healthcare (SE1141) and fibre pads (Figure 2-2). Air bubbles were removed by gently rolling over the sandwiched gel and membrane. The transfer cassette was properly orientated within the transfer tank and filled with transfer buffer. Protein transfer occurred at 100 volts for 60 minutes or 25 volts for 16 hours. After transfer, the membrane was stained with Ponceau S to confirm successful protein transfer and then washed with water to remove the staining.

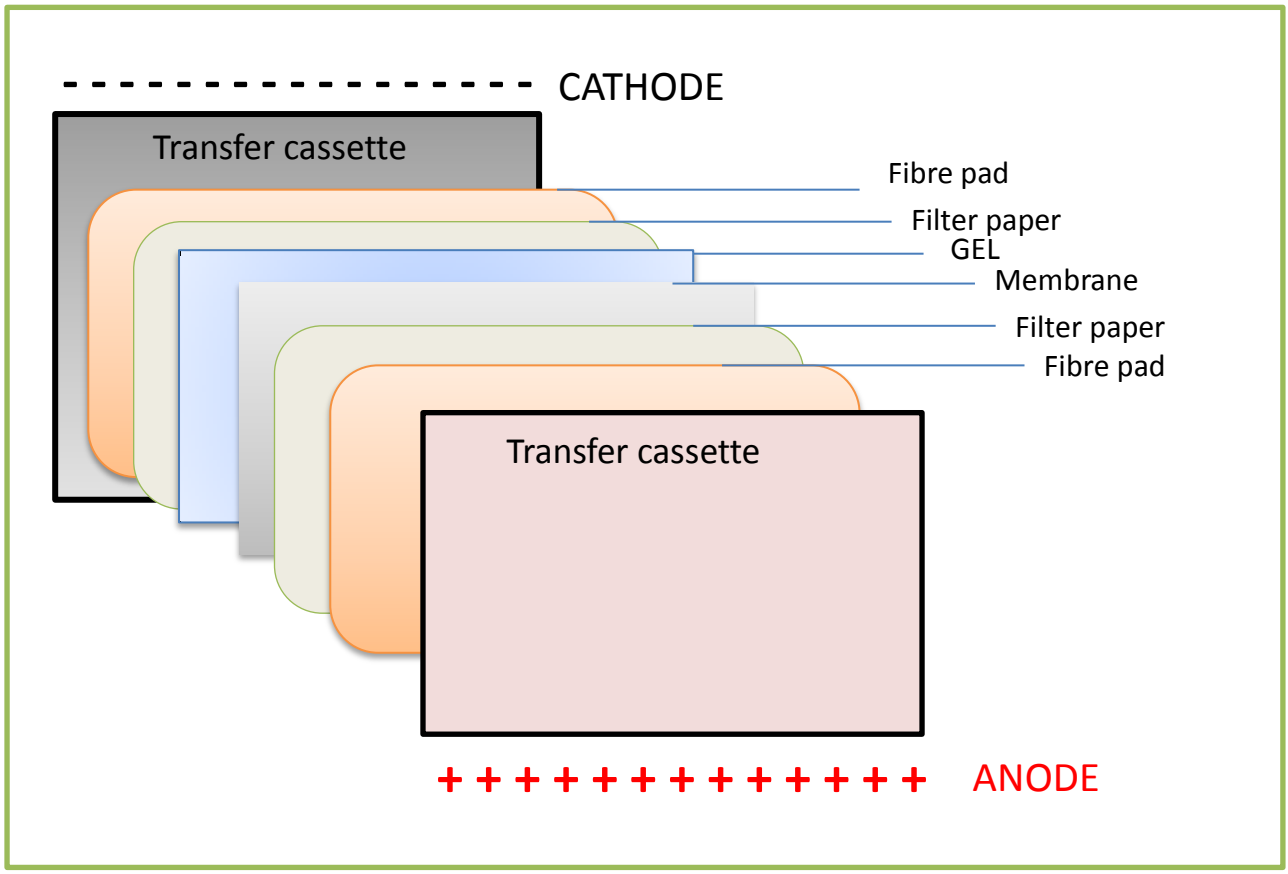


Figure 2-2: Schematic overview of Western blotting protein transfer system.

2.5.1.6 WESTERN BLOT ANALYSIS

The Immobilon-P PVDF transfer membrane was removed from the cassette and incubated in TBS-T milk for 1hour. The membrane was then washed with TBS-T and probed with primary antibody in TBS-T/BSA for 1hour or overnight at 4 °C. Afterward, membranes were washed 3 times in TBS-T for 5 min at room temperature and incubated for 1 hr in TBS-T/Milk containing secondary antibody. An ECL kit was used for detection of protein according to manufacturer's instructions. The presence of chemiluminescence was detected using the CL-XPosure Film from Thermo Scientific (PN34089), in the dark room with the AGFA Curix 60 film developer (Figure2-3). The duration of film exposure was specific for each proteins and antibody used.

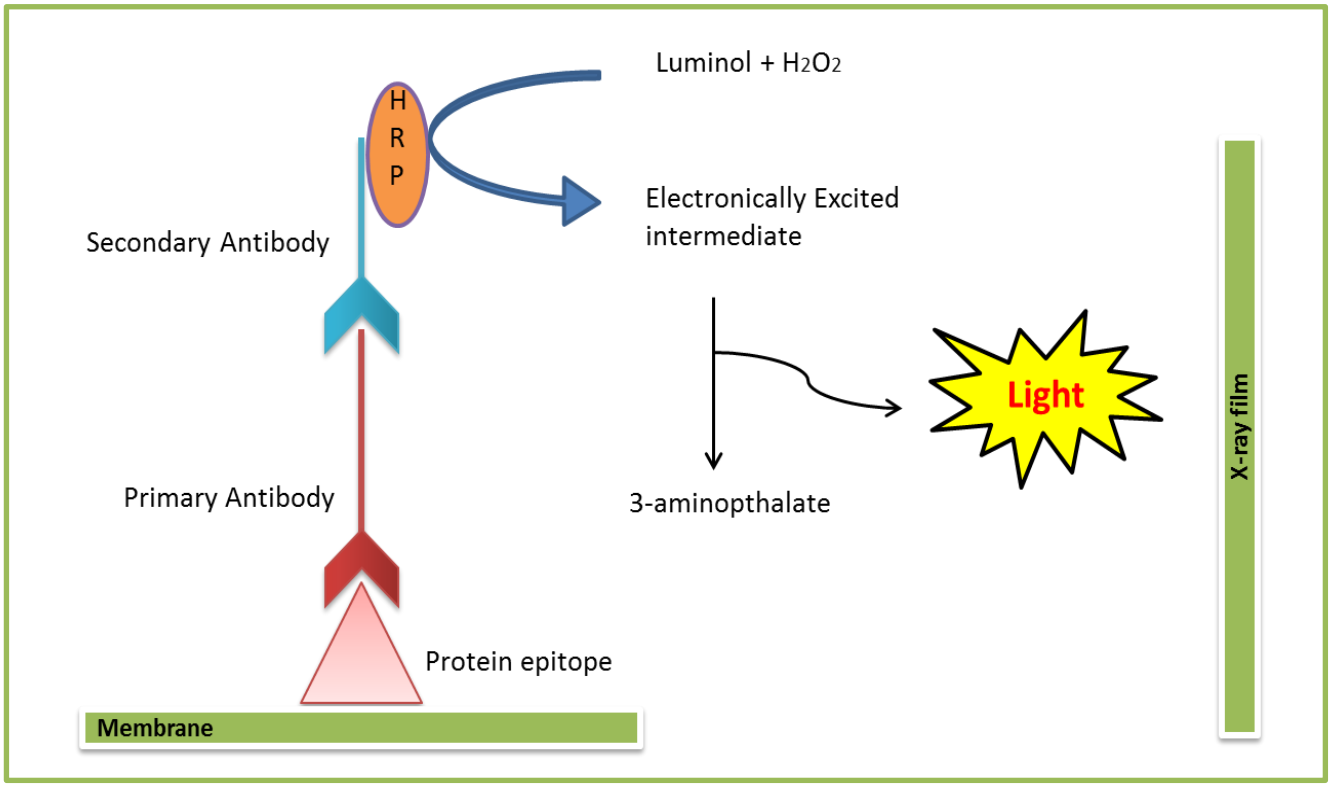


Figure 2-3: Schematic of protein detection by ECL reaction.
The schematic represents the ECL reaction when the primary antibody, specific to the protein of interest, is bound by a species-specific secondary antibody, which was conjugated to horse radish peroxidase (HRP). HRP is able to catalyse the chemiluminescent reaction of luminal in the presence of hydrogen peroxide to produce nitrogen plus light, allowing the immobilised protein to be visualised. (Marquette & Blum, 2006).

2.6 LUCIFERASE ASSAY

Cells were initially transfected with the appropriate luciferase reporter construct, the β-galactosidase construct and targeted plasmid (Section 2.2.3 and Table 2-4). Following an incubation of 48 hours, cells were obtained from the tissue culture incubator and processed for the luciferase and β-galactosidase assays. Cells were washed twice with PBS at room temperature and collected by scraping into a fresh 1.5 ml microcentrifuge tube. Collected cells were centrifuged at 1000 rpm for 5 minutes and supernatant was removed. Three freeze thaw cycles were carried out using (dry-ice containing ethanol/IMS and then in water bath at 37^{o}C). Pellets were re-suspended in 50 µl of 1x reporter lysis buffer (Promega, Cat. No. 32495201) and incubated at room temperature for 15 minutes. Lysis was centrifuged at 13,200rpm for 3 minutes. 5 µl of the supernatant were added into a fresh luminometer tube and the assay was started by injecting 300µl of luciferase assay solution and recording luminescence. For the β- Galactosidase Assay, 5µl of cell lysates from luciferase assay were added to 270 µl of assay master mix for each sample and incubated at 37^{o}C for 10-20 minutes or until the assay turned a faint brown colour. The absorbance was measured at 450nm (EL x808 Ultra Microplate Reader, Biotek instrument USA).

2.7 FACS ANALYSIS

Cells were cultured in 1x10cm dish (Thermo Scientific No. 130182) until 90% confluent and then washed with PBS, trypsinised and pelleted. The trypsinised cells were re-suspended in PBS and transferred to the flow cytometry tube (Sarstedt 5ml No.55.1578). The tube was spun at 1,200rpm for 6 minutes and the supernatant removed. 2 ml of 70% ethanol was added to re-suspend cells by vortexing gently and placed on ice. Cells were stored at 4^{o}C for 4-48 hours in 70% ethanol before spinning down at 1,200rpm for 6 minutes and re-suspending in 200µl PBS. 100 µl RNAse (3.76 mg/ml Sigma Ribonuclease A no.R5503) was added and the cells vortexed and incubated at 37^{o}C in a gently shaking water bath for 20 minutes. Cells were stained with 400µl propidium Iodide (PI 50µg/ml), vortexed and incubated shaking at 37^{o}C for 20 minutes. Finally, samples were read on the Becton Dickinson Facscan machine.

2.8 IMMUNOFLUORESCENCE

Immunofluorescent techniques were originally optimised to determine the protocol for co-visualisation of both nuclear protein, for example ZEB1 and ZEB2, and membranous protein, E-cadherin, staining. Cells plated on cover slips from VWR (18×18 mm; thickness no.1; borosilicate glass) (631-0120) were washed twice with PBS and submerged in 1 ml of 4% PFA and rinse with 1 ml PBS. 1 ml of 0.5% Triton X-100 was applied for 5 minutes then washed twice for 5 minutes. Cover slips were removed and air dried for 10 minutes. The cover slips were then washed with 2 ml PBS prior to the application of 150 µl of primary antibodies, at the required dilution (Table 2-3), in 3% BSA/PBS. The cover slips were incubated for 1 hour. The cover slips were then washed 3 times for 5 minutes each in PBS on a rocker. The secondary antibodies (Table 2-4) were then applied in 3% BSA/PBS to the cover slips. These were again incubated for 1 hour. The cover slips were then washed 3 times in PBS, each time for 5 minutes. The second PBS wash included a 1 in 25,000 dilution of DAPI (5 mg/ml in water). The cover slips were then reverse mounted onto microscope slides (Fisher scientific, 76mm x 26mm 1.0-1.2 mm thick) (11572203), containing a drop of Fluoromount G to ensure attachment. The slides were allowed to dry and the edges sealed with varnish to prevent movement. The cells were then visualised using a fluorescence microscope.

CHAPTER 3: TRANSCRIPTIONAL REGULATION OF ZEB2 IN CANCER CELLS

3.1 INTRODUCTION

Cancers are characterised by disruption of normal cell proliferation, differentiation and survival. The initiation and maintenance of cancer often involves the aberrant expression of genes that are associated with normal development. The process of EMT, a fundamental feature of normal development, is also utilised by tumour cells progressing towards malignancy. For instance, the invasive fronts of colorectal tumours are characterised by epithelial dedifferentiation, loss of intercellular adhesion, and an increase in cell migration. This is associated with the activation of transcription factors related to EMT (Spaderna et al., 2006)

It has been found that transcriptional regulation and function of EMT factors differ depending on cancer types, including malignant melanoma (Caramel et al., 2013). The role of the different master regulation of EMT (MR-EMT) in carcinomas is well known (Morel et al., 2012) but is relatively less well studied in neural crest-derived tumours. The loss of E-cadherin is the major hallmark of EMT and a number of EMT transcription factors, such as the SNAIL family and the ZEB family, are considered to be responsible for repressing E-cadherin by inducing EMT (Peinado et al., 2007). These transcription factors, such as the ZEB proteins, are able to work as gene activators or gene repressors once they bind to co-regulators that either repress or activate transcription. As such, both ZEB1 and ZEB2 function as transcriptional activators and regulate TGFβ/BMP through interacting with p300 and P/CAF, while they conversely repress E-cadherin in the presence of the CtBP co-repressor (Postigo et al., 2003, Shi et al., 2003). Both ZEB1 and ZEB2 work as an E-cadherin repressor by binding to the two conserved E2-boxes that are located in the canine and human E-cadherin promoter (Comijn et al., 2001), and also via its interaction with the NuRD co-repressor complex (Verstappen et al., 2008), whilst ZEB1 is a sufficient repressor of E-cadherin by interacting with the SWI/SNF chromatin-remodeling complex (Sanchez-Tillo et al., 2010).

ZEB2 is regulated at different levels, including miRNA, transcription, translation and splicing. A previous study showed that ZEB2 expression is controlled by novel long-range regulatory sequences in combination with miRNA (El-Kasti et al., 2012). As such, microRNAs have been

found to regulate EMT by targeting ZEB1 and ZEB2. The well-studied type of microRNA that targets ZEB2 is the microRNA-200 family (Park et al., 2008). Additionally, and interestingly, Y-box binding protein, which activates cap-independent translation of mRNAs encoding EMT factors, is involved in the down-regulation of ZEB2 (Evdokimova et al., 2009). ZEB2, and other EMT transcription factors, are induced by TGFβ, which activates Smad-independent pathways that result in the modulation of ZEB2 (Nawshad et al., 2005).

It also has been demonstrated that ZEB2 transcription is repressed by the NANOG and OCT4 stem cell factors that are responsible for the maintenance of self-renewal. The regulatory element located at -4kb in the *ZEB2* promoter is at the position where these factors bind, and where repression is more likely to occur. The expression of these pluripotency factors is repressed by ZEB2, which highlight its ability to shift embryonic stem cells from pluripotency to differentiation (Chng et al., 2010).

3.2 AIMS AND OBJECTIVES

The aim of this chapter was to investigate the transcriptional regulation of the ZEB2 EMT-TFs during EMT and in cancer cells, and to study whether RAS pathway correlates with the ZEB2 protein and the stem cells factors NANOG, OCT4 and SOX2 in melanoma cells.
The specific objectives for this chapter were as follows:

- To analyse the activity of the ZEB2 U5 and E1 promoters in melanoma cells by measuring the luciferase activity.
- To test whether MEK signalling has an influence on the stability of the EMT-TFs, particularly the -4kb regulatory element in ZEB2 or whether it confers its regulation by treating melanoma cell line with U0126.
- To study the role of the stem cell factors NANOG, OCT4 and SOX2 in the regulation of ZEB2 in melanoma cell lines, and by using the MEK inhibitor, investigate whether the active RAS pathway influences the expression and activity of stem cell factors in melanoma cells.

3.3 RESULTS

3.3.1 THE ACTIVITY OF THE ZEB2 U5 AND E1 PROMOTERS IN A MELANOMA CELL LINES

Analysis of the mouse ZEB2 5'-UTR identified nine untranslated exons (U1-U9), which are located upstream of the first translated exon, exon 1. Three possible promoter regions were identified, promoter P1 sited upstream of exon U5, promoter P2 sited upstream of exon U1, and promoter P3 sited upstream of exon 1 (Figure 3-1) (Nelles et al., 2003).

According to the publicly available databases, there are two promoters U5 and E1. As the ZEB2 gene potentially contains variant promoters, the relative luciferase activity of both the U5 and E1 promoters was examined in a melanoma cell line, A375M, after they were cloned into the pGL3 vector and relative luciferase activity compared to the pGL3-Basic vector, which contained no promoter. The experiments showed a highly significant increase ($p=<0.0001$) in the activity of the U5 promoter when it was transfected into the A375M melanoma cell line when compared to both the empty vector and E1 promoter. Additionally, the transfected cells were treated with U0126, which has been previously shown to result in the transcriptional repression of ZEB2 (Caramel et al., 2013), but was shown to have no influence on either promoters (Figure 3-2).

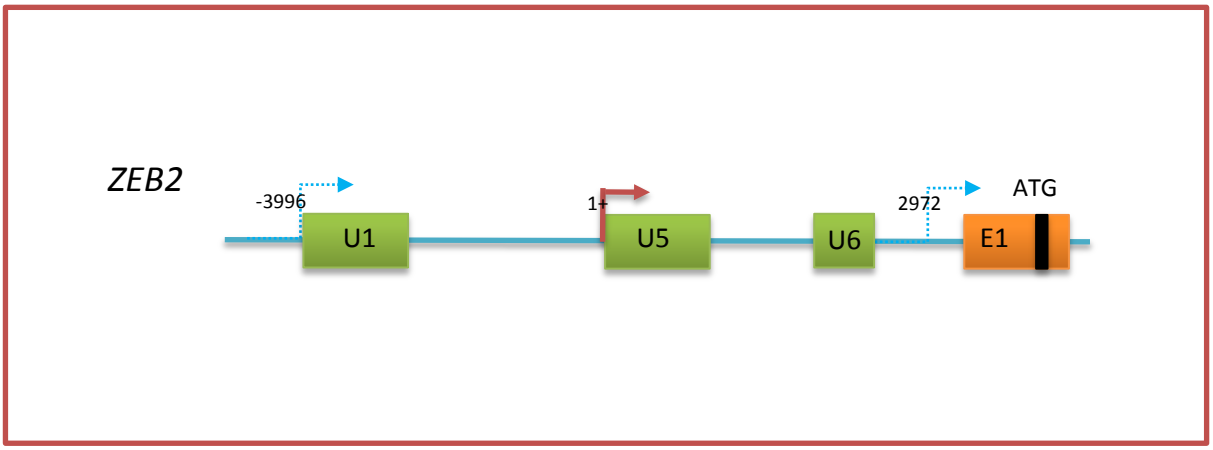


Figure 3-1: Schematic of the ZEB2 5'- UTR.
Three potential promoters were observed within ZEB2 gene structure, promoter P1 sited upstream of exon U5, promoter P2 sited upstream of exon U1 and promoter P3 sited upstream of exon 1. Both U5 and E1 are highly conserved in sequence and are considered as the primary ZEB2 gene (Nelles et al., 2003).

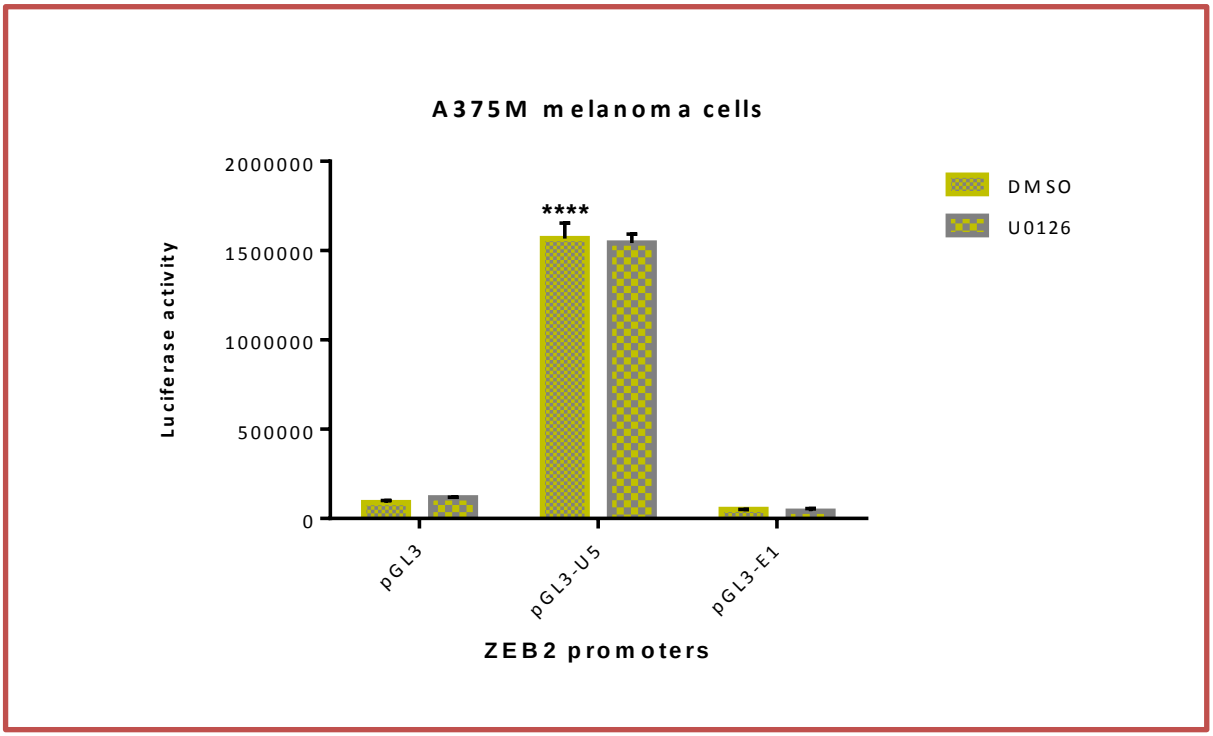
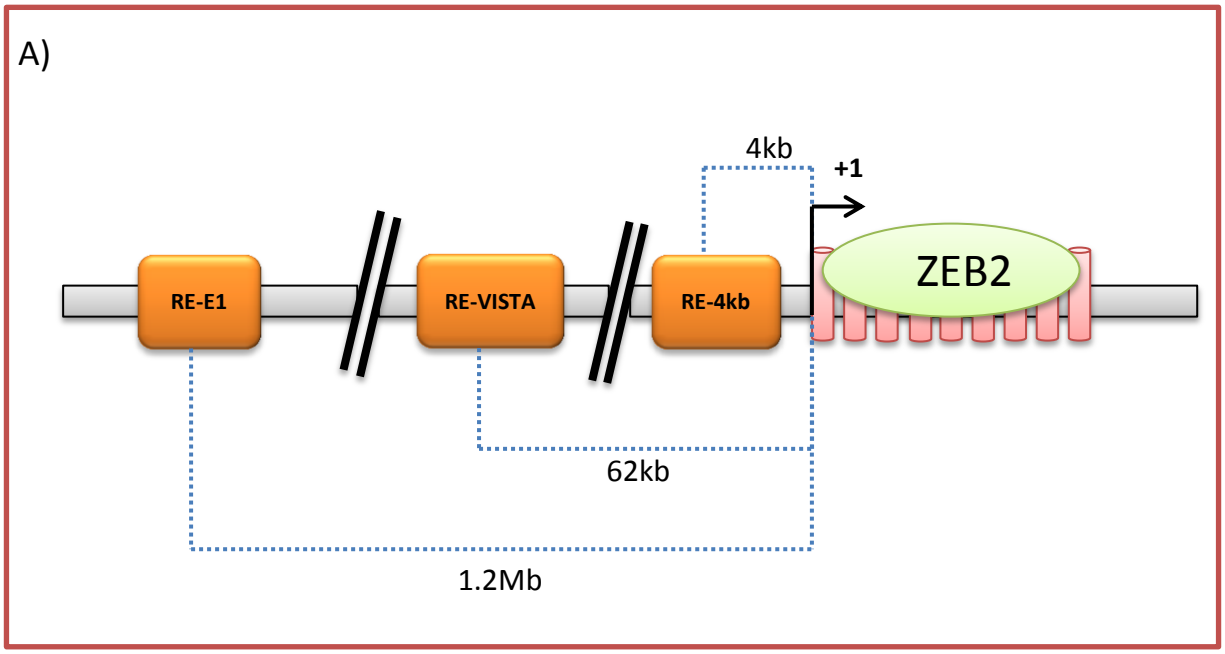


Figure 3-2: The relative luciferase activity of U5 and E1 promoters.
The luciferase activity from the pGL3-U5 and pGL3-E1 ZEB2 reporters was determined after transient transfection into the melanoma cell line, A375M -/+ U0126. Three independent experiments were performed, with the standard error of the mean presented for each promoter. Significance was tested via one-way ANOVA (****=p≤0.0001).

3.3.2 THE ACTIVITY OF ZEB2-DERIVED REPORTERS IN A MELANOMA CELL LINE

Previous experiments suggested that the U5 and E1 promoters may influence ZEB2 regulation. Therefore, the luciferase assay was chosen to measure ZEB2 regulatory element (RE) activity in the A375M cell line. Three different sequences located upstream of the U5 promoter were chosen and each cloned individually into the pGL3 basic vector which contained the U5 promoter (p-1047Luc). Specifically, these inserts were RE-4 construct, which included sequence 4 KB from the ZEB2 transcription start site, RE/E1, containing a ZEB2 enhancer that was identified in the rat and located at 1.2MB from the transcription start site (El-Kasti et al., 2012), and finally RE/Vista, which was selected from the UCSC human genome browser as the sequence was unique to the ZEB2 gene and located 62 kb from the transcriptional start site (Figure 3-3A). To detect some of the ZEB2 activity, a short promoter region fragment p-395 was amplified from ZEB2 and inserted into the p-1047Luc (Figure 3-3B).



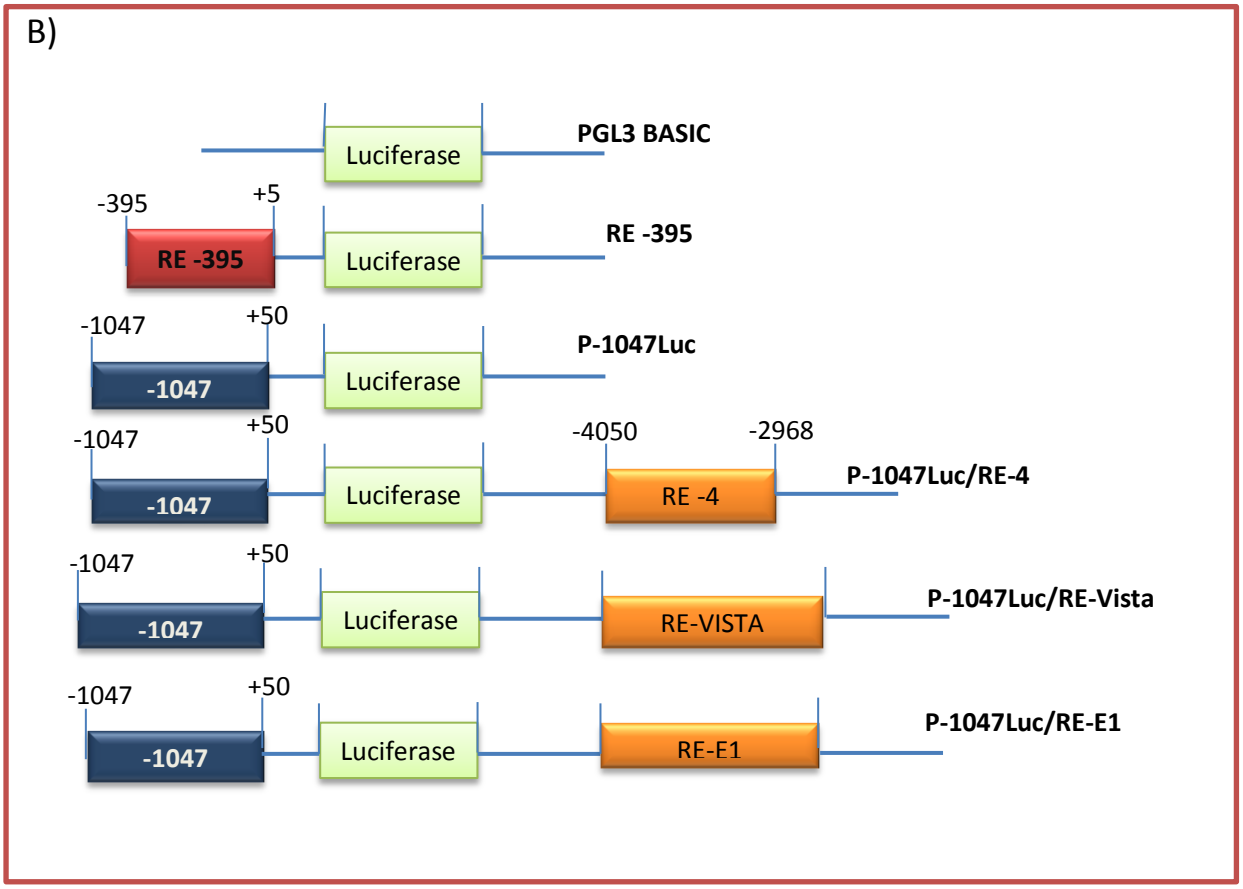


Figure 3-3: Generation of ZEB2 promoters.
A) The map indicates the upstream ZEB2 regulatory elements of the region E1, Vista (El-Kasti et al., 2012) and RE -4 constructs. B) Schematic of the two promoters (-395 and -1047) in pGL3-Basic vector, with the regulatory elements cloned into the -1047Luc plasmid.

The ZEB2-derived reporters, p-1047Luc/RE-4, p-1047Luc/RE-Vista, and p-1047Luc/RE-E1, which all contain an enhancer sequence, and p-395Luc as well as p-1047Luc, were transfected into A375M cells. A pCMVluc construct (Cohn et al., 2001), in which luciferase reporter expression was driven by a viral promoter, was used as a positive control. Cells were transfected with the empty pGL3 basic as a negative control. The results of three independent experiments are shown in Figure 3.4. After all constructs were normalised against p-1047Luc, it was identified that the activities of the p-1047Luc and p-395Luc constructs are approximately 10-fold higher than that of the promoterless pGL3 basic plasmid, indicating that the U5 ZEB2 promoter is active in A375M cells. Moreover, the

regulatory element contained within the RE-4 constructs produces a significantly higher luciferase activity in melanoma cells, approximately 2.5 fold higher than p-1047/Luc. Finally, the p-1047Luc/RE-Vista was significantly more active in A375M cells when compared to p-1047Luc promoters. Interestingly, all three elements produced stronger luciferase activity than the U5 promoter alone, with a 1.8, 2.5 and 3 fold increase for RE-E1, RE-4 and RE-Vista constructs, respectively.

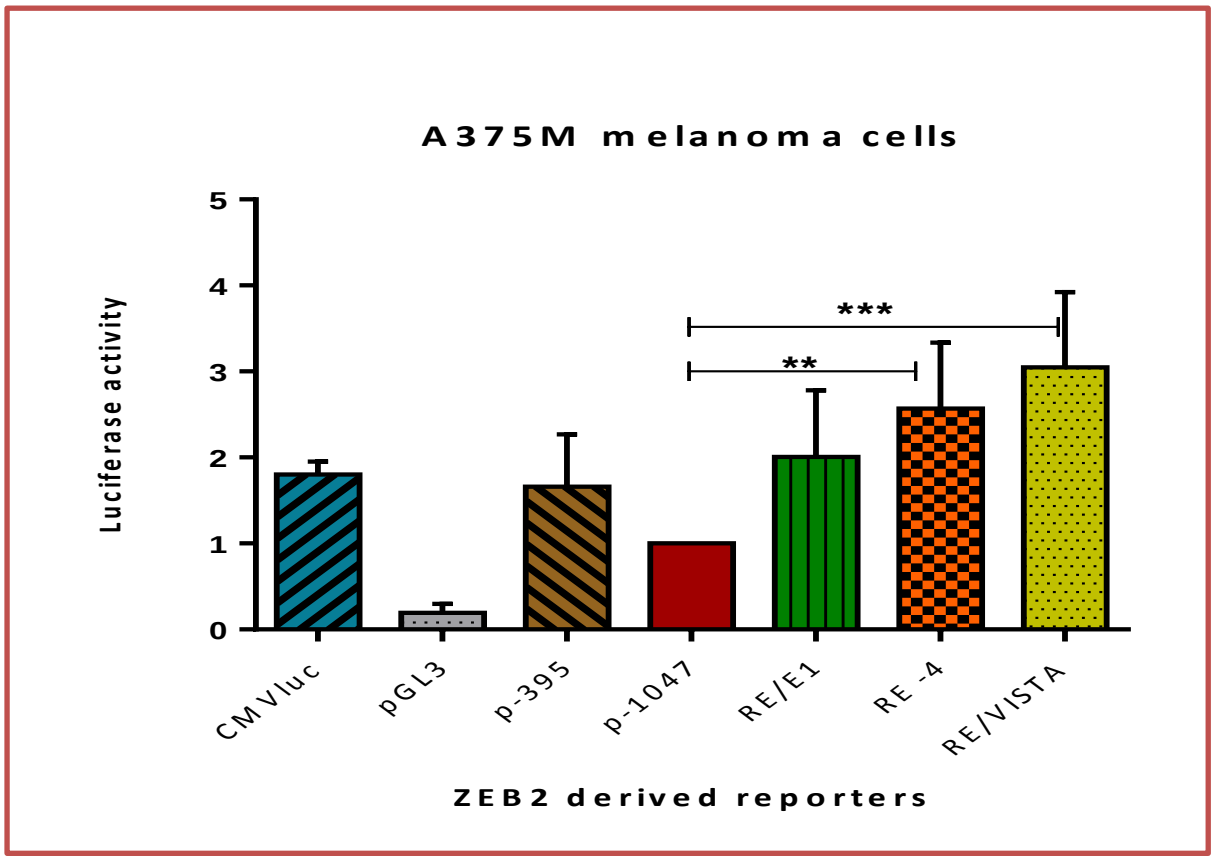


Figure 3-4: Luciferase assay of ZEB2 reporter's activity.
Graph shows an increased luciferase activity of ZEB2 regulatory elements p-1047Luc/RE-4kb, p-1047Luc/RE-E1 and p-1047Luc/RE-Vista when compared to the control RE -1047Luc. Significance was tested via one-way ANOVA (** p = ≤ 0.01, *** p = ≤ 0.001).

3.3.3 IS ZEB2 REGULATED BY STEM CELLS CORE FACTORS?

The network between EMT and cancer stemness has been suggested due to evidence that the EMT process generates cells with stem-like properties (Hollier et al., 2009, Mani et al., 2008). Moreover, the expression of the pluripotency factor NANOG is regulated via SMAD2/3, proteins implicated in the EMT process (Vallier et al., 2009). Additionally, ZEB2 expression is controlled via cooperation between Activin-Nodal signalling and NANOG, OCT4 and SOX2, with binding occurring within the RE-4 region identified within ZEB2, with this seeming to be active in melanoma (Chng et al., 2010).

Western blot analysis was carried out to analyse the expression of stem cell factors and EMT transcription factors in different melanoma cell lines. The relative expression of the stem cell factors and ZEB1/2 was examined and compared to neonatal human epidermal melanocytes (NHEMs) (Figure 3-5).

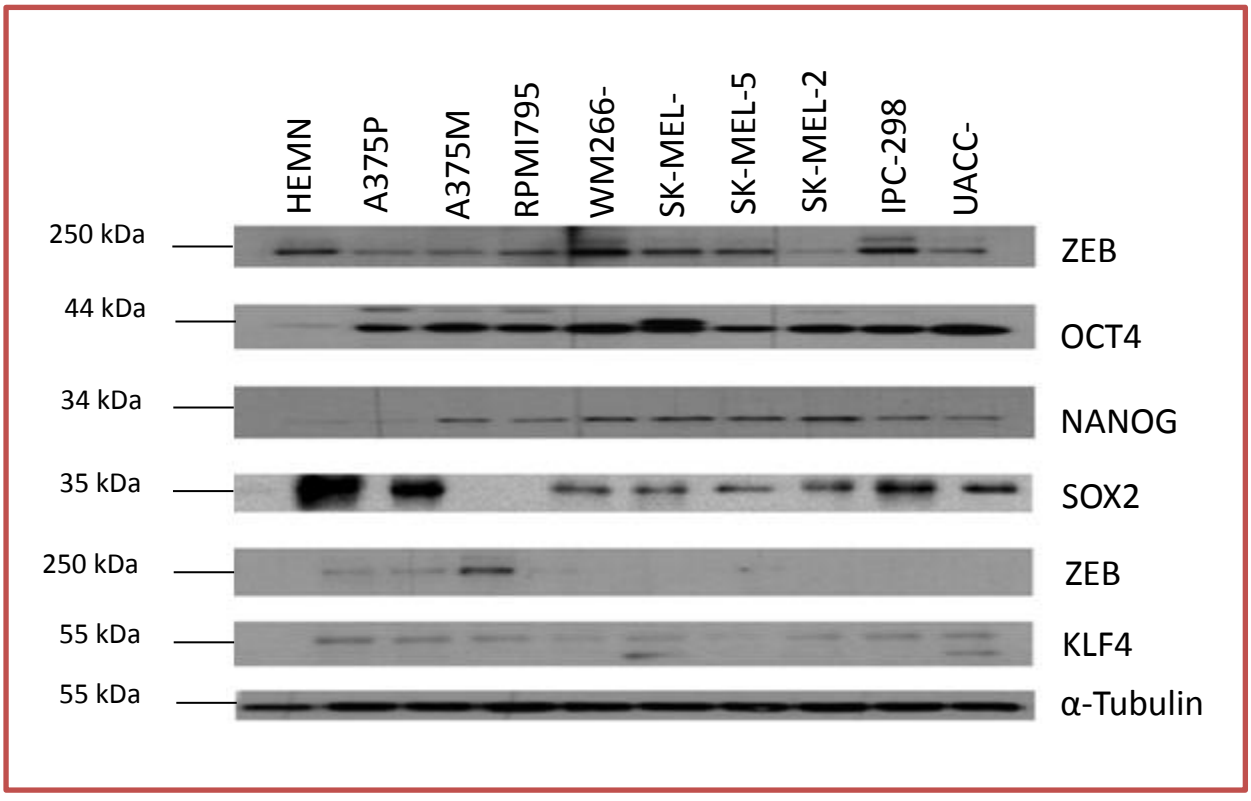


Figure 3-5: The expression of ZEB proteins and stem cells factors in certain melanoma cell lines.
Cells were cultured to 70-80% confluence and proteins analysed via western blotting, with a protein loading control, α-Tubulin. Representative images are presented; ZEB2 was expressed in the NHEMs and maintained in the melanoma cell lines. Core transcriptional factors were absent in the NHEMs but expressed in other melanoma cell lines.

NHEMs expressed ZEB2, OCT4, SOX2 and NANOG but not ZEB1. However, the expression of stem cell factors is increased in the melanoma cell lines compared with the NHEM. As such, the B-RAF mutant A375P cells showed a high expression of OCT4 and SOX2 but not NANOG, whilst the B-RAF mutant A375M cell line retained a high expression of both OCT4 and SOX2, but showed the lowest expression of both ZEB1, ZEB2 and NANOG proteins. Moreover, a lack of ZEB1 expression was evident in a subset of the melanoma cell lines. Interestingly, the result represents the correlation between the expression of ZEB1, OCT4, SOX2 and ZEB2 in the A375P and A375M cell lines, wherein A375P cells expresses ($ZEB2^{low}$, $ZEB1^{low}$, $OCT4^{high}$, $SOX2^{high}$) and A375M cells express also ($ZEB2^{low}$, $ZEB1^{low}$, $OCT4^{high}$, $SOX2^{high}$), which suggests a link between ZEB2 and stem cells factors. Kozlowski pointed out that the metastatic capacity of A375M cells was more than the capacity of A375P cells (Kozlowski et al., 1984). The three regulatory core transcription factors, OCT4, SOX2 and NANOG, play roles in maintaining the stemness in melanoma. For instance, SOX2 is expressed in 50% of melanoma and the silencing of SOX2 causes apoptosis and inhibits growth in melanoma cell lines, as well as regulating self-renewal (Santini et al., 2014).

MEK-ERK pathway regulates the expression of the EMT-TFs network in melanoma (Caramel et al., 2013). Indeed, we showed a correlation between the EMT-TFs, particularly ZEB2 expression and the activation of MEK. We analysed here the expression of ZEB1 and ZEB2 by using the MEK inhibitor U0126 at different time points for 24 hours to inhibit the MEK pathway in the mutant B-RAF cell lines, A375P and A375M (Figure 3-6). This confirms the up-regulation of ZEB2 and the down-regulation of ZEB1 by MEK inhibition.

To address whether BRAF-MEK or NRAS-MEK oncogenic signalling is implicated in the regulation of stem cell factors in melanoma, we treated A375M cells with the U0126 MEK inhibitor at different time points and determined the expression of OCT4 and SOX2 by western blot. We demonstrated that SOX2 and OCT4 are down-regulated after 24 hours of U0126 treatment, following the same pattern as ZEB1 (Figure 3-7). To test whether the influence of MEK inhibition on the expression of OCT4 and SOX2 has a link with ZEB1/2, knockdown of OCT4 and SOX2 was performed in A375M cells which resulted in no effect on the expression of ZEB1 and ZEB2 (Figure 3-8).

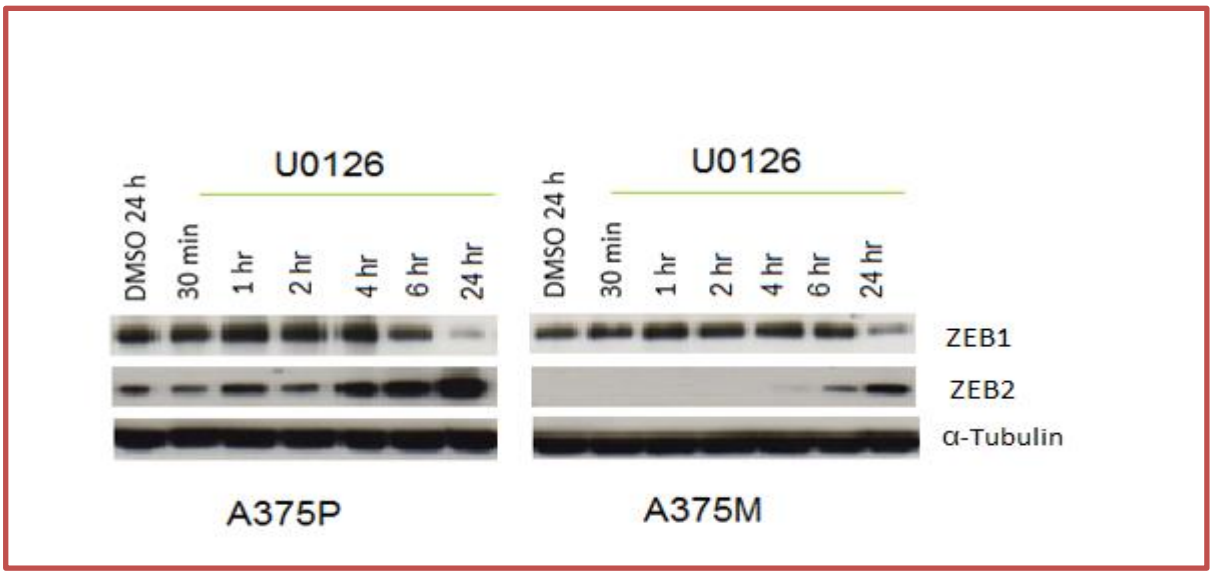


Figure 3-6: Hierarchical expression of ZEB proteins.
10µM of U0126 was added to A375M and A375P cells and incubated at varying times from 30 minutes to 24 hours. Lysed cells were used to analyse protein by western blotting. Decrease of ZEB1 protein level required 24 hours while increased ZEB2 expression was noted after 4 hours. α-tubulin was used as a protein loading control.

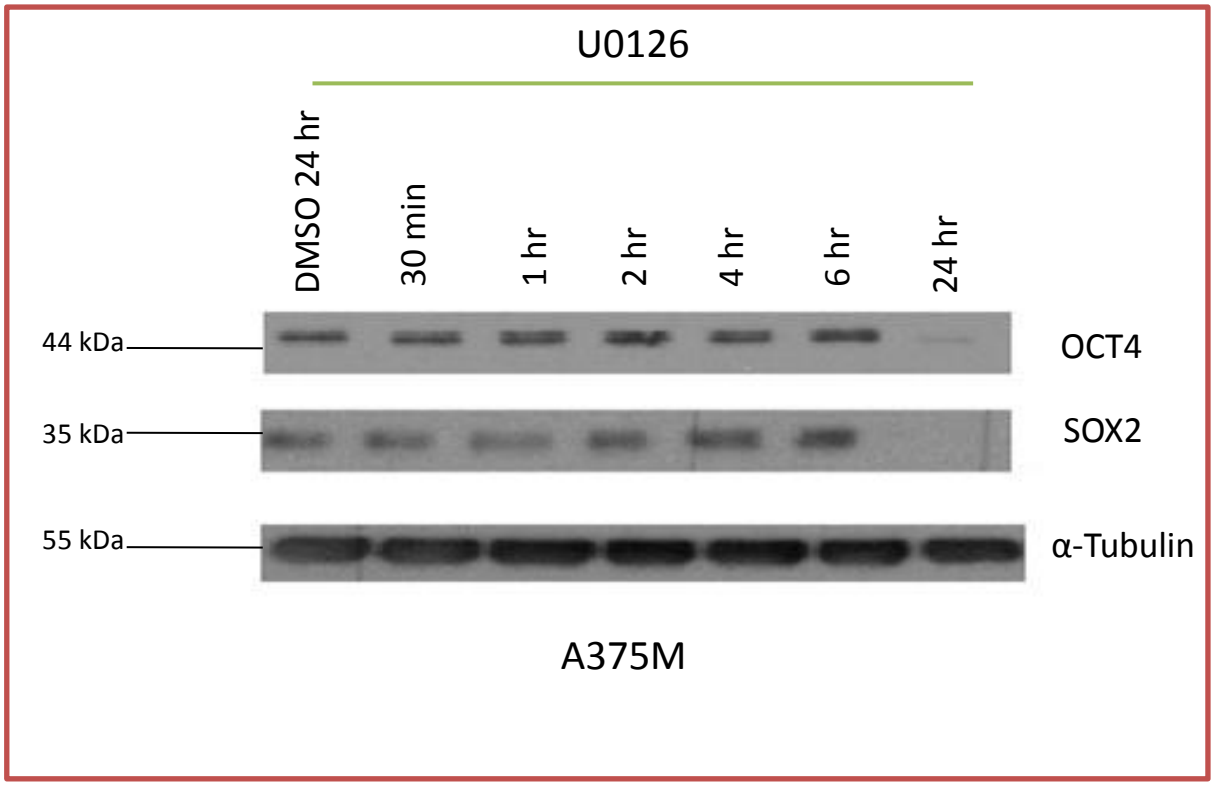


Figure 3-7: Western blotting analyses of the U0126 treatment.
The A375M melanoma cells was treated with MEK inhibitor U0126 at different times and stained for OCT4, SOX2 and tubulin. Results show that both OCT4 and SOX2 are down-regulated after 24 hours. α-tubulin was used as a loading control.

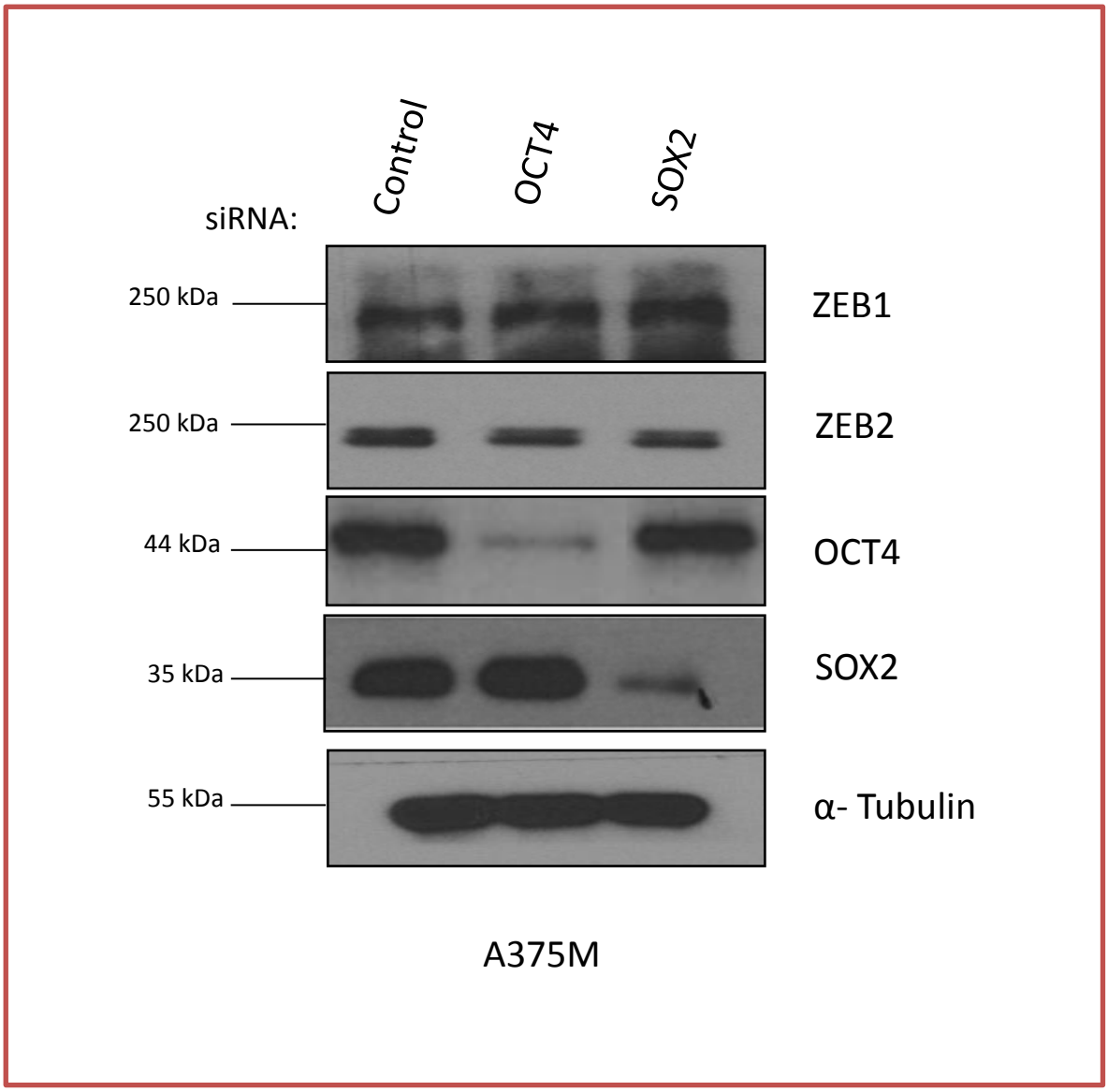


Figure 3-8: Knockdown of core stem cell factors.
An A375M cell was transfected with the various siRNAs and incubated for 72 hours. Knockdown of OCT4 and SOX2 resulted in no influence in the expression of either ZEB1 or ZEB2. α-tubulin was used as a protein loading control.

3.4 DISCUSSION

The cloning of the ZEB2 regulatory elements into the p-1047Luc plasmid was not expected to be straightforward because of the inherent challenges of cloning experiments, as well as the large size of each DNA fragment that contains the regulatory element. These fragments were used to measure the ZEB2 regulatory element activity in the A375M melanoma cells. Our data show that these results are consistent with the results previously generated in the EMT laboratory showing that U5, but not E1, is a ZEB2 gene promoter utilised in melanoma cells. Only minor differences in the activities of p-1047Luc and p-395Luc constructs were demonstrated, indicating that there are no cis-elements between the -395 and -1047 positions that are significantly contributing to the ZEB2 gene transcriptional control. Although the effect of the insertion of the E1 fragment on reporter activity was minor (comparing p-1047Luc and p-1047Luc/RE-E1), the addition of the -4kb and VISTA elements activated the reporter. Therefore, we conclude that the expression of ZEB2 is controlled by two relatively weak transcriptional enhancers in A375M cells, located within the -4kb and VISTA fragment (Figure 3-3). As the regulatory element contained within the -4kb fragment has the capacity to be controlled by stem cell transcription factors, we subsequently analysed their role in melanoma cells (Figure 3-4).

The data show that the RAS pathway regulates the expression of transcriptional factors in malignant melanoma cell line. Indeed, MEK inhibition leads to inhibition of the BRAF pathway in malignant melanoma through the down-regulation of ZEB1/TWIST1 and up-regulation of ZEB2/SNAIL2 (Caramel et al., 2013). Furthermore, stem cell factors NANOG, OCT4 and SOX2 bind within the RE-4 element, ZEB2 transcription is repressed by OCT4 and NANOG, while NANOG is in turn repressed by ZEB2 (Chng et al., 2010). Additionally, ZEB2 represses ZEB1, with ZEB1 being the only true repressor of E-cadherin, which is an EMT marker. Here, it was shown that the protein expression of the stem cell factors OCT4 and SOX2 follow the same pattern as that of ZEB1 and TWIST1 (Figure 3-6). Firstly, the protein expression of OCT4 and SOX2 is down-regulated after 24 hours of treatment with MEK inhibitor U0126. Therefore, I hypothesise a link between RAS pathway activation, EMT factors and the stem cell factors OCT4, NANOG and SOX2 in melanoma cells.

Expression of the stem cell factors, however, varied in the melanoma cell lines. For instance, the NANOG protein was expressed at very low levels so it was excluded from further analysis. Finally, it was suggested that knocking-down the high expression level of OCT4 and SOX2 in A375M did not influence either ZEB1 or ZEB2.

CHAPTER 4: REGULATION OF ZEB2 TRANSLATION IN CARCINOMA CELLS

4.1 INTRODUCTION

ZEB proteins have different functions in melanoma; ZEB1 is pro-tumorigenic, and ZEB2 activates differentiation and possesses tumour suppressive features (Caramel et al., 2013). Emerging evidence suggests that ZEB1 and ZEB2 exhibit opposing properties in carcinoma, although both proteins are capable of inducing EMT in different epithelial cell lines. Indeed, cyclin D1 and hTERT are down-regulated by ZEB2 which can induce cell cycle arrest and senescence, a feature that has never been reported for ZEB1. On the other hand, ZEB1 cooperates with classical oncogenes in malignant transformation, which is in contrast to ZEB2 for which oncogenic activity has never been reported. Data suggest that cell fate might depend upon which EMT-TFs are driving EMT, and this may represent a determinant of whether a program of senescence or oncogenic transformation will prevail. Therefore, better understanding of how the ZEB1/2 balance is regulated at different levels is important.

The lab has generated data suggesting that in addition to several microRNA species, regulation of ZEB proteins synthesis may form a double negative feedback loop that regulates EMT and MET equilibrium. According to these data, a protein motif adjacent to the smad-binding domain within the ZEB2 protein induces ribosome stalling and compromises translation. The activity of this motif is dependent on triplets of rare codons, Leu(UUA)-Gly(GGU)-Val(GUA), whereas introducing these stretches in the homologous region of ZEB1 has no effect on protein expression.

4.2 AIMS AND OBJECTIVES

The aims of this study were:

1. **To gain further insight into the mechanism of translational control of ZEB2 protein**, and the following objective were set:
   - To analyse expression of ZEB proteins in a panel of cancer cell lines to clarify the importance of translational control in ZEB protein regulation.
   - To assess the effects of individual codons in the rare codon triplets.
   - To assess whether the lysine residues adjacent to the rare codon triplets take part in translational regulation.
   - To investigate whether the presence of rare codons affects negative regulation of ZEB2 by the ubiquitin E3 ligase FBXO45.

2. **To investigate how EMT influences ZEB regulation**, and the objectives of this aim were:
   - To generate breast carcinoma epithelial cell lines expressing ZEB1 or ZEB2 proteins in Doxycycline (DOX)-regulated manner.
   - To characterise EMT programs in the abovementioned cellular models.
   - To investigate how the ZEB1 or ZEB2 driven EMT programs affect regulation of the endogenous ZEB2 gene.

4.3 RESULT

4.3.1 EXPRESSION OF ZEB2 PROTEINS IN CANCER CELL LINES

The aim in this study was to investigate the expression levels of ZEB2 in several cancer cell lines and compare these to the expression level of ZEB1. Data in our lab have shown that ZEB1 protein is expressed in the majority of carcinoma cell lines while ZEB2 remains uncommon; however, both genes are found to be transcribed in the majority of the mesenchymal cell lines. Western blots analysis of the two categories of cell lines were examined for the expression of ZEB1 or ZEB2 proteins using whole protein lysates from cells culture, epithelial cells (MDA-MB-468, MCF-7, T47D, ZR-75-1 and RT112), and mesenchymal cells (MDA-MB-231, J82, T24, UMU-C3, H1299, U2Os, SaOs-2, A375P and UACC) (see table 2-5). The epithelial marker E-cadherin was used to distinguish between the two cell line groups, while the A375P human melanoma cell line was loaded as a positive control for both proteins ZEB1 and ZEB2 (Figure 4-1). The initial experiment confirmed the inverse correlation between ZEB1 and E-cadherin as seen in previous studies. For instance, in the melanoma UACC-257 cells, repression of ZEB1 was shown to result in the activation of E-cadherin which supports the idea that E-cadherin is repressed by ZEB1 (Eger et al., 2005). On the other hand, the expression level of ZEB2 was undetectable in the most of the selected cells. However, the sarcoma cell line SaOs-2 showed the highest expression level among all of other cell lines. These data show that ZEB2 is absent in the majority of carcinoma cell lines which is in agreement with the speculation that ZEB2 may have a tumour suppressor role in carcinoma genetic background.

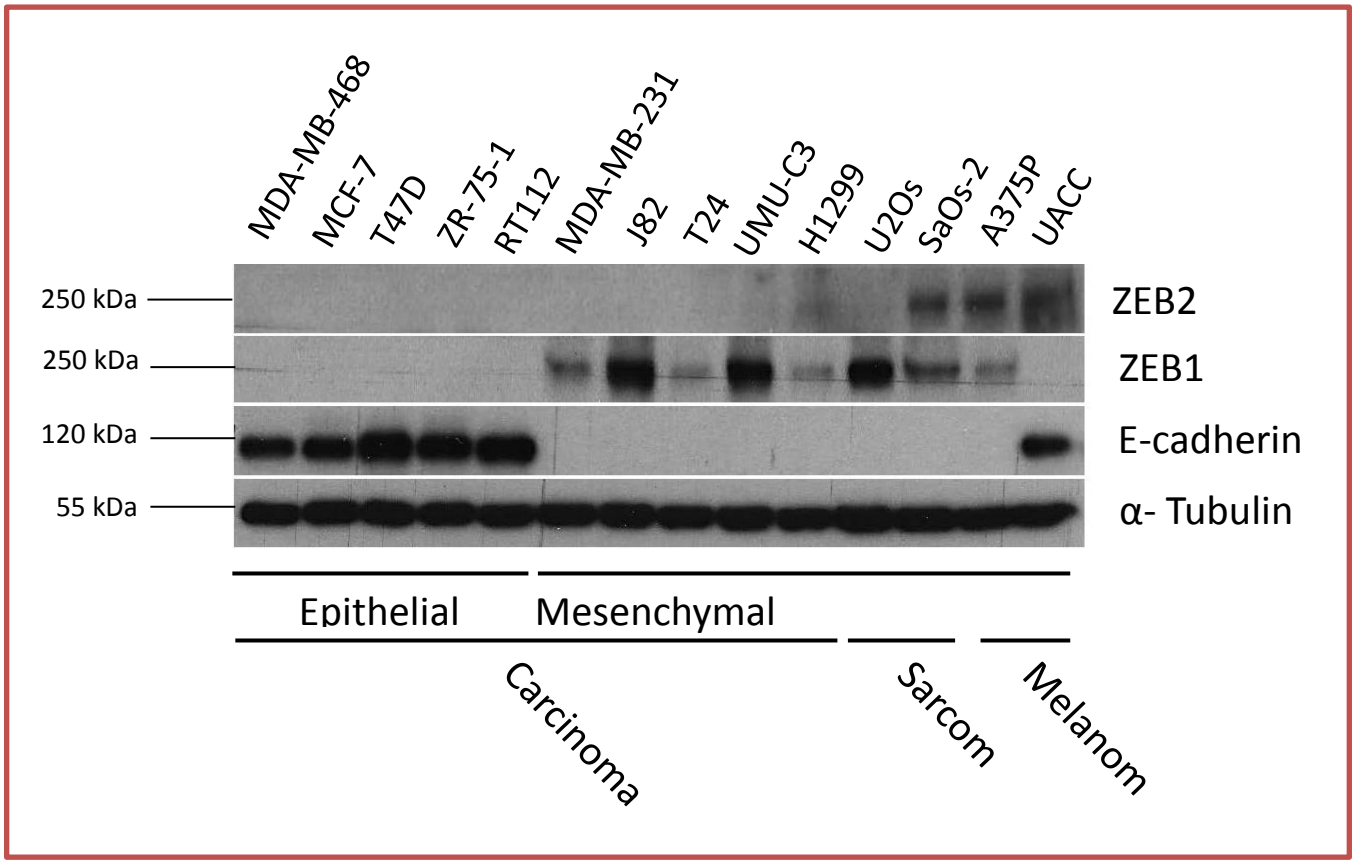
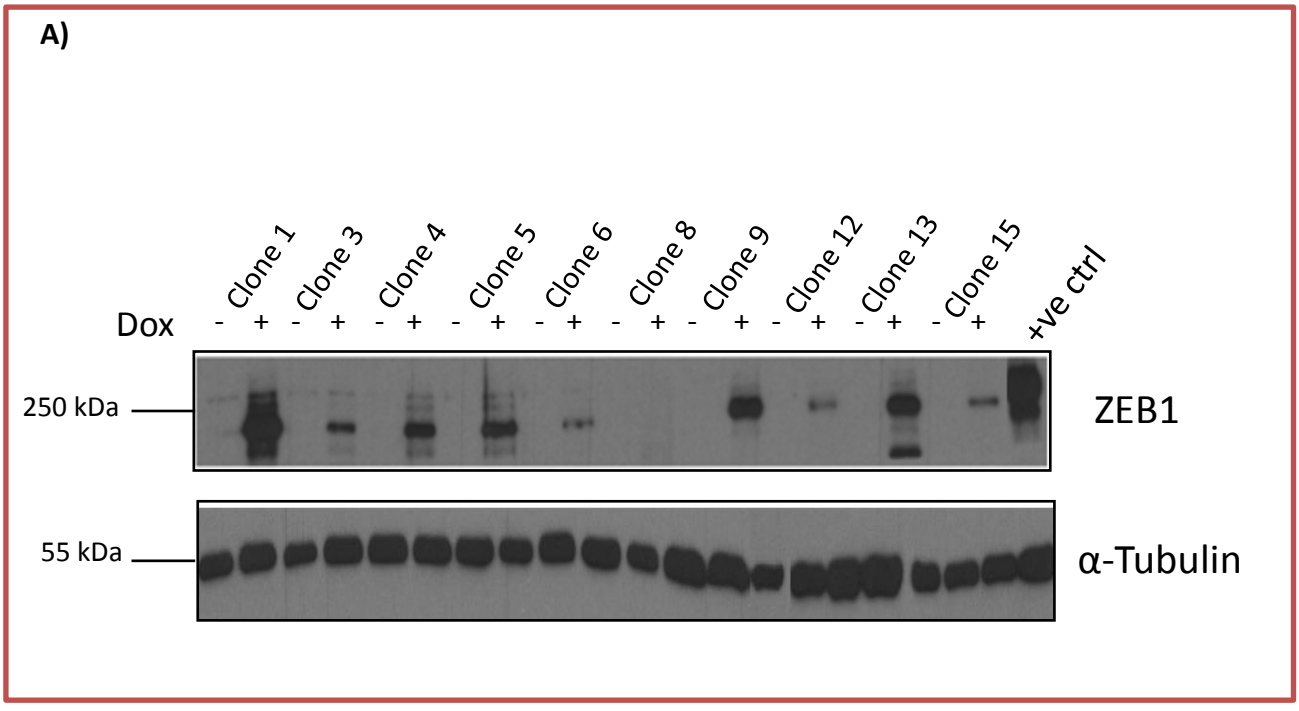


Figure 4-1: ZEB1 and ZEB2 expression in various epithelial and mesenchymal cells.
Cells were cultured to 70% confluence and proteins expression was analysed by western blotting. α-Tubulin was used as a protein loading control.

4.3.2 GENERATION OF MCF7 CELL LINES WITH DOX-REGULATED EXPRESSION OF EGFP-TAGGED ZEB1 OR ZEB2 PROTEINS

The function of ZEB proteins in different epithelial cell lines has been addressed in a number of studies. However, no attempts so far have been made to compare their features in the same experimental settings. A model of EMT has been established in human epithelial breast adenocarcinoma cells, which expresses the tetracycline-regulated activator and allows any gene of interest to be expressed in this cell line by inducing the targeted gene upon treatment with doxycycline (DOX). DOX-regulated expression of EMT-TFs is broadly used in studies addressing different aspects of EMT (Vandewalle et al., 2005). Four different constructs ZEB1, ZEB2, EGFP-tagged ZEB1 and EGFP-tagged ZEB2 were ectopically induced into MCF-7. After induction, several clones for each vector were selected via western blotting and stained for a specific protein to confirm induction (Figure 4-2). Clones with an EGFP-tag were confirmed using fluorescent microscope after doxycycline treatment for a certain length of time (Figure 4-3). Here it shows that ZEB2 is a difficult-to-express protein, whereas typically ectopic ZEB2 expression is much lower than that of ZEB1.



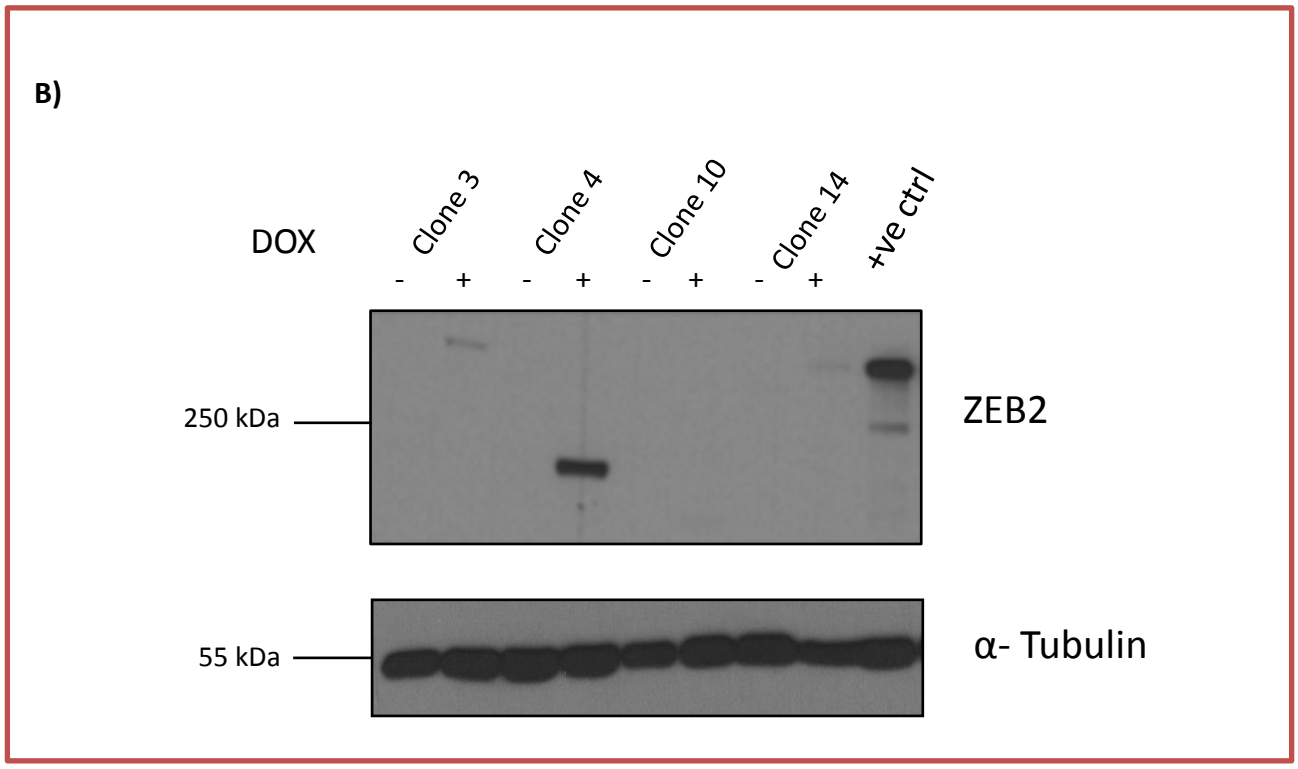


Figure 4-2: Generation of novel cellular models of EMT.
A) Several clones were selected and treated with doxycycline to induce targeted EMT model MCF7-ZEB1. Fifteen clones were chosen and western blotting was carried out to examine the expression of ZEB1 and confirm its expression in these clones. Different expression levels were noticed among clones. A375M was used as positive control. B) MCF7-ZEB2 clones were selected and protein samples were analysed by western blotting to identify the correct clones.

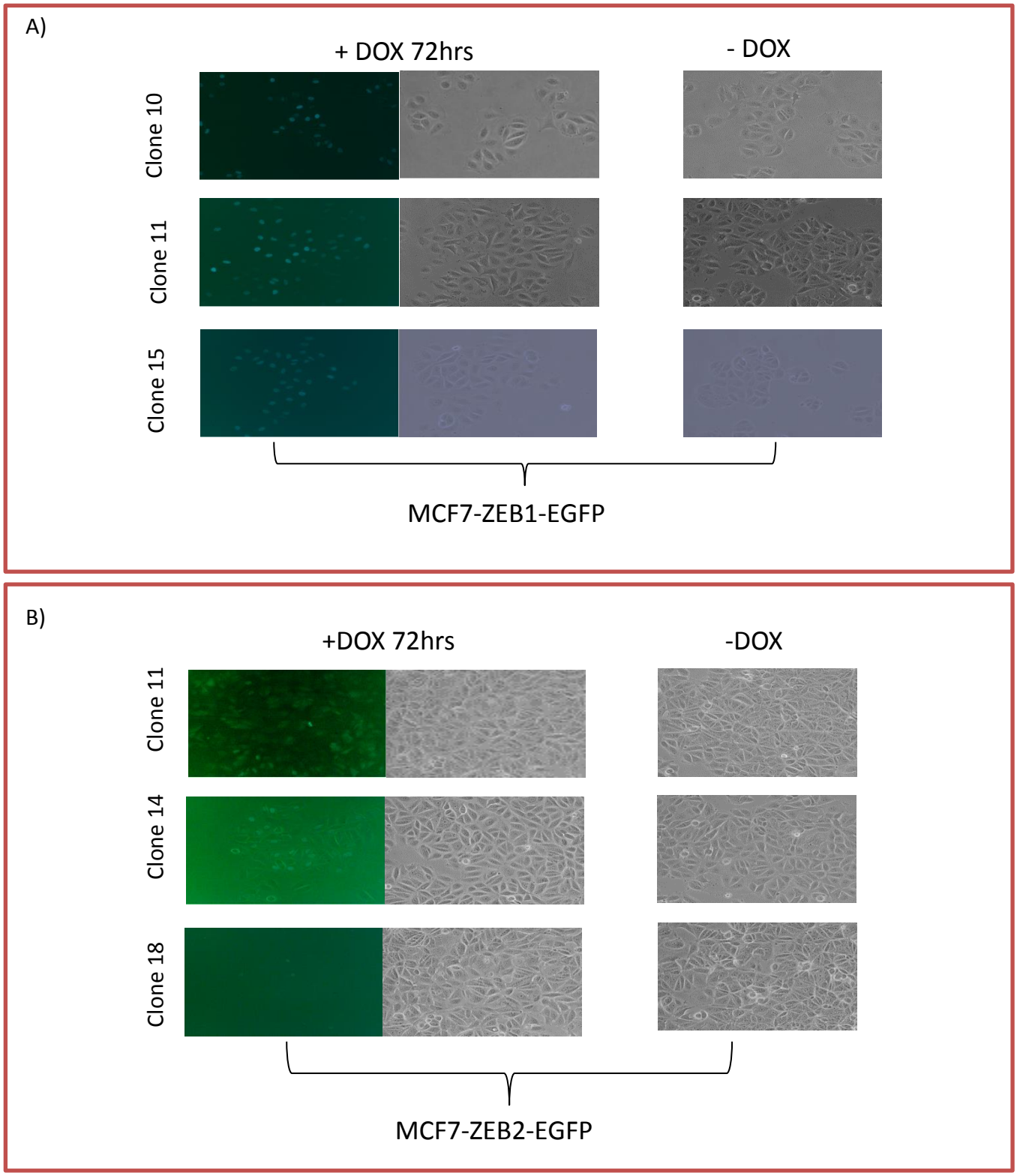


Figure 4-3: Induced ZEB1 and ZEB2 double tagged with EGFP into MCF7.
MCF7-ZEB1-EGFP (A) or MCF7-ZEB2-EGFP (B) clones were selected and cultured in the presence/absence of doxycycline for 72hrs. Cells were examined by fluorescent microscope for EGFP-ZEB expression and photographed using IF and phase contrast.

As previously stated, a range of inducible clones were examined for expression of ZEB1 and ZEB2. Next, more investigations were carried out on these models to confirm their properties as EMT models in addition to a successfully inducible system which will be used for further work. Western blot analysis of cell lysates in the presence and absence of doxycycline were performed using conditioned medium (Figure 4-4). The expression level of different proteins was found to vary among the different clones. For instance, both ZEB1 and EGFP expression showed high levels in MCF7-EGFP-ZEB1 models particular in clone number 10. The low level of ZEB2 and EGFP was more evident in MCF7-ZEB2-EGFP models, but not in clone 18. These data are consistent with the view that decreased ZEB2 expression is determined by the intrinsic features of ZEB2 ORF. To characterise the generated EMT cell models known ZEB1 and ZEB2 targets were analysed. I was not able to detect any significant effect on E-cadherin protein levels in any of the clones analysed. However, P-cadherin was down-regulated in all clones except for a ZEB1-expressing clone 15. Given that all ZEB1-expressing clones contain higher levels of EGFP-ZEB1 fusion in comparison with ZEB2 clones (Figure 4-4). These observations suggest that in MCF7 cells, the ZEB2 protein is a more efficient EMT inducer than ZEB1. Consistent with a previous study which has shown that ZEB1 and ZEB2 proteins function as cyclin D1 inhibitors and induce Rb hypophosphorylation (Mejlvang et al., 2007), it was shown here that cyclin D1 is repressed in EGFP-ZEB1 clone number 10 and EGFP-ZEB2 clone number 14. These clones also exhibited hypophosphorylation of Rb tumor repressor in response to ZEB protein induction.

Previous studies have proven that human breast cancer MCF7 cells express NANOG, OCT4 and SOX2, but their role in human breast cancer remains unclear (Ling et al., 2012).The analysis of the EMT models MCF7-ZEB1-EGFP and MCF7-ZEB2-EGFP has shown that stem cells core factors are expressed at different levels in these cells. NANOG was observed in both models with some differences among clones, and different clones exhibited different expression levels of OCT4 and SOX2. It was demonstrated here that NANOG, but not OCT4 and SOX2, are down-regulated to some extent due to the activation of either ZEB1 or ZEB2.

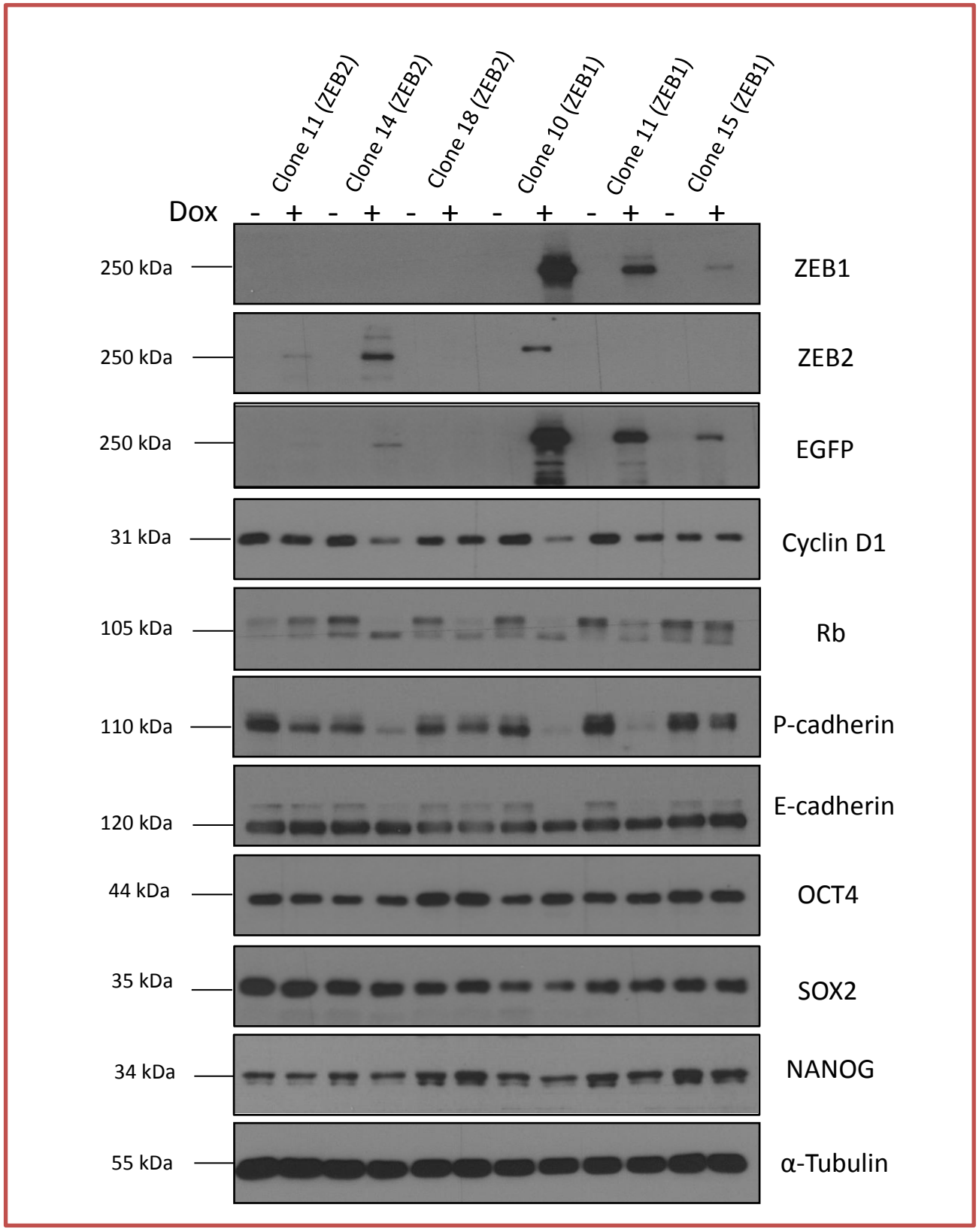


Figure 4-4: Western blot analysis of MCF7 clones expressing ZEB1-EGFP or ZEB2-EGFP fusion in DOX-regulates manner.

MCF7-ZEB1-EGFP and MCF-ZEB2-EGFP clones were cultured in the presence and absence of doxycycline for 72 hrs. Cells were lysed and protein analysed by western blotting. Both ZEB1- and ZEB2-expression vectors have similar structures, N-terminal GFP tags with the optimised Kozak sequence. Note, the expression level of ZEB1 is much higher than that of ZEB2. α-tubulin was used as a protein loading control.

4.3.3 SHORT STRETCH OF RARE CODON CONTROLS TRANSLATION OF ZEB2

According to the data obtained in the EMT lab, two stretches of rare codons LGV are located adjacent to the Smad Binding Domain (SBD) within the ZEB2 ORF. These triplets induce ribosome stalling and reduce the efficacy of ZEB2 protein translation. One of the triplets is present within the 372-437 fragment expressed as a fusion with EGFP by the construct pEGFP-ZEB2 (372-437). To examine this hypothesis, a vector including ZEB2 (372-437) fragment with fused EGFP was generated (Figure 4-5). Ribosome stalling during protein synthesis can be inflicted by mRNA secondary structure; that is, mRNA-associated proteins or codon usage. Examination of the ZEB2 (372-437) ORF using a codon frequencies search program (http://www.molbiol.ru/eng/scripts/01_11.html) revealed a single rare codon cluster comprising three adjacent codons corresponding to the amino acids leucine, glycine and valine ($L^{426}G^{427}V^{428}$). This cluster was a part of 372-437 aa sequence that was essential for the expression-inhibition activity of the 372-437 aa ZEB2 protein fragment fused with GFP. In addition, a correlation between tRNA and codon frequencies has been confirmed in the breast cancer cell line (Pavon-Eternod et al., 2009). The synonymous substitution of rare codons UUA GGU GUA by a common codon CUG GGC GUG triplet has been shown to enhance the expression level of ZEB2-EGFP fusion in different cell lines. To examine the contribution of individual codons, three more constructs were generated, in which double (rare L and V replaced by common L and V) or single (rare V or G replaced by common V or G) substitution were introduced (figure 4-6). MDA-468 or A431 cells were transfected with these constructs and incubated for 48 hours and proteins were lysed. The data show that single rare to common codon substitution has little or no effect on protein yield. Substitution of two rare codons apparently enhanced translation efficacy. Therefore, the data suggest that rare codons within the ZEB2 ORF may compromise efficacy of protein translation when they form clusters, and three rare codons in a row is sufficient to effect this process (figure 4-7a). DLD-ZEB2 cells were used to examine the effect of rare to common codon substitution (figure 4-7b). It was suggested that ribosome stalling may result in the degradation of the nascent peptide through a mechanism possibly involving the ubiquitin proteasome proteolytic pathway. This hypothesis is in agreement with a recent study implicating SPF^{Fbxo45} E3 ligase in the regulation of ZEB2 protein expression in several cancer cell lines (Xu et al., 2015a). Two lysines are localised in the vicinity of LGV triplets and

form part of consensus sumoylation motif. According to a previous study, these two lysines are modified by sumoylation, which influence protein function as a transcriptional repressor (Long et al., 2005). It was proposed that the nascent peptide can be ubiquitinated at this site leading to its degradation and reduced protein production. To test this, lysines K^{389} and K^{391} were substituted with chemically related arginine residues, which cannot be modified by ubiquitination (figure 4-6). This mutant fusion protein was transiently expressed in A431 and MDA-468 cell lines. The data show that these lysine substitutions to arginine had no effect on protein yield (Figure 4-7a).

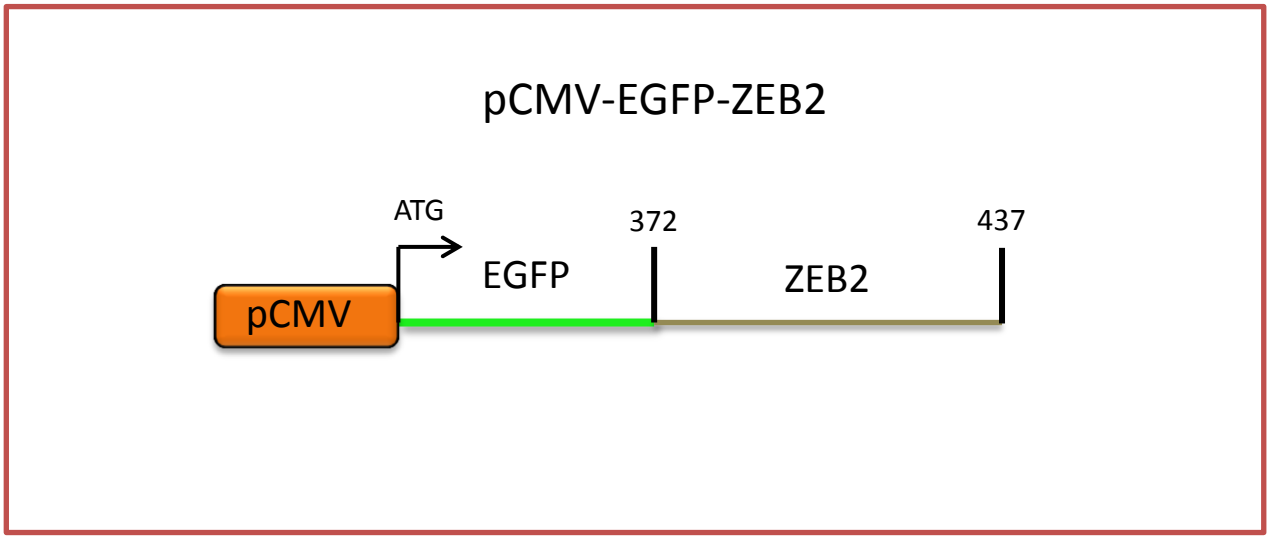


Figure 4-5: Schematic of pCMV-EGFP-ZEB2-C1 plasmid.
The ZEB2 (372aa-437aa) was cloned into the pCMV-EGFP vector at the C-terminal.

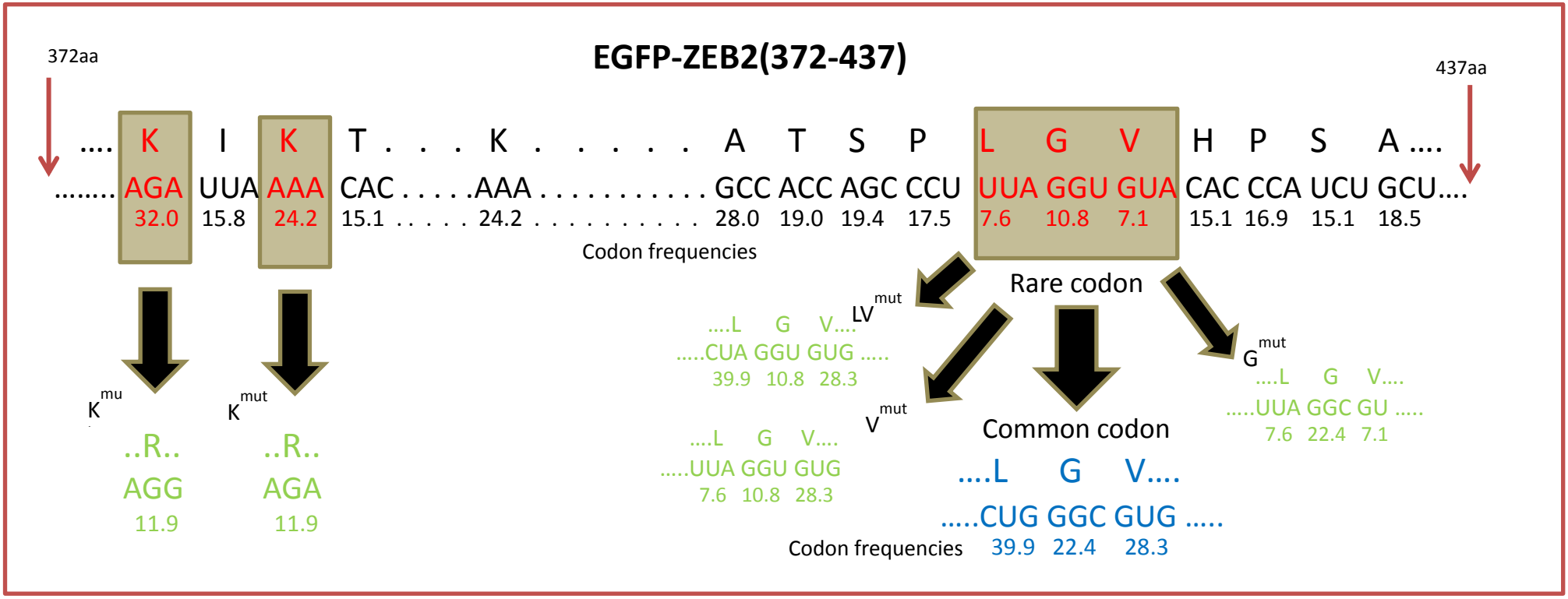


Figure 4-6: Illustration of ZEB2 372-437 fragment showing the position of targeted codons.
Triplet' mutations (blue) were carried out to substitute the rare codon with frequencies 7.6, 10.8, and 7.1 by a common codon with frequencies 39.9, 22.4, and 28.3. Individual mutations (green) of LV, V and G amino acids were subsequently performed from rare to common codons, and codon frequencies were changed accordingly. Two lysine residues K389 and K391 were substituted with chemically related arginine residues.

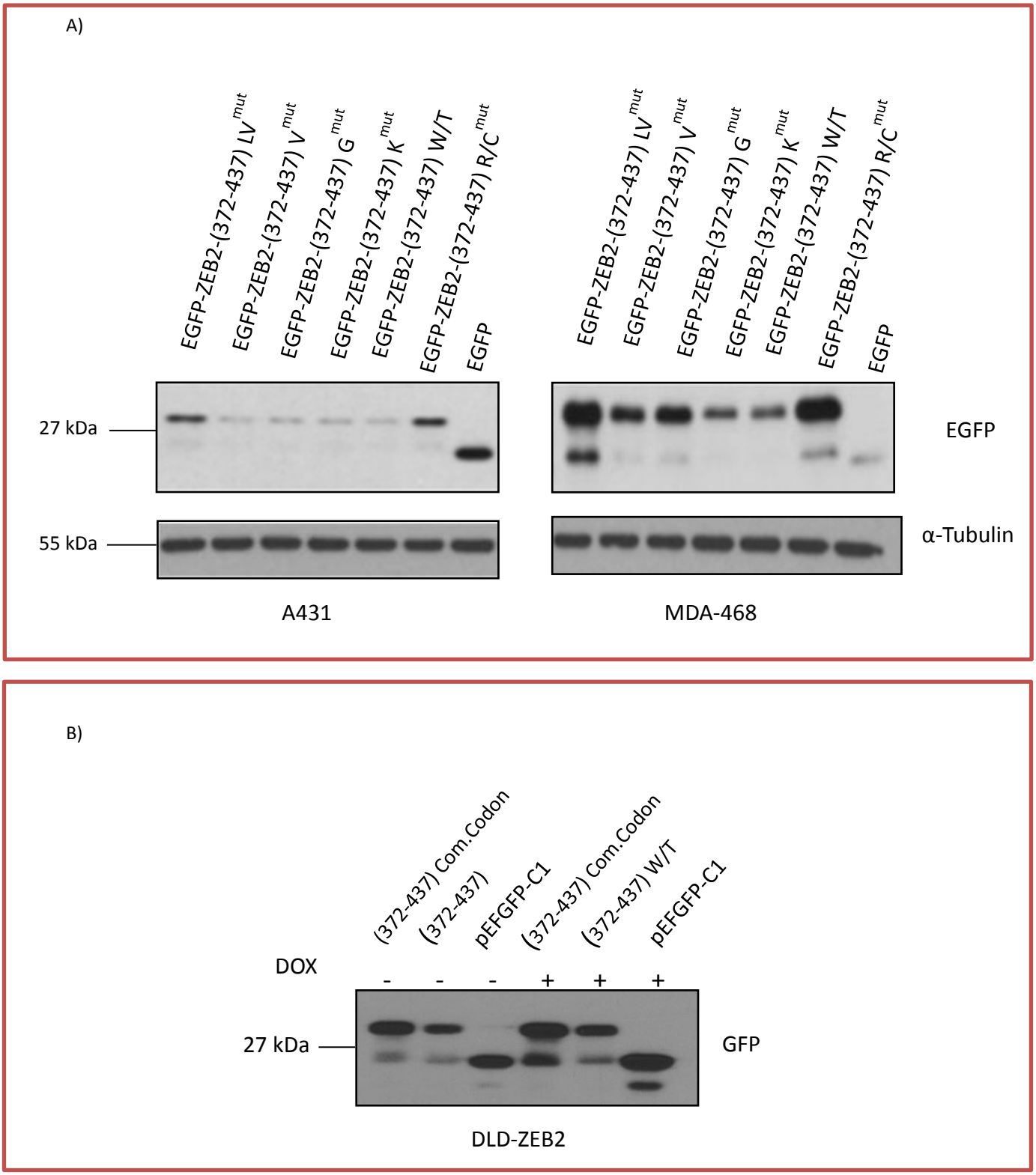
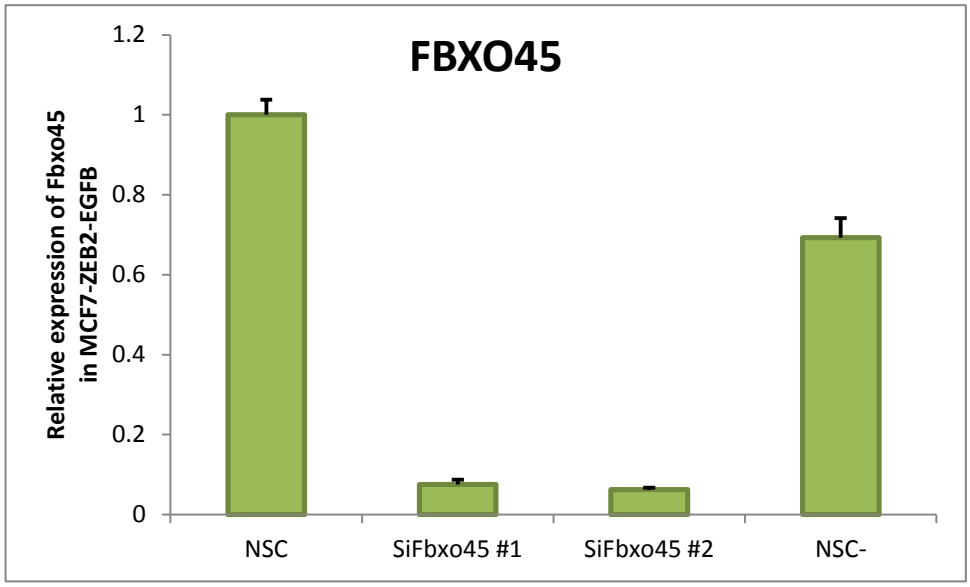


Figure 4-7: LGV influence ZEB2 expression level.
A) 431 and MDA-468 cells were transfected with differently mutated EGFP-ZEB2 (372-437) and incubated for 48 hours. B) DLD-ZEB2 cells were transfected, after seven days of doxycycline treatment, with LGV mutated plasmid for 24 hours for a maximum of eight days of doxycycline treatment. Cells were lysed and the protein analysed via western blotting. Transfected A431 did not show any difference among mutated clusters. MDA-468 and DLD-ZEB2 showed that shifting from a rare codon to common codon particularly in the LGV cluster influence the expression of the ZEB2 gene. α-tubulin was used as a protein loading control.

4.3.4 UBIQUITIN E3 LIGASE CONTROLS ZEB2

It has been previously shown that EMT transcriptional factors can be influenced by atypical ubiquitin E3 ligase complex. Three subunits of E3 ligase include Skp1, Pam and Fbxo45 were found to affect EMT through targeting both ZEB1 and ZEB2 proteins for proteolytic degradation via the ubiquitin proteasome pathway. In particular, Fbxo45 was found to bind to SBD within ZEB2 and cause its ubiquitination (Xu et al., 2015a). The expression of Fbxo45 was inhibited by RNA interference, using two different siRNAs, in Dox-treated MCF7-ZEB2-EGFP and the effect of siRNA was validated via qPCR (Figure 4-8a). Next, I assessed how these siRNAs influence ZEB2 expression levels in these cells. Consistent with the previous report (Xu et al., 2015a), knockdown of Fbxo45 resulted in up-regulation of ZEB2 in MCF7-ZEB2-EGFP cells (Figure 4-8b). This suggests that Fbxo45 has the potential to negatively regulate the expression of ZEB2. According to the data previously obtained in the EMT lab, ZEB2 is a very stable protein in melanoma cell lines. An experiment was carried out to analyse the effect of Fbxo45 depletion on the stability of ZEB2 in MCF7 cells. MCF7-ZEB2-EGFP was maintained in the presence of cycloheximide, lysed at different time points and analysed for the expression of ZEB2. Consistent with the result obtained in melanoma cells, ZEB2 appeared to be a very long-lived protein in MCF7 cells. Significantly, knockdown of Fbxo45 had no effect on ZEB2 half-life (Figure 4-9). Given that the predominant localisation of the SPF^{Fbxo45} E3 ligase is the cytoplasm (Salat et al., 2015), it was proposed that this enzyme might decrease the expression level of ZEB2 by ubiquitination ZEB2 protein during its synthesis. It was also proposed that ribosome stalling might be required for the ubiquitination and subsequent degradation of the nascent peptide. To test this, the effect of Fbxo45 depletion was investigated on the expression level of full-length ZEB2 protein mutant (ZEB-R/C), in which a rare codon within the LGV clusters were substituted with a common codon. ZEB2-R/C protein was expressed in MCF7-ZEB2-EGFP cells along with the control or Fbxo45-specific siRNA. Transfected cells were maintained without DOX for 72 hours, and ZEB2 expression was assessed in western blotting (Figure 4-10). Consistent with our hypothesis, the mutant ZEB2 expression level was not affected by the knockout of Fbxo45. These data suggest that SPF^{Fbxo45} E3 ligase-mediated regulation of ZEB2 expression is coupled with the kinetics of the translation of this protein.

A)



B)

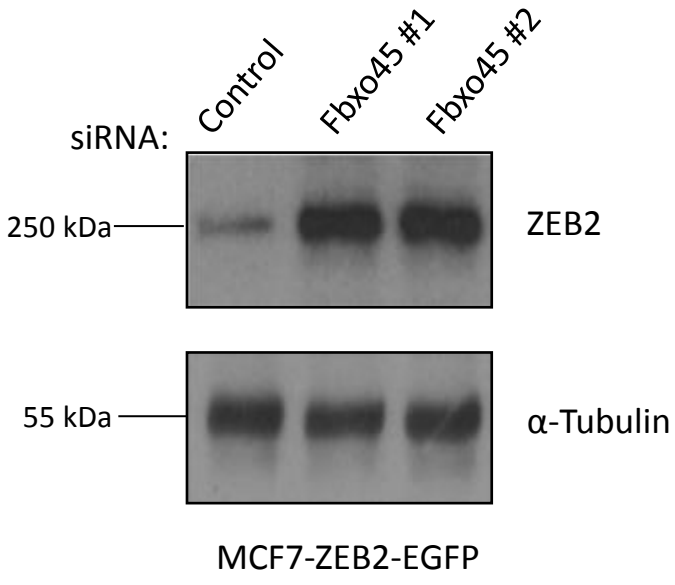


Figure 4-8: Knockdown of Fbxo45 increased ZEB2 expression level.
A) Relative expression values of siFbxo45 gene in MCF7-ZEB2-EGFP using two different siRNAs compared to siControl +/- doxycycline. B) MCF7-ZEB2-EGFP cells were transfected with the various siRNAs and incubated for 72 hours in the presence of doxycycline. Western blot analysis showed that Fbxo45 knockdown resulted in ZEB2 up-regulation. α-tubulin was used as a protein loading control.

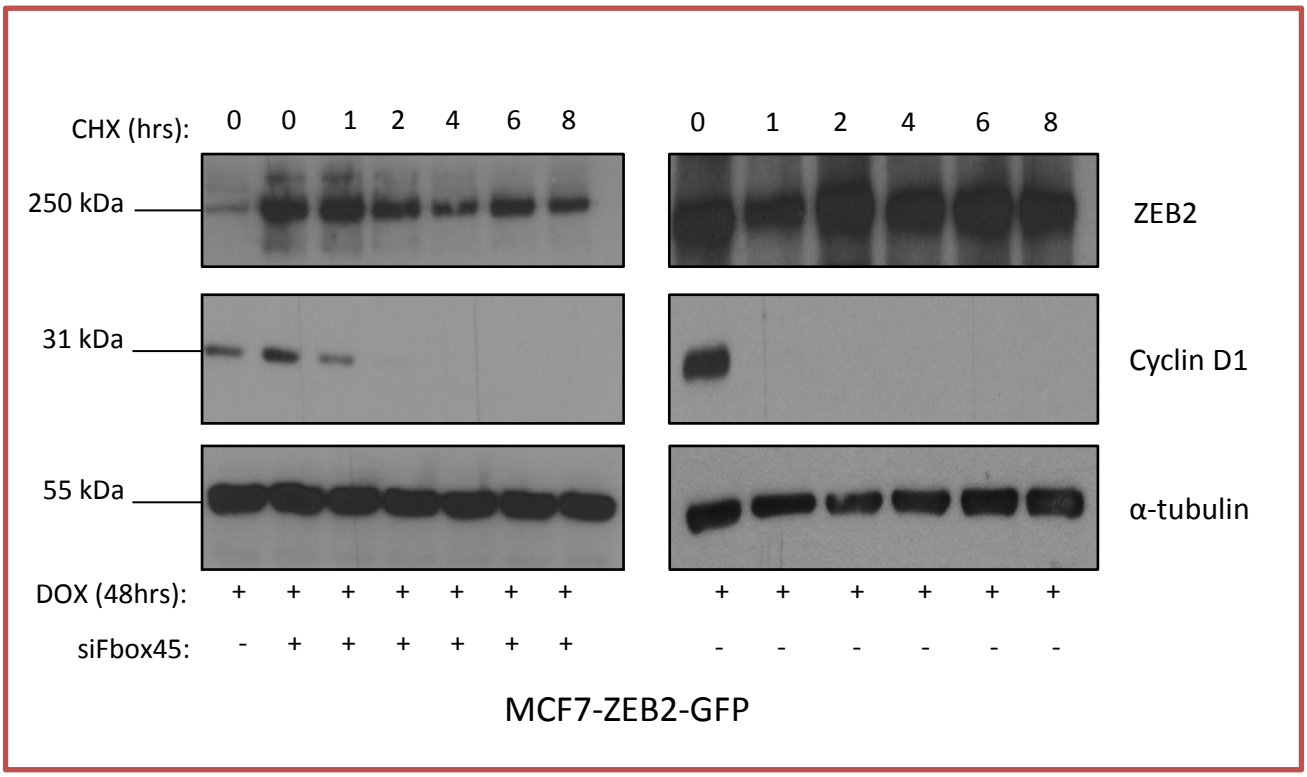


Figure 4-9: ZEB2 is a stable protein.
MCF7-ZEB2-EGFP cells were treated with cycloheximide for different time periods, and the expression of ZEB2 protein was analyzed by westernblotting. Cells were also transfected with siFBxo45 in different experiment. The stability of ZEB2 at -/+ siFbox45 shows a long-lived ZEB2 protein. Expression of short-lived Cyclin D1 protein was analyzed to control for the efficacy of cycloheximide treatment. α-tubulin was used as a protein loading control.

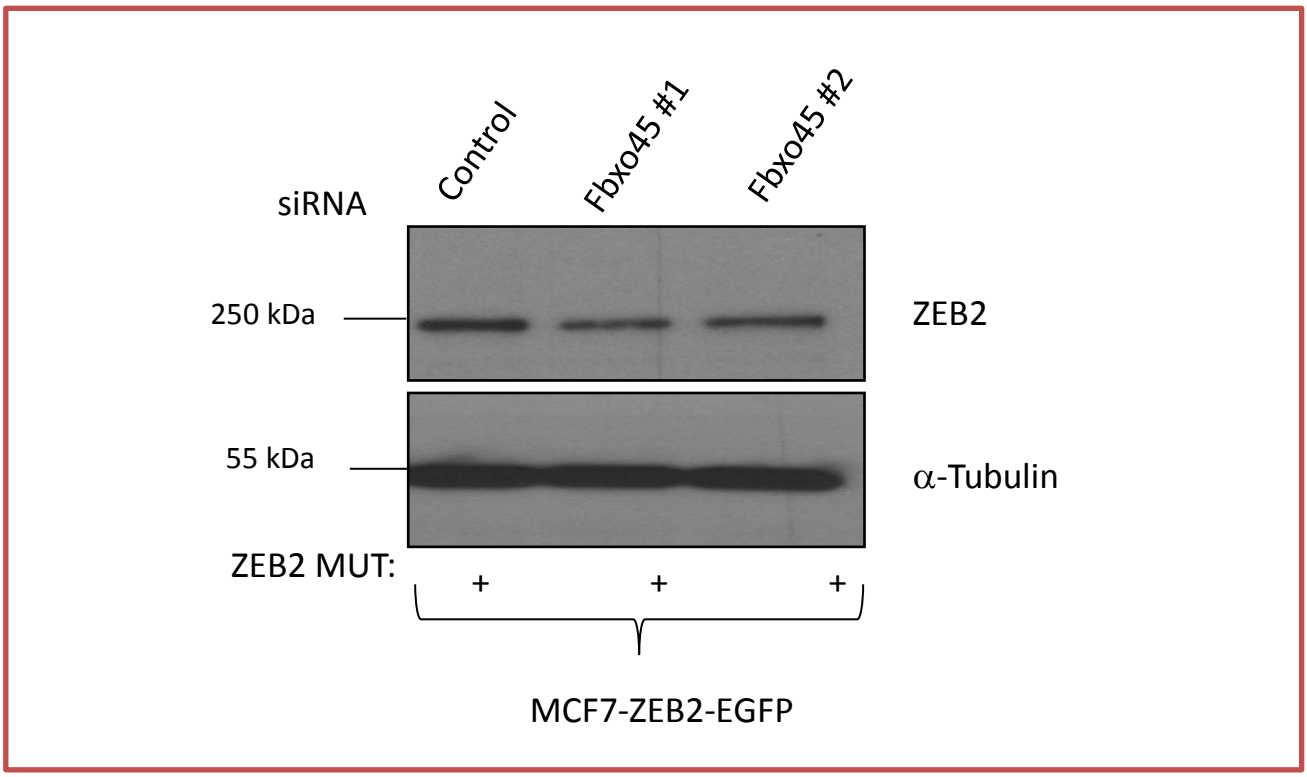


Figure 4-10: ZEB2-MUT expression level.
ZEB2-MUT was co-transfected with siFbxo45 in the MCF7-ZEB2-EGFP cell line. Two siRNA of Fbxo45 were used alongside the siControl, incubated for 72 hours and proteins isolated. Western blot analysis showed that ZEB2-MUT expression was not affected by the knockdown of Fbxo45. α-tubulin was used as a protein loading control.

4.3.5 EFFECT OF EMT ON THE REGULATION OF THE EXPRESSION OF ZEB2 PROTEIN

Often activated by oncogenic pathways, EMT programs may cooperate or counteract the oncogenes in malignant transformation. As ZEB2 and ZEB1 are proposed to have opposing roles in this process, I aimed to analyse how ZEB2 regulation is affected by EMT programs in carcinoma cells. To this end, an analysis was undertaken to determine whether EMT programs perpetrated by ectopically expressed ZEB1 or ZEB2 proteins influence ZEB2 regulation at the translational level. MCF7-ZEB1-EGFP or MCF7-ZEB2-EGFP cells were transfected with the mutant ZEB2 (rare to common) in parallel with wild-type constructs in the presence or absence of DOX, incubated for 48 hours and proteins analysed (Figure 4-11). Consistent with results obtained from MCF7-ZEB1-EGFP and MCF7-ZEB2-EGFP, data were confirmed in DLD-ZEB2 cells (Vandewalle et al., 2005) (Figure 4-12). The data suggest that rare-to-common codon mutations produce similar effects on the yield of the EGFP-ZEB2 chimeras suggesting that translational regulation of ZEB2 was affected by neither ZEB2 nor ZEB1.

The influence of ZEB1- or ZEB2-activated EMT programs on the activity of *ZEB2* gene transcriptional promoter and enhancer RE-4 analysed in chapter 3 was also investigated. MCF7-ZEB2-EGFP or MCF7-ZEB2-EGFP was transfected with both the promoter and RE-4 enhancer, incubated for 48 hours and cells collected for determination of luciferase activity (Figure 4-13). Cells were transfected with pGL3 basic as a negative control. Interestingly, although ectopic ZEB2 had no effect on the activity of its own promoter, it significantly repressed the RE-4 enhancer element. These data might reflect the existence of a negative feedback mechanism maintaining low expression of ZEB2. In contrast, ZEB1 up-regulates the activity of both the transcriptional promoter and RE-4 enhancer of the ZEB2 gene. To our knowledge, to date there are no reports documenting opposing effects of ZEB proteins on the activity of transcriptional regulatory elements.

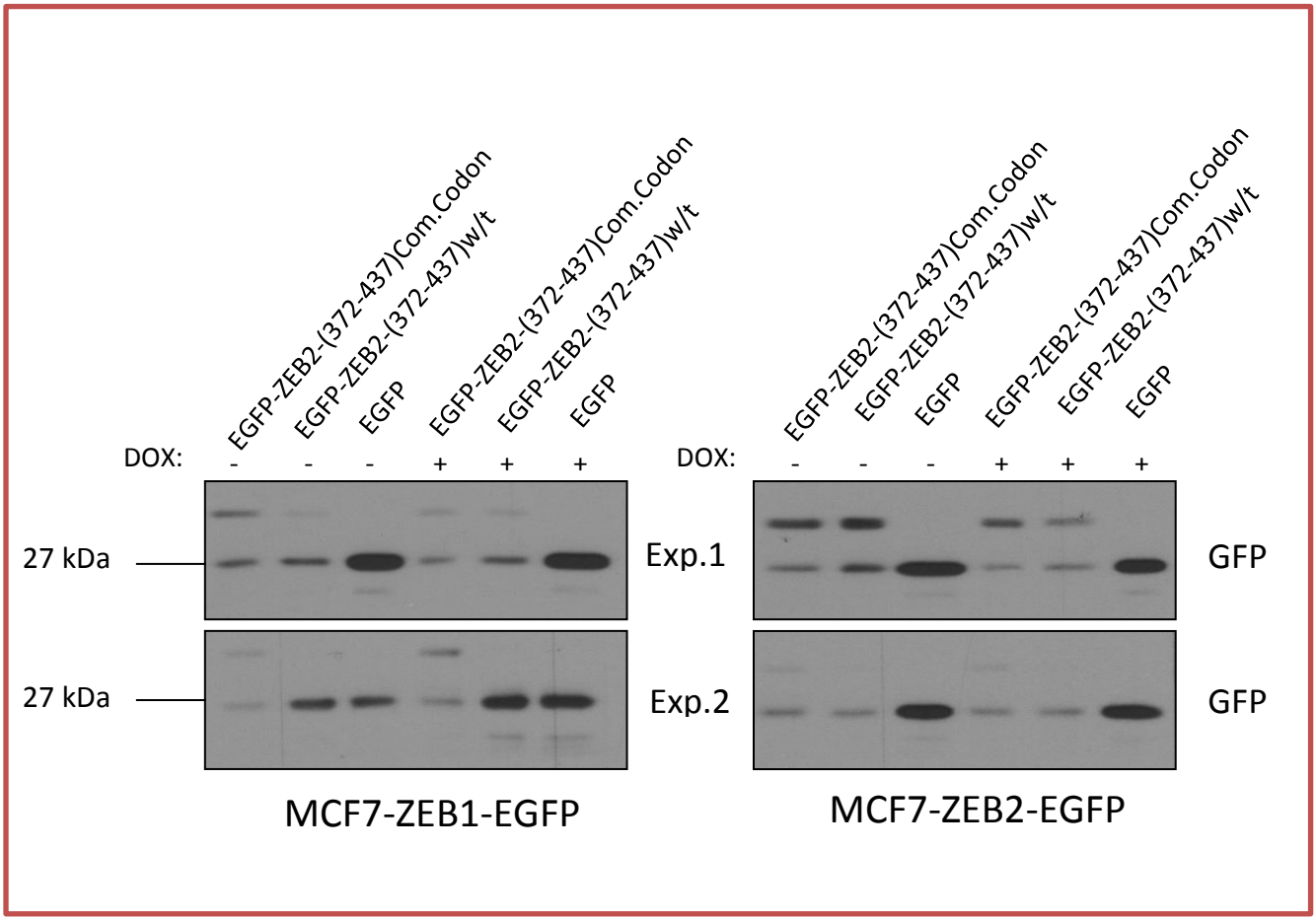


Figure 4-11: EMT effect EGFP-ZEB2 regulation.
MCF7-ZEB1-EGFP and MCF7-ZEB2-EGFP cells were transfected with mutant ZEB2 (372-437) (rare to common), ZEB2 (372-437) wild-type and EGFP in the presence and absence of DOX. 0.05µl of EGFP plasmid were added to each to control transfection. Experiments were performed in triplicate and two were considered.

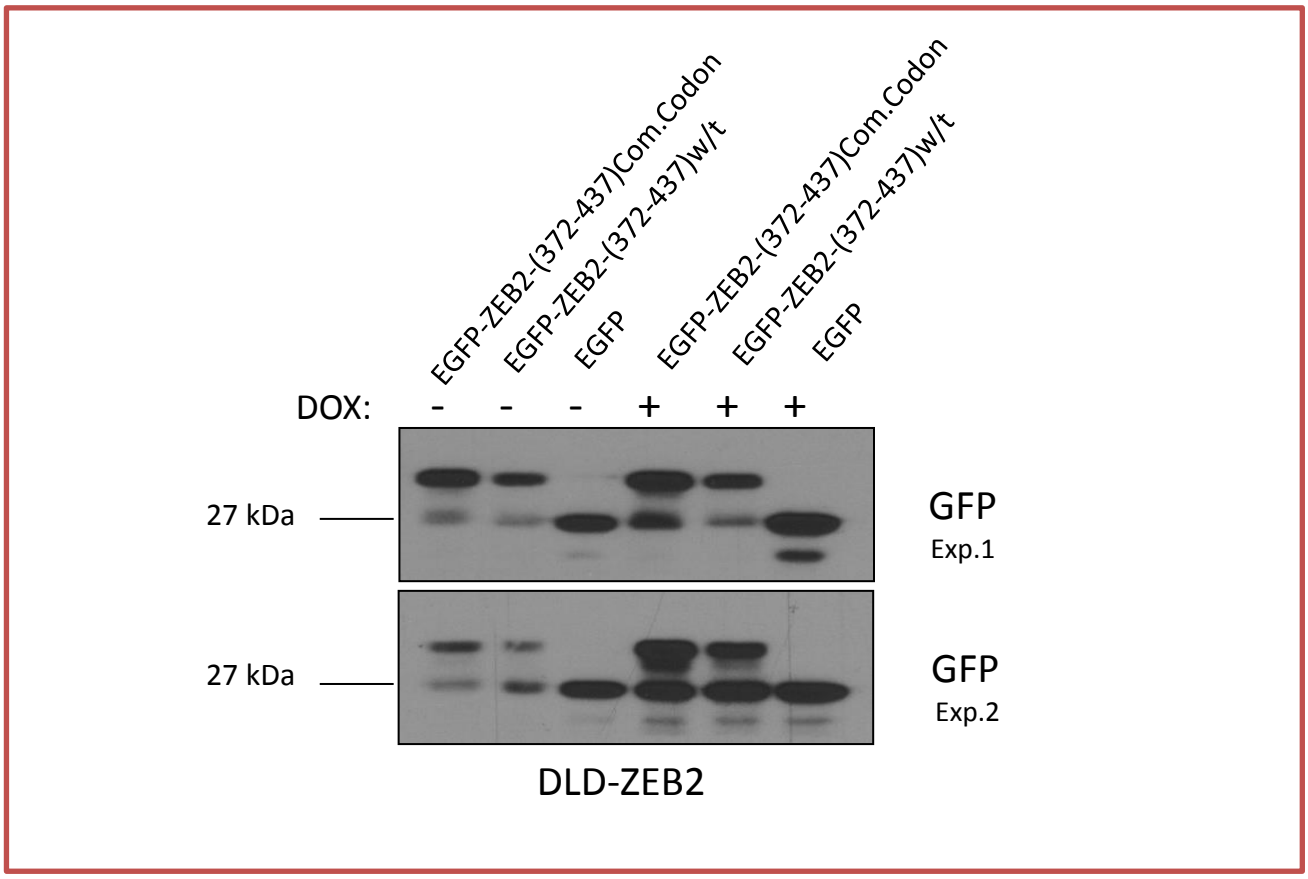


Figure 4-12: EMT influence ZEB2 regulation.
Treated DLD-ZEB2 cells for seven days were transfected with mutated rare to common ZEB2 (372-437), wild-type ZEB2 (372-437) and EGFP plasmid for 24 hours for a maximum of eight days of doxycycline treatment. Cells were lysed and the protein analysed via western blotting. 0.05µl of EGFP plasmid was added to each to control transfection.

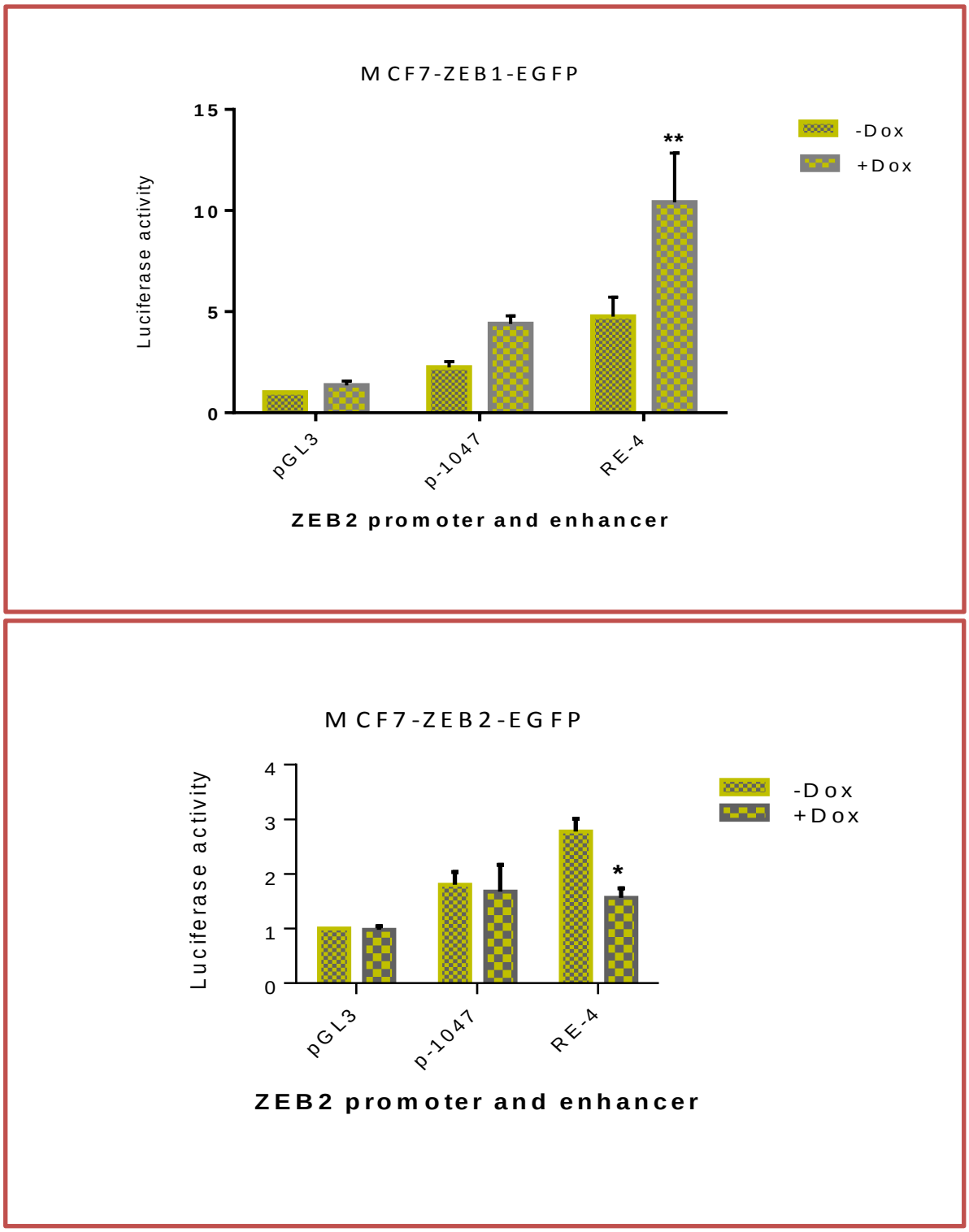


Figure 4-13: The differential sensitivity of ZEB2 enhancer and promoter during EMT.
MCF7-ZEB1-EGFP and MCF7-ZEB2-EGFP cells were transfected with p-1047 promoter and RE-4 enbancer, incubated for 48 hours. Relative luciferase activity were presented as the mean of duplicate readings, with each condition replicated in three independent experiments. pGL3 basic vectro was used as negative control. Significance was tested via a two-way ANOVA (* $p = \leq 0.05$,** $p = \leq 0.01$).

4.4 DISCUSSION

By studying the expression of both ZEB1 and ZEB2 in different cell lines, it was shown that the level of both proteins vary significantly. Apart from in epithelial cells, ZEB1 was detected in most mesenchymal carcinomas, while ZEB2 was not found to be expressed in carcinoma but was expressed at different levels in sarcoma and melanoma (Figure 4-1). Indeed, it was difficult to study the role of ZEB2 expression in cancer cells. Therefore, EGFP-tagged cell line modules that express ZEB1 and ZEB2 were established. The induction was not expected to be straightforward as the procedure required the Tet-on or Tet-off system and many additional critical stages (Gossen and Bujard, 1992). Inducible models of MCF7-ZEB1-EGFP and MCF7-ZEB2-EGFP were then analysed in relation to the EMT process, which is considered to be activated when ZEB1 and ZEB2 are induced. The results show that there is a higher abundant of ZEB1 than of ZEB2 with agreement in different cell lines (MDA-468, A431 and DLD-ZEB2) (figure 4-7a/b). However, the expression level of ZEB2 in cancer cell lines remains contradictory.

Possible mechanisms that play an important role in ZEB2 protein synthesis in the cancer cell lines were investigated. It was shown that the substitution of ZEB2 homologous protein pairs within ORF influenced gene expression. It was specifically shown that the presence of common codon triplets $L^{426}G^{427}V^{428}$ lead to higher ZEB2 translation than its translation in the presence of the rare LGV codon. Interestingly, the data suggests that this rare codon must form clusters in order to influence ZEB2. In addition, a single substitution of L, G and V from the rare codon to the common codon had an important influence on EGFP-ZEB2 expression. However, the data shows that there was no significant difference of the singular substitution in the MDA-468 and the A431 cell lines.

It was also shown that ubiquitin E3 ligase had an effect on ZEB2 regulation (Figure 4-8). In particular, how the E3 ligase subunit Fbxo45 associated with ZEB2. The data show that a high level of ZEB2 is induced when using siRNAs for knockdown of Fbxo45, which explains the loss of E3 ligase function. Moreover, the data on Fbxo45 expression in correlation with mutated EGFP-ZEB2 presented in chapter4 indicates that the role of Fbxo45 is affected by the change in triplets of the rare codons, LGV, which are localised at the border of the ZEB2

SBD domain where Fbxo45 binds. This may indicate that E3 ligase degrades the nascent peptide during translation. These data are consistent with a previous study which showed that Notch activity is terminated through ubiquitination by E3 ligase (Gupta-Rossi et al., 2001). Additionally, Hey proteins mediate nuclear translocation of Fbxo45 (Salat et al., 2015), suggesting that ZEB2 expression as well as the ZEB1/2 balance may be affected.

CHAPTER 5: RETROVIRAL EXPRESSIONS ENHANCE ZEB2 AND PROMOTES EMT

5.1 INTRODUCTION

In chapter 4, it was shown that in comparison with ZEB1, ZEB2 is a difficult protein to express. It was also shown that replacing the rare LGV codon triplets with common codons within ZEB2 ORF enhanced its expression. The kinetics of protein translation has an important regulatory role. Specifically, among other factors (interaction of nascent peptides with chaperons, modifications of the nascent peptide) co-translational folding depends on ribosome pausing (Komar, 2009). It is, therefore, unclear how the presence of rare LGV clusters would influence ZEB2 protein function by affecting the kinetic of its synthesis. If the presence of rare codon clusters affects ZEB2 function through co-translational folding pathways, this would indicate that the availability of rare isoacceptor tRNAs may determine the configuration of an EMT pathway activated in a particular cell type by a particular signal. In normal melanocytes and low-grade melanoma, high levels of ZEB2 are detectable in E-cadherin positive cells (Caramel et al., 2013). These observations may indicate that rapid and efficient translation might influence co-translational folding and affect ZEB2 functions as an EMT inducer.

5.2 AIMS AND OBJECTIVES:

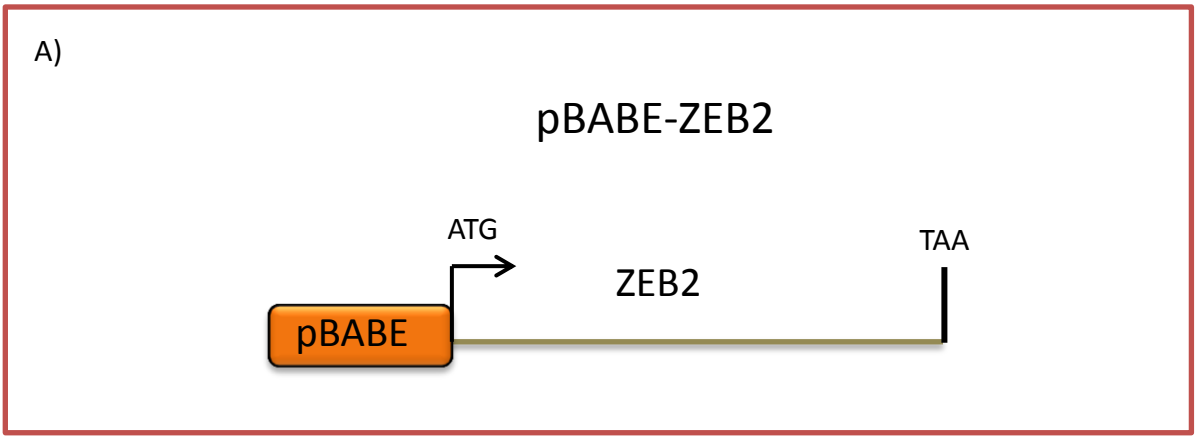
This study aimed to address whether the presence of rare codon clusters influence the activity of ZEB2 as an EMT-TF and our objectives as follow:

- To obtain viral vectors expressing either wild-type ZEB2 or ZEB2 with rare to common codons mutations.
- To analyse activities of wild-type and mutant protein as inducers of EMT.

5.3 RESULTS

A retroviral vector pBABE expressing ZEB2 was previously generated by Dr Gareth Browne (Morel et al., 2012). Two triplets of rare codons within ZEB2 ORF were substituted for the common codons using QuikChange Lightning Multi Site-Directed Mutagenesis kit (Agilent Technology, cat.No.210515), to generate a vector expressing the ZEB2-R/C mutant (see section 2.3.8) (Figure 5-1). Human mammary epithelial cells (HMEC) immortalized by ectopic expression of the human Telomerase Reverse Transcriptase hTERT (HMEC-hTERT) are highly responsive to EMT-inducing signals and broadly used in EMT-TFs studies. HMEC-hTERT cells were infected with retroviral vectors expressing wild-type ZEB2, ZEB2-R/C mutant or an empty vector. Additionally, the ZEB1-expressing pBABE vector was used as a positive control. Infected cells were selected in the medium containing 0.5 µg/ml puromycin and after 5, 8 or 12 days in culture, cell morphology was assessed using phase-contrast microscopy (Figure 5-2). Whereas HMEC-hTERT cells infected with an empty vector exhibited a typical pattern of epithelial growth, infection with pBABE-ZEB1 resulted in cell elongation, dissociation and scattering. The morphology and growth pattern of the cells expressing the wild-type ZEB2 or ZEB2-R/C mutant was intermediate at day 5. By day 8, the cells became phenotypically similar with pBABE-ZEB1 expressing cells. Next, the expression of EMT-TFs and EMT markers were analysed in the infected populations of HMEC-hTERT cells. Replacement of rare codon triplets by common codons within the ZEB2-R/C mutant resulted in the enhanced expression levels of the protein (Figure 5-3). In comparison with the wild-type ZEB2 protein, ZEB1 was a more efficient inducer of the expression of mesenchymal markers, such as N-cadherin or vimentin. Interestingly, R/C mutation increased the efficacy of the induction of mesenchymal markers analysed, N-cadherin and vimentin. In fact, ZEB2-R/C mutant was as efficient as ZEB1 in activating expression of these markers.

Loss of E-cadherin is considered as possibly the most important hallmark of EMT. Of note, whereas ectopic expression of ZEB1 leads to the apparent repression of E-cadherin, no or little effect was observed in cells infected with vectors harbouring ZEB2 wild-type or ZEB2-R/C mutant inserts. It has been established that EMT-TFs cooperate with the oncogenic pathways to induce full EMT response including loss of E-cadherin (Morel et al., 2012).



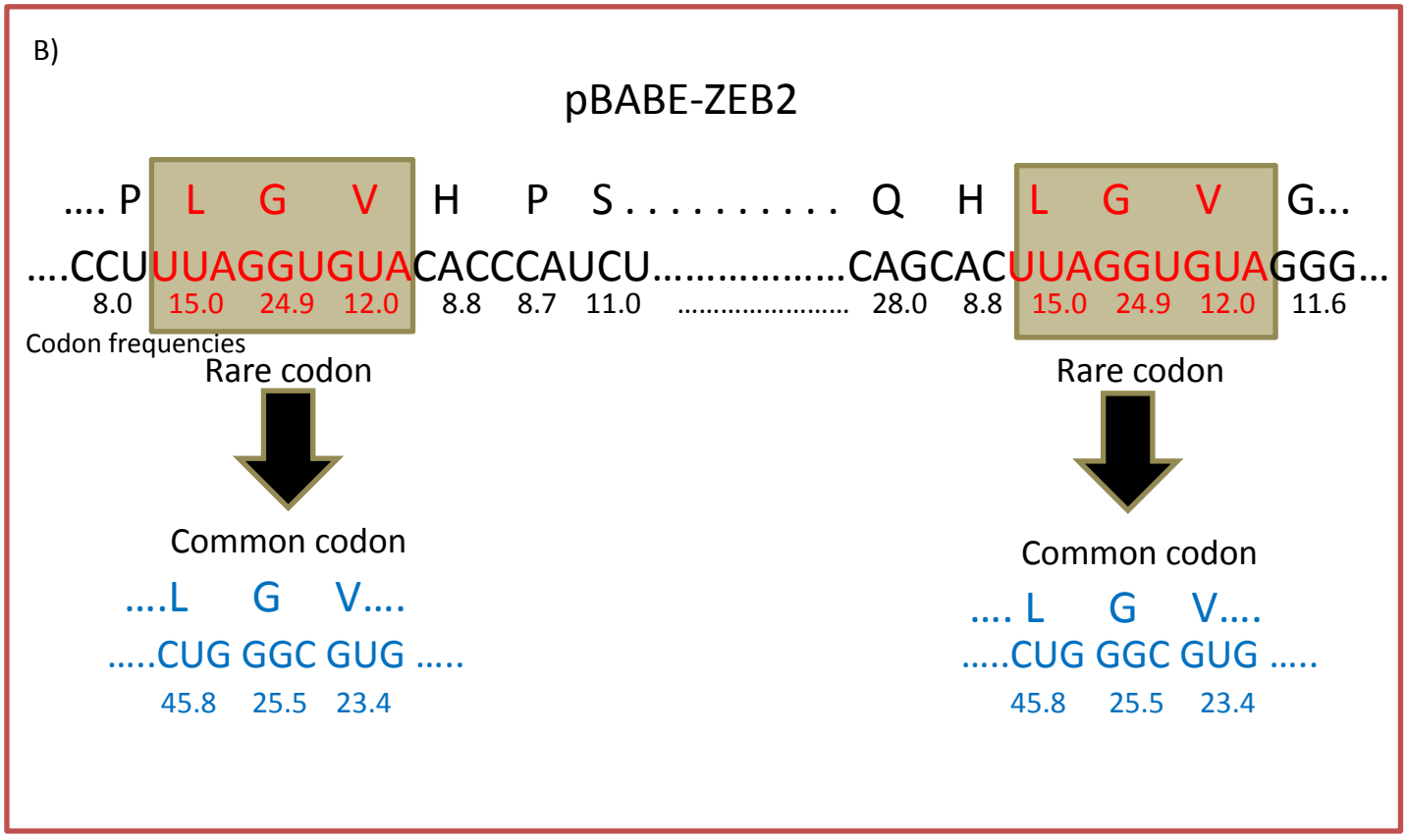


Figure 5-1: Illustration of pBABE-ZEB2 plasmid and the position of substituted codons.
A) ZEB2 was cloned into the retroviral pBABE vector at the C-terminal. B) Schematic of two triplets (LGVs) mutated from rare codon to common codon within ZEB2 ORF. Codon and codon frequencies are presented.

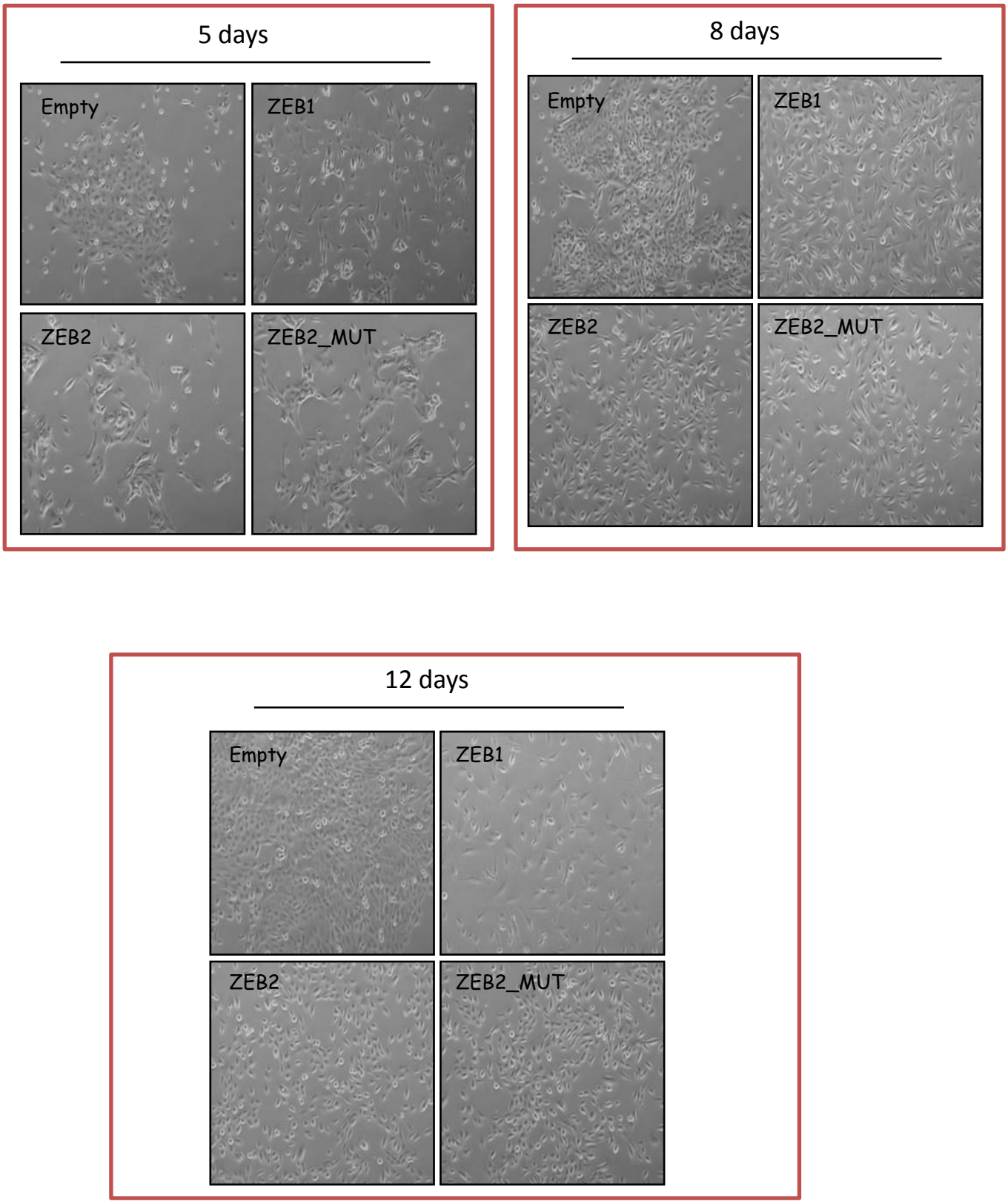


Figure 5-2: Post-transfection of HMEC cells.
HMEC-hTERT cells were infected with retroviral vector expressing ZEB1, ZEB2 or ZEB2-R/C. Cells were analysed by phase-contrast microscopy and photographed at five, eight and 12 days post-selection. Data provided by Dr Louise Hill.

Therefore, it was assessed how the wild-type and R/C mutants cooperate with the oncogenic RAS (HA-RAS^{G12V}). HMEC-hTERT cells were first infected with the neomycin-resistant retroviral vector harbouring HA-RAS^{G12V}. Two days post-infection, cells were infected again with the pBABE vectors containing ZEB1, ZEB2 or ZEB2-R/C mutant. Then cells were maintained in the presence of puromycin and neomycin, and expression of E-cadherin and vimentin was analysed in selected cell populations by western blotting. As expected, both ZEB proteins cooperated with HA-RAS^{G12V} in the induction of EMT including down-regulation of E-cadherin. It is noteworthy that no difference between ZEB2 wild-type and ZEB2-R/C mutant was observed in HA-RAS^{G12V}-transformed cells with regards to the activation of vimentin or down-regulation of E-cadherin (Figure 5-4).

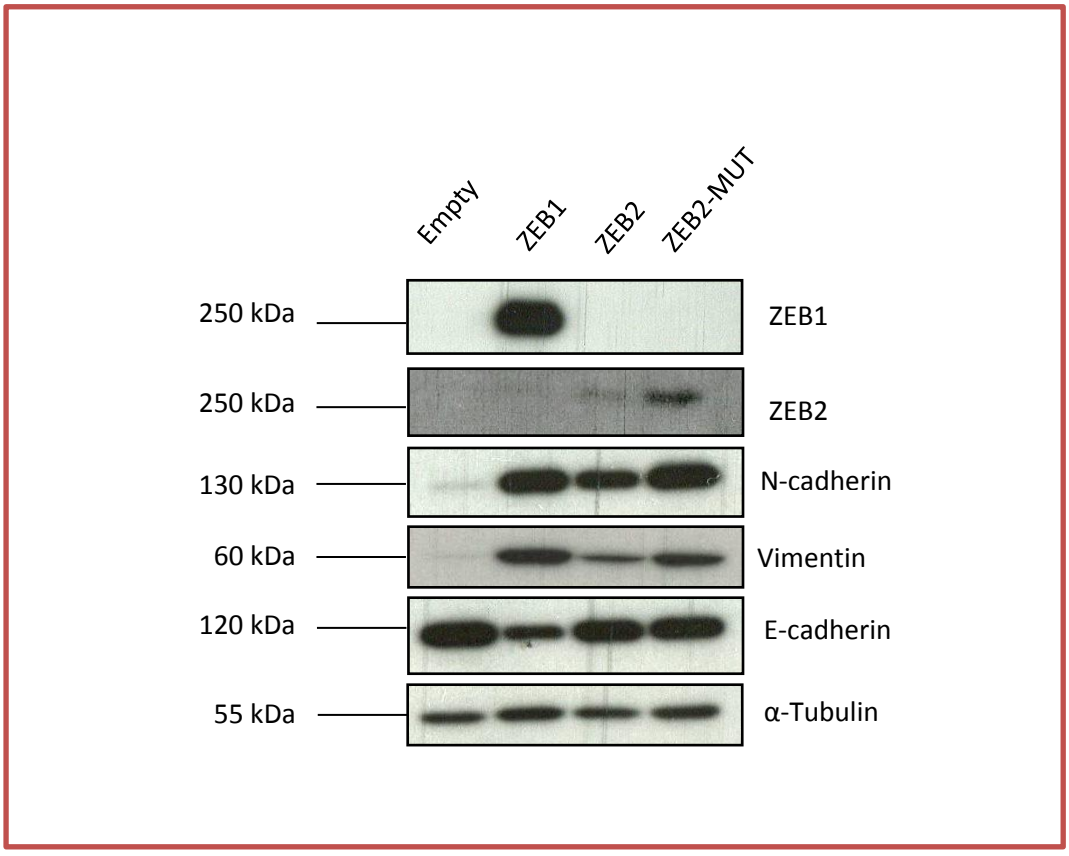


Figure 5-3: Western blot analysis at 6 days post-transfection HMEC cells with ZEB1, ZEB2, and ZEB2-mut. HMEC-hTERT cells were transfected and lysed. Proteins examined via western blot to check for expression levels of ZEB1/2, ZEB2–mut and mesenchymal markers. α-tubulin was used as loading control.

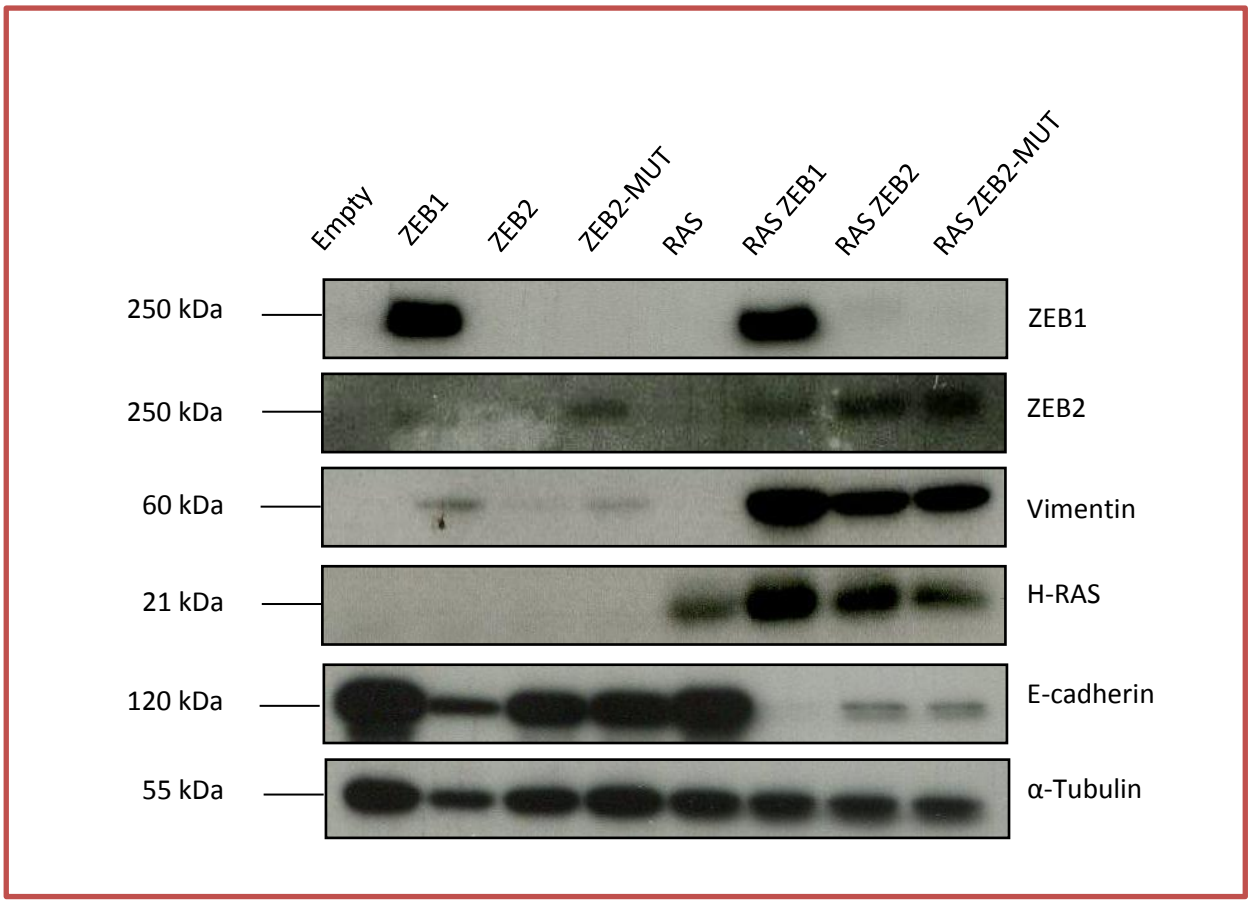


Figure 5-4: Introducing RAS promotes EMT.
Western blot analysis of HMEC cells transfected with retroviral ZEB1 and ZEB2 constructs with and without RAS. Level of ZEB1 is higher compared to ZEB2 with loss of E-cadherin and increased level in N-cadherin and Vimentin. H-RAS was used as transfection control while α-tubulin was used as a loading control.

5.3 DISCUSSION

Through the analysis of the regulation of ZEB2 protein synthesis, two stretches of rare codons ($L^{426}G^{427}V^{428}$) and ($L^{439}G^{440}V^{441}$) were characterised, which are located at the border of SBD and induce ribosome pausing (Figure 5-1). These codon clusters are responsible for the reduced expression of chimeric proteins composed of EGFP and short ZEB2-derived fragments (Chapter 4). However, it was important to establish a role for LGV elements in the context of the full-length protein. Experiments with the retroviral vectors expressing wild-type ZEB2 or ZEB2-R/C mutant (Figure 5-2) have shown that introducing common codons in these triplets enhances protein yield concomitantly with more efficient activation of mesenchymal markers (Figure 5-3). This finding indicates the existence of a novel regulatory mechanism which is dependent on the availability of a rare isoacceptor tRNAS among other factors (expression and subcellular of localisation of FBXO45, and possibly other component of ubiquitin-proteasome system; expression level of cytoplasmic chaperons, etc.). A recent study has demonstrated that eukaryotic cells contain two distinct pools of tRNA isoacceptor (Gingold et al., 2014). These two pools of tRNAs carry anticodons corresponding to the codons more often utilised either in proliferation/cancerous or differentiation/arrested cell states. Interestingly, both rare isoacceptor tRNAs (tRNA-L^{UUA} and tRNA-V^{GUA}) are part of the tRNA gene expression signature characteristics for the differentiated/arrested state. This indicates that this state is permissive for the expression of ZEB2 and point to the tumour suppressive ZEB2 features possessed by some genetic backgrounds. In line with these speculations, the ZEB-R/C mutant was expressed at a higher level than its wild-type counterpart in hTERT-immortalised proliferative HMEC cells. This difference was however abandoned in HA-RAS^{G12V}-transformed HMEC cells. Whether the expression of tRNA-L^{UUA} and tRNA-V^{GUA} gene is regulated by the oncogenic RAS deserves further investigation.

It is becoming increasingly clear that co-translational folding pathways have an important role in the regulation of protein function (Kimchi-Sarfaty et al., 2007). This phenomena could be explained if ZEB2 function is regulated by co-translational folding and co-expression with E-cadherin in normal human and mouse melanocytes (Caramel et al., 2013), and in some forms of human cancer such as transitional cell carcinoma of the bladder (Sayan et al., 2009). However, currently we have no data to support this theory. Indeed, the

ability to activate mesenchymal markers correlates with the expression level of ZEB2 in HMEC-hTERT cells. On the other hand, ZEB2 function as an E-cadherin repressor is independent of LGV elements, but is determined by RAS transformation. Whether oncogenic induces ZEB2 modifications or activates some co-factors which are important for the repression of E-cadherin gene remains to be determined in future studies.

CHAPTER 6: GENERAL DISCUSSION

Development EMT pathways are responsible for the generation of individually-migrating mesenchymal- like cells from epithelial tissue. This process is reminiscent of the detachment of cancer cells from a tumour mass and intravasation followed by the formation of secondary tumours at distant sites. In earlier studies the implication of developmental EMTs in tumour progression has been considered primarily in light of this analogy. During the last decade, it has been established that the role of EMT programs in cancer is much broader and not limited to the alterations in adhesive and migratory features of tumour cells. EMT programs affect the majority of all hallmarks of cancer highlighted in Hanahan and Weinberg's reviews (Hanahan and Weinberg, 2011). In particular, the interrelation between EMT and cancer cell stemness has been the focus of recent research. Established in two independent studies published in 2008, this link unveiled a mechanism of generation of highly tumorigenic breast carcinoma cells through EMT pathways involving EMT-TFs of TWIST, SNAIL and ZEB families. Among the criteria for stem-like characteristics these studies used expression levels of CD24 and CD44, cell surface markers whose expression pattern ($CD24^{low}/CD44^{high}$) is associated with both human breast CSCs and normal mammary epithelial stem cells (Mani et al., 2008). In contrast, manipulating the expression levels of canonical Yamanaka pluripotency transcription factors in prostate and bladder carcinoma cells has shown that these factors support epithelial phenotype, and EMT suppressed pluripotency in these cell models (Celia-Terrassa et al., 2012). Likewise, ZEB2 drives the neuroectodermal differentiation of human embryonic stem cells, and directly represses NANOG and OCT4. NANOG, OCT4 and SOX2 in turn repress ZEB2 transcription (Chng et al., 2010). These data obtained in prostate and bladder carcinoma cells and normal hESCs are in line with the observation that adult fibroblasts experience MET to undergo reprogramming and acquire self-renewing capacity (Li et al., 2010). However, there are numerous reports showing overexpression of pluripotency factors in cancerous tissues (Jeter et al., 2011, Sun et al., 2014), and a cooperation between these factors and EMT-TFs has been reported (Uthaya Kumar et al., 2015). Taken together, these data indicate that the interrelationship between EMT and stemness is complex, whereas EMT program promote features of adult stem cells, they may antagonise or support pluripotency, likely dependent on cell context.

In my work, I attempted to address the interrelationship between EMT-TFs and embryonic stem cell factors in malignant cutaneous melanoma cell lines. EMT is a biological

programme characterised by loss of E-cadherin expression during cancer. Indeed, many oncogenic pathways, including the RAS pathway, are known to play a vital role during EMT. Malignant Melanoma, for example, has the ability to undergo EMT process at the invasive front, rendering it capable of invasion and metastasis. Another critical concept of melanoma development and progression is the theory of cancer stem cells. These are initiating cells in many types of cancer, including malignant melanoma (Boiko et al., 2010, Klein et al., 2007). Malignant melanoma is driven through the activation of MEK-ERK signalling via the mutations in the oncogenes B-RAF or N-RAS (Caramel et al., 2013). By studying the functions of EMT-TFs in melanoma cell lines, data from our lab has demonstrated that the use of B-RAF and MEK inhibitors resulted in the regulation of EMT-TFs expression. Consistent with these data, this study demonstrated that inhibition of B-RAF pathway lead to down-regulation of ZEB1 and TWIST1 and up-regulation of ZEB2 and SNAIL2. Remarkably, data shows that both embryonic stem cells core transcriptional factors OCT4 and SOX2, which bind to and repress ZEB2 via RE-4, are following the same pattern of ZEB1 and TWIST1 in the melanoma cell line. Using these methods I was, however, not able to demonstrate the link between stem cell factors and EMT-TFs.

The down-regulation of ZEB2 suggesting a possible function in supressing the tumour by oncogenic activation. In agreement with this suggestion, a recent study has shown that ZEB2 modulates PTEN expression in melanoma via cooperation with miRNA (Karreth et al., 2011). From a genomic structure perspective, both ZEB1 and ZEB2 are similar, but their function and regulation in cancers remain unclear. Although ZEB2 is sited up-stream of ZEB1 and negatively regulates its expression, their genomic organisations are complex. It is interesting in this context to note that both genes are found to be controlled by different transcriptional enhancers. For instance, a recent study has reported that ZEB2 expression is regulated by two enhancers located at 1.2 Mb and 60 KB upstream of *ZEB2* gene (El-Kasti et al., 2012). In addition, two promoters U5 and E1 were identified within ZEB2 (Nelles et al., 2003). The activity of these ZEB2 two promoters in malignant melanoma can be linked with the activity of its enhancers. Indeed, the activity of the U5 promoter in the melanoma cell line has been confirmed in this study, while comparing different regulatory elements show that they are active to some extent.

The link between ZEB proteins and miRNAs species has been demonstrated in several studies. For example, the inhibition of both ZEB1 and ZEB2 expression via miRNA-200 family and miRNA-205 causes high expression level of E-cadherin and induced MET with epithelial characteristics (Gregory et al., 2008a). By investigating the expression of both ZEB1 and ZEB2 in a number of different cell lines we showed that their expression level varies significantly. Despite the common properties between both proteins, ZEB1 is expressed in almost all mesenchymal cells while ZEB2 remains uncommon. Consistent with this data, our strategy which aims to study the expression of ZEB proteins in the same setting by establishing a new model of EMT show that ZEB1 is still expressed at high levels compared to ZEB2. These data has led to consider that post-translational and post-transcriptional regulation may be responsible for the delimited expression level of ZEB2.

There is also limited literature on the potential role of translational regulation of ZEB2 in cancer cells. Analysis of ZEB2 reported that its ORF contains codons clusters which influence ZEB2 translation. An interesting observation by a recent study showed that 60 gene pairs with high protein identity differ in rare codon density and their expression was affected by codon bias (Lampson et al., 2013). In this context, ZEB2 included two rare codon LGV triplets located at the border of smad binding domain. Noteworthy, it has been found that replacing these rare codons with common codons enhanced the expression of ZEB2, while substitution of a single rare to common codon has no significant effect on ZEB2 expression levels. Moreover, such an experiment using full length of ZEB2 including two stretches of rare codons (LGV) and using retroviral vector expression was carried out in HMEC-hTERT cells. I have clearly presented evidence that ZEB2 protein yield is promoted with efficiently notable activation of mesenchymal markers. Indeed, this indicates the importance of rare isoacceptor tRNAs among other EMT factors.

In the context of post-translational modification, ubiquitination is a post-translational modification system which has vital function in degrading proteins. The link between ubiquitination and ZEB proteins remains little studied. However, a subsequent study using a trophoblast cell has reported that ZEB1 is regulated by cullin7 E3 ligase (Fu et al., 2010). Additionally, in a very recent study such a role of ubiquitination on ZEB2 was found to be dependent on the E3 ligase complex SPF^{Fbxo45} (Xu et al., 2015a). Consistent with this study, our data shows that SPF^{Fbxo45} mediate ZEB2 expression at the level of protein translation.

Therefore, it was proposed that the ribosome stalling promotes the degradation of the nascent peptide through ubiquitin-proteasome pathway, and involves the FBXO45/PAM/SKP1 E3 ligase activity (Figure 6-1).

Overall, investigating the link between EMT-TFs and cancer stemness as well as EMT-TFs and tRNA requires further studies. Focusing particularly on ZEB2 translation may provide a better understanding of the fundamental principle of ZEB2 expression and its role during EMT.

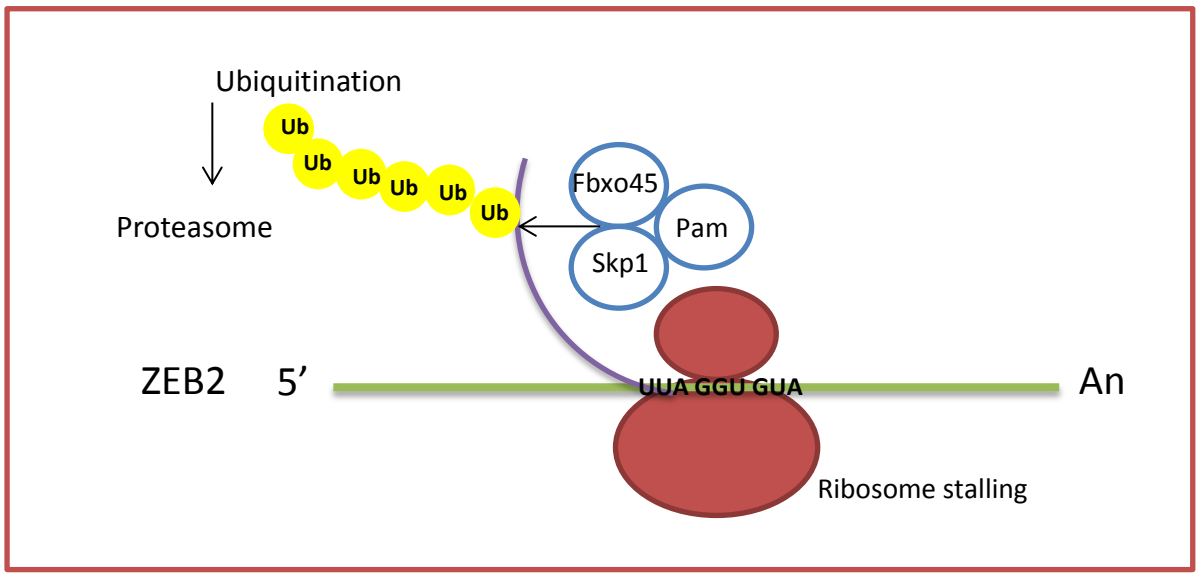
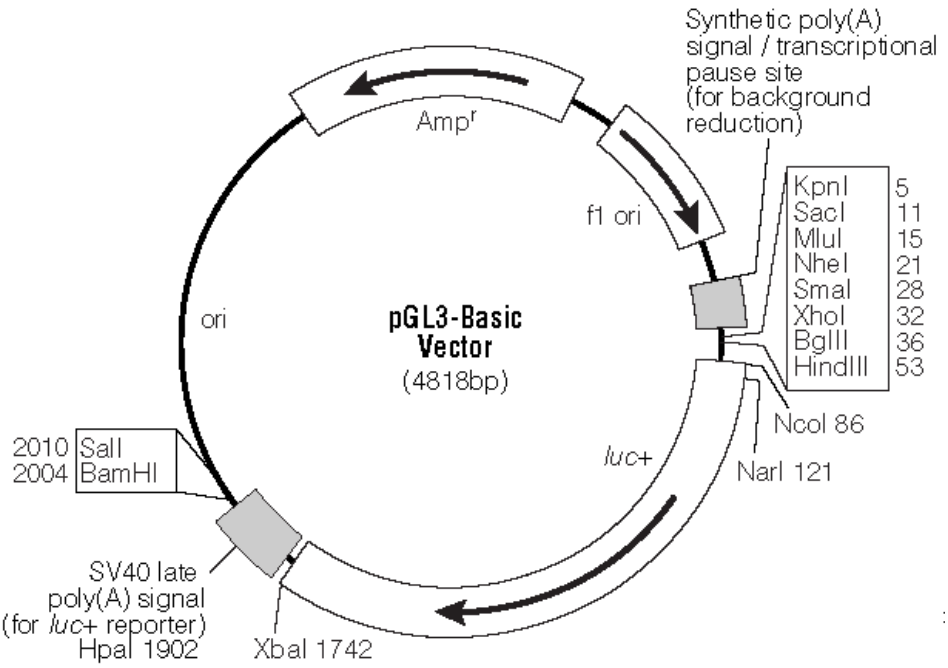


Figure 6-1: A hypothetical scheme illustrating translational control of ZEB2 protein expression.
Ubiquitin E3 ligase complex FBXO45/PAM/SKP1 strongly affects ZEB2 expression level, but has no influence on the protein half-life. The ribosome stalling promotes the degradation of the nascent peptide through ubiquitin-proteasome pathway, and involves the FBXO45/PAM/SKP1 E3 ligase activity.

APPENDIXES

Appendix 1: Map of the pGL3 basic cloning vector. Promega (cat. no. E1751)



Appendix 2: ZEB2 RE-4 reporter sequence**.**

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CCGAAAGAGATCAGT

Appendix 3: ZEB2 E1 reporter sequence**.**

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Appendix 2: ZEB2 short enhancer -395 sequence**.**

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Appendix 4: ZEB2 VISTA enhancer sequence.

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Appendix 5: Sequence alignment of EGFP-ZEB2 (RC-CC) and EGFP-ZEB2 showing the RC to CC substitutions of (LV^{mut}).

```
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                         ||||||||||||||||||||||||||||||||||||||||||||||||||
EGFP-ZEB2        1700 ACAGAACCACTAGACTTCAATGACTATAAAGTTCTTATGGCAACACATGG   1749

EGFP-ZEB2(LVmut) 149 GTTTAGTGGCAGCAGTCCCTTTATGAACGGTGGGCTTGGAGCCACCAGCC    198
                         ||||||||||||||||||||||||||||||||||||||||||||||||||
EGFP-ZEB2        1750 GTTTAGTGGCAGCAGTCCCTTTATGAACGGTGGGCTTGGAGCCACCAGCC   1799

EGFP-ZEB2(LVmut) 199 CTCTGGGTGTGCACCCATCTGCTCAGAGTCCAATGCAG------------    236
                         ||.|.||||||.|||||||||||||||||||||||||||
EGFP-ZEB2        1800 CTTTAGGTGTACACCCATCTGCTCAGAGTCCAATGCAGCACTTAGGTGTA   1849
```

Appendix 6: Sequence alignment of EGFP-ZEB2 single (RC-CC) and EGFP-ZEB2 showing the RC to CC substitutions of (V^{mut}).

```
EGFP-ZEB2(Vmut)   100 ACAGAACCACTAGACTTCAATGACTATAAAGTTCTTATGGCAACACATGG    149
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||
EGFP-ZEB2        1700 ACAGAACCACTAGACTTCAATGACTATAAAGTTCTTATGGCAACACATGG   1749

EGFP-ZEB2(Vmut)  150 GTTTAGTGGCAGCAGTCCCTTTATGAACGGTGGGCTTGGAGCCACCAGCC    199
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||
EGFP-ZEB2        1750 GTTTAGTGGCAGCAGTCCCTTTATGAACGGTGGGCTTGGAGCCACCAGCC   1799

EGFP-ZEB2(Vmut)  200 CTTTAGGTGTGCACCCATCTGCTCAGAGTCCAATGCAG------------    237
                      ||||||||||.||||||||||||||||||||||||||
EGFP-ZEB2        1800 CTTTAGGTGTACACCCATCTGCTCAGAGTCCAATGCAGCACTTAGGTGTA   1849
```

Appendix 7: Sequence alignment of EGFP-ZEB2 single (RC-CC) and EGFP-ZEB2 showing the RC to CC substitutions of (G^{mut}).

```
EGFP-ZEB2(Gmut)    98 ACAGAACCACTAGACTTCAATGACTATAAAGTTCTTATGGCAACACATGG    147
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||
EGFP-ZEB2        1700 ACAGAACCACTAGACTTCAATGACTATAAAGTTCTTATGGCAACACATGG   1749

EGFP-ZEB2(Gmut)  148 GTTTAGTGGCAGCAGTCCCTTTATGAACGGTGGGCTTGGAGCCACCAGCC    197
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||
EGFP-ZEB2        1750 GTTTAGTGGCAGCAGTCCCTTTATGAACGGTGGGCTTGGAGCCACCAGCC   1799

EGFP-ZEB2(Gmut)  198 CTTTAGGCGTACACCCATCTGCTCAGAGTCCAATGCAG------------    235
                      ||||||||.||||||||||||||||||||||||||||
EGFP-ZEB2        1800 CTTTAGGTGTACACCCATCTGCTCAGAGTCCAATGCAGCACTTAGGTGTA   1849
```

Appendix 8: Sequence alignment of EGFP-ZEB2 (K^{mut}) and EGFP-ZEB2 showing the substitution of two lysines K^{389} and K^{391} with chemically related arginine.

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EGFP-ZEB2(Kmut)    36 AAAATGGAAAACCaCTTAGCAtgTCTGAGCAGACAGGCTTACTTAGGATT     85
                      ||||||||||||||||||||||||||||||||||||||||||||||.||||
EGFP-ZEB2        1647 AAAATGGAAAACCACTTAGCATGTCTGAGCAGACAGGCTTACTTAAGATT   1696

EGFP-ZEB2(Kmut)    86 AGAACAGAACCACTAGACTTCAATGACTATAAAGTTCTTATGGCAACACA    135
                      |.|||||||||||||||||||||||||||||||||||||||||||||||||
EGFP-ZEB2        1697 AAAACAGAACCACTAGACTTCAATGACTATAAAGTTCTTATGGCAACACA   1746

EGFP-ZEB2(Kmut)  136 TGGGTTTAGTGGCAGCAGTCCCTTTATGAACGGTGGGCTTGGAGCCACCA    185
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||
EGFP-ZEB2        1747 TGGGTTTAGTGGCAGCAGTCCCTTTATGAACGGTGGGCTTGGAGCCACCA   1796

EGFP-ZEB2(Kmut)  186 GCCCTTTAGGTGTACACCCATCTGCTCAGAGTCCAATGCAG---------    226
                      |||||||||||||||||||||||||||||||||||||||||
EGFP-ZEB2        1797 GCCCTTTAGGTGTACACCCATCTGCTCAGAGTCCAATGCAGCACTTAGGT   1846
```

Appendix 9: Sequence alignment of pBABE-ZEB2 double (RC-CC) and pBABE-ZEB2 showing the RC to CC substitutions of (LGV^{mut}).

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