Y-BOX BINDING PROTEIN-1 IS ESSENTIAL FOR THE GROWTH AND SURVIVAL OF HER2 OVER-EXPRESSING BREAST CANCERS AND MEDIATES TRASTUZUMAB RESISTANCE BY INDUCING CD44

by

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ABSTRACT

Y-box binding protein-1 (YB-1) is an oncogenic transcription/translation factor expressed in 40% of all subtypes of invasive breast carcinomas, where its expression is correlated with relapse and poor survival. *HER2* amplifications are a frequent genetic abnormality observed in approximately 25% of breast cancers where its over-expression is associated with poor clinical outcome and decreased disease free survival. We recently reported that HER2 over-expressing breast cancers are dependent on YB-1 for growth and survival. In HER2 positive tumours we implicated YB-1 in sustaining cancer cells by its involvement in the STAT3 signalling pathway. The development of trastuzumab, a targeted therapy against HER2, has provided substantial advances in the care and treatment of patients whose tumours over-express HER2. Unfortunately, the development of acquired resistance to trastuzumab remains a prevalent challenge in the treatment of patients whose tumours express HER2. Since YB-1 is also linked to drug resistance in other types of cancer, we addressed its possible role in trastuzumab insensitivity. Employing an *in vivo* model of acquired resistance, we demonstrated that resistant cell lines have elevated levels of P-YB-1^{S102} and its activating kinase P-RSK and that these levels are sustained following trastuzumab treatment. Further, to demonstrate the importance of YB-1 in mediating drug resistance, the expression of the active mutant YB-1^{S102D} rendered the BT474 cell line insensitive to trastuzumab. Questioning the role of tumour initiating cells (TICs) and their ability to escape cancer therapies, we investigated YB-1's involvement in inducing the cancer stem cell marker CD44. Notably, the resistant cells expressed more CD44 mRNA and protein compared to BT474 cells, which correlated with increased mammosphere formation. Expression of YB-1^{S102D} in the BT474 cells increased CD44 protein levels, resulting in enhanced mammosphere formation. Further, exposing BT474 cells to trastuzumab selected for a resistant subpopulation enriched for CD44. Conversely, siRNA inhibition of CD44 restored trastuzumab sensitivity in the resistant cell lines. Our findings provide insight on a novel mechanism employed by tumour cells to acquire the ability to escape the effects of trastuzumab and suggest that targeting YB-1 may overcome resistance by eliminating the unresponsive TIC population, rendering the cancer sensitive to therapy.

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4-OHT	4-hydroxy tamoxifen
ABC	ATP-binding cassette
ADCC	Antibody dependent cellular cytotoxicity
ALDH	Aldehyde dehydrogenase
AML	Acute myeloid leukemia
ATP	Adenosine triphosphate
CDK2	Cyclin dependent kinase-2
CGH	Comparative genomic hybridization
ChIP	Chromatin immunoprecipitation
CML	Chronic myelogenous leukemia
COC	ChIP-on-chip
CRS	Cytoplasmic retention site
CSC	Cancer stem cell
CSD	Cold shock domain
ECD	Ectodomain
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELB	Egg lysis buffer
ER	Estrogen receptor
ERE	Estrogen response element
ESA	Epithelial surface antigen
FACS	Fluorescent Activated Cell Sort
FBXW7	F-box and WD repeat domain-containing 7
GIST	Gastrointestinal stromal tumour
HA	Hyaluronan
HCS	High content screening
HER2	Human epidermal growth factor receptor-2
HR	Trastuzumab resistant
IGF-IR	Insulin like growth factor-I receptor
IR	Insulin receptor
JAK	Janus kinase
JNK	C-jun kinase
MAPK	Mitogen-activated protein kinase
MDR1	Multi-drug resistance-1
MET	Mesenchymal epithelial transition
MHC II	Major Histocompatibility Class II
MMTV	Mouse mammary tumour virus
MRP1	Multi-drug resistance protein-1
mTOR	Mammalian target of rapamycin
MUC4	Mucin-4
MVP	Major vault protein
Myr	Myristolated
NK	Natural killer
NLS	Nuclear localization signal
NOD/SCID	Non-obese diabetic mice with severe combined immunodeficiency

	disease
NSCLC	Non-small cell lung cancer
PBS	Phosphate buffered saline
PCNA	Proliferative cell nuclear antigen
PI3K	Phosphatidylinositol -3-kinase
Pin1	Peptidyl-prolyl cis/trans isomerise
PLK1	Polo-like kinase-1
RSK	p90 ribosomal S6 kinase
RTK	Receptor tyrosine kinase
SERM	Selective estrogen receptor modulator
SH2	SRC homology-2
STAT	Signal transducer and activator of transcription
TBP	TATA-box binding protein
TIC	Tumour-initiating cell
TKI	Tyrosine kinase inhibitor
TMA	Tumour microarray
TSP1	Thrombospondin-1
VEGF	Vascular endothelial growth factor
YB-1	Y-box binding protein-1

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To my parents.

CO-AUTHORSHIP STATEMENT

Chapter 2:
The work described in Chapter 2 was published in *Cancer Research* as a Priority Report in November 2008. The manuscript presented within the Chapter was designed by Sandra Dunn, Cathy Lee and me. I designed, optimized, completed, and analyzed all work associated with querying the mechanism of YB-1 mediated apoptosis. I assessed signalling changes following YB-1 knockdown in BT474-m1 and Au565 cells by western blot, confirmed apoptosis following STAT3 inhibition, characterized *in vitro* STAT3 binding to an *MCL* promoter by electrophoretic mobility shift assays, completed the STAT3C rescue experiment and also determined mTOR transcript levels with YB-1 inhibition by q-RT-PCR in the BT474-m1 and Au565 cell lines. The majority of the data presented in Chapter 2 was completed by Cathy Lee. She completed YB-1 knockdown western blots showing changes in EGFR and HER2 levels, YB-1 timecourse knockdown experiment demonstrating the induction of apoptosis, FACS analysis of Annexin V with YB-1 inhibition, soft agar assays, and *in vivo* experiments. Kaiji Hu completed the highcontent screening to assess growth with YB-1 knockdown in a panel of breast cancer cells and also completed the three channel apoptosis screen. Michelle Wang completed the animal experiments. Eugene Park performed the combined Taxol and YB-1 inhibition experiments. Arezoo Astanehe assessed mTOR transcript levels following YB-1 knockdown in the SUM149 cell line. The manuscript was written and prepared by Sandra Dunn, Cathy Lee, Connie Eaves, and me.

Chapter 3:
The manuscript described in Chapter 3 was recently submitted to a peer-reviewed journal. The focus of the experimental work was designed by me and Sandra Dunn. I designed, optimized, performed, and analyzed all cell line/YB-1 knockdown/drug treatment western blots, soft agar assays, growth assays, establishment of the BT474 stable clones, quantitative real-time PCRs, mammosphere assays, immunofluorescence analysis, and luciferase assays. Arezoo Astanehe completed the ChIP experiment, established the BT474LT cultures and also assisted in various aspects of the research plan. Cathy Lee completed the soft agar assay with YB-1 knockdown and trastuzumab treatment in the BT474-m1 cells. Abbas Fotovati performed the immunofluorescence staining of CD44 in the BT474, HR5, and HR6 cells, helped to optimize the staining protocol for mammospheres and also assisted with imaging and general staining techniques. Kaiji Hu analyzed growth experiments employing the Cellomics ArrayScan VTI. The manuscript was written and prepared in collaboration with Sandra Dunn and me.

CHAPTER ONE: INTRODUCTION

1.1 BREAST CANCER

Cancer arises from the ability of a single cell to bypass the normal homeostatic mechanisms regulating proliferation, differentiation, and death resulting in abnormal and uncontrolled cell growth. A myriad of processes which insult the genome of a single cell, whether genetic or epigenetic, are sufficient to expand and select for these deviant cells, initiating tumour formation (Hahn and Weinberg, 2002). The acquisition of genomic instability arises through a number of mechanisms including inherited mutations and exposure to harmful chemical and/or physical mutagens. Although complex, cancers conform to six classical hallmarks: the ability to evade apoptosis, self-sufficiency in growth signals, insensitivity to anti-growth signals, sustained angiogenesis, limitless replicative potential, and the ability to invade tissue and metastasize (Hanahan and Weinberg, 2000).

Breast cancers are diverse; no one tumour being the same. The cellular origin of breast malignancies remains controversial as the mammary cellular hierarchy is only partially understood. It has been speculated that the various tumour types arise from distinct breast epithelial cells which have different susceptibilities for oncogenic hits (Visvader and Lindeman, 2008). Nevertheless, breast tumours are composed of an assortment of cell types, they vary in anatomic extent, and give rise to malignancies that have various clinical outcomes (Vargo-Gogola and Rosen, 2007). Loss of tumour suppressor genes including germline mutations in the high-penetrance cancer susceptibility genes *BRCA1* and *BRCA2* (Nathanson *et al.*, 2001) or the activation of oncogenes such as c-Myc (*MYC*) (Nass and Dickson, 1997), and cyclin D1

(*CCND1*) (Steeg and Zhou, 1998) have all been implicated in breast malignancies as their deregulation can lead to cellular chaos. Although these common genetic aberrations have been recognized for their contribution to the initiation and progression of breast cancer, an exclusive genetic malfunction has not been identified.

More recently, comprehensive cDNA microarray experiments employing a large cohort of primary tumour samples categorized invasive ductal carcinomas into five distinct molecular classes based on differences in gene expression patterns (Perou *et al.*, 2000; Sorlie *et al.*, 2003). Breast cancers are first classified for their expression of the estrogen receptor (ER). Tumours lacking ER are classified as basal-like which accounts for approximately 11% of breast cancers (Kwan *et al.*, 2009), the HER2+ subgroup which is discussed later, in addition to the normal breast-like. ER positive tumours are associated with a favourable prognosis and are categorized into the prevalent Luminal A and also the Luminal B subtype (Kwan *et al.*, 2009). These studies made evident that breast tumours are molecularly heterogeneous, directly impacting the clinical course of the disease. The HER2+ and basal-like subgroups have the least favourable prognosis and clinical outcome compared with ER positive subclasses which have a relatively good clinical outcome (Sorlie *et al.*, 2001). The revelation of the molecular complexity in breast cancer and its influence on tumourigenic potential and disease outcome emphasizes how important individualized therapies will be in the future. Given that breast cancer is not a single disease, therapeutic strategies can be altered to best combat the tumour at hand and hence improve patient care and clinical outcomes.

1.2 CURRENT THERAPEUTIC STRATEGIES

Breast cancer is the most frequently diagnosed malignancy in Canadian women (Canadian Cancer Society). In 2010, over 20 000 women will be diagnosed with the disease in Canada alone (Canadian Cancer Society) and according to the World Health Organization, over one million new cases will be diagnosed globally. Metastasis of breast tumours, commonly to the bone, lungs, and liver, is the primary cause of disease related mortalities (Chambers *et al.*, 2002). Advancements in early screening technologies and innovative therapeutic options have provided an 87% five year survival rate (Canadian Cancer Society). Although the death rates from breast cancer have declined, it remains the second leading cause of cancer related deaths (Kamangar *et al.*, 2006). Disease recurrence is a common clinical burden women encounter following an initial remission. Often, cancer relapse is associated with a more aggressive and therapeutically unresponsive tumour where patients are left with unfavourable survival rates. Hence, enhanced therapeutic options are essential to treat and prevent disease recurrence and relapse.

There are an abundance of options to treat breast malignancies dependent on tumour biology variables, such as its size and grade. Regardless, a combinatory therapeutic approach is often taken to control and treat the disease. Surgery, including lumpectomies, mastectomies, and axillary dissections are the primary treatment options to remove cancerous masses (Baselga and Norton, 2002). To most effectively treat the disease, chemotherapy, radiotherapy, hormonal therapy, and biological therapy are utilized in conjunction with surgery to remove residual malignant cells and prevent recurrence (Baselga and Norton, 2002). Given the diverse array of characteristics amongst tumours, treatment regimes have become personalized to target the unique biology of the tumour.

Traditional breast cancer therapy includes the use of a variety of chemotherapeutic agents which function to kill cancer cells while ideally leaving normal cells unharmed. However, as these therapies target rapidly dividing cells, normal cells are also affected leading to the many adverse side effects associated with chemotherapy. These cytotoxic chemicals act as cellular poisons by interfering with cell cycle progression (Wright, 1984). Cancer cells are highly proliferative making them particularly vulnerable to chemotherapeutic agents that cause DNA damage such as doxorubicin. This further relies on the cells retention of normal check point controls which in many instances is not the case (Gralow, 2005; Hortobagyi, 2000). For early stage breast carcinoma, adjuvant treatment options include the combinatory use of alkylating agents (cyclophosphamide), anthracyclines (doxorubicin), antimetabolites (5-fluorouracil), and antimicrotubule agents (paclitaxel and docetaxel) (Gralow, 2005; Hortobagyi, 2000). New and improved therapies with more potent activity are employed to treat advanced malignancy in the adjuvant setting including vinca alkaloids (vinorelbine), pyrimidine analogs (gemcitabine), and new antimetabolies (capecitabine) (Gralow, 2005). Although normal cells have regulatory mechanisms capable of recognizing the insults of chemotherapy, they do not always escape the effects of these chemicals, hence toxicity is a common symptom.

Estrogen, a steroidal sex hormone, mediates a variety of processes in normal female physiology. However, prolonged cycling of the hormone has been implicated in the development of breast pathogenesis given its ability to induce cellular proliferation (Clarke *et al.*, 1997). The function of estrogen is mediated by its receptor ER, a nuclear receptor which acts as a transcription factor upon ligand binding (MacGregor and Jordan, 1998). Activated ER is capable of inducing a variety of genes whose promoters contain estrogen response elements (EREs) (MacGregor and Jordan, 1998). Approximately 60% of breast tumours express ER (Ali and Coombes, 2000),

hence endorcrine therapies are a critical entity in the treatment of hormonally responsive tumours and contribute to the positive clinical outcomes for patients with these tumour types. Tamoxifen, a selective estrogen receptor modulator (SERM) was the first anti-ER agent used in the clinical setting (MacGregor and Jordan, 1998; Martino *et al.*, 2004). Administered as a prodrug, the active form of the antiestrogen, 4-hydroxytamoxifen (4-OHT) is generated following first pass metabolism in the liver and competes with endogenous estrogen for ER ligation. In the breast, 4-OHT acts as an ER antagonist and inhibits ER from regulating the transcription from EREs. Unfortunately, tamoxifen has both antagonist and agonist properties, depending on the target cell, therefore increasing the risks of adverse effects including endometrial cancer (Jordan, 2004). More recently, raloxifene, a new SERM with antiestrogenic activity in both the breast and uterus, has been approved for the treatment of ER+ breast cancers (Jordan, 2004).

A second approach to fighting ER positive breast cancer is by inhibition of the enzyme aromatase, which functions to convert androgens to estrogen. The aromatase inhibitors, anastrozole and letrozole function by preventing estrogen synthesis and have been approved for the treatment of postmenopausal patients with ER positive tumours (Din *et al.*, 2010). An additional strategy utilized to treat ER positive tumours is administration of fulvestrant, a selective estrogen receptor downregulator, which functions by downregulating and degrading the ER (Dowsett *et al.*, 2005).

Not all tumours respond to cytotoxic and hormonal therapies as cancer cells are able to acquire mechanisms of resistance. A contributing factor to the failure of achieving a therapeutic

response to tamoxifen has been linked to the loss of ER expression (Johnston *et al.*, 1995). Further to this, growth factor receptor signalling crosstalk with ER has also been implicated in resistance. Signalling via the mitogen-activated protein kinase (MAPK), phosphatidylinositol -3-kinase (PI3K)-AKT, and p38 MAPK pathways by activation of the epidermal growth factor receptor (EGFR), the human epidermal growth factor-2 receptor (HER2), or the insulin-like growth factor-I receptor (IGF-IR) and/or insulin receptor (IR) are capable of activating ER by a number of phosphorylation events which allows for continued ER mediated transcription in the presence of tamoxifen (Johnston and Dowsett, 2003). We have previously demonstrated that inhibiting activation of IGF-IR/IR with BMS-536924 impeded the growth of tamoxifen resistant MCF-7 Tam-R cells (Law *et al.*, 2008).

Identifying specific molecules which have become deregulated in cancer is important in the design and development of innovative treatments specifically targeting dysfunctional molecular entities (Gibbs, 2000; Strebhardt and Ullrich, 2008). Tyrosine kinases play a fundamental role in normal cellular processes by their involvement in cellular signal transduction. This occurs through the activation of proteins via transfer of a phosphate moiety from adenosine triphosphate (ATP) to the hydroxyl group of a tyrosine residue (Gschwind *et al.*, 2004). Aberrant tyrosine kinase activity aids in cellular transformation and carcinogenesis. Therefore, blocking their activity serves as an attractive target in hindering the progression of their message to the nucleus (Gschwind *et al.*, 2004). The development of tyrosine kinase inhibitors (TKIs) has provided success in the treatment of patients with a wide variety of malignancies. Imatinib (Gleevec), a small molecule inhibitor against the BCR-ABL oncoprotein, was the first TKI therapy approved for clinical use (Druker *et al.*, 1996). The majority of chronic myelogenous leukemia (CML) patients harbour the classic Philadelphia chromosome and express the resulting BCR-ABL

protein, a kinase with constitutive activity (Goldman and Melo, 2008). Imatinib functions as a competitive inhibitor for the kinase active site of ABL and therefore prevents its natural substrates access to the site (Druker *et al.*, 1996). Clinical applications of imatinib were later expanded to the treatment of gastrointestinal stromal tumours (GISTs) (Joensuu *et al.*, 2001) after discovering its ability to also inhibit c-Kit, a tyrosine kinase receptor crucial in the pathogenesis of GISTs (Buchdunger *et al.*, 2000). The expression of c-Kit and its ligand, stem cell factor, has been reported in breast cancer (Hines *et al.*, 1995). However, a study employing imatinib to treat patients whose tumours expressed c-Kit demonstrated no response to therapy as all experienced disease progression (Cristofanilli *et al.*, 2008). Lapatinib (Tykerb), a TKI, with dual specificity for EGFR and HER2 has demonstrated clinical success and has been approved by Health Canada for the treatment of breast cancer in combination with capecitabine (Gomez *et al.*, 2008). Furthermore, erlotinib (Tarceva) and gefitinib (Iressa), TKIs targeting the kinase activity of EGFR, which is over-expressed in large cohorts of breast cancer patients (Fox *et al.*, 1994; Klijn *et al.*, 1992; Sainsbury *et al.*, 1987), have demonstrated success in preclinical studies (Campiglio *et al.*, 2004; Takabatake *et al.*, 2007; Yamasaki *et al.*, 2007). However, independent phase II clinical trials with erlotinib and gefitinib demonstrated little success as single agents since only a partial response was observed in a minority of patients (Lin and Winer, 2004). However, these agents have been successfully implemented in the treatment protocols for non-small cell lung cancers (NSCLCs) (Comis, 2005), as well as for the treatment of pancreatic cancer (erlotinib) (Starling *et al.*, 2009).

Targeting biological molecules which have been recognized for their importance in the signalling circuitry programs of cancer cells have also been identified as promising druggable targets (Gibbs, 2000). As these proteins are necessary for the maintenance of the hallmarks of a

cancer cell, inhibiting their actions appear to be a rational idea for drug development. The mammalian target of rapamycin (mTOR) is involved in cell growth, proliferation, survival, transcription, and translation (Hay and Sonenberg, 2004), hence, agents including rapamycin (sirolimus), and its derivatives RAD001 (everolimus) (Miller *et al.*, 2009) and CCI-779 (temsirolimus) (Yu *et al.*, 2001), have been developed to inhibit mTOR activity and have demonstrated success in preclinical studies for use in breast cancer.

1.3 TRANSCRIPTION FACTORS IN CANCER

Transcription factors function to modulate gene expression by accepting signalling cues in the cytoplasm and transmitting them to the DNA. Interestingly, the human genome project identified transcription factors as one of the largest classes of molecules; represented by approximately 2000 genes (Venter *et al.*, 2001). Furthermore, transcription factors have been implicated for their role in cancer, in fact a significant proportion of oncogenes and tumour suppressors are transcription factors (Darnell, 2002). Commonly mutated or over-expressed transcription factors include members of the following families: STAT, Ets, CREB, API, FoxO, Myc, NF-κB, and E2F (Brennan *et al.*, 2008). Strategies to interfere with the function of transcription factors are in development and include dimerisation blockage via peptides, decoy oligonucleotides, and displacing transcription factors from DNA by blocking their binding site (Brennan *et al.*, 2008; Darnell, 2002). For example, a peptide targeting STAT3 has demonstrated success in preventing STAT3 dimer formation, and therefore inhibition of STAT3 mediated gene induction (Turkson *et al.*, 2001). More recently, peptide stapling has become an area of interest to convert peptides into bioactive ligands. The technique employs a number of modifications to the typical features of a peptide while maintaining its functionality, with the

ultimate goal of making a more potent moiety than the native peptide. Moellering *et al* have demonstrated successful targeting of the NOTCH transcription factor complex with these synthetic peptides (Moellering *et al.*, 2009). Transcription factors serve as powerful players in oncogenesis in that a single factor is capable of regulating a number of different genes. Therefore, targeting this group of molecules may provide an additional approach for targeted therapies.

1.4 Y-BOX BINDING PROTEIN-1 (YB-1)

The Y-box binding protein-1 (YB-1) is a cellular entity with a constellation of functions including transcriptional regulation, translation, DNA repair, drug resistance and stress responses. Y-box proteins are members of the highly conserved cold-shock domain (CSD) family of proteins and are composed of three unique structural features, a CSD sandwiched between N-terminal and C-terminal domains, each with distinct functions, as illustrated in Figure 1.1. The variable N-terminal domain, composed of proline and alanine residues, functions in transactivation (Kohno *et al.*, 2003). The C-terminal domain of YB-1 mediates protein-protein interactions via the alternating basic and acidic residues within the domain (Kohno *et al.*, 2003). Further, the C-terminal domain also harbours a nuclear localization signal (NLS) in addition to a cytoplasmic retention site (CRS), dictating the cellular localization of the protein (Kohno *et al.*, 2003).

Figure 1.1. The Structure and Functions of YB-1. YB-1 is composed of three unique domains. The N-termainl domain is essential for transactivation, while the C-terminal functions in protein-protein interactions. The cold shock domain (CSD) interacts with nucleic acids and also binds to DNA regions containing the inverted CAAT box. YB-1 is also phosphorylated at serine 102 which resides within the CSD by either AKT or RSK. This activation is crucial for nuclear import and DNA binding.

The evolutionary conserved CSD functions to interact with both DNA (Hasegawa *et al.*, 1991) and RNA (Bouvet *et al.*, 1995) and was later identified as an essential requirement in YB-1's affinity for nucleic acids (Kloks *et al.*, 2002). The CSD of YB-1 has a unique DNA recognition motif that recognizes and binds to DNA regions containing an inverted CAAT box (Didier *et al.*, 1988; Sakura *et al.*, 1988). Furthermore, the CSD also functions in the nuclear trafficking of YB-1 in co-operation with the C-terminal domain (Jurchott *et al.*, 2003). YB-1 was first identified as a transcription factor regulating the promoter of the Major Histocompatibility Class II (MHCII) gene (Didier *et al.*, 1988) and in the same year it was also shown to interact with the EGFR enhancer and HER2 promoter (Sakura *et al.*, 1988). Since then it has been extensively characterized in its role in gene regulation. However, YB-1 also functions in DNA repair via its interaction with proliferating cell nuclear antigen (PCNA) (Ise *et al.*, 1999) in addition to its involvement in mRNA transport and translation (Evdokimova *et al.*, 2006). Provided that the focus of our research looks at YB-1's function in transcription, this aspect will be the focus of discussion.

YB-1 is an important factor in cancer initiation and progression by having the ability to alter the gene expression pattern of cells, which may ultimately induce an oncogenic switch in signal transduction. Both the AKT and MAPK signalling cascades are well established pathways studied in cancer biology. In fact, our laboratory identified YB-1 as a novel target of AKT for phosphorylation at its serine 102 residue which resides in the CSD (Sutherland *et al.*, 2005). More recently, we have also described p90 ribosomal S6 kinase (RSK) as being capable of phosphorylating the same serine 102 residue of YB-1 (Stratford *et al.*, 2008). A schematic representation of YB-1 signalling is presented in Figure 1.2.

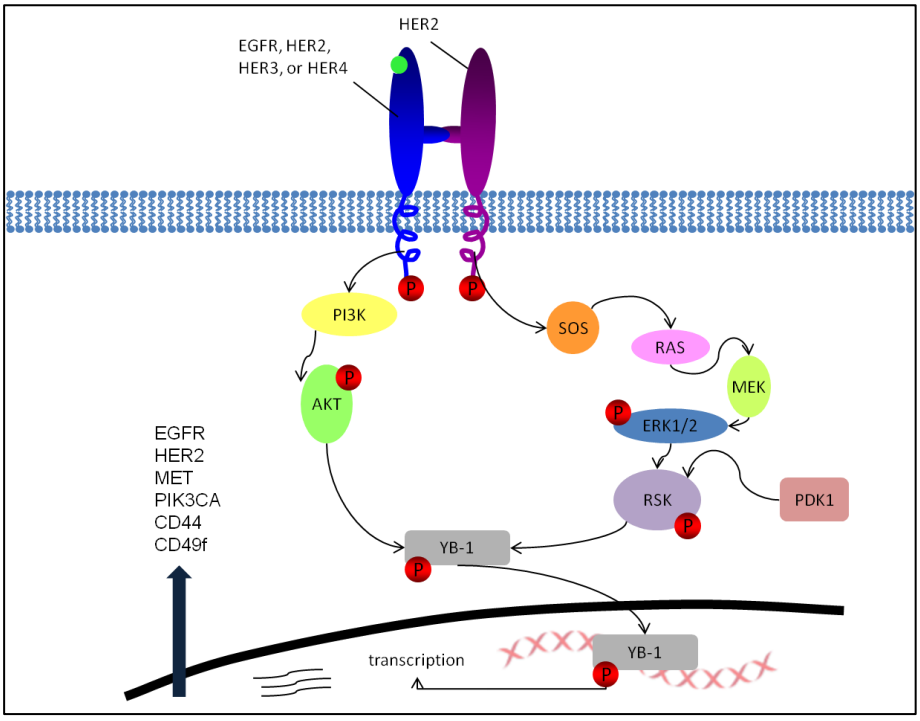

Figure 1.2. Schematic model of YB-1 signalling. YB-1 is activated by phosphorylation of its serine 102 residue by either AKT or RSK via output signals from growth factor receptors. Nuclear P-YB-1^{S102} functions as a transcription factor by binding to CAAT elements within promoters and regulates a number of genes including *EGFR*, *HER2*, *MET*, *PIK3CA*, *CD44*, and *CD49f*.

In fact, it has been demonstrated that YB-1 is preferentially activated by RSK using *in vitro* kinase assays as YB-1 has a higher affinity for interaction with RSK over AKT (Stratford *et al.*, 2008). Activation of YB-1 by phosphorylation at its serine 102 residue is critical to its function within the nucleus. Exogenous expression of YB-1^{S102A}, which eliminates the capacity to be phosphorylated at that site in the CSD, attenuates the nuclear translocation of YB-1 and also affects its role as a transcription factor since only 50% of the mutant protein is found within the nuclear compartment (Sutherland *et al.*, 2005). More importantly, the phosphorylation of the serine residue is crucial in YB-1's ability to interact with DNA and induce gene expression (Wu *et al.*, 2006). Expression of Flag-YB-1^{S102A} completely abolished binding to the -1kb of the *EGFR* promoter where as binding was evident in cells with wild type YB-1 (Wu *et al.*, 2006).

Taken together, AKT and RSK function to phosphorylate serine 102 and enhance YB-1's ability to enter the nucleus and its affinity for DNA. There, YB-1 has been shown to be involved in regulating cyclin A (*CCNA*) (Jurchott *et al.*, 2003), cyclin B (*CCNB*) (Jurchott *et al.*, 2003), topoisomerase II alpha (*TOP2A*) (Shibao *et al.*, 1999), and DNA polymerase alpha (*DPA*) (En-Nia *et al.*, 2005). Further to its induction of the mentioned growth promoting genes, YB-1 also transcriptionally controls matrix metalloproteinase-2 (*MMP-2*) (Mertens *et al.*, 1997), matrix metalloproteinase-12 (*MMP-12*) (Samuel *et al.*, 2005), collagen type I alpha 1 (*COL1A1*) (Norman *et al.*, 2001), and collagen type I alpha 2 (*COL1A2*) (Higashi *et al.*, 2003). Hence, YB-1 also indirectly controls metastatic potential by its regulation of genes whose products are involved in cell adhesion, motility, and invasion. This collectively indicates that YB-1's function as a transcription factor is capable of inducing cancer with an aggressive phenotype. YB-1 also has the ability to indirectly modify gene expression via protein-protein interaction. It is able to bind to the tumour suppressor p53, preventing its regulation of *p21* and therefore

stimulate aberrant cell cycle progression (Okamoto *et al.*, 2000). It is clear that YB-1 functions to switch a cell's normal regulatory machinery into overdrive and therefore provoke changes to acquire a malignant phenotype.

YB-1 expression is limited to rapidly growing cells including those found in various embryonal and fetal tissues (Uchiumi *et al.*, 2006). However, its expression is silenced in most adult tissues except for those which are highly proliferative such as the glandular epithelial cell zone of normal colorectal mucosa (Shibao *et al.*, 1999). In fact, the generation of YB-1 knockout mice results in impaired cell proliferation resulting in embryonic lethality (Uchiumi *et al.*, 2006). Moreover, YB-1 has been associated with highly proliferative cells in a range of cancer types. It correlates with increased Ki67 staining in primary tissue samples of osteosarcoma (Oda *et al.*, 1998) and breast cancer (Wu *et al.*, 2006). In addition, YB-1 positively correlates with PCNA expression, a marker for highly proliferative cells in colorectal cancer (Shibao *et al.*, 1999). Direct evidence for a role of YB-1 in proliferation came from a gene knock down study where the loss of one allele in the chicken lymphocytic cell line DT40 impeded growth by greater that 70% (Swamynathan *et al.*, 2002). Interestingly, elevated levels of YB-1 have been recognized in a wide variety of malignancies including cancers of the breast (Bargou *et al.*, 1997), prostate (Gimenez-Bonafe *et al.*, 2004), lung (Shibahara *et al.*, 2001), ovary (Yahata *et al.*, 2002), bone (Oda *et al.*, 1998), myelomas (Chatterjee *et al.*, 2008), rhabdomyosarcoma (Oda *et al.*, 2008) and glioblastoma (Faury *et al.*, 2007) while the normal matched tissues had little or no YB-1 expression. Further, RNA antisense against YB-1 in melanoma, adenocarcinoma, hepatoma, fibrosarcoma, and colon cancer cells induced cell death (Lasham *et al.*, 2003). Since then, we have also confirmed the role of YB-1 in the growth and survival of breast cancers (Lee *et al.*,

2008). Taken together, a wide range of malignant tissues not only highly express YB-1 but also depend on it.

In the breast, YB-1 has been established as an oncogene. YB-1 expression is confined to malignant breast tissue as it is not detected in normal breast (Bargou *et al.*, 1997; Rubinstein *et al.*, 2002). Furthermore, targeted expression of YB-1 in the mammary gland employing a transgenic mouse model induced invasive tumour formation with 100% penetrance (Bergmann *et al.*, 2005). We have demonstrated the requirement of wild type YB-1 for growth in monolayer and in 3-dimensional anchorage independent assays where as expression of YB-1^{S102A} or YB-1 inhibition with siRNA impeded cell growth (Finkbeiner *et al.*, 2009; Lee *et al.*, 2008; Sutherland *et al.*, 2005; Wu *et al.*, 2006). We have also identified YB-1 in transcriptionally regulating the expression of *EGFR* (Stratford *et al.*, 2007; Wu *et al.*, 2006), *HER*2 (Wu *et al.*, 2006), *MET* (Finkbeiner *et al.*, 2009), and *PIK3CA* (Astanehe *et al.*, 2009), all of which are commonly over-expressed or mutated in cancer. Furthermore, Chapter Two provides evidence for the requirement of YB-1 for *in vivo* tumourigenesis (Lee *et al.*, 2008). Although the levels of P-YB-1^{S102} vary in the various types of breast cancer cell models studied, the levels of total protein remain constant amongst breast cancer subtypes (Finkbeiner *et al.*, 2009). Of note, the over-expression of YB-1 in breast cancers is not due to chromosomal amplifications on account that a comparative genomic hybridization (CGH) array analysis on primary basal-like breast cancer specimens did not identify any changes in the *YB1* locus (1p34.2) (Stratford *et al.*, 2007). Since there are no known mutations and YB-1 is not amplified, its expression likely results from transcriptional activation. C-Myc for example is aberrantly expressed in breast cancers (Liao and Dickson, 2000). Interestingly, the YB-1 promoter contains sequences specifically recognized by c-Myc which directly regulates the YB-1 promoter in collaboration with p73

(Uramoto *et al.*, 2002). More recently, TWIST has been shown to induce YB-1 (Shiota *et al.*, 2008). Undoubtedly, YB-1's direct regulation of a wide range of oncogenes implicates it as a critical mediator in many established cancer pathways.

In addition to the above mentioned myriad of functions, YB-1 also plays a significant role in drug resistance. It facilitates the expression of the multi-drug resistance-1 gene (*MDR1*) (Goldsmith *et al.*, 1993; Stein *et al.*, 2001), multi-drug resistance related protein-1 (*MRP1*) (Stein *et al.*, 2001), and the major vault protein (*MVP*) (Stein *et al.*, 2005), all which function in drug resistance. MDR1, a P-glycoprotein, is a member of the ATP-binding cassette (ABC) transporters which functions to efflux compounds out of the cell. Unfortunately, this includes ridding toxic agents from cancer cells and their expression is often elevated following chemotherapy (Choudhuri and Klaassen, 2006). Cisplatin (Ise *et al.*, 1999) and paclitaxel (Fujita *et al.*, 2005) have been implicated in inducing YB-1 mediated expression of MDR-1 resulting in a multi-drug resistant phenotype. Furthermore, antisense against YB-1 in human epidermoid cancer cell lines increased their sensitivity to cisplatin, mitomycin C, and UV radiation (Ohga *et al.*, 1998). Besides, through induction of *MDR1*, YB-1 may also mediate drug resistance by its involvement in DNA repair as it has been identified to preferentially bind cisplatin damaged DNA in association with PCNA (Shibahara *et al.*, 2004). Clinical evidence further supports the *in vitro* chemoresistant phenotype maintained by YB-1. In an analysis of breast cancer specimens from patients receiving post-operative chemotherapy, those with high YB-1 expression had a 66% relapse rate compared to 0% relapse in those with low YB-1 expression (Janz *et al.*, 2002). In addition, work in our lab implicated YB-1 as a predictive biomarker of relapse and poor survival across all breast cancer subtypes in a cohort of 4049 samples (Habibi *et al.*, 2008). Moreover, YB-1 was also predictive of decreased breast cancer specific survival in a

subset of patients treated with tamoxifen (Habibi *et al.*, 2008). Collectively, this suggests that YB-1's ability to induce chemoresistance may contribute to its correlations with poor clinical outcome.

1.5 SIGNAL TRANDUCERS AND ACTIVATORS OF TRANSCRIPTION (STATs)

Signal transducers and activators of transcription (STATs) are a family of latent cytoplasmic transcription factors essential for cell growth, survival, differentiation, and motility (Bromberg and Darnell, 2000). Since their initial identification (Schindler *et al.*, 1992), a total of seven family members have been described: STAT1 to STAT4, STAT5a, STAT5b, and STAT6 (Darnell *et al.*, 1994). The activation of STAT3 is achieved by cytokine and growth factors that have receptors with intrinsic or associated tyrosine-kinase activity. Activated Janus kinase (JAK) family members, SRC kinases, EGFR, and HER2 interact with STAT3 through their SRC homology 2 (SH2) domain resulting in phosphorylation of tyrosine 705 which allows for STAT3 dimer formation via reciprocal phosphotyrosine-SH2 interactions between monomers (Levy and Darnell, 2002). Furthermore, the active dimers undergo an additional phosphorylation event within their transactivation domain at serine 727 mediated by ERK (Chung *et al.*, 1997), p38 (Goh *et al.*, 1999), c-jun kinase (JNK) (Haq *et al.*, 2002), PKCδ (Jain *et al.*, 1999), mTOR (Yokogami *et al.*, 2000), and Pin1 (Lufei *et al.*, 2007). It has been suggested that this serine phosphorylation functions to enhance STAT3 mediated transcription (Wen *et al.*, 1995). However, at present the functions of both phosphorylation at tyrosine 705 and serine 727 remain controversial. It has been reported that phosphorylation at tyrosine 705 is essential for dimer formation, nuclear transport, and DNA binding while activation at serine 727 enhances STAT3's potency as a transcription factor by increasing its affinity for DNA in addition to recruiting co-

activators (Bowman *et al.*, 2000). Conversely, evidence suggests that STAT3 is able to function in the absence of the above mentioned phosphorylation events. STAT3 has been reported to achieve nuclear transport independent of tyrosine phosphorylation (Liu *et al.*, 2005). Furthermore, acetylation events at particular lysine residues have been reported to induce STAT3 dimer formation where transcription activity remains persistent in the absence of phosphorylation thereby contradicting the requirement for tyrosine 705 phosphorylation for dimerisation (Lee *et al.*, 2009; O'Shea *et al.*, 2005). Regardless, STAT3 serine phosphorylation is necessary for oncogenesis since expression of a dominant negative mutant where the serine 727 residue is substituted for an alanine prevents transformation of v-*src* NIH-3T3 cells (Bromberg *et al.*, 1998).

Persistent activation of STATs, STAT3 and STAT5 in particular, has been found in a significant proportion of solid tumours, as well as, haematological malignancies where their role in oncogenesis is substantial (Yu and Jove, 2004). STAT3 is commonly found to be constitutively activated in breast cancer as a result of deregulated and overactive upstream kinase activity. At this time no known *STAT3* gene mutations have been identified (Yu and Jove, 2004). Further, STAT3 has been demonstrated to function as an oncogene since it is capable of transforming fibroblasts measured by *in vitro* anchorage independence and *in vivo* tumour growth assays (Bromberg *et al.*, 1999). The ability of STAT3 to induce oncogenic transformation is a direct result of it's role in regulating genes involved in survival, cell cycle progression and motility including cyclin D1 (*CCND1*) (Bromberg *et al.*, 1999), $p21^{WAF1}$ (Sinibaldi *et al.*, 2000), $Bcl\text{-}x_L$ (Bromberg *et al.*, 1999), *MCL1* (Liu *et al.*, 2003), *survivin* (Gritsko *et al.*, 2006), *MYC* (Bromberg *et al.*, 1999), and *TWIST* (Lo *et al.*, 2007). Moreover, *in vivo* RNA interference against STAT3 suppressed tumour growth in both breast (Ling and Arlinghaus, 2005) and

prostate (Gao *et al.*, 2005) tumours. Beyond its role in carcinogenesis, STAT3 has also been reported to be involved in drug resistance in breast and ovarian cancers (Bourguignon *et al.*, 2008; Real *et al.*, 2002). Interestingly, STAT3 activity has been demonstrated to be essential for the maintenance of breast cancer stem-like cells (Zhou *et al.*, 2007), suggesting that it plays a more global role in malignant transformation by its involvement in tumour progression and perhaps recurrence.

1.6 MEMBRANE RECEPTORS

Membrane receptors are essential in normal cell growth and development as they are responsible for governing proliferation, migration, metabolism, differentiation, and survival by relaying extracellular mitogenic growth signals at the cell surface to the cytoplasm (Gschwind *et al.*, 2004). Numerous growth factor receptors have intrinsic kinase activity within their cytoplasmic tails where as integrins impart signals via indirect association with intracellular kinases. Regardless, these receptors orchestrate the many cellular regulatory pathways governing cell fate through their interaction with growth factors, extracellular matrix (ECM) components, and cell-cell adhesion/interaction molecules (Hanahan and Weinberg, 2000). In cancer, deregulation of membrane receptors by over-expression or mutations renders them constitutively active and allows for abnormal intracellular signalling capable of potent oncogenic transformation (Weinberg, 2007). Consequently, cell-surface receptors serve as attractive targets for cancer therapy (Gschwind *et al.*, 2004). Presently, therapeutic agents targeting membrane receptors are used in the clinic to treat breast cancer, GISTs, and NSCLCs (Gschwind *et al.*, 2004).

1.7 HUMAN EPIDERMAL GROWTH FACTOR RECEPTOR-2 (HER2)

HER1/EGFR/ErbB1, HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4 constitute a superfamily of receptor tyrosine kinases (RTKs) with potent roles in tumourigenesis. These four distinct receptors function in collaboration with one another to dictate a network of signalling pathways involved in cell division, apoptosis, invasion, and adhesion (Yarden and Sliwkowski, 2001). A variety of ligands specifically bind to the epidermal growth factor (EGF) receptors leading to either homo- or heterodimerisation. This results in the activation of intrinsic kinase activity ensuing in the phosphorylation of tyrosine residues which are present within the cytoplasmic tails of these receptors (Yarden and Sliwkowski, 2001). However, HER2 has little to no affinity for any particular ligand and achieves activation through heterodimerisation. Despite its inability to bind ligands, HER2 is the preferential heterodimerisation partner of the remaining activated ligand bound family members resulting in powerful signalling outputs (Graus-Porta *et al.*, 1997).

The importance of the human epidermal growth factor receptor-2 (HER2) was realized in the 1980s by a number of hallmark studies. In 1981, Weinberg's laboratory identified unique DNA sequences isolated from rat carcinomas and neuroblastomas with the ability to transform the mouse fibroblast cell line NIH-3T3, which they referred to as *neu* (Shih *et al.*, 1981). Subsequently, they demonstrated *neu* had substantial homology to the established *ERBB* gene and also, its protein product (p185) shared similarities to EGFR (Schechter *et al.*, 1984). Next, the functional importance of *neu* was demonstrated when monoclonal antibodies (mAb) against p185 reverted transformation in *neu* transfected NIH-3T3 cell lines (Drebin *et al.*, 1984). Later, *HER2*, the human counterpart of *neu* was identified by two independent research studies using

DNA hybridization studies employing v-*ERBB* probes (Coussens *et al.*, 1985; King *et al.*, 1985). In fact King *et al* identified *HER2* as being amplified five to ten fold in the mammary cancer cell line MAC117 (King *et al.*, 1985), providing the impetus to further explore HER2 in breast cancers.

Landmark studies conducted by Dennis Slamon in collaboration with Genentech later identified *HER2* as an extremely important factor in breast cancer (Slamon *et al.*, 1987; Slamon *et al.*, 1989). *HER2* was found to be amplified two to twenty fold in approximately 25% to 30% of primary human breast cancer samples (Slamon *et al.*, 1987). The genetic lesion had a direct clinical correlation with outcome as amplification was associated with decreased overall survival and time to relapse (Slamon *et al.*, 1987). It was later noted that the incidence of *HER2* amplification was directly linked to the over-expression of HER2 in both breast and ovarian cancer (Slamon *et al.*, 1989). The aggressive phenotype associated with HER2 can be explained by its involvement in cell proliferation and survival mechanisms as its activity regulates PI3K-AKT, MAPK, SRC, and STAT signalling events (Hynes and Lane, 2005). The potency of HER2 cannot be attributed to activating mutations since they are absent in primary human breast cancer samples (Lemoine *et al.*, 1990). Further, transgenic mice models with targeted expression of unactivated *HER2* under the mouse mammary tumour virus (MMTV) promoter had the capacity to induce latent focal mammary tumours. Many mice also harboured metastases to the lungs (Guy *et al.*, 1992). Evidently, HER2 functions in orchestrating cellular events which promote the initiation and progression of aggressive metastatic breast cancers with poor clinical outcome. The identification of one novel factor frequently over-expressed in a proportion of breast cancer patients was groundbreaking and provided the rationale for the development of targeted therapies to treat breast cancers.

1.8 TRASTUZUMAB (HERCEPTIN™)

The vast evidence supporting the role of HER2 in breast cancer development and progression prompted the development of novel therapeutic agents against the oncogenic membrane receptor. Trastuzumab (Herceptin™), a humanized monoclonal antibody that binds to the extracellular domain of HER2 was created by Genentech in 1992 (Carter *et al.*, 1992). Initial proof of concept studies demonstrated efficacy of the antibody as trastuzumab was able to bind HER2 with high affinity. This resulted in decreased cell growth *in vitro* (Carter *et al.*, 1992) as well as suppression of *in vivo* tumour growth in xenograft models (Baselga *et al.*, 1998). Initial clinical trials employing trastuzumab as a single agent for the treatment of HER2 positive metastatic breast cancers demonstrated clinical benefit with 12 to 34% of tumours responding to treatment (Baselga *et al.*, 1996; Cobleigh *et al.*, 1999; Vogel *et al.*, 2002). Later trials employed a strategy of combining trastuzumab with either paclitaxel (Slamon *et al.*, 2001) or docetaxel (Esteva *et al.*, 2002). The combinatory therapeutic regime achieved increased response rates, time to disease progression, and overall survival. The results were far superior compared to trastuzumab monotherapy. Trastuzumab was approved by the Food and Drug Agency (FDA) in 1998 for women with metastatic breast cancer whose tumours over-express HER2.

The direct mechanism by which trastuzumab achieves tumour regression remains elusive, however several mechanisms have been proposed. HER2 receptor blockage results in a dampening of downstream HER2 mediated AKT and MAPK signalling cascades (Dubska *et al.*, 2005; Nagata *et al.*, 2004). This results in the accumulation of $p27^{kip1}$ and hence sequestration of cyclin dependent kinase 2 (CDK2), promoting cell cycle arrest and apoptosis (Lane *et al.*, 2001). Furthermore, trastuzumab has also been linked to HER2 receptor degradation and internalization

(Cuello *et al.*, 2001), yet contrary studies have suggested trastuzumab has no effect on HER2 surface expression (Arnould *et al.*, 2006). Also, proteolytic cleavage of HER2 yields an intact membrane receptor with enhanced kinase activity in addition to an ectodomain (ECD) fragment (Christianson *et al.*, 1998). *In vitro* studies have demonstrated trastuzumab mediated inhibition of HER2 cleavage, preventing the creation of a more potent RTK (Molina *et al.*, 2001). Another mechanism which has been implicated is the reduction of tumour angiogenesis by decreasing vascular endothelial growth factor (VEGF) levels while increasing thrombospondin-1 (TSP1) (Izumi *et al.*, 2002). Further, *in vivo* studies of HER2 over-expressing breast cancers with trastuzumab demonstrated a significant reduction in tumour volume as well as decreased microvessel density (Izumi *et al.*, 2002; Petit *et al.*, 1997). Lastly, mechanisms regulated by the immune system have also been linked to cytotoxic events induced by trastuzumab. Natural killer (NK) cells express the Fc gamma receptor which binds to the Fc domain of trastuzumab. Hence, trastuzumab recruits NK cells to tumours which then initiate cancer cell lysis in an antibody dependent cellular cytotoxic (ADCC) manner. This has been demonstrated in several breast cancer cell lines (Cooley *et al.*, 1999; Lewis *et al.*, 1993).

Trastuzumab has provided clinical benefit when administered in combination with conventional chemotherapies. Unfortunately, in some instances the therapeutic efficacy of trastuzumab is futile, a subset of patients do not respond to initial therapy while others develop resistance within one year (Esteva *et al.*, 2002; Slamon *et al.*, 2001). A variety of hypotheses regarding resistance have been proposed, however they have not provided definitive answers to overcoming trastuzumab insensitivity. One theory proposed in mediating resistance is alterations in HER2 receptor-trastuzumab interactions. The membrane associated glycoprotein mucin-4 (MUC4) has been shown to interact with HER2 and in doing so preventing interaction between HER2 and

trastuzumab (Nagy *et al.*, 2005; Price-Schiavi *et al.*, 2002). Another idea suggests increased cell signalling from compensatory pathways during trastuzumab treatment may aid in escaping its effect. EGFR, HER3, HER4 and IGFR-1R signalling have been suggested as alternative pathways activated during therapy thereby aiding tumour cells in overriding the effects of trastuzumab (Lu *et al.*, 2001; Sergina *et al.*, 2007). For example, the expression of constitutively active AKT in HER2 over-expressing breast cancer cell lines BT474 and SKBR3 rendered them insensitive to trastuzumab mediated apoptosis (Yakes *et al.*, 2002). Furthermore, Nagata *et al* demonstrated that PTEN inhibition reversed the antitumour activity of trastuzumab which was further validated when it was shown that patients with HER2 over-expressing tumours which are PTEN deficient have a poor response to therapy (Nagata *et al.*, 2004). More recently, a large scale RNA interference screen also implicated *PTEN* as well as mutant *PIK3CA* in the development of trastuzumab resistance (Berns *et al.*, 2007). Clearly, a more thorough understanding of the molecular mechanisms that limit the efficacy of trastuzumab would help to identify patients at risk of being nonresponsive and also aid in the development of better therapeutic options.

1.9 CD44

CD44 is a class I transmembrane glycoprotein expressed across a range of cell types where it primarily functions as an adhesion molecule. CD44 binds hyaluronan (HA) in addition to serving as a docking site for collagen, laminin, and fibronectin thereby mediating cellular migration through the extracellular matrix (Ponta *et al.*, 2003). Various CD44 isoforms exist as a result of alternative splicing of pre-mRNA species and extensive post-translational modifications including glycosylation (Screaton *et al.*, 1992). Thus the functions of CD44 are

intimately related to the structural modifications encountered by the molecule as they dictate the receptor's interactions with various extracellular components.

Initial interest in understanding the receptor's role in cancer was sparked by the identification of CD44 having a role in the metastasis of rat pancreatic cancer cell models (Gunthert *et al.*, 1991). Although CD44 lacks intrinsic signalling activity, its role in tumourigenesis can be contributed to its extracellular interactions with the mesenchymal epithelial transition (MET) receptor (Nestl *et al.*, 2001) and all four ErbB receptors (Bourguignon *et al.*, 1997; Sherman *et al.*, 2000; Yu *et al.*, 2002). Thus CD44's ability to serve as a co-receptor mediates downstream survival and growth pathways through MAPK and PI3K-AKT signalling. Interestingly, in the trastuzumab resistant JIMT-1 breast cancer cell line, the CD44-HER2 interaction circumvents trastuzumab access to its receptor. This is a result of HER2 being masked by HA binding to CD44 (Palyi-Krekk *et al.*, 2007). Beyond its indirect role in influencing cell signalling by serving as a co-receptor, CD44 also mediates other signalling events by interacting with SRC (Lee *et al.*, 2008), Rho GTPase (Bourguignon, 2008), Rho kinase (Bourguignon *et al.*, 2006), LCK (Ilangumaran *et al.*, 1998), FYN (Ilangumaran *et al.*, 1998), and PKC (Legg *et al.*, 2002). Despite lacking kinase activity in its cytoplasmic tale, the intracellular segment of the protein has two serine residues whose phosphorylation is regulated by PKC (Legg *et al.*, 2002). The phosphorylation status of CD44 has been associated with its ability to interact with ankryn, ezrin, radixin, and moesin (Legg *et al.*, 2002) and thus impact actin cytoskeletal dynamics which in pathogenesis are dysfunctional. More recently, a novel role of CD44 in DNA transcription has been described (Lee *et al.*, 2009). Lee *et al* report that following receptor activation, CD44 is internalized and associates with STAT3 and p300, allowing for STAT3 dimerisation by acetylation of its lysine 685 residue (Lee *et al.*, 2009). This complex then localizes to the nucleus where it binds and

regulates the cyclin D1 promoter, resulting in cell cycle progression (Lee *et al.*, 2009). Thus, CD44 has been implicated in a wide range of cancers where it appears to afford cells with a growth advantage.

CD44 has most recently been identified as a marker which defines a subset of breast cancer cells with the ability to drive tumourigenesis and generate a tumour with a heterogeneous cell population, referred to as tumour-initiating cells (TICs) (Al-Hajj *et al.*, 2003). Subsequent studies in cancers of the prostate (Collins *et al.*, 2005), ovary (Zhang *et al.*, 2008), colon (Dalerba *et al.*, 2007), stomach (Takaishi *et al.*, 2009), and blood (Jin *et al.*, 2006) validated $CD44^+$ cells as being highly associated with tumour initiation. In fact, cells positive for CD44 expression have also been implicated in conferring resistance to radiotherapy (Phillips *et al.*, 2006) and chemotherapy (Bourguignon *et al.*, 2008; Li *et al.*, 2008; Marangoni *et al.*, 2009). Complementary to this, gene expression studies comparing $CD44^+$ tissues to normal mammary epithelium identified genes associated with invasion, metastasis, angiogenesis and known stem cells markers to be upregulated in $CD44^+$ samples (Liu *et al.*, 2007; Shipitsin *et al.*, 2007). Moreover, the gene profiles of these tumourigenic cells strongly associated with poor clinical outcome, providing evidence for CD44 serving as a direct prognostic marker (Liu *et al.*, 2007; Shipitsin *et al.*, 2007). Therefore, targeting the TIC population which is defined by its expression of CD44 would perhaps help to improve the success of cancer therapies.

1.10 CANCER STEM CELLS

Solid tumours are composed of a variety of cell types which differ in morphology, cell surface antigens, genetic aberrations, proliferation rates, response to drugs, and in their ability to initiate

tumour formation in *in vivo* transplant assays (Dick, 2008). There exist two hypotheses which attempt to explain the observed heterogeneity in tumours. The traditional paradigm known as the clonal model suggests that genetic lesions within a single cell confers superior growth and survival advantages which allows for the divergent expansion of these mutated cells thus initiating tumour formation (Reya *et al.*, 2001). In this model, cells composing the tumour are considered functionally equivalent and the acquisition of behavioural heterogeneity is achieved by intrinsic and extrinsic influences. Alternatively, a second theory implicates cancer stem cells as the culprits of carcinogenesis. Normal stem cells are a rare population that are multipotent, that is they have the ability to differentiate to give rise to a progeny of various cell types. In addition, these cells have the ability to self-renew and generate new stem cells, thereby maintaining the stem cell pool. The cancer stem cell theory argues that a unique and rare subset of cells, referred to as cancer stem cells (CSCs) and/or TICs, drives tumour initiation, progression, and recurrence. CSCs are thought to be the progeny of transformed normal stem cells and hence share similar characteristics to normal stem cells. On the other hand, it has also been argued that cancer cells mutate to adopt stem cell characteristics. The exact mechanisms by which CSCs arise thus remains to be further explored. Therefore, a cell within a tumour, capable of tumour initiation through its indefinite self-renewal and differentiation properties, and able to generate a heterogeneous tumour is termed a CSC (Clarke *et al.*, 2006). Thus, experimentally, *in vivo* transplant assays are utilized to identify this class of cells. Cells are selected for specific surface antigens capable of generating a tumour composed of various cell types and that recapitulates the parent tumour. This suggests that they possess differentiation properties while the cell's capacity for self-renewal is demonstrated by its ability to form additional tumours when serially passaged into a second host (Clarke *et al.*, 2006). In summary, the capacity for self-renewal and the ability to differentiate and produce a heterogeneous progeny of cells are the hallmarks of CSCs and/or TICs.

Evidence supporting the cancer stem cell model was first identified in acute myeloid leukemia (AML). A pioneering study by Dick *et al* demonstrated that $CD34^+/CD38^-$ cells isolated from AML patient samples were extremely rare, however they were the only cells capable of inducing AML in mice when transplanted into non-obese diabetic mice with severe combined immunodeficiency disease (NOD/SCID) (Bonnet and Dick, 1997). Interestingly, the $CD34^+/CD38^-$ phenotype had been previously established to identify normal stem cells in the hematopoietic system (Larochelle *et al.*, 1996). Therefore, for the first time, a subset of cancer cells related to normal stem cells, were identified to be present in cancer and also initiate disease. More recently, tumourigenic breast cancer cells have been identified by a $CD44^+/CD24^{-/low}/ESA^+$ (Al-Hajj *et al.*, 2003) and/or $ALDH^+$ (Ginestier *et al.*, 2007) phenotype. Cells possessing this phenotype were present albeit rare in human tumour samples. Strikingly, as few as one hundred $CD44^+/CD24^{-/low}$ cells were able to induce tumour formation in mice whereas thousands of cells with varying phenotypes were necessary for cancer initiation (Al-Hajj *et al.*, 2003). The prospective identification of these highly tumourigenic cells thus provides a new avenue of exploration to elucidate the pathways involved in disease initiation, progression, and recurrence.

Following the initial identification of TICs in the breast, progress has been made in understanding their role beyond disease initiation. Gene profiling studies of $CD44^+/CD24^{low/-}$ breast cancer samples implicated them with an invasive gene signature, thus associating TIC-like cells with cancer progression (Liu *et al.*, 2007; Shipitsin *et al.*, 2007). Furthermore, it has been demonstrated that TICs are resistant to radiotherapy (Phillips *et al.*, 2006) and a variety of chemotherapies (Li *et al.*, 2008). For example, enrichment in the proportion of $CD44^+/CD24^{low/-}$ cells was observed in primary tumour samples following neoadjuvant chemotherapy suggesting

that TIC-like cells are unresponsive to therapy (Li *et al.*, 2008). In addition, $CD44^{+}CD24^{low/-}$ breast tumours also correlate with poor clinical outcome (Liu *et al.*, 2007). Taken together, an emerging concept is that cancer recurrence is a consequence of ineffective targeting of TICs.

1.11 THESIS HYPOTHESIS AND OBJECTIVES

Hypothesis:

YB-1 provides an enhanced growth and survival phenotype in HER2 over-expressing breast cancer cells where its aberrant expression confers acquired resistance to trastuzumab therapy by inducing a tumour initiating phenotype through its regulation of *CD44*.

Objectives:

I. To characterize the molecular mechanism regulated by YB-1 which protects HER2 over-expressing breast cancer cells against apoptosis.
II. To determine YB-1's role in the growth and survival of HER2 over-expressing cells resistant to trastuzumab.
III. To validate the role of activated P-YB-1^{S102} in mediating a trastuzumab resistant phenotype.
IV. To query the relationship between YB-1 and the tumour-initiating associated receptor CD44 in acquired trastuzumab resistance.

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CHAPTER TWO: TARGETING YB-1 IN HER2 OVER-EXPRESSING BREAST CANCER CELLS INDUCES APOPTOSIS VIA THE mTOR/STAT3 PATHWAY AND SUPPRESSES TUMOUR GROWTH IN MICE*

This chapter introduces the necessity of YB-1 for the growth and survival of breast cancer cells. We demonstrate that siRNA targeting YB-1 significantly suppressed growth in a panel of breast cancer cells representative of the aggressive triple negative and HER2 positive subtypes. Importantly, in the HER2 subtype, it was demonstrated that inhibition of YB-1induced apoptosis evidenced by the phosphorylation of H2AX, Annexin V staining, and a triple immunofluorescence apoptosis assay. Further, we were able to implicate STAT3 as a facilitator in the YB-1 mediated survival advantage in HER2 over-expressing breast cancer cells. In addition, we show that inhibiting YB-1 prevents colony formation in anchorage independent growth assays as well as suppressing tumour formation *in vivo*. Taken together, this study implicates YB-1 as a critical factor involved in the tumourigenic capacity of HER2 positive breast cancer cells.

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2.1 INTRODUCTION

The over-expression of the human epidermal growth factor receptor-2 (HER2) is clearly associated with one of the most aggressive types of breast cancer (Sorlie *et al.*, 2001). Equally challenging are those in the triple negative or basal-like subtype (Sorlie *et al.*, 2001). HER2 has become a desirable molecular target for breast cancer that has led to the development of therapies designed to inhibit it such as Herceptin, pertuzumab and more recently lapatinib. Thus far, the success of these agents is initially very good despite that ~30% of patients do not respond and those that do are often faced with the development of resistance. We have identified a second factor expressed in aggressive types of breast cancer, the Y-box binding protein-1 (YB-1) that induces growth promoting genes such as *HER2, EGFR,* proliferating nuclear antigen (*PCNA*), cyclin A (*CCNA*) and cyclin B (*CCNB*); review (Wu *et al.*, 2007). Further to this, the presence of YB-1 specifically in the nuclear compartment of breast cancer cells is associated with HER2 based on the examination of primary tumours by immunohistochemistry (Fujii *et al.*, 2008). YB-1 is activated by kinases such as AKT (Sutherland *et al.*, 2005) also known to be linked to breast cancer (Wu *et al.*, 2007). When YB-1 is highly expressed in the mammary gland, transgenic mice develop tumours with 100% penetrance indicating that it is a *bona fide* oncogene (Bergmann *et al.*, 2005). Because YB-1 is commonly expressed in breast cancers (Wu *et al.*, 2006), we questioned whether they were indeed dependent upon it for growth and survival. We therefore inhibited YB-1 using small interfering RNAs as a novel way of potentially blocking the growth of breast cancers. Inhibiting YB-1 suppressed the growth of 6 out of 7 breast cancer cell lines that were either triple negative or had *HER2* amplifications. Following this, we focused on those that had *HER2* amplifications given the many clinical challenges that currently prevent the successful treatment of this aggressive type of breast cancer.

2.2 MATERIALS AND METHODS

Inhibition of YB-1 with small interfering RNAs

BT474-m1 cells were obtained from MC Hung, MD Anderson Cancer Center. SUM149 cells were obtained from Astrand (Ann Arbor, Michigan). 184htrt cells were a gift from Dr. J. Carl Barett (National Insitute of Health, USA). All other cell lines were purchased from the American Type Culture Collection (Rockwood, MD). The sequence for siYB-1#1 was as previously reported (Wu *et al.*, 2006), siYB-1#2 was designed using the following sequence (CCACGCAAUUACCAGCAAAdTdT) and the control siRNA oligonucleotide was (UUCUCCGAACGUGUCACGUdTdT). Each of the cell lines were plated at a density of 1000 cells per 96 well, transfected with siYB-1#2 (5nM), we confirmed that knock-down was >75% by detecting YB-1 protein levels by immunofluorescence after 48 hours. Following this tumour cell growth was assessed after 72 hours by Hoechst staining as previously described using the ArrayScan VTI (Cellomics, Pittsburg PA) (To *et al.*, 2007). For the siRNA transfections, where changes in signal transduction were monitored, the cells ($3x10^5$ per 6-well dish to $3.5x10^5$ per 6-well dish) were transfected with 5nM of control or siRNA oligonucleotides (Dharmacon, IL) according to the manufacturer's protocol.

Apoptosis assays

BT474-m1 cells were treated with control siRNA, siYB-1#1 or siYB-1#2 oligonucleotides for 1-4 days and analyzed for P-H2AX^{S139} (1:500 dilution; AbCam) by immunoblotting. For Annexin V staining, the manufacturer's protocol was followed (Promega) and the cells were analyzed on a FACSCalibur (BD). Analysis of chromatin condensation, propidium iodide uptake and P-H2AX^{S139} was performed as previously described (To *et al.*, 2007). For propidium iodide

staining, the cells were collected after being treated for 4 days with siRNA to YB-1, washed, stained with 30µg/mL of PI (Sigma, St. Louis, MO), and suspended in 500µL of 1% FBS containing PBS before being analyzed on a FACSCalibur (BD).

Pathway evaluation of apoptosis induction

BT474-m1 or Au565 cells were treated with siYB-1 for up to 96 hours and then they were lysed in ELB buffer (To *et al.*, 2007) and the proteins were evaluated by immunoblotting using antibodies to the following diluted to 1:1000 unless otherwise indicated: YB-1 (1:10,000; gift from Dr. Colleen Nelson, University of British Columbia**),** P-ERK1/2$^{T202/Y204}$ (Cell Signalling Technology (CST), Danvers, MA), P-STAT3^{S727} (CST), P-STAT3^{Y705} (CST), STAT3 (CST), MCL-1 (Santa Cruz Biotechnology, Santa Cruz, CA), survivin (CST), P-H2AX^{S139} (1:500; AbCam), EGFR (StressGen, San Diego, CA), HER2 (AbCam), P-AKT^{S473} (CST), and P-GSKβ^{S9} (CST). Vinculin (1:2000; clone Vin 11-5, V4505 antibody, Sigma) and pan-actin (CST) antibodies were used as loading controls.

The activity of STAT3 was evaluated via gel shift by isolating nuclear proteins from BT474-m1 cells and a MCL-1 probe (Liu *et al.*, 2003), was used to detect activity according to our previously reported methods (Stratford *et al.*, 2007). We used 1µg of STAT3 antibody (Santa Cruz, Cat# SC-482x) in the competition experiment. STAT3 was silenced using QIAGEN HP validated siRNA in addition to a second siRNA oligonucleotide (Konnikova *et al.*, 2003). BT474-m1 and Au565 cells ($3x10^5$ per 6-well dish or $3.5x10^5$ per 6-well dish) were treated with transfection reagent alone, control siRNA (50nM) or siSTAT3#1 oligonucleotide (50nM, Dharmacon Research Inc) or siSTAT3#2 (50nM, QIAGEN) for 96 hours. For the rescue

experiments, 2μg of Flag:STAT3C (Bromberg *et al.*, 1999) was co-transfected into BT474-m1 cells ($5.5x10^5$ per 6- well dish) with 5nM of siYB-1#2 or control siRNA using Lipofectamine 2000 with a DNA:lipofectamine ratio of 1:3, harvested 72 hours later and immunoblotted for P-$H2AX^{S139}$, Flag (1:2000 M2 antibody, Sigma) and YB-1 (as described above). mTOR mRNA was evaluated using Assay on Demand (Applied Biosystems).

Soft agar assays

YB-1 was silenced as described above in the BT474-m1 and MDA-MB-453 cells and 24 hours later the cells were plated into the soft agar. Colony formation was performed as previously described (Sutherland *et al.*, 2005). Briefly, BT474-m1 ($1x10^4$ per 6-well dish) or MDA-MB-453 ($1.5x10^5$ per 6-well dish) cells were added to a 1:1 mixture of 2x DMEM and a solution of 0.6% agarose (Invitrogen) was prepared and the colonies were counted 28 days later.

Inhibition of YB-1 in vivo

BT474-m1 cells were transfected with 5nM of control siRNA or YB-1 siRNA oligonucleotides for 24 hours, harvested, and washed twice with Hanks Balanced Salt Solution (Invitrogen). They were then mixed ($1x10^6$ cells) with Matrigel (BD) at a 1:1 ratio yielding a total volume of 200μL which was injected subcutaneously into the right lower hind flank of six female *nu/nu* mice per treatment group. Tumour growth was measured using calipers and body weight was measured twice a week. Differences in tumour incidence were evaluated using the student's *t* test. Tumours from the termination of the study (week 3) were evaluated for YB-1 protein levels.

2.3 RESULTS AND DISCUSSION

Initially we silenced YB-1 with siRNA in a panel of breast cancer cell lines and cell growth was evaluated 96 hours later using a high content screening (HCS) platform (Figure 2.1 A, representative images of YB-1 knockdown). Seven breast cancer cell lines were screened which had *HER2* amplifications or were triple negative. Silencing YB-1 with siYB-1#2 inhibited the growth of the HER2 over-expressing BT474-m1 and Au565 cells by ~50% (Figure 2.1 B), yet the 184htrt immortalized breast epithelial cells and MDA-MB-453 cells were insensitive to the effect of YB-1. Beyond this, YB-1 knockdown also suppressed the growth of triple negative breast cancer cell lines by 40-80% (Figure 2.1 B). To understand the underlying reason for growth suppression in the HER2 over-expressing models of breast cancer, we show that inhibiting YB-1 in the BT474-m1 (Figure 2.1 B, right panel) and Au565 cells (data not shown) decreased HER2, EGFR, and ERK1/2 signalling while there was no effect on the P-AKT^{S473} or P-GSKβ^{S9} pathway. For unknown reasons siYB-1 failed to inhibit the ERK pathway in MDA-MB-453 cells which may explain why their growth was not suppressed (data not shown). Over a 1-4 day time course with siYB-1 the BT474-m1 cells underwent apoptosis that was detectable after 2 days based on the induction of P-H2AX^{S139} and increased annexin V staining (Figure 2.1 C-D). In support of this, cells treated for 3 days with siYB-1#2 had enhanced chromatin condensation, propidium iodide uptake and P-H2AX^{S139} at the cellular level as detected in HCS in both BT474-m1 and Au565 cells compared to the scrambled control (Supplemental Figure 2.1 A-B). Treating BT474-m1 cells for 4 days with siYB-1#1 or siYB-1#2 increased propidium iodide uptake as much as 30-fold based on flow cytometry (Supplemental Figure 2.1 C). Because YB-1 inhibition altered the apoptotic threshold of the BT474-m1 cells as indicated above, we examined the possibility that this would enhance their sensitivity to taxol. To this effect, we showed that silencing YB-1 improved the growth inhibitory effect of taxol (0.1-10

nM) on the BT474-m1 cells compared to cells treated with the control oligonucleotide (Supplemental Figure 2.1 D). Our studies therefore indicate that breast cancers that express high levels of HER2 as well as those that are triple negative are dependent upon YB-1 for growth and survival. Furthermore, combining YB-1 inhibition with taxol improves cell killing.

To investigate the underlying mechanism of siYB-1 induced apoptosis, we queried the STAT3 pro-survival pathway (Gritsko *et al.*, 2006). This was a candidate pathway because it was previously reported that STAT3 is phosphorylated at serine 727 in breast cancer cells (Yeh *et al.*, 2006) as an event downstream of HER2 (Fernandes *et al.*, 1999) and its dimerisation partner EGFR (Garcia *et al.*, 1997) which are both transcriptionally regulated by YB-1 (Wu *et al.*, 2006). As yet, a link between YB-1 and STAT3 has not been previously documented. Inhibiting YB-1 in BT474-m1 or Au565 cells for 3 days decreased P-STAT3^{S727} and its downstream gene MCL-1, however P-STAT3^{Y705} was undetectable and total STAT3 levels were unaffected (Figure 2.2 A). The STAT3 downstream gene survivin did not change. This correlated with decreased signalling through P-ERK1/2$^{T202/Y204}$ and P-mTOR^{S2448}. Surprisingly, total mTOR was also decreased (Figure 2.2 A). In addition, following YB-1 knock-down the decrease in mTOR protein levels as noted in other cell lines, namely SUM149 (triple negative breast cancer) and SF188 (pediatric glioblastoma) cells (Supplemental Figure 2.2 A-B). However, mTOR transcript level did not decrease following YB-1 inhibition (Supplemental Figure 2.2 C-D) ruling it out as a direct transcriptional target. Additional studies are underway to investigate whether YB-1 regulates the rate of mTOR translation or sustains its protein stability. Independent of how mTOR is altered by YB-1, it could have an important impact on the STAT3 pathway because it is known to phosphorylate STAT3 ^{S727} (Yokogami *et al.*, 2000).

Following cytokine stimulation, STAT3 is phosphorylated at tyrosine 705 leading to co-operation with serine 727 for maximal transcriptional activation (Wen *et al.*, 1995). However, NOTCH signalling leads to P-STAT3^{S727} in the absence of P-STAT3^{Y705} (Androutsellis-Theotokis *et al.*, 2006). Similarly, P-STAT3^{S727} is essential for the survival of macrophages in the absence of P-STAT3^{Y705} (Liu *et al.*, 2003). Given our data, we suspected that STAT3 was able to protect the BT474-m1 and Au565 cells from apoptosis upon phosphorylation at serine 727 while it appears that phosphorylation of tyrosine 705 was not required. In support of this, we confirmed the absence of tyrosine 705 phosphorylation by comparing the BT474-m1 cells to the MDA-MB-231 cells which are known to express high levels of STAT3^{Y705} (Berishaj *et al.*, 2007) (data not shown). We determined STAT3 was indeed active by isolating nuclear proteins from BT474-m1 cells and then probing an MCL-1 promoter sequence using gel shift assays. Nuclear isolates bound to the MCL-1 promoter, which was inhibited with unlabelled oligonucleotide or by pre-incubating the nuclear extracts with an antibody to STAT3 (Figure 2.2 B, lanes 1-4). Similar to the effect of siYB-1, inhibition of STAT3 expression using siRNA decreased expression of MCL-1 protein and thereby increased P-H2AX^{S139} (Figure 2.2 C, left and right columns, respectively). Finally, transfection of a constitutively activated form of STAT3 (STAT3C) (Bromberg *et al.*, 1999) partially rescued the cells from siYB-1#2 induced apoptosis based on reduced P-H2AX^{S139} (Figure 2.2 D). Taken together we concluded that YB-1 engages the STAT3 pro-survival pathway to protect breast cancer cells from apoptosis.

Given this, we addressed whether inhibiting YB-1 could suppress the tumourigenic potential of HER2 over-expressing breast cancer cells by examining growth in soft agar and then in mice. To do so, we demonstrated that inhibition of YB-1 with siYB-1#1 and siYB-1#2 silenced the expression of the target protein for up to 14 days (Figure 2.3 A). In soft agar, YB-1 inhibition

prevented colony growth by 85-92% compared to the control (Figure 2.3 B-C, left column). Au565 cells responded similarly (data not shown). Colony formation was inhibited in the MDA-MB-453 cells but to a lesser degree (~50%) (Figure 2.3 C, right column). Finally, we characterized the tumour growth of BT474-m1 cells in mice, and determined that >80% of the mice develop tumours within one week making this a convenient model for studying the effect of relatively short-lived siRNAs *in vivo*. We therefore transfected the cells with siYB-1#1 or siYB-1#2 for 24 hours and then injected 1 million cells into the hind flank of nude mice. Inhibition of YB-1 suppressed tumour formation throughout the first 2 weeks (n=6, Figure 2.4 A). By the third week, smaller but detectable tumours were apparent in 4 of 6 and 5 of 6 mice injected with siYB-1#1 and in siYB-1#2-treated cells, respectively (Figure 2.4 A-B, inset, representative images of tumours). As we suspected, the small tumours that developed by the third week from the cells pre-treated with siYB-1, re-expressed YB-1, indicating that the siRNA was no longer active (Figure 2.4 C). Taken together, our data demonstrates that inhibiting YB-1 disrupts the tumour initiating potential of HER2 over-expressing breast cancer cells likely via sensitizing the cells to apoptosis by interfering with the STAT3 pathway.

It is noteworthy that in a recent study the PTEN/mTOR /STAT3 pathway reportedly promotes the growth of tumour initiating cells in breast cancer cells (Zhou *et al.*, 2007). In that study, STAT3 was specifically phosphorylated at serine 727 based on reverse phase array profiling. Importantly, inhibition of STAT3 with siRNA or a small molecule referred to as IS3 295 selectively blocked the growth of these cells and perturbed the establishment of tumours in mice (Zhou *et al.*, 2007). Because we find that inhibiting YB-1 interferes with this pathway it seems reasonable that it could also suppress the growth of tumour initiating cells by altering this network.

To conclude, these studies provide pre-clinical rationale for targeting YB-1 in HER2 over-expressing or triple negative breast cancers setting forward the idea that it may be a good molecular target across different tumour subtypes.

2.4 FIGURES

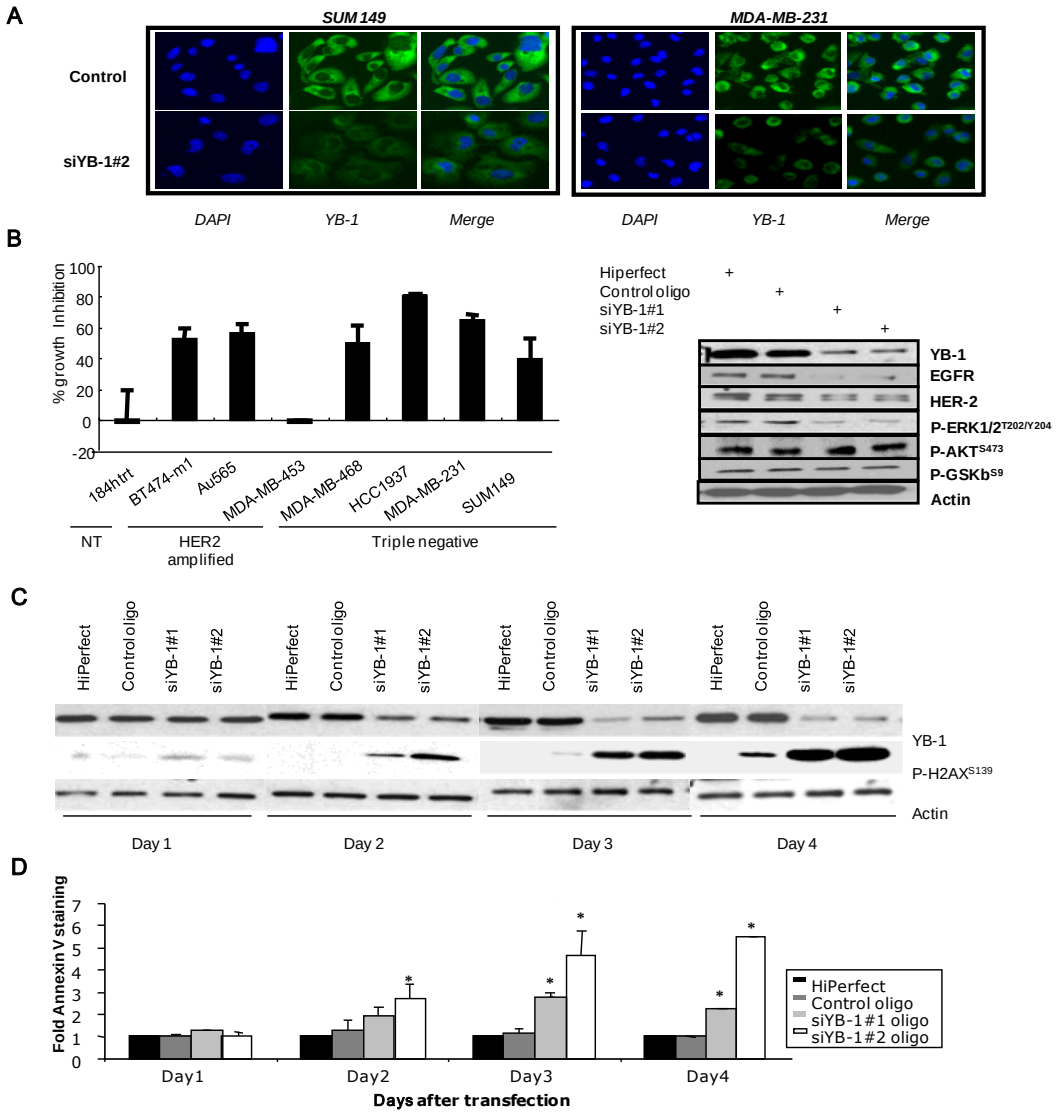


Figure 2.1. Inhibition of YB-1 generally suppresses the growth of breast cancer cell lines and leads to the induction of apoptosis. In a high content screen, YB-1 was silenced for three days with siYB-1#2. **A.** The loss of YB-1 expression following treatment with siYB-1#2 was monitored by immunofluorescence. Representative images for SUM149 and MDA-MB-231 cell are shown (Magnification 10x, ArrayScan VTI). **B.** Inhibiting YB-1 suppressed the growth of 6 out of 7 breast cancer cell lines representing cancers that had *HER2* amplifications or were triple negative. The loss of YB-1 had no effect on the immortalized breast epithelial cell line 184htrt or MDA-MB-453 cells. Silencing YB-1 in the BT474-m1 cells for 72 hours also resulted in decreased signalling through the HER2/EGFR/ERK pathway based on immunoblotting. **C.** BT474-m1 cells were transfected with control siRNA or siRNA oligos targeting YB-1 and harvested 1, 2, 3 and 4 day after transfection. Proteins were isolated from the cells and the lysates were subjected to Western blotting to examine the level of P-H2AX^{S139}. Pan-actin was used as loading control. **D.** Small interfering RNAs against YB-1 induced annexin V staining after treatment for 4 days. Changes in annexin V were monitored by flow cytometry.

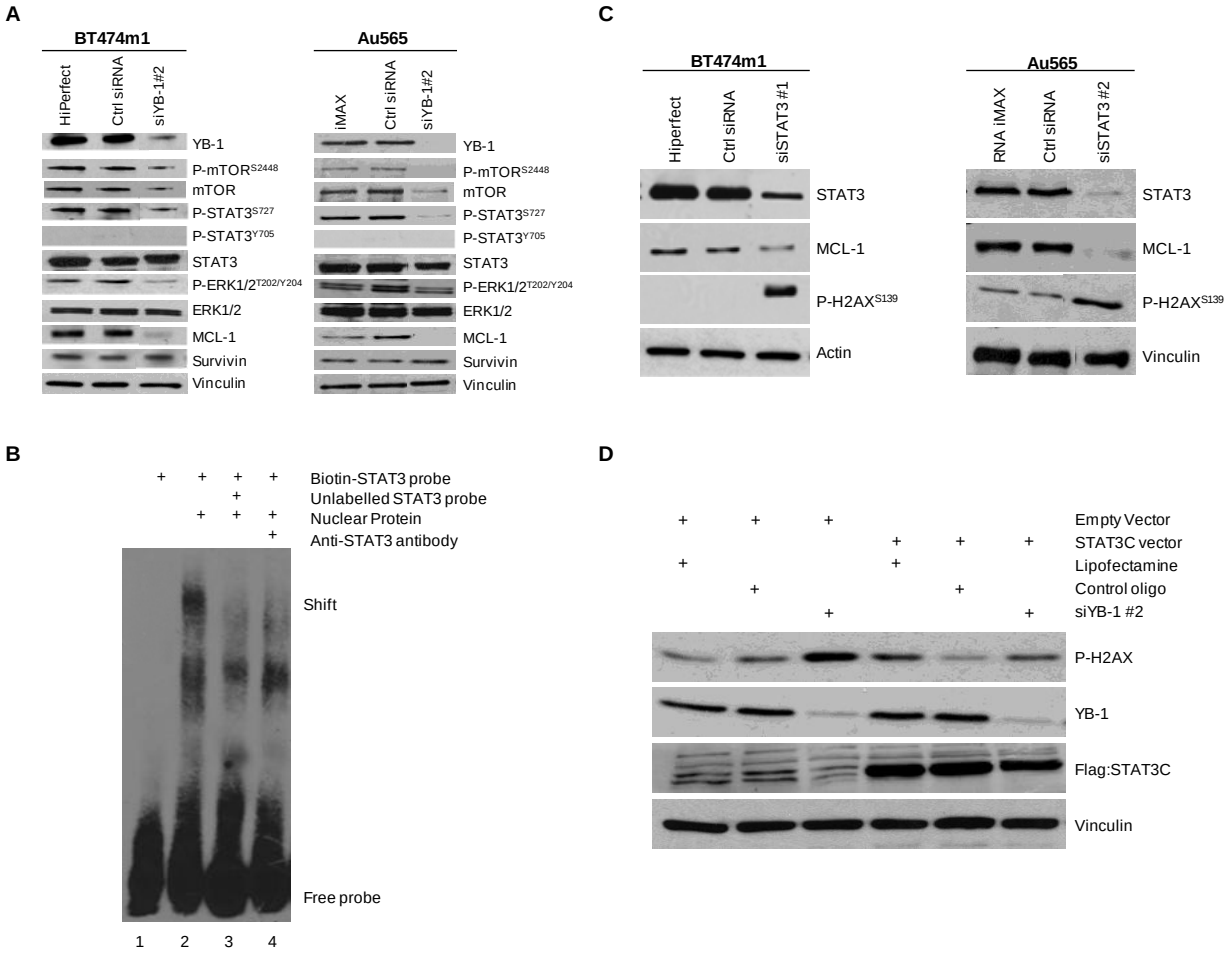


Figure 2.2. Loss of YB-1 decreased signalling through the STAT3 pathway. A. Loss of YB-1 expression with siYB-1#2 (72 hours) was associated with a decrease in P-STAT3^{S727} and MCL-1 relative to the transfection reagent control (lane 1) or control siRNA (lane 2) in the BT474-m1 cells (left panel). There was no change in total levels of STAT3 or survivin. STAT3^{Y705} was undetectable. Actin or vinculin was used as a control for equal sample loading. The extracts were also evaluated for P-mTOR^{S2448}, mTOR, and ERK1/2. This was also consistently observed in the Au565 cells (right panel). **B.** Nuclear extracts were isolated from BT474-m1 cells to confirm that STAT3 was active in these cells lacking tyrosine 705 phosphorylation. In the absence of nuclear protein, no binding was observed (lane 1). The addition of nuclear proteins resulted in binding to a MCL-1 promoter sequence containing the STAT3 binding site (lane 2), which could be inhibited with cold competitive oligonucleotide (lane 3) or an antibody specific to STAT3 (1µg) (lane 4). **C.** BT474-m1 and Au565 cells were treated with tranfection reagent, control siRNA (50 nM) or STAT3 siRNA (50 nM) for 96 hours. As a consequence, STAT3 was silenced, MCL-1 decreased and P-H2AX^{S139} was induced in BT474-m1 (left panel) and Au565 cells (right panel). **D.** To examine the possibility that STAT3 could rescue the induction of apoptosis by YB-1 we introduced Flag:STAT3C and siYB-1#2 by co-transfection. The cells were then examined 3 days later for changes in P-H2AX^{S139}. The ectopic expression of STAT3C rescued YB-1 induced apoptosis given that P-H2AX^{S139} was reduced.

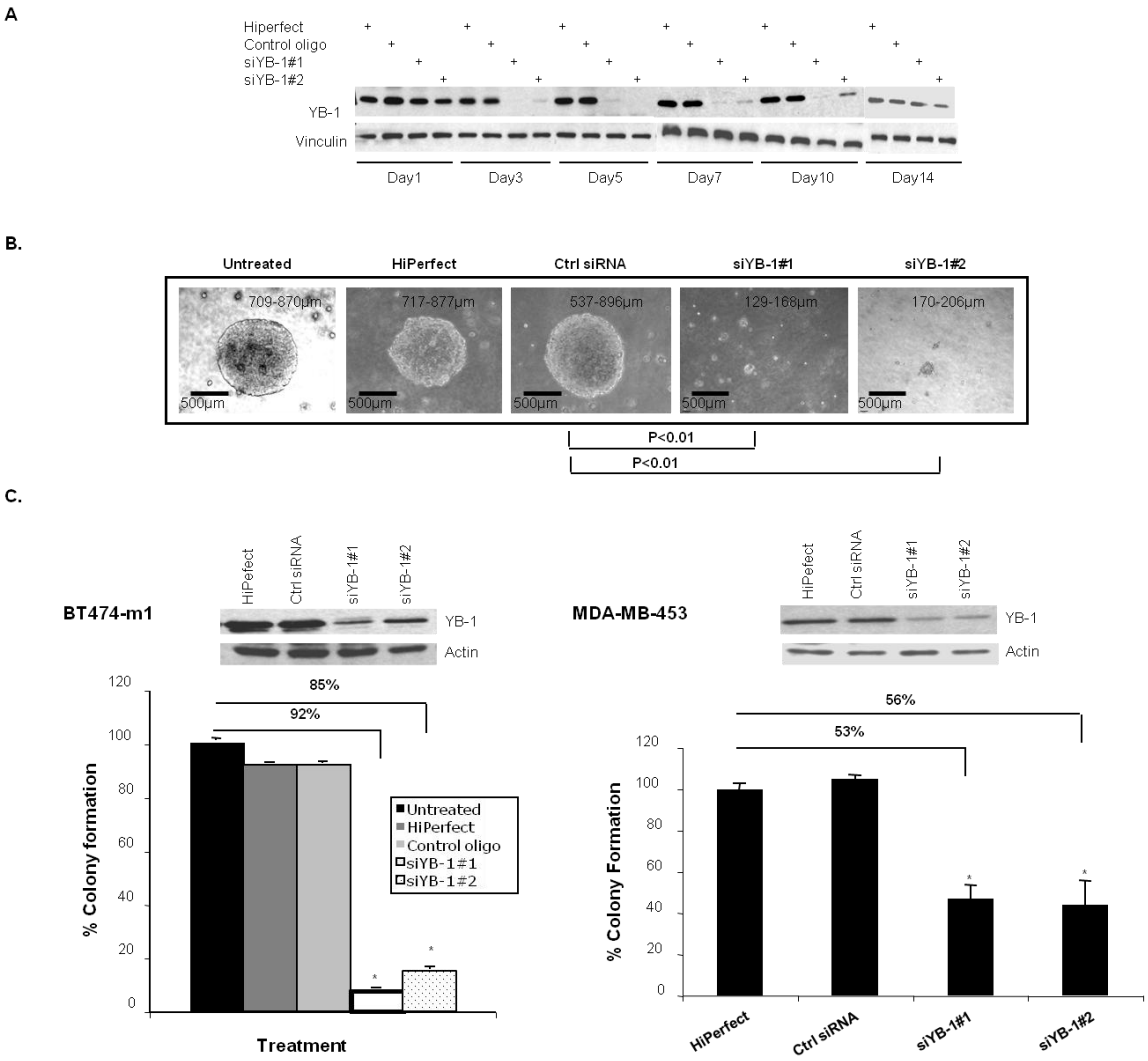


Figure 2.3. Inhibition of YB-1 suppresses tumour growth in soft agar. A. Time-course study (1-14 days) was conducted to examine the long-term stability of the YB-1 siRNA oligonucleotides. Vinculin antibody was used as loading control. YB-1 inhibition was sustained out to 10 days with some detectable inhibition still at 14 days. **B**. The impact of knocking down YB-1 was evaluated in soft agar. BT474-m1 cells were treated with siYB-1 for 24 hours and colony growth was assessed after 28 days. Photomicrographs of representative colonies were taken. YB-1 inhibition decreased the size of colonies compared to the control siRNA. **C.** BT474-m1 and MDA-MB-453 cells were treated with YB-1 siRNA for 48 hours and anchorage-independent growth of the cells was assessed by soft agar assays. Quantification of the number of colonies is shown in the bar charts. On average the BT474-m1 cells produced larger colonies ie greater than 300-800 microns and 10-12 could be counted per field. The MDA-MB-453 cells developed smaller colonies of approximately 50 microns in size but were more numerous. On average there were 40-50 colonies per field. * treatment was significantly different from the controls based on student's *t* tests.

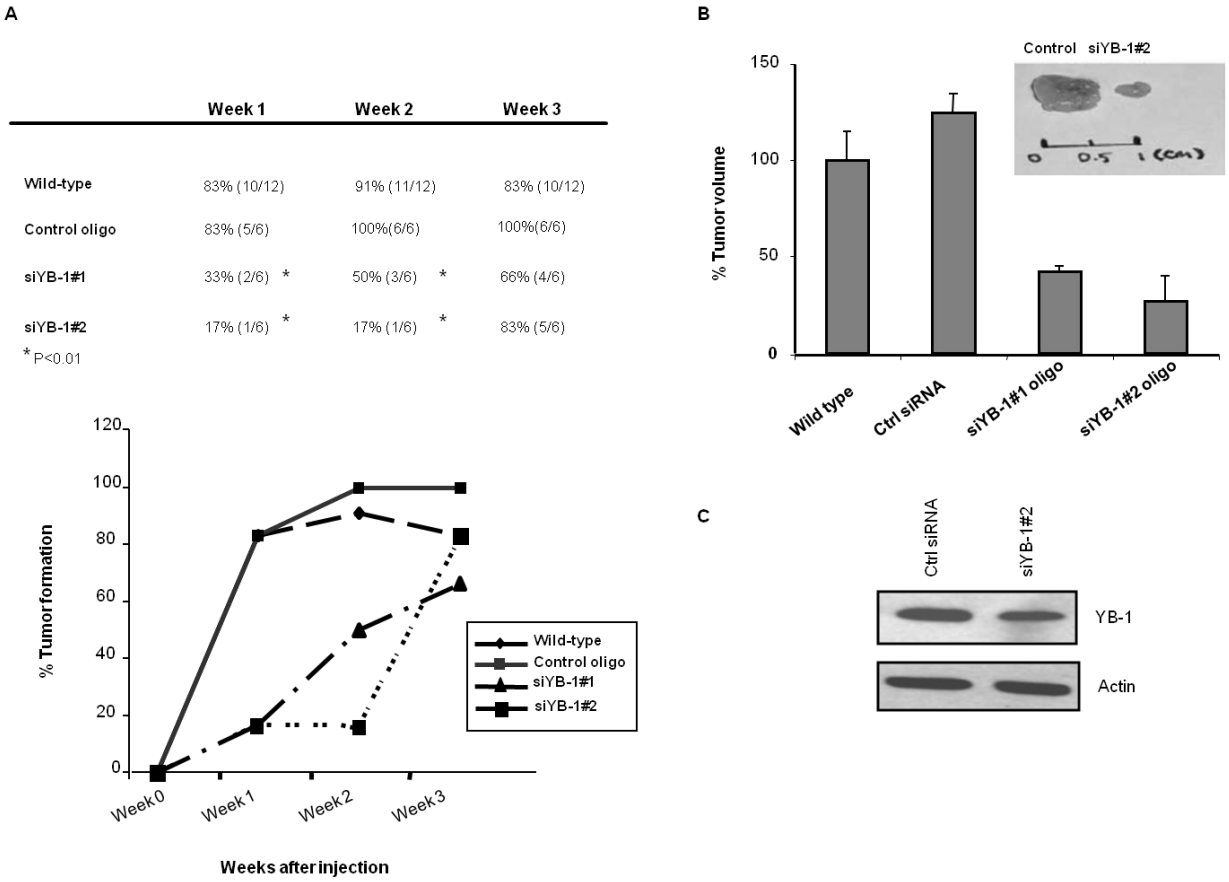
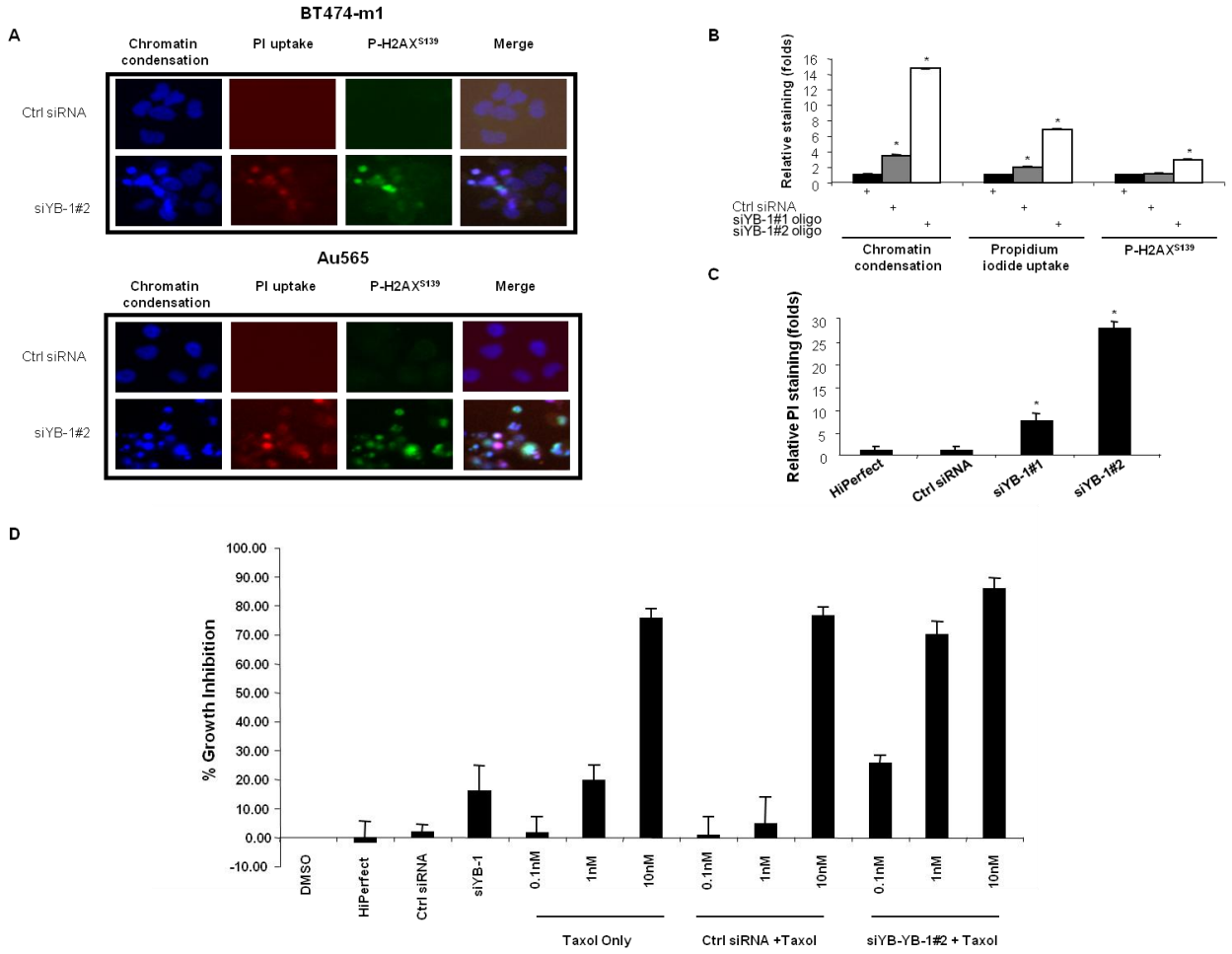
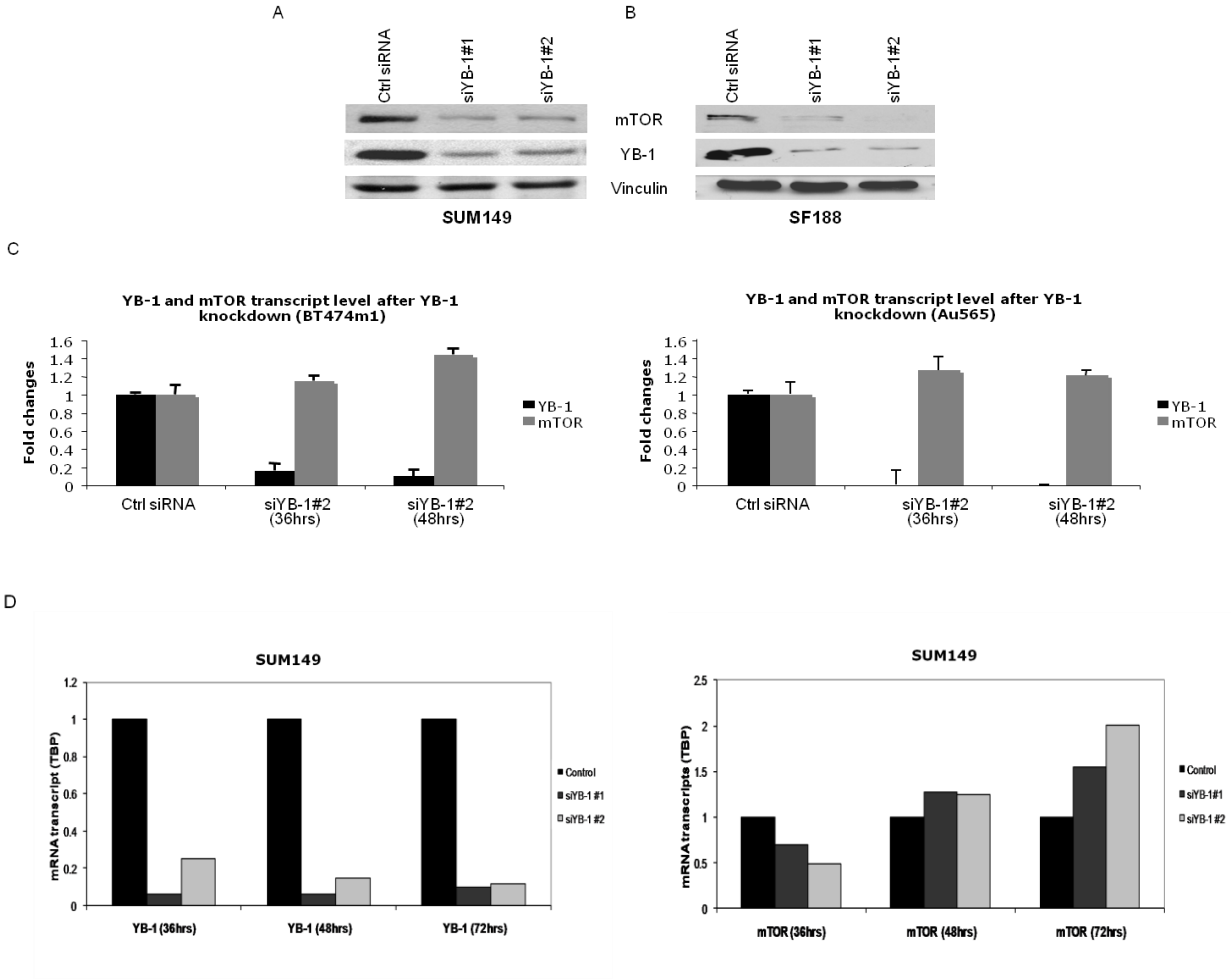


Figure 2.4. Inhibition of YB-1 suppressed the development of tumours in mice. A. BT474-m1 cells were transfected with control siRNA, siYB-1#1 or siYB-1#2 for 24 hours and then the cells were injected subcutaneously into nu/nu mice. The incidence of tumour formation was on average 83% in the mice treated with either the wild-type cells or the control siRNA oligonucleotide in the first week. Conversely, tumour incidence was only 33% or 17% when mice were injected with cells transfected with siYB-1#1 or siYB-1#2, respectively ($P<0.01$). Similarly, the loss of YB-1 expression suppressed tumour formation in the second week as well ($P<0.01$). **B.** By the third week, tumours developed in the mice however they were remarkably smaller in size compared to the control siRNA treated mice, the tumours that arose in siYB-1#1 or siYB-1#2 were ~50-67% smaller ($P<0.01$) respectively. Significance was determined using a student's *t* test. *<0.01. **C.** The re-expression of YB-1 was confirmed in the small tumours that eventually arose in the cells transfected with siYB-1#2. ** indicates $P<0.01$ compared to the control siRNA oligonucleotide control.

2.5 SUPPLEMENTAL FIGURES



Supplemental Figure 2.1. Inhibition of YB-1 induces apoptosis and enhances growth suppression in combination with Taxol. A. BT474-m1 and Au565 cells were transfected with 5nM of Ctrl siRNA or siYB-1 oligonucleotides in a 96 well plate and incubated for 3 days. Following cells were permeabilized and stained with Hoechst dye, propidium iodide, or P-H2AX^{S139}. The plates were then analyzed and images were acquired on the ArrayScanVTI Reader. Representative images demonstrate apoptosis in cells treated with siYB-1. **B.** Relative staining intensities of Hoechst, propidium iodide, and P-H2AX^{S139} were compared between the control and treatment groups in the BT474-m1 cells. **C.** PI staining was quantified in the Au565 cells comparing the control and treatment groups. **D.** BT474-m1 cells were treated with siYB-1#2 in combination with taxol for 72 hours. Cell growth was analyzed on the ArrayCan VTI. The combined treatment had an enhanced effect on growth inhibition.



Supplemental Figure 2.2. YB-1 inhibition decreases mTOR protein levels but not transcript levels. A. SUM149 (triple negative breast cancer cells) and SF188 (pediatric glioblastoma cells) were treated with 20nM of either Ctrl siRNA, siYB-1#1, or siYB-1#2 for 96 hours. Following, YB-1, mTOR, and Vinculin levels were assessed by immunoblotting. **B.** Quantitative real-time PCR of BT474-m1 and Au565 cells harvested following 36 or 48 hours of 5nM of Ctrl siRNA, siYB-1#1, or siYB-1#2 transfection. Data demonstrates mTOR transcript levels do no correlate with decrease in YB-1 mRNA levels. **D.** SUM149 cells were transfected with 20nM of Ctrl siRNA, siYB-1#1, or siYB-1#2 for 48 or 72 hours and analyzed by quantitative real-time PCR. mTOR transcript levels did not decrease with inhibition of YB-1.

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CHAPTER THREE: THE EXPRESSION OF ACTIVATED Y-BOX BINDING PROTEIN-1 SERINE 102 MEDIATES TRASTUZUMAB RESISTANCE IN BREAST CANCER CELLS BY INCREASING CD44 EXPRESSION**

This chapter describes the importance of P-YB-1^{S102} in a model of acquired trastuzumab resistance. We demonstrate that the trastuzumab resistant cell lines, HR5 and HR6, have increased levels of P-YB-1^{S102} and its activating kinase P-RSK in comparison to the sensitive BT474 cells. The importance of activated YB-1 in mediating trastuzumab resistance is demonstrated by the expression of a constitutively active mutant of YB-1, Flag-YB-1^{S102D} in the trastuzumab sensitive BT474 cell line which rendered them insensitive to treatment. In addition, we provide evidence that trastuzumab resistance is brought about by P-YB-1^{S102} mediated induction of CD44 expression, given the HR5 and HR6 cells have increased levels of CD44 in comparison to the BT474 cells which correlates with the levels of P-YB-1^{S102}. Overall, this study reveals a novel role of YB-1 in mediating trastuzumab resistance by inducing a tumour initiating phenotype which is unresponsive to therapy.

**A version of this chapter has been submitted for publication. Dhillon J, Astanehe A, Lee C, Fotovati A, Hu K, Dunn SE. The expression of activated Y-box binding protein-1 serine 102 mediates trastuzumab resistance in breast cancer cells by increasing $CD44^+$ cells.

3.1 INTRODUCTION

Molecular targeted therapies have revolutionized the field of cancer therapeutics (Sawyers, 2004). Although they have proved to be successful in the treatment of cancer patients, there remain many pitfalls associated with their use. The human epidermal growth factor receptor-2 (HER2), an established proto-oncogene over-expressed in 20 to 30% of breast cancer patients, defines aggressive disease and is associated with poor prognosis, a shorter disease free period and poor survival (Slamon *et al.*, 1987). The development of trastuzumab (Herceptin), a humanized monoclonal antibody targeting HER2, has provided clinical benefit when administered in combination with conventional chemotherapies (Cobleigh *et al.*, 1999; Slamon *et al.*, 2001). Unfortunately, the therapeutic efficacy of trastuzumab has been low in many instances, as many patients do not respond to initial treatment or develop resistance to therapy within one year (Slamon *et al.*, 2001; Vogel *et al.*, 2002). A number of mechanisms have been implicated in the acquisition of resistance; however they have not provided definitive answers to overcoming the problem.

In this study, we queried the role of YB-1, a transcription/translation factor that is over-expressed in 40% of human breast cancers, in trastuzumab resistance because of its established association with poor survival, disease recurrence, and relapse, specifically in the HER2 subtype (Habibi *et al.*, 2008). Its function in breast cancer growth and survival is eminent by its regulation of *EGFR, HER2, MET, PIK3CA* expression in addition to its mediation of drug resistance by induction of *MDR1* (Astanehe *et al.*, 2009; Bargou *et al.*, 1997; Finkbeiner *et al.*, 2009; Stratford *et al.*, 2007; Wu *et al.*, 2006). We have previously identified a role for YB-1 in the growth and survival of HER2 positive breast cancers and demonstrated that it was necessary

for *in vivo* tumourigenesis (Lee *et al.*, 2008). More recently, our laboratory has confirmed a novel role for YB-1 in the maintenance of a TIC phenotype (To *et al.*, 2010).

Given the recent evidence implicating HER2 as a regulator of TICs and that these cell types are resistant to common chemotherapies (Korkaya *et al.*, 2008; Li *et al.*, 2008), we questioned whether YB-1 plays a role in the development of acquired trastuzumab resistance by inducing a TIC phenotype. Since the mode of resistance remains poorly understood, this study provides a novel mechanism by which cancer cells acquire the ability to escape the effects of trastuzumab and thus a rationale to target YB-1 in the treatment of trastuzumab resistance.

3.2 MATERIALS AND METHODS

Cell Culture and Reagents

Human breast cancer cells, trastuzumab sensitive BT474, and the resistant counterparts HR5 and HR6 were a generous gift from Dr. Carlos L. Arteaga (Vanderbilt-Ingram Comprehensive Cancer Center, Vanderbilt University School of Medicine, Nashville, Tennessee). Cell line models mimicking acquired trastuzumab resistance (HR) were created from BT474 xenografts established in athymic nude mice which were given a course of trastuzumab therapy. HR5 and HR6 cell lines were established from tumours which recurred during treatment (Ritter *et al.*, 2007). BT474-m1 cells were obtained from MC Hung, M.D. Anderson Cancer Center and AU565 cells were purchased from the American Type Culture Collection. BT474, BT474-m1, HR5 and HR6 cells were maintained in F12/DMEM supplemented with 10% fetal bovine serum (Invitrogen, Burlington, ON, Canada) and AU565 cells were maintained in 10% RPMI at 37ºC, 5% CO_2. Trastuzumab was purchased from the BC Cancer Agency Pharmacy (Vancouver, BC, Canada).

Protein extraction and Immunoblot analysis

Protein was isolated from log-growing cells which were lysed in Egg Lysis Buffer (ELB) as previously described (Wu *et al.*, 2006), supplemented with protease and phosphatase inhibitors, and quantified using the Bradford assay (Biorad, Hercules, CA, USA). Immunoblotting techniques were completed as described previously (Wu *et al.*, 2006). The primary antibodies used for protein detection were as follows: YB-1, 1:2000 (Abcam, Cambridge, MA, USA), P-YB-1^{S102}, 1:1000 (Cell Signalling Technology, Danvers, MA, USA), EGFR, 1:1000 (Stressgen, San Diego, CA, USA), HER2, 1:200 (Abcam), ERK, 1:1000 (Cell Signalling Technology,

Danvers, MA, USA), P-ERK$^{Thr202/Tyr204}$, 1:1000 (Cell Signalling Technology), AKT, 1:1000 (Cell Signalling Technology), P-AKT^{S473}, 1:1000 (Cell Signalling Technology), pan-RSK, 1:1000 (Cell Signalling Technology), P-RSK^{S380}, 1:500 (Cell Signalling Technology), P-RSK$^{S221/S227}$, 1:1000 (Invitrogen), P-γH2AX^{S139}, 1:1000 (Abcam), Pan-actin, 1:1000 (Cell Signalling Technology), Vinculin, 1:1000 (Upstate, Temecula, CA, USA), PARP, 1:1000 (Cell Signalling Technology).

Trastuzumab treatment

BT474, HR5, and HR6 cells were seeded at a density of 3 x 10^5 cells in a six-well plate. Cells were treated with 20μg/mL of trastuzumab 24 hours after seeding; cells were harvested for immunoblotting 72 hours post treatment. BT474 Flag-EV, Flag-YB-1^{WT}, Flag-YB-1^{S102D}, and Flag-YB-1^{S102A} were plated at a density of 3.5 x 10^4 cells/well into a 12-well plate and treated with 20μg/mL of trastuzumab for seven days. Cells were then stained with Hoechst33342 (Sigma Aldrich, Oakville, ON,Canada) and cell numbers were analyzed with the Cellomics ArrayScan VTI instrument (Cellomics, Pittsburgh, PA, USA) to assess growth.

siRNA transfection

BT474, HR5, and HR6 were seeded into a 6-well plate at 3 x 10^5 cells/well 24 hours prior to transfection. Five nM of either control siRNA (AllStars Negative Control siRNA, #1207820, Qiagen, Hilden, Germany) or YB-1 oligonucleotide #1 or oligonucleotide #2 (Astanehe *et al.*, 2009) was transfected using Lipofectamine RNAiMAX (Invitrogen) for 96 hours.

Growth Inhibition Analysis

BT474, HR5, and HR6 cells were seeded at a density of 5.5 x 10^4 cells/well in a 24-well plate and allowed to adhere for 24 hours. Cells were then treated with 5nM of either control siRNA or YB-1 siRNA or 20nM of CD44 siRNA (CD44 siRNA (Hs_CD44_5_HP Validated siRNA, #SI00299705, Qiagen). After 24 hours, cells were either grown in regular growth media or media supplemented with 20µg/mL of trastuzumab for 72 hours. Following treatment, cell proliferation was evaluated using the CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA). YB-1 knockdown was concurrently confirmed by immunoblot analysis of whole cell extracts and q-RT-PCR was employed to verify CD44 inhibition.

Soft agar anchorage independent growth assay

To evaluate anchorage independent growth with YB-1 inhibition, BT474, HR5 and HR6 cells were transfected with either control siRNA or YB-1 siRNA as described above. Forty eight hours post transfection, cells were trypsinized and suspended into 0.3% agar and plated at a density of 5 x 10^3 cells/well in a 24-well plate on top of a layer of 0.6% agar. For the combined YB-1 inhibition and trastuzumab soft agar studies, trastuzumab was added to the top layer of agar at final concentrations of 0µg/mL, 0.2µg/mL, 2.0µg/mL, and 20µg/mL. Soft agar solutions were prepared with DMEM supplemented with 10% FBS, except for the studies employing AU565 cell where RPMI supplemented with 10% FBS was used instead. Colonies were counted after 21 – 28 days.

Stable transfectant cell lines

BT474 cells were plated at a density of 5.5 x 10^5 cells/well into a 6-well plate. After 24 hours, cells were transfected with 2µg of Flag-EV, Flag-YB-1^{WT}, or Flag-YB-1^{S102D} plasmid constructs as described previously (Finkbeiner *et al.*, 2009) with 6µL of Lipofectamine 2000 Reagent (Invitrogen), employing the manufacturer suggested DNA:transfection reagent ratio of 1:3. Transfection reagent and DNA complexes were replaced with fresh growth media 5 hours post transfection. The transfected cells were then expanded into 10cm dishes, at approximately 80% confluency, 400µg/mL of G418 (Calbiochem, EMD Chemicals, San Diego, CA, USA) was added to the growth media and replaced every 4 days to select for cells expressing the transgene. Cells were continuously maintained in G418 and split at low density. For each construct, four clones were picked and further expanded. Clonal cells were then evaluated for transgene expression by immunoblot analysis and quantitative real-time PCR. Clones expressing the highest levels of the transgene were then selected to carry out further experiments.

Quantitative real-time PCR

RNA isolation, cDNA synthesis, and real-time PCR experiments were performed as described previously (Astanehe *et al.*, 2009). TaqMan Universal Master Mix was used with TaqMan Gene Expression Assays designed against CD44 (Hs00153304_m1, Applied Biosystems, Foster City, CA, USA) and TATA-box binding protein (TBP) endogenous control (Part No. 4326322E, Applied Biosystems, Foster City, CA, USA) were used to detect transcript levels on an ABI Prism 7000 Sequence Detector.

Immunofluorescence analysis

Cells were seeded onto glass coverslips at a density of 5 x 10^5 cells/well and allowed to adhere. Next, cells were washed with phosphate buffered saline (PBS) and then fixed with 2% formalin for 20 minutes and rinsed three times with PBS. Following, the coverslips were incubated with CD44 conjugated to phycoerythrin, 1:100 (Clone G44-26, Becton Dickinson (BD) Pharmingen, Franklin Lakes, NJ, USA) diluted in immunofluorescence buffer (PBS with 2% goat serum, 1% BSA, and 1% saponin) for 1 hour at room temperature. Following, coverslips were washed three times with PBS and then incubated in secondary Alexa Fluor 546 goat anti mouse IgG (Invitrogen) diluted 1:1000 in immunofluorescence buffer for 1 hour at room temperature. Lastly, coverslips were washed with PBS three times and then mounted onto glass slides using Prolong Gold antifade reagent with DAPI (Invitrogen). Cells were imaged on an Olympus Fluorescence BX61 Microscope.

Mammosphere non-adherent growth assay

Single cells at a density of 5 x 10^3 were plated into Ultra-Low Attachment coated 6-well culture plates (Corning, Lowell, MA, USA) in a 1:1 DMEM/F12 basal media freshly supplemented with human basic fibroblast growth factor (Invitrogen), epidermal growth factor (Invitrogen), heparin (Sigma Aldrich), and B27 supplement without Vitamin A (Sigma Aldrich). Mammospheres greater than 15 cells in size were counted at seven days.

Immunofluorescence analysis of mammospheres

Mammospheres were grown and cultured as described above. After seven days of growth, spheres were transferred into a 15mL conical tube and centrifuged at 1000 rpm for 2 minutes. Spheres were then washed once with PBS and resuspended into a 1:1 solution of methanol:acetone and spotted onto glass slides, slides were placed at -20ºC for 10 minutes and then air dried. Spheres were stained with CD44 conjugated to phycoerythrin, 1:25 (Clone G44-26, Becton Dickinson (BD) Pharmingen, Franklin Lakes, NJ, USA) diluted in immunofluorescence buffer (PBS with 2% goat serum, 1% BSA, and 1% saponin) overnight at 4ºC. Slides were then washed 3 times with PBS and then incubated with secondary antibodies as described above for 1 hour at room temperature. Next, slides were washed twice with PBS and then nuclei were stained with Hoechst33342 (Sigma Aldrich, Oakville, ON, Canada). Slides were then washed once with PBS and mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Spheres were visualized by confocal micrsocopy using the Fluoview FV1000 laser scanning microscope (LSI3-FV1000-inverted).

Chromatin immunoprecipitation (ChIP)

BT474, HR5, and HR6 cell lines were plated at a density of 1×10^7 in a 15cm dish for each of control chicken IgY and YB-1 immunoprecipitation antibody pull-down. YB-1 promoter complexes were isolated by chromatin immunoprecipitation using a polyclonal chicken antibody as previously described (Wu *et al.*, 2006). DNA was purified using the QIAquick PCR purification kit (Qiagen). A primer set designed to flank a YB-1 binding site (ATTG) in the first two kilobases of the *CD44* promoter was designed with the following sequence, annealing

position, and optimized annealing temperature (T_a): -1657 to -1900, F – TCAAACACAATTTTGCTTTTAGTAATG, R – TGGCTGCTTCTTAGTTGTGTG, T_a = 59ºC.

CD44 luciferase assay

To compare the direct effect of YB-1 levels on *CD44* promoter activity, cell lines were transfected with a 2kB promoter construct (Addgene) and a renilla expression vector, pRL-TK (Promega). Cells were plated in 6-well plates (5.5×10^5 cell/well) and transfected with a total of 1.5µg of DNA using FuGene HD Transfection Reagent (Roche, Indianapolis, IN, USA). Cells were harvested 48 hours post transfection in 1 x PLB buffer (Promega) and luciferase activity was measured. All luciferase measurements were normalized to the renilla readings for the matching sample.

Statistical Analysis

The results are reported as the mean ± standard deviation. Significance was determined using paired Student's *t*-test and p-values < 0.05 were considered significant and represented by a *.

3.3 RESULTS AND DISCUSSION

P-YB-1^{S102} is highly expressed in trastuzumab resistant cell lines.

A recent study by Ritter *et al* demonstrated the *in vivo* generation of cell lines with acquired trastuzumab resistance that serve as an attractive model to study as they emulate a typical course of clinical therapeutic resistance (Ritter *et al.*, 2007). Moreover, these resistant cell lines, HR5 and HR6, were shown to express increased levels of the YB-1 target gene *EGFR* (Ritter *et al.*, 2007), which we have extensively characterized (Stratford *et al.*, 2007; Wu *et al.*, 2006). Interestingly, we observed that protein levels of P-YB-1^{S102} and its activating kinase P-RSK (Stratford *et al.*, 2008) were significantly higher in the HR5 and HR6 cell lines in comparison to their parental BT474 counterpart (Figure 3.1 A). Importantly, P-RSK and P-YB-1^{S102} levels were persistent in the HR5 and HR6 cell lines following 72 hours of trastuzumab treatment, but dropped considerably in the trastuzumab sensitive cell line BT474 (Figure 3.1 B). Surprisingly, P-AKT^{S473} levels also diminished with trastuzumab exposure in all three cell lines suggesting that is not the kinase responsible for maintaining YB-1 phosphorylation.

Trastuzumab resistant cell lines depend on YB-1 for growth and survival.

We have previously reported on the dependence of HER2 positive breast cancer models on YB-1 for cell survival, growth *in vitro* and *in vivo* tumourigenesis (Lee *et al.*, 2008). Consequently, we inhibited YB-1 with targeting siRNA and found that the loss of YB-1 initiated apoptosis as indicated by increased levels of P-H2AX^{S139} and PARP cleavage in all three cell lines (Figure 3.1 C). Furthermore, we evaluated anchorage independent growth in the BT474, HR5, and HR6 cell lines with YB-1 depletion and found that colony formation was considerably hindered by 60% -

90%, suggesting that it is also essential for the growth and survival of both trastuzumab sensitive and resistant cell lines (Figure 3.1 C).

YB-1 inhibition further sensitizes cells to trastuzumab and can be used to overcome resistance.

To determine whether the expression of YB-1 contributes to a cells response to trastuzumab, we inhibited YB-1 with siRNA with simultaneous drug treatment and measured cell viability in the BT474, HR5, and HR6 cell lines. Interestingly, depletion of YB-1 alone resulted in a 40% reduction in viable cells (Figure 3.1 D). Further, as displayed in Figure 3.1 D, an approximate 60% reduction in proliferation was observed when cells were treated with trastuzumab with YB-1 inhibition. In fact, with the loss of YB-1, both sensitive BT474 and resistant HR5 and HR6 cell lines had similar responses to trastuzumab treatment. Additionally, inhibiting YB-1 expression with siRNA suppressed the growth of the BT474-m1 and AU565 cells in soft agar, which are HER2 positive and trastuzumab sensitive, by ~60%, moreover siYB-1 enhanced the sensitivity of these cells to trastuzumab (Supplemental Figure 3.1). To elaborate on this, in the BT474-m1 cells, it took 20µg/mL of trastuzumab to suppress colony formation by 95%. In contrast, only 0.2µg/mL of trastuzumab was required to have the same effect with simultaneous YB-1 inhibition. Also, pharmacological inhibition of YB-1 phosphorylation with BI-D1870, a RSK inhibitor, had a striking effect on growth and resulted in cell death in both sensitive and resistant cell lines (Figure 3.2 A-B). These findings suggest that a RSK dependent mechanism preserves YB-1 activation to promote resistance to trastuzumab therapy.

BT474 Flag-YB-1^{S102D} stable clones are trastuzumab resistant.

BT474 cells expressing Flag-YB-1^{S102D}, a phospho-mimic mutant of YB-1 was employed with the intention of generating a cell line capable of phenocopying acquired resistance. We therefore compared the response of all stable cell lines to trastuzumab in a monolayer growth assay. The BT474 YB-1^{S102D} mutant was capable of rendering the cell line insensitive to trastuzumab in contrast to the BT474 EV cell line (Figures 3.2 C-D). However, the inactive mutant, YB-1^{S102A} did not, providing further evidence that signal transduction pathways regulated by phosphorylated YB-1 are critical to the ability of the cells to escape the effects of trastuzumab.

Trastuzumab resistant cell lines have increased expression of CD44 which is regulated by YB-1.

In breast cancers, cells with a CD44^{+}/CD24$^{-/low}$/ESA^{+} phenotype have been defined as TICs (Al-Hajj *et al.*, 2003). These cells are more tumourigenic as demonstrated by *in vivo* serial dilution tumour formation assays in addition to their enhanced mammosphere forming abilities when compared to their non-TIC counterparts. More recently, TICs have been implicated in drug resistance and disease recurrence (Li *et al.*, 2008; Marangoni *et al.*, 2009). A study comparing paired tumour biopsies of patients before and after conventional chemotherapy demonstrated that breast cancer cells resistant to treatment were enriched in CD44^{+}/CD44$^{-/low}$ cells and in fact these resistant cells had higher mammosphere formation capability compared to the pre-treatment samples (Li *et al.*, 2008). We have previously implicated YB-1 as a regulator of a set of common genes associated with a stem cell signature (Finkbeiner *et al.*, 2009), including *CD44*. More recently, we demonstrated that YB-1 promotes tumour initiation by inducing *CD44*, an established TIC marker (To *et al.*, 2010). Here we find the HR5 and HR6 cell lines express

more CD44 transcripts as well as protein as compared to the BT474 cells (Figure 3.3 A) which correlates with increased mammosphere formation (Figure 3.3 B). There was also more P-YB-1^{S102} found in HR5 and HR6 cells grown as mammospheres (Figure 3.3 B). Further, when grown as mammospheres, the HR5 and HR6 cells expressed more CD44 as demonstrated by immunofluorescence analysis of whole mount mammospheres (Figure 3.3 C) whereas inhibition of YB-1 impaired mammosphere formation (Figure 3.3 D).

We next employed ChIP assays to demonstrate that YB-1 in fact was responsible for the regulation of *CD44* by showing promoter binding in our model of resistance, in fact the resistant cell lines displayed increased binding which also correlated to increased *CD44* promoter activity (Figure 3.4 A). The stable expression of YB-1^{S102D} in BT474 cells had similar effects by increasing CD44 protein levels (Figure 3.4 B). Similarly to the HR5 and HR6 models, the BT474 YB-1^{S102D} mutant cell line was more efficient in its ability to form mammospheres and also demonstrated a significant increase in *CD44* promoter activity (Figure 3.4 B). We assessed CD44 levels in the BT474 cell line following trastuzumab treatment for seven days to ask whether the remaining cells were CD44 positive. Interestingly, the remaining cells that survived treatment had a two fold increase in CD44 transcript levels (Figure 3.4 C). Further, when trastuzumab sensitive BT474 cells were grown as mammospheres in the presence of trastuzumab, they were unresponsive to therapy, again suggesting that TIC like cells are resistant to trastuzumab (Supplemental Figure 3.2). This suggests that a subset of cells exists within the bulk population which is intrinsically resistant to trastuzumab. Hence, we inhibited CD44 with siRNA with coinciding trastuzumab treatment and demonstrated that the combinatory treatment was capable of significantly reducing the number of viable cells (Figure 3.4 D).

Taken together, we have implicated YB-1 as a mediator of acquired trastuzumab resistance. The ability of YB-1 to induce a TIC phenotype in HER2 positive breast cancer cells desensitizes them to the effects of trastuzumab. Given YB-1's established association with cancer relapse, the ability of this transcription factor to regulate such a tumourigenic subpopulation sheds light on its ability to confer drug resistance. In a recent prospective study, high YB-1 expression signified a subset of HER2 positive patients that benefited from more aggressive chemotherapy (Gluz *et al.*, 2009). Our findings complement current literature correlating cancer recurrence to the inability of therapies to effectively terminate the TIC population within the bulk tumour.

To conclude, this study provides insight into a novel mechanism by which cancer cells acquire the ability of resistance to trastuzumab therapy. We nominate YB-1 as a candidate for molecularly targeted therapy with the hopes of treating acquired trastuzumab resistance by eliminating the TICs responsible for cancer recurrence and hence relapse.

3.4 FIGURES

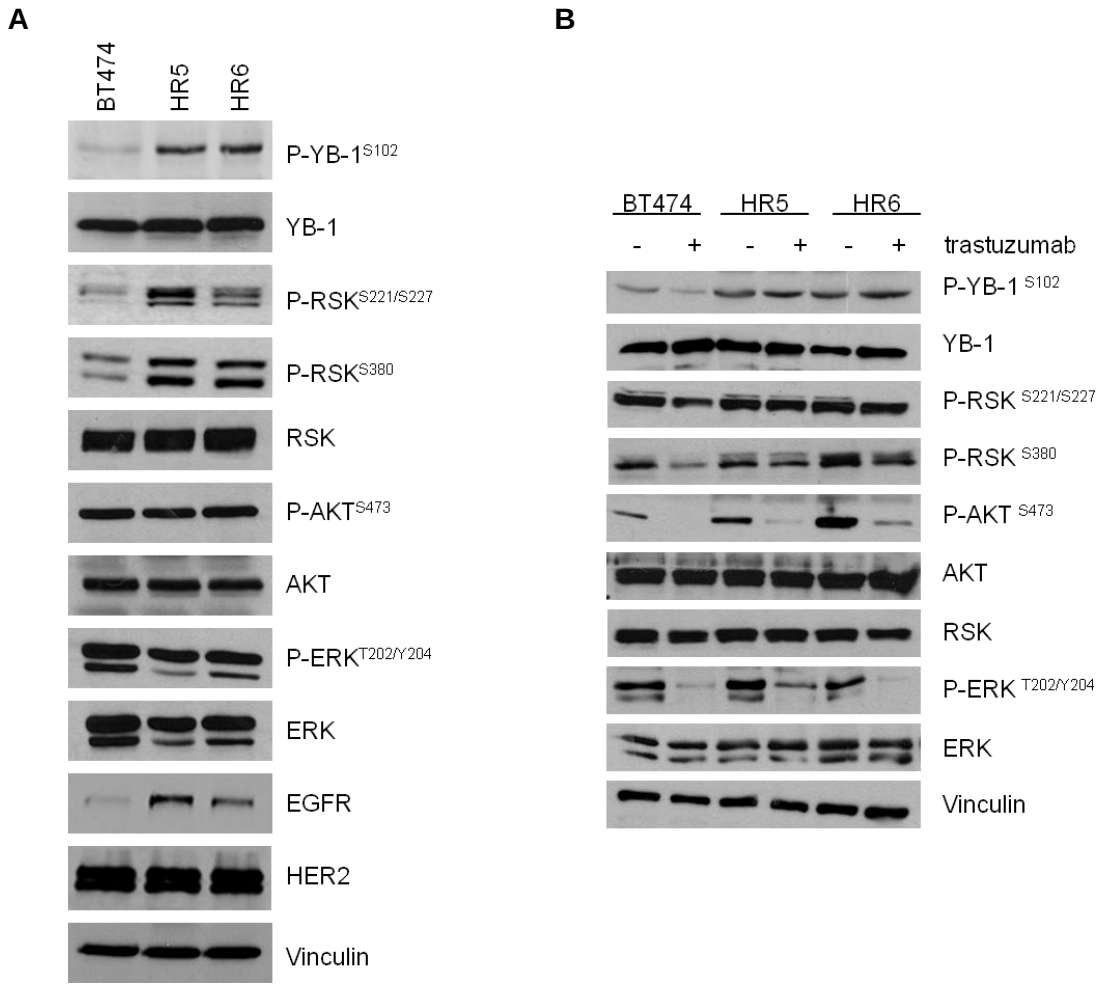
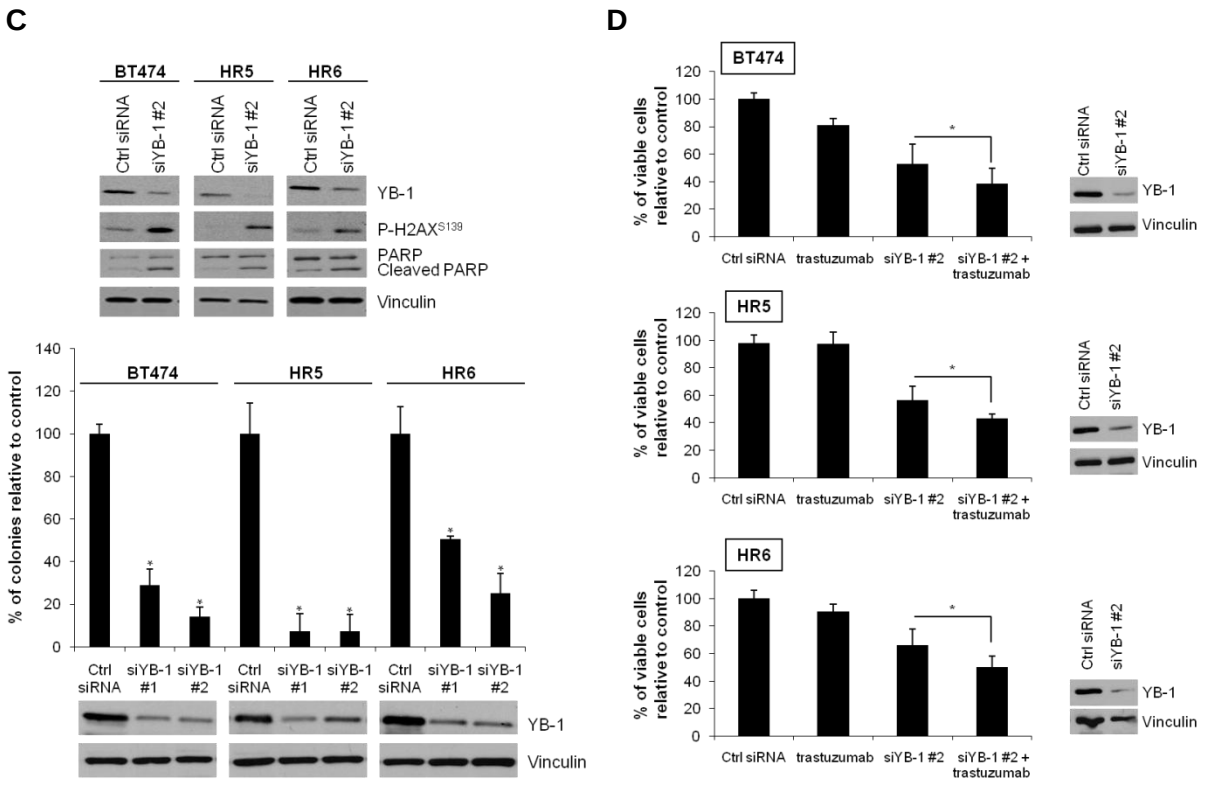


Figure 3.1. Trastuzumab resistant cell lines have increased levels of P-YB-1^{S102} and depend on YB-1 for growth and survival. A. BT474, HR5, and HR6 cells were lysed and protein levels were evaluated by immunoblotting. The trastuzumab resistant cells, HR5 and HR6, express increased levels of P-YB-1^{S102} protein and its activating kinase P-RSK compared to the sensitive BT474 cell line. **B.** BT474, HR5, and HR6 cells were treated with trastuzumab (20µg/mL) for 72 hours. Following, cells were harvested and protein expression was analyzed by immunoblotting.



C. Cells were transfected with 5nM of either control siRNA or siYB-1#2 for 72 hours. Following, cells were harvested and protein levels were determined by immunoblotting. Silencing YB-1 with siRNA induced apoptosis in all three cell lines as suggested by phosphorylation of H2AX and PARP cleavage. Also, following 24 hours of transfection, cells were seeded into soft agar. Inhibition of YB-1 markedly attenuated colony formation. YB-1 inhibition was confirmed by immunoblotting 72 hours post transfection. **D.** BT474, HR5, and HR6 cells were transfected with 5nM of either control siRNA or siYB-1#2 for 24 hours, after which trastuzumab (20µg/mL) was added to the growth media. Cell proliferation was analyzed 96 hours post transfection via MTS assay.

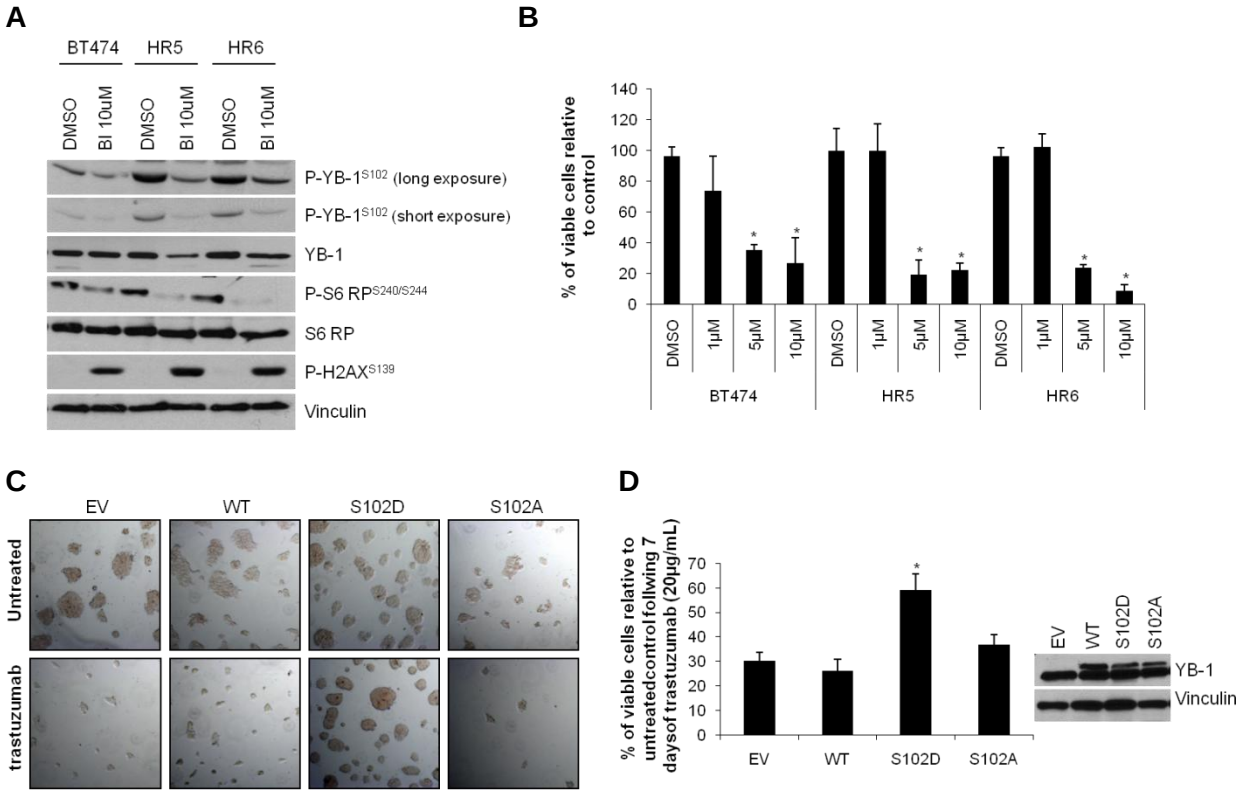


Figure 3.2. Phosphorylation of YB-1 is essential for cell viability and also promotes resistance to trastuzumab therapy. A. 72 hours of BI-D1870 treatment (10μM) decreased P-YB-1^{S102} protein levels and induced apoptosis as demonstrated by immunoblotting. Successful targeting of RSK was demonstrated by decreased levels of P-S6RP$^{S240/244}$, an established RSK substrate. **B.** Cells were treated with 1μM, 5μM, and 10μM of BI-D1870 for 72 hours after which cell growth was evaluated by the Cellomics ArrayScanner. BI-D1870 affected cell growth across all cell lines at 5μM and 10μM. **C.** BT474 stable cells were treated with trastuzumab (20μg/mL) for seven days. Representative photomicrographs of cells following treatment demonstrate cells expressing YB-1^{S102D} were less sensitive to trastuzumab. **D.** Following trastuzumab treatment, cell growth was quantified by nuclei staining and analysis by the Cellomics Arrayscanner. Immunoblot illustrates transgene expression in the BT474 stable cell lines.

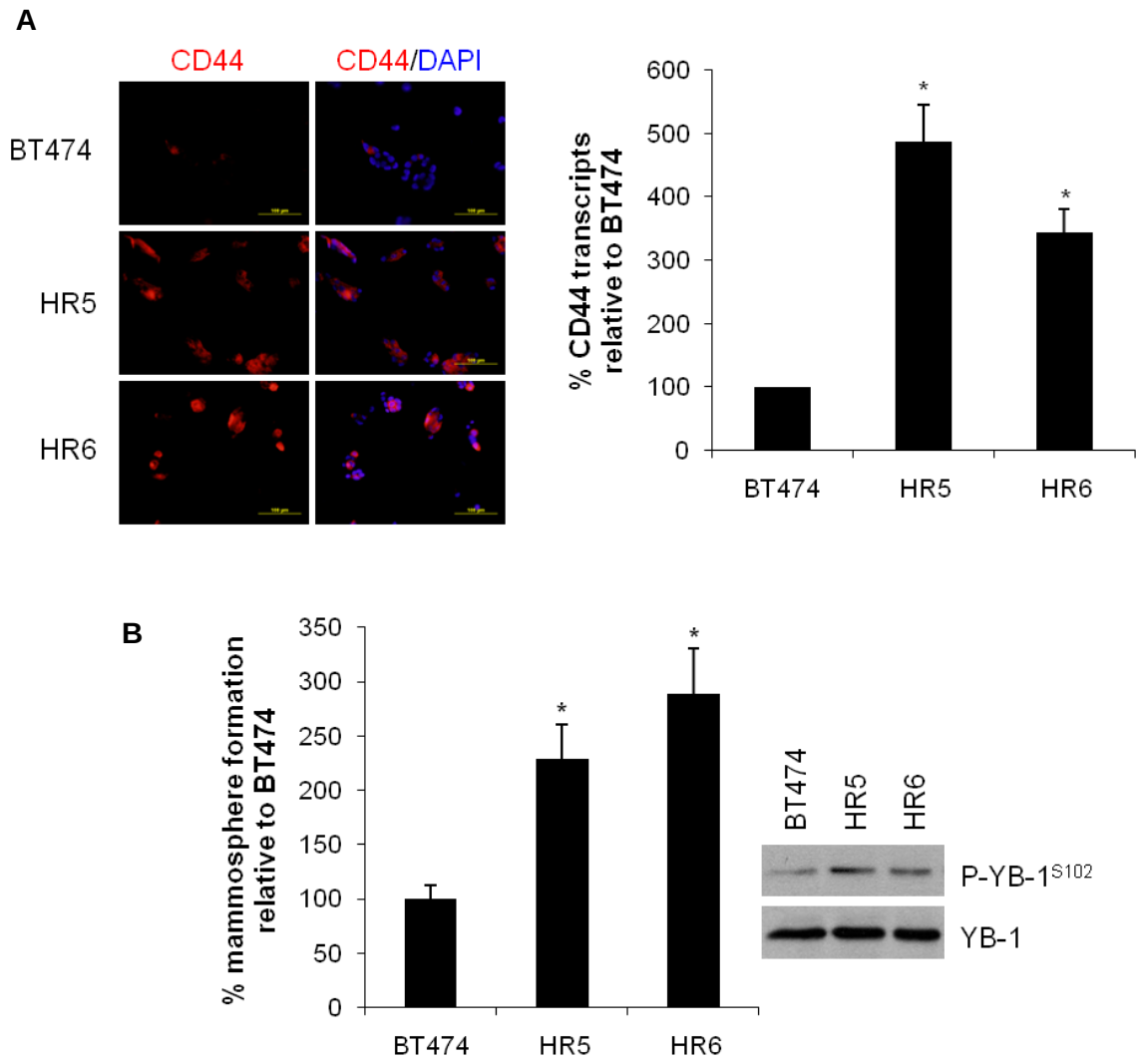
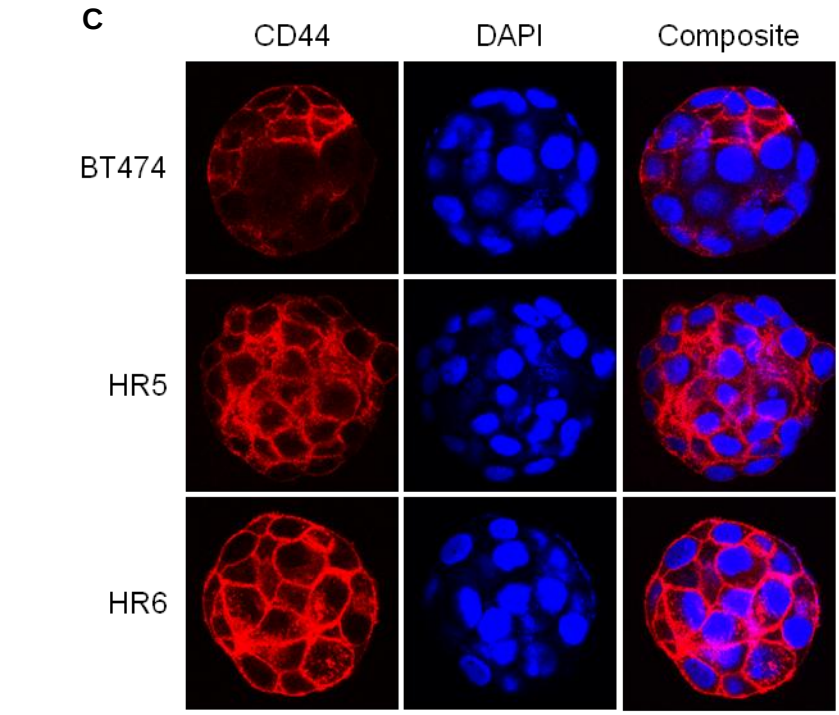
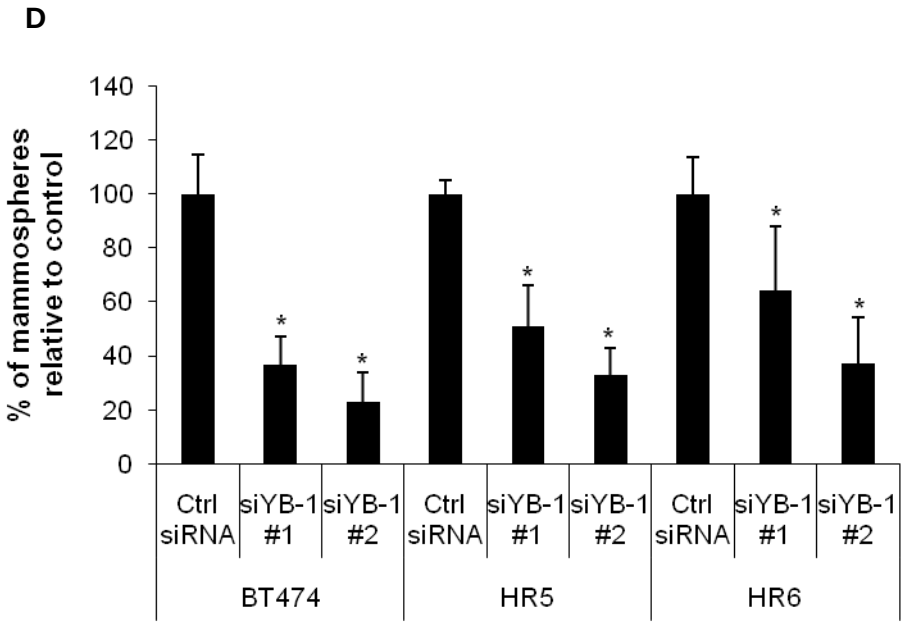


Figure 3.3. Trastuzumab resistant cell lines have increased levels of CD44. A. BT474, HR5 and HR6 cells were grown on glass coverslips. Following, cells were fixed and stained for CD44. Cells were imaged on the Olympus Fluorescence BX61. CD44 transcript levels were also characterized from RNA isolated from log growing cells. Trastuzumab resistant cell lines, HR5 and HR6, have increased CD44 protein as demonstrated by immunofluorescence and increased CD44 transcript levels as quantified by q-RT-PCR. **B.** Single BT474, HR5, and HR6 cells were seeded into the mammosphere assay at a density of 5 x 10^3 cells per 6-well dish. Mammospheres greater than 15 cells in size were counted at seven days. Mammospheres were subsequently isolated and lysed. Protein analysis of mammospheres shows increased levels of P-YB-1^{S102} in the resistant cell lines.





C. Cells grown as mammospheres were isolated and stained for CD44. Spheres were resuspended into a 1:1 solution of methanol:acetone and spotted onto glass slides and placed at -20ºC for 10 minutes and then air dried. Following, cells were stained at 4ºC with anti-CD44 overnight. Spheres were next washed, incubated in secondary antibody and mounted. Confocal microscopy illustrates increased expression of CD44 in spheres formed by the resistant cell lines. **D.** Cells were transfected with 5nM of control siRNA, siYB-1#1, or siYB-1#2 for 24 hours and then seeded into the mammosphere assay. Spheres were quantified 7 days later. Inhibiting YB-1 with siRNA decreased sphere formation efficiency.

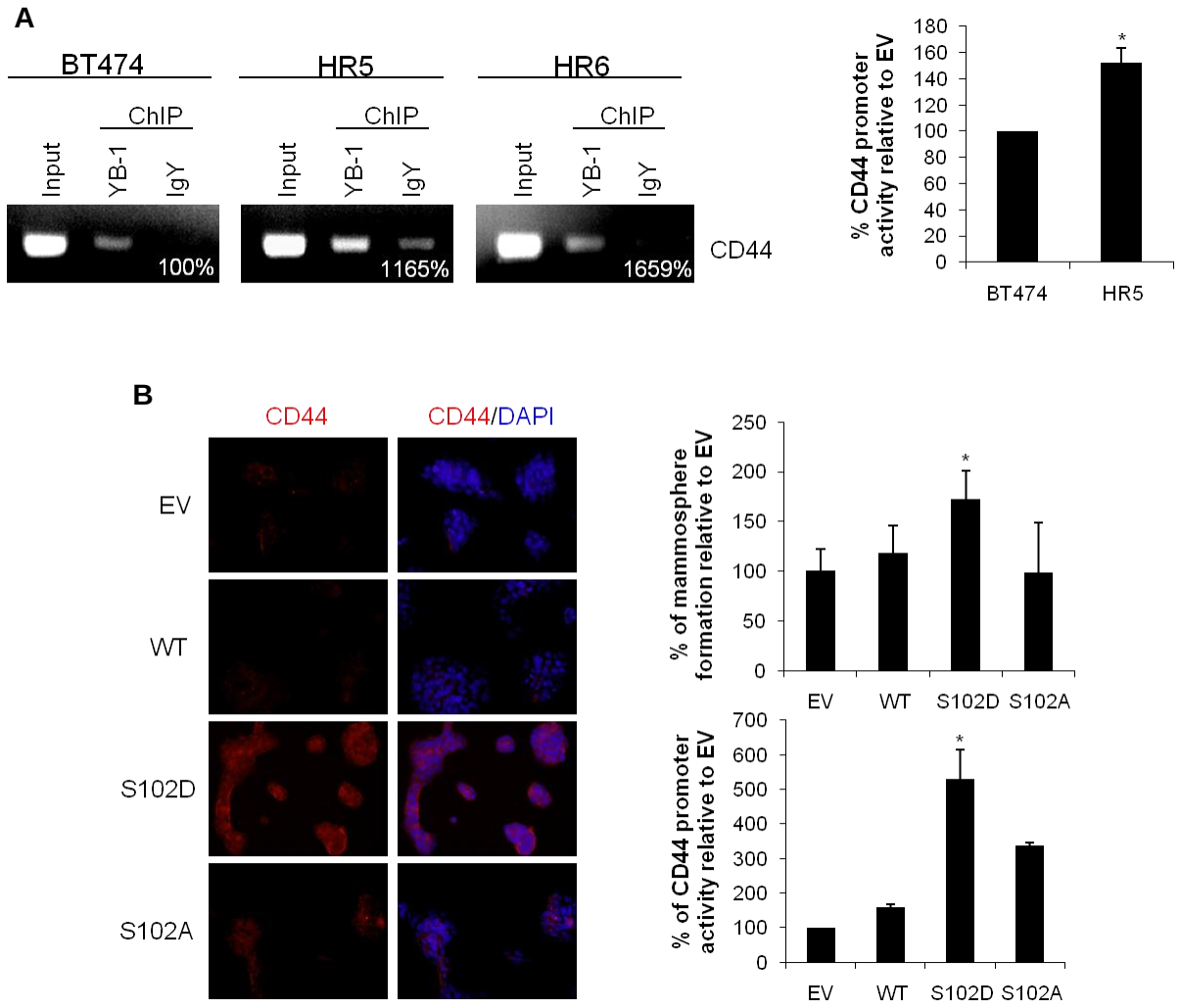
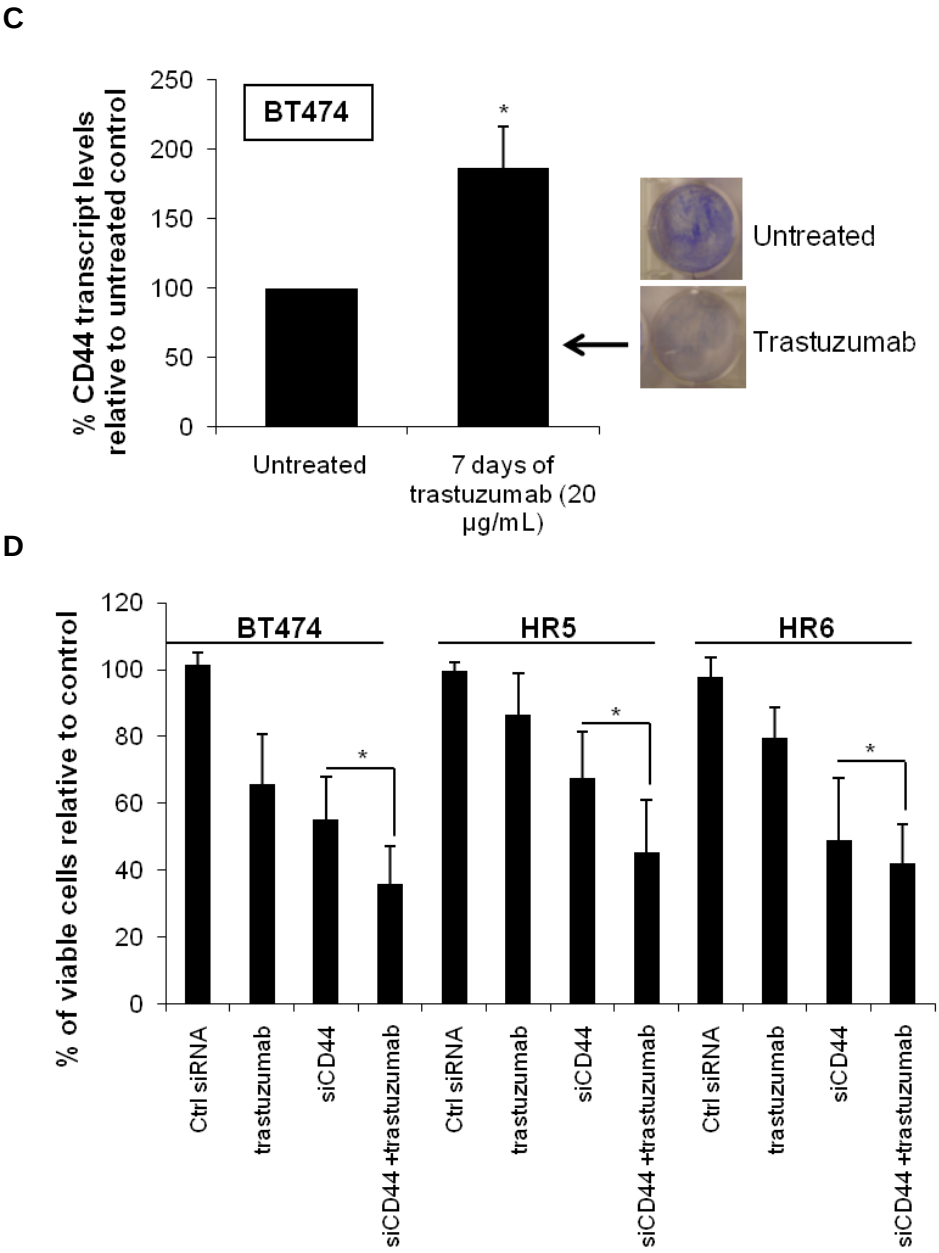
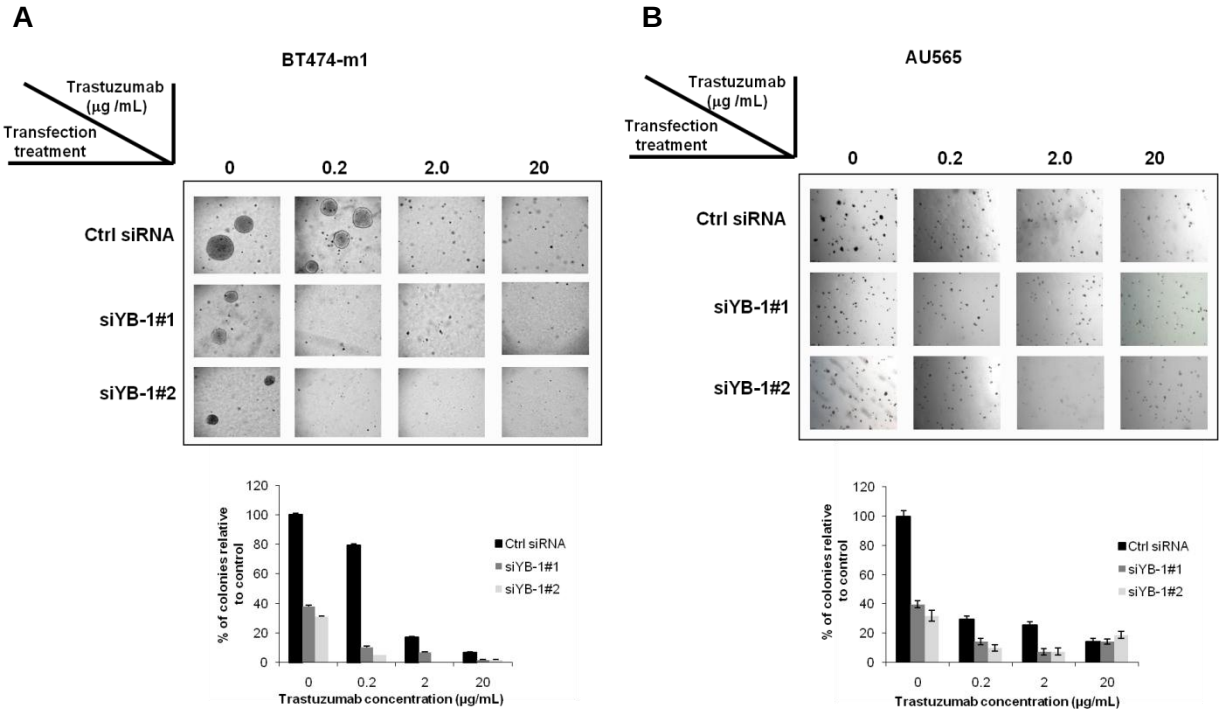


Figure 3.4. YB-1 binds to and regulates the *CD44* promoter thereby maintaining trastuzumab resistant cells. A. DNA complexes pulled down with either a YB-1 or non-immune IgY were amplified by PCR reactions using a primer set flanking the human *CD44* promoter. Representative ethidiom bromide stained agarose gel results demonstrate enhanced binding in the resistant cell lines. The images were quantified by taking into account nonspecific binding to the IgY. The BT474 and HR5 cells were transfected with a *CD44* promoter construct and a *Renilla* expression vector. Luciferase activity was measured 48 hours after transfection and all luciferase measurements were normalized to the renilla readings for the matching sample. The HR5 cells exhibited a 1.6 fold increase in *CD44* promoter activity compared to the BT474 cell line. **B.** The BT474 YB-1^{S102D} cell lines express higher levels of CD44 protein as demonstrated by immunofluorescence and also exhibit an enhanced potential to form mammospheres in addition to a significant increase in *CD44* promoter activity.

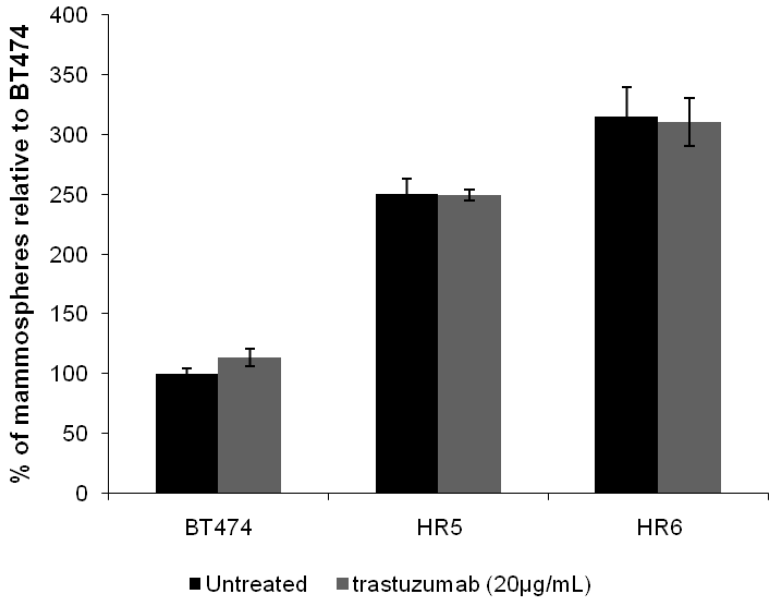


C. BT474 cells were treated with trastuzumab (20µg/mL) for seven days after which CD44 mRNA levels were assessed by q-RT-PCR. Viable cells following trastuzumab treatment were enriched two-fold in CD44 levels compared to the untreated control. **D.** BT474, HR5, and HR6 cells were transfected with 20nM of either control siRNA or siCD44 for 24 hours. Following, trastuzumab (20µg/mL) was added to the growth media for 72 hours. Cell viability was assessed following 96 hours by MTS assay.

3.5 SUPPLEMENTARY FIGURES



Supplemental Figure 3.1. Inhibition of YB-1 enhances the suppression of colony formation in soft agar in combination with trastuzumab. HER2 positive, trastuzumab sensitive cells BT474 (**A**) and AU565 (**B**) cells were transfected with control siRNA or siRNA against YB-1 for 48 hours and then seeded into soft agar with trastuzumab at concentrations of 0, 0.2, 2, and 20 µg/mL. Colonies were quantified 30 days later. Representative photomicrographs demonstrate the additive effects of YB-1 depletion with trastuzumab treatment on colony inhibition and are quantified in the bar graphs.



Supplemental Figure 3.2. Trastuzumab is ineffective at preventing mammosphere formation. BT474, HR5, and HR6 cells were seeded into the mammosphere assay at 5000 cells/well and treated with 20µg/mL of trastuzumab. Mammospheres were counted 7 days later. Trastuzumab was ineffective at hindering sphere formation.

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CHAPTER FOUR: DISCUSSION AND FUTURE DIRECTIONS

4.1 SIGNIFICANCE OF YB-1 IN HER2 OVER-EXPRESSING BREAST CANCER

Breast cancer is a disease characterized as being molecularly heterogeneous. While the gain and loss of chromosomes may seem random, amplifications in *HER2* are recurrent with a frequency of 25-30% of reported cases (Slamon *et al.*, 1987). The resultant receptor over-expression typifies cancer cells with an aggressive and metastatic phenotype associated with unfavourable clinical outcomes (Slamon *et al.*, 1987; Slamon *et al.*, 1989). The ability of HER2 to transform cells rests on its influence on a multitude of cellular processes. At the cell surface, HER2 interacts with a range of membrane receptors including EGFR (Yarden and Sliwkowski, 2001), MET receptor (Khoury *et al.*, 2005), CD44 (Bourguignon *et al.*, 1997b), IGF-IR (Balana *et al.*, 2001) and integrins (Falcioni *et al.*, 1997), allowing for the activation of divergent signal transduction cascades that elicit aberrant cellular growth and survival, thereby mediating pathogenesis.

Studies presented herein characterize the function of YB-1 in the HER2 subtype of breast malignancy. The data in Chapter Two introduces the necessity of YB-1 for the growth and survival of both HER2 positive and the triple negative subtypes of breast cancer cells. Moreover, in HER2 over-expressing breast cancer cells, YB-1 inhibition induced apoptosis. A molecular mechanism involving the STAT3 pro-survival pathway was identified to be dampened with YB-1 silencing which altered the apoptotic threshold of the cells. Furthermore, we reveal that silencing YB-1 suppressed *in vitro* anchorage independent growth and also inhibits tumourigenesis *in vivo*. Related to these findings, we next explored the role of YB-1 in a model

of acquired trastuzumab resistance. Given YB-1's association with the HER2 subtype and the common clinical hurdle of resistance to trastuzumab, a specific HER2 targeting therapy, we questioned whether this transcription factor also contributes to the acquisition of a resistant phenotype. Within Chapter Three, we demonstrate that protein levels of P-YB-1^{S102} are elevated in two trastuzumab resistant cell lines, in addition to its activating kinase, P-RSK. Further, the RSK/YB-1 signalling pathway remains engaged following trastuzumab treatment while it is hindered in the sensitive counterpart. The significance of P-YB-1^{S102} in contributing to resistance was made evident by successfully establishing a cell line mimicking the resistant phenotype by expressing a constitutively active mutant of YB-1. Furthermore, we provide evidence that P-YB-1^{S102} regulates *CD44*, an established stem cell associated factor which is found to be elevated in the resistant models. The increased CD44 levels in the trastuzumab resistant cells contribute to their enhanced propensity for mammosphere formation. This work implicates cells with tumour-initiating properties as the culprit population responsible for escaping trastuzumab therapy. We have demonstrated that P-YB-1^{S102} sustains tumour-initiating cells as defined by their CD44 expression and hence promotes a resistant phenotype. Overall, the data presented in this thesis provide evidence for the requirement of YB-1 in HER2 positive breast cancer and suggests that it may be a contributing factor to the poor prognosis and tumour recurrence associated with this subtype.

4.2 IMPLICATIONS AND FUTURE DIRECTIONS

YB-1 regulates STAT3 mediated pro-survival signalling

Having observed that the loss of YB-1 led to the induction of apoptosis, we sought to understand why this occurred at the molecular level. We queried the function of STAT3 in altering the

apoptotic threshold of BT474-m1 cells following the loss of YB-1 function via siRNA treatment. The activation of STAT3 has been demonstrated to be an event downstream of HER2 (Fernandes *et al.*, 1999) and EGFR (Garcia *et al.*, 1997), both of which are YB-1 target genes (Stratford *et al.*, 2007; Wu *et al.*, 2006). Furthermore, Tan *et al* demonstrated inhibition of *in vivo* tumourigenesis in xenograft models of HER2 over-expressing breast cancer cells employing a cell permeable peptide blocking STAT3 activity (Tan *et al.*, 2006). In fact, STAT3 suppression has been shown to evoke apoptosis in cancers of the breast (Gritsko *et al.*, 2006), and prostate (Lee *et al.*, 2004), and in gliomas (Iwamaru *et al.*, 2007) and astrocytomas (Konnikova *et al.*, 2003). This ability stems from STAT3's regulation of *survivin* (Gritsko *et al.*, 2006), *BCL2* (Real *et al.*, 2002), *Bcl-x_L* (Bromberg *et al.*, 1999), and *MCL1* (Liu *et al.*, 2003), proteins that have anti-apoptotic functions. Traditionally, the function of STAT3 has been attributed to its phosphorylation at tyrosine 705 which leads to protein dimerisation (Levy and Darnell, 2002), while subsequent phosphorylation at serine 727 has been suggested to enhance nuclear transport and DNA binding (Wen *et al.*, 1995). Interestingly, we show decreases in P-STAT3^{S727} with YB-1 inhibition whereas P-STAT3^{Y705} was not detectable in any of the HER2 over-expressing breast cancer cell lines under investigation (Appendix I), which is further supported by Berishaj *et al* (Berishaj *et al.*, 2007). YB-1 itself does not have kinase activity, therefore decreases in P-STAT3^{S727} must be the result of changes in the activity of kinases which are associated with YB-1 signalling pathways or are directly regulated by the transcription factor. ERK (Chung *et al.*, 1997) and mTOR (Yokogami *et al.*, 2000) are two potential kinases that have previously been reported to phosphorylate STAT3 at serine 727. We show that inhibiting YB-1 with siRNA in HER2 over-expressing breast cancer cell lines resulted in decreased levels of both P-ERK$^{T202/Y204}$ and P-mTOR^{S2448}. To identify the kinase responsible for the phosphorylation of STAT3 at serine 727, we inhibited mTOR with rapamyacin, as well as ERK with PD98058 in the BT474-m1 cell line. A reduction in P-STAT3^{S727} was observed with pharmacological

inhibition of either ERK or mTOR, suggesting that both kinases are involved in the activation of STAT3 (Appendix II). To further characterize STAT3 phosphorylation and validate the drug inhibition studies, a rescue experiment should be performed. This would entail inhibiting YB-1 with siRNA and attempting to rescue phosphorylation of STAT3 at serine 727 with ectopic expression of either mTOR or ERK.

Interestingly, total levels of mTOR decreased with the loss of YB-1. Transcriptional regulation of *MTOR* was ruled out since mTOR transcript levels did not decrease with siRNA inhibition of YB-1. The mechanism by which total mTOR protein levels decrease is an avenue open for exploration as the regulation of mTOR expression has not been characterized. Yet the importance of mTOR in cancer is evident (Guertin and Sabatini, 2007). Protein levels of mTOR have been shown to be directly regulated by the tumour suppressor F-box and WD repeat domain-containing 7 (FBXW7) (Mao *et al.*, 2008). FBXW7 binds to and marks mTOR for ubiquitination and subsequent degradation (Mao *et al.*, 2008). Therefore, YB-1 may directly or indirectly be involved in hindering the expression of FBXW7; hence with YB-1 depletion, FBXW7 has the opportunity to control total mTOR levels. It would be of interest to assess FBXW7 levels in the HER2 over-expressing cells BT474-m1 and Au565 following YB-1 inhibition to query whether the observed decrease in total mTOR is potentially the result of protein ubiquitination mediated by FBXW7. Given YB-1's role in translation, it would be interesting to explore its involvement in the translational control of mTOR. Of note, cytoplasmic YB-1 has been demonstrated to interact with a number of mRNA species, where it functions to suppress translation (Evdokimova *et al.*, 2006a). Upon activation by phosphorylation at serine 102, YB-1 is shuttled to the nucleus, thereby allowing for the translation of a number of oncogenic transcripts including VEGF and FOS (Evdokimova *et al.*, 2006b). Therefore, it is

unlikely that decreased mTOR protein is a direct result of YB-1 functioning in translation. In fact if this was the case, mTOR protein levels would be expected to rise with the loss of YB-1. We observed that silencing of YB-1 leads to a downregulation of mTOR and phosphorylation of STAT3, however, we cannot determine whether they are related events at this time.

STAT3 has been shown to promote tumour cell survival and the development of malignancies. How this occurs is of great debate. Classically, it was thought that STAT3 required phosphorylation at serine 727 and tyrosine 705 in order to optimally function. However, STAT3 is reportedly capable of functioning as a transcription factor in the absence of phosphorylation at tyrosine 705 in macrophage survival (Liu *et al.*, 2003) and also in NOTCH signalling in stem cells (Androutsellis-Theotokis *et al.*, 2006). Moreover, the human prostate cancer cell line LNCaP expressing mutated STAT3, where the tyrosine 705 residue was changed to an phenylalanine and the serine 727 mutated to glutamic acid, was capable of *in vivo* tumourigenesis, suggesting that it retains its oncogenic abilities, independent of tyrosine phosphorylation (Qin *et al.*, 2008). Furthermore, Pin1 mediated activation of STAT3 at its serine 727 residue has also been demonstrated to recruit p300 (acetyltransferase), a STAT3 co-factor, which allows for P-STAT3^{S727} dependent gene transcription (Lufei *et al.*, 2007). Interestingly, intrinsic transferase activity of p300 regulates STAT3 dimer formation via acetylation of lysine at residue 685 within the SH2 domain (Yuan *et al.*, 2005). Given this, it is plausible that YB-1 may also physically interact with STAT3 in a complex with p300 given that a YB-1/p300 interaction has been described (Higashi *et al.*, 2003). If so, YB-1 inhibition may allow p300 to interact with its preferential binding partners and in doing so, decrease STAT3 transcriptional activity. It would be worthwhile to assess YB-1/STAT3/p300 interactions by co-immunoprecipitation studies. Also, we show that MCL-1 protein levels are decreased with the

loss of YB-1 and STAT3. Furthermore, we used electrophoretic mobility shift assays to demonstrate nuclear STAT3 binding to a representative binding site within the *MCL1* promoter. MCL-1 regulation can also occur in a transcriptionally independent mechanism mediated by glycogen synthase kinase-3β (GSK3β) (Ding *et al.*, 2007). GSK3β directly phosphorylates MCL-1, marking it for proteosome mediated turnover (Ding *et al.*, 2007). However, with YB-1 inhibition, we did not observe changes in P-GSK3β^{S9} or its activating kinase P-AKT^{S473}. Beyond *MCL1*, additional STAT3 downstream targets such as *BCL2* (Real *et al.*, 2002), *BCL-x$_L$* (Bromberg *et al.*, 1999), and *survivin* (Gritsko *et al.*, 2006) function as regulators of apoptosis. We did not observe changes in survivin levels with decreased P-STAT3^{S727} and preliminary evaluation of BCL-x$_L$ provided inconclusive results. Therefore, it would be worth analyzing additional STAT3 targets which function as mediators of apoptosis following YB-1 knockdown. With the current set of data, we implicate STAT3 in promoting a survival advantage in HER2 positive breast cancer cells by its transcriptional regulation of *MCL1*.

YB-1 and acquired trastuzumab resistance

The benefits of directly targeting HER2 with trastuzumab have been demonstrated (Cobleigh *et al.*, 1999; Slamon *et al.*, 2001; Vogel *et al.*, 2002); however the molecular mechanism by which the antibody exerts its anti-tumour effect remains to be fully understood. Further to this, many patients with *HER2* amplifications fail to respond to initial trastuzumab treatment or escape the effects of the antibody within one year, suggesting both intrinsic and acquired mechanisms of therapeutic resistance. Various molecular theories have been proposed to describe how tumour cells gain the ability to escape the effects of trastuzumab, however they have failed to identify a reliable biomarker which would aid in predicting one's response to therapy.

We investigated the role of P-YB-1^{S102} in an *in vivo* model of acquired trastuzumab resistance generated by Ritter *et al* which emulates a typical course of antibody therapy (Ritter *et al.*, 2007). Initial studies ruled out changes in *HER2* receptor levels as a possible explanation (Ritter *et al.*, 2007). They also explored antibody insensitivity to antibody-dependent cellular cytotoxicity as a potential mechanism of resistance but this did not turn out to be the cause. A defect in molecular signalling was next considered. As such, they report that the resistant cells express higher levels of EGFR (Ritter *et al.*, 2007). Since *EGFR* is a YB-1 target gene (Stratford *et al.*, 2007; Wu *et al.*, 2006), we queried the function of P-YB-1^{S102} in trastuzumab resistance. In our work, we provide evidence for the requirement of YB-1 in both resistant and sensitive breast cancer cell lines as demonstrated in monolayer and soft agar assays. Moreover, we suspect P-YB-1^{S102} as a causative agent in generating a resistant phenotype as activated protein levels withstand trastuzumab treatment in the HR5 and HR6 cell lines. Supportive of this, over-expression of a constitutively active mutant of YB-1 rendered the trastuzumab sensitive BT474 cell line resistant to therapy whereas the inactive mutant YB-1^{S102A} failed to convey drug resistance.

Considering YB-1's function as a transcription factor that regulates a number of cancer related genes, a rational idea for its contribution to trastuzumab resistance would be its modulation of gene expression and the resultant changes in protein levels. We have evidence that following trastuzumab treatment, P-YB-1^{S102} nuclear levels are sustained in the resistant cell lines whereas levels decrease in the sensitive BT474 cells (submitted). A related research project in the lab investigated this further by performing a ChIP-sequencing study in the BT474, HR5, and HR6 cells following trastuzumab treatment to identify changes in gene expression in the resistant models by sequencing YB-1 bound DNA complexes. ChIP-sequencing is more advantageous

than a ChIP-on-chip study as the former technique is capable of an in depth genome wide analysis whereas microarray platforms are limited to specific genomic regions. To further support the current set of gene expression changes, it would be important to also complete the ChIP-sequencing study with the BT474 YB-1^{S102D} cells. The binding profiles could then be compared to those identified in the resistant cell lines. We have previously confirmed the ability of YB-1^{S102D} to induce *MET* (Finkbeiner *et al.*, 2009) and *PIK3CA* (Astanehe *et al.*, 2009), hence changes similar to those observed in the HR5 and HR6 cells should be expected in the BT474 YB-1^{S102D} cells. The MET receptor was in fact identified in the ChIP-Sequencing study of YB-1 target genes in the HR5 and HR6 cells. Furthermore, the resistant cells were confirmed to have greater amounts of MET receptor as compared to the BT474 cells (submitted). Interestingly, the MET receptor has been previously implicated in trastuzumab resistance (Shattuck *et al.*, 2008). Therefore, YB-1 mediated trastuzumab resistance may also be a result of its induction of *MET*. Taken together, these findings suggest that YB-1's regulation of the transcriptional circuitry of a cell is capable of rendering it insensitive to trastuzumab by bringing about changes to a vast array of signalling pathways.

A recent study utilizing the HR5 and HR6 cells to study acquired trastuzumab resistance implicated mTOR as a mediator of drug insensitivity. Miller *et al* demonstrated that mTOR inhibition with RAD001 significantly impeded the growth of both sensitive BT474 and resistant HR5 and HR6 cells (Miller *et al.*, 2009). During the preliminary characterization phase of the BT474, HR5, and HR6 cells, we did not observe increased levels of activated mTOR in the resistant models compared to their sensitive counterpart, however we did not look at mTOR's downstream target p70 S6 kinase, which was shown to be elevated by Miller *et al*. Interestingly, in Chapter Two, we provide evidence for the downregulation of mTOR with YB-1 inhibition.

Perhaps the sensitization of the HR5 and HR6 cells to trastuzumab amidst YB-1 exhaustion (Chapter Three) was an effect mediated by decreased signalling via the mTOR pathway. It would be of interest to further examine mTOR in the HR5 and HR6 cells. Initially, mTOR protein levels should be assessed following YB-1 inhibition. If levels do decrease, the functional value of mTOR could be further investigated in trastuzumab resistance.

Altered signalling seems to be a causative explanation for trastuzumab resistance based on EGFR over-expression. This was reported by Ritter *et al* and confirmed independently by us. The events following EGFR receptor activation in these models is where our results differ from Ritter *et al*. They show increased levels of P-AKT^{S473} in the resistant HR5 and HR6 cell lines, but we do not, rather we find higher activation of the related serine/threonine kinase RSK. This can be attributed to the elevated EGFR levels in the HR5 and HR6 cells, as activation of EGFR by EGF has been shown to lead to RSK phosphorylation (Nam *et al.*, 2008). Further, we suggest that the MAPK pathway promotes a resistant phenotype by RSK's phosphorylation of YB-1 at serine 102. AKT mediated YB-1 activation was ruled out since phosphorylated levels of YB-1 were sustained while P-AKT^{S473} decreased. These studies prompted the question as to whether trastuzumab resistant cells rely on RSK signalling.

To address whether RSK was important to the growth of the trastuzumab resistant cells, it was inhibited with either a small molecule inhibitor or siRNA. Direct inhibition of RSK with BI-D1870, an ATP competitive inhibitor, suppressed phosphorylation of YB-1 and also induced apoptosis. However, BI-D1870 is not completely specific to RSK alone as it also targets Aurora B and polo-like kinase-1 (PLK1) (Sapkota *et al.*, 2007). To complement the BI-D1870 studies

and confirm apoptosis was induced by RSK inhibition, we targeted both RSK1 and RSK2 with siRNA and demonstrated an induction of cell death, thereby eliminating the potential involvement of additional kinases (Appendix III). In our RSK knockdown experiment, we were unable to achieve a substantial RSK1 knockdown. However, decreases in RSK2 alone were enough to induce apoptosis. It would be necessary to optimize the siRNA transfection conditions for RSK1 and evaluate its role in apoptosis. Also, since RSK2 knockdown alone was sufficient to induce cell death, it would be necessary to evaluate RSK1 and RSK2 inhibition separately to figure out their individual contributions. Interestingly, we have demonstrated that with an effective inhibition of RSK1 and RSK2, the trastuzumab resistant cells have an increased response to trastuzumab (submitted). To further substantiate our proposal of the RSK/YB-1 signalling pathway in trastuzumab resistance, it would be important to transform the sensitive BT474 cell line with myristolated (myr) RSK, a constitutively active mutant of the kinase, and assess cell growth in the presence of trastuzumab. In addition to this, cell growth and apoptosis should be evaluated in the stable cell lines following BI-D1870 to determine whether YB-1^{S102D} expression is capable of rescuing the downstream effects of RSK inhibition thereby confirming RSKs involvement in trastuzumab resistance.

Finally, to supplement our *in vitro* evidence implicating P-YB-1^{S102} in trastuzumab resistance, an *in vivo* study would be necessary to absolutely confirm YB-1 as a biomarker for drug resistance. This could be completed by establishing xenografts employing the BT474 YB-1^{S102D} stable cell lines in mice, followed by trastuzumab treatment. Tumour regression would then be monitored and the response of the BT474 YB-1^{S102D} cells would be evaluated after observation of complete tumour regression in the BT474 EV xenografts. We expect that BT474 YB-1^{S102D} tumours would be unresponsive to trastuzumab treatment.

Resistance to therapy by YB-1's induction of tumour-initiating cells

YB-1's contribution to drug resistance is evident by its regulation of *MDR1* (Goldsmith *et al.*, 1993; Stein *et al.*, 2001), *MVP* (Stein *et al.*, 2005), and *MRP1* (Stein *et al.*, 2001). Further, upon treatment with paclitaxel, there appears to be an increase in nuclear localization of YB-1, with a concordant increase in P-glycoprotein levels in primary breast cancer patient samples (Fujita *et al.*, 2005). Supportive of this, upon trastuzumab treatment, HR5 and HR6 resistant cell lines maintain nuclear P-YB-1^{S102} levels. These findings suggest YB-1 is able to retain its function as a transcription factor in the presence of therapy targeting tumour cells and thereby perhaps implement their escape from treatment. In fact gene expression analysis of residual tumour cells following docetaxel treatment identified the expression of genes associated with cell survival and the cell cycle to be increased in comparison to the tumour prior to drug therapy (Chang *et al.*, 2005), findings which corroborate genes regulated by YB-1.

More recently, drug resistance has been associated with tumour-initiating cells (TICs). These cells represent a minor fraction of a tumour and have unique stem-cell like qualities which allow them to regenerate disease via their capacity for self-renewal and differentiation (Reya *et al.*, 2001). Breast TICs are identified by their unique cell surface marker profile $CD44^{+}/CD24^{-/low}$ (Al-Hajj *et al.*, 2003) and also increased aldehyde dehydrogenase-1 (ALDH1) activity (Ginestier *et al.*, 2007). ALDH1 is an intracellular detoxifying enzyme that functions to oxidize aldehydes (Sophos and Vasiliou, 2003). In stem cells, ALDH1 has been associated with differentiation by its function in the oxidation of retinol to retinoic acid (Chute *et al.*, 2006). A current model to explain drug resistance in cancer is the ability of TICs to survive therapy as they are inherently resistant, thus allowing them to regenerate disease, hence providing a rationale for cancer

recurrence and relapse (Dean *et al.*, 2005). The theory has been validated in the clinical setting by the demonstration of an elevation in the TIC population, defined by $CD44^{+}/CD24^{low/-}$ following neoadjuvant chemotherapy in patients with advanced breast cancer (Li *et al.*, 2008). Subsequent studies have further characterized this subpopulation of cells as being resistant to therapy (Creighton *et al.*, 2009; Fillmore and Kuperwasser, 2008; Marangoni *et al.*, 2009). TICs therefore represent a logical target for improving the treatment of cancer.

Several lines of evidence point toward YB-1 in the regulation of TICs. We investigated YB-1 in a cohort of >4000 breast cancer cases representative of all tumour subtypes and correlated its expression with relapse and poor survival (Habibi *et al.*, 2008). Moreover, in a subset of the cohort under investigation, YB-1 was also predictive of decreased survival in patients treated with tamoxifen (Habibi *et al.*, 2008). In addition, we also determined *YB1* expression in bipotent progenitor cells from reduction mammoplasty samples, suggesting YB-1 plays a function in the normal primitive stem cell (Finkbeiner *et al.*, 2009). Further to this, a ChIP-on-chip analysis using promoter arrays in a basal like breast cancer model identified a number of YB-1 target genes, including *CD44*, *CD49f*, *NOTCH*, and *WNT*, which are associated with stem cells (Finkbeiner *et al.*, 2009). In addition, HER2 has been demonstrated to regulate breast stem cells (Korkaya *et al.*, 2008) and TICs have also been isolated from tumours generated in Neu-MMVT transgenic mice models (Liu *et al.*, 2007a). Besides, activation of YB-1 by RSK lies downstream of HER2 signalling. Hence, YB-1's relationship to TICs was explored as a potential mechanism of acquired trastuzumab resistance.

To corroborate the hypothesis that TICs are drug resistant, we observed increased levels of CD44 protein and mRNA in the trastuzumab resistant cells which paralleled their enhanced efficiency for mammosphere formation. Correspondingly, expression of the YB-1^{S102D} mutant in BT474 cells caused the cells to acquire insensitivity to trastuzumab which correlated to their increase in CD44 protein, *CD44* promoter activity, as well as an elevation in mammosphere formation efficiency. Coupled to the *in vitro* evidence of a TIC phenotype in the trastuzumab resistant cell lines, we found that trastuzumab was ineffective in eliminating TICs. In mammosphere assays, trastuzumab had no effect on sphere formation in all three cell lines suggesting that it lacks the ability to function on this subset of cells. Furthermore, after challenging the sensitive BT474 cells with the antibody, the residual cells were enriched for CD44. Additionally, we established an *in vitro* model of a trastuzumab resistant cell line from the BT474 cells by long-term treatment with trastuzumab (described as BT474LT). The resultant population expressed increased levels of CD44 protein and mRNA (Appendix IV) which correlated with increased levels of P-YB-1^{S102}, P-RSK^{S380}, and P-$RSK^{S221/S227}$ (data not shown). We therefore propose that trastuzumab is incapable of exerting its anti-tumourigenic effects against TICs. Contrary to our hypothesis, Magnifico *et al* report that trastuzumab is successful in eradicating TICs (Magnifico *et al.*, 2009). However, they fail to employ established markers such as CD44 to identify potential TICs and rely on the mammosphere assay as their method of TIC identification. Further, the trastuzumab concentrations used in their studies are extremely high and do not represent drug levels achievable in patients. Nevertheless, our data and recent literature supports the idea that TICs are unresponsive to therapies. Hence targeting this population of cells may aid in improving the therapeutic response of patients to trastuzumab.

Collectively, the data presented herein demonstrate that YB-1 mediated signalling sustains a TIC population. Given that our *in vitro* model of trastuzumab resistance, BT474LT, has increased levels of activated RSK and YB-1 and that the BT474 YB-1^{S102D} recapitulates a phenotype similar to the HR5 and HR6 cells, the RSK/YB-1 pathway should be further investigated for its role in the maintenance of TICs. To assess RSKs activation of YB-1 and the resulting impact on the TIC population, it would be of interest to characterize mammosphere formation efficiency and CD44 levels in BT474 cells expressing myr-RSK (described above). In addition, CD44 levels and the propensity for mammosphere formation should be evaluated following RSK inhibition with either siRNA and/or BI-D1870 in both the *in vivo* models of acquired resistance as well as those established *in vitro* to ascertain RSKs involvement in TICs. Korkaya *et al* have demonstrated that expression of HER2 in normal mammary epithelial cells increases the expression of ALDH and also increases mammosphere formation efficiency. Further to this, they also showed that a number of stem cell regulatory genes are upregulated with HER2 over-expression (Korkaya *et al.*, 2008). Lastly, they demonstrate that in trastuzumab resistant cell lines, drug treatment has no effect on altering the percentage of ALDH cells, suggesting that trastuzumab has no influence on these stem like cells. Contrary to our results regarding CD44 in trastuzumab sensitive cells, they show that trastuzumab is capable of decreasing ALDH positive cells in sensitive models (Korkaya *et al.*, 2008). Therefore, to further supplement our current set of data, it would also be interesting to evaluate ALDH1 levels in the sensitive BT474 and resistant HR5 and HR6 cell lines and query whether enzyme levels correlate to the CD44 status.

In addition, to further assess our current hypothesis, it would be valuable to perform a Fluorescent Activated Cell Sort (FACS) analysis for CD44 in the BT474 cells and assess cell growth of the sorted populations after exposure to trastuzumab. Also, the P-YB-1^{S102} levels

should be compared in the $CD44^{-/low}$ and $CD44^{+}$ populations of BT474 cells and these populations should then be assessed for mammosphere formation and the sphere formation efficiency of the $CD44^{+}$ population should be compared to that of the HR5 and HR6 resistant models. Further to this, it would be expected that mammosphere formation would be inhibited in the $CD44^{-/low}$ population as the tumour initiating capacity of the cell fraction is eliminated.

Beyond being a positive marker for tumour-initiating cells, functional roles for CD44 have also been described in ovarian cancer (Bourguignon *et al.*, 1997a), acute myeloid leukemia (Jin *et al.*, 2006), and breast cancer (Marangoni *et al.*, 2009). We also demonstrate that trastuzumab treatment amidst CD44 inhibition decreases cell viability in the BT474, HR5, and HR6 cells. This in part may be due to a loss in HER2-CD44 receptor interaction. Previous studies have indicated that HER2 and CD44 physically interact with one another and in fact in the JIMT-1, trastuzumab resistant cell line, high CD44 levels correlate with a decreased rate of HER2 downregulation with trastuzumab (Nagy *et al.*, 2005; Palyi-Krekk *et al.*, 2007; Robinson *et al.*, 2006). They also demonstrate that inhibiting CD44 with siRNA enhances the effects of trastuzumab. Thus, it would be of interest to investigate receptor interactions in the HR5 and HR6 cells, first via a preliminary immunofluorescence screen followed by co-immunoprecipitation studies. To further evaluate CD44 and HER2 interactions, live cell imaging technology could be utilized to investigate the receptor interactions in real time with and without trastuzumab treatment. In addition, flow cytometric fluorescence resonance energy transfer (FRET) analysis can also be used to evaluate associations between the two receptors.

Lastly, to substantiate our proposed model of acquired trastuzumab resistance, a correlation to clinical outcome would be essential. At present, there exists a tumour microarray (TMA) prepared by Dr. Karen Gelmon, composed of 153 cases of HER2 positive breast cancers treated with trastuzumab (Robinson *et al.*, 2006). Staining consecutive slices of the TMA block for P-YB-1^{S102} and CD44 and correlating protein expression to various clinical outcomes such as survival and relapse would validate our novel mechanism. CD44 expression has been previously associated with poor prognosis in breast cancer (Liu *et al.*, 2007b; Shipitsin *et al.*, 2007), lung cancer (Liu *et al.*, 2007b), and prostate cancer (Liu *et al.*, 2007b; Noordzij *et al.*, 1997). Contradictory to this, CD44 expression has also been linked to a favourable clinical outcome in patients with ovarian cancer (Sillanpaa *et al.*, 2003), while a study suggests that CD44 expression is implicated in metastasis with no influence on clinical outcome in breast cancer (Abraham *et al.*, 2005). At present, no study has evaluated CD44 expression in prospective breast tumour samples; hence the importance of evaluating P-YB-1^{S102} and CD44 expression in these samples and correlating it to clinical behaviour. This would provide further impetus to target these factors in the clinical setting. Albeit, CD44 is an essential factor in normal cells where it is involved in haematopoiesis, the immune system, and organogenesis; its over-expression is associated with pathology (Ponta *et al.*, 2003). Further to this, transgenic CD44 null mice are fully viable with a slightly perturbed phenotype (Schmits *et al.*, 1997). Therefore, interfering with its expression would not be expected to have robust effects in normal cells. More importantly, given that YB-1 expression is limited to disease and its regulation of a number of established oncogenes, it serves as an ideal target for cancer therapy.

STAT3 and acquired trastuzumab resistance

Another point of discussion is the function of STAT3 in trastuzumab resistance. STAT3 has previously been associated with resistance to chemotherapy by its induction of *BCL2* (Real *et al.*, 2002) and *MDR1* (Bourguignon *et al.*, 2008a). Interestingly, a study describing trastuzumab induced apoptosis mediated by a reduction in MCL-1 expression gained our attention as *MCL1* is a STAT3 target gene (Henson *et al.*, 2006). Since we have linked STAT3 signalling to YB-1 (Chapter Two), it was worth investigating the transcription factor in acquired resistance. We show that the HR5 and HR6 cells have increased levels of P-STAT3^{S727} in comparison to the sensitive BT474 cells, however mTOR levels do not correlate with the levels of activated STAT3, suggesting it is not associated with the elevated P-STAT3^{S727} levels in the resistant cell lines. Furthermore, following trastuzumab treatment activated STAT3 levels are sustained in the resistant cell lines whereas they decrease in the sensitive cell line (Appendix V). We therefore attempted to induce resistance in the BT474 cell by transfection of a constitutively active mutant of STAT3, referred to as STAT3C. To our surprise, STAT3C was incapable of rescuing the BT474 cells from trastuzumab induced growth inhibition (Appendix V). However, we explored this with transient transfections and the generation of a cell line that stably expresses STAT3C may provide better insight into STAT3's ability to confer resistance. Given that STAT3C functions as an active P-STAT3^{Y705} mutant, it may be necessary to use a P-STAT3^{S727} mutant where the serine residue is mutated to a glutamic acid to assess the significance of the serine 727 site of STAT3 in trastuzumab resistance. On another note, breast cancer stem like cells require P-STAT3^{S727} for viability (Zhou *et al.*, 2007). In addition, neural stem cells also rely on P-STAT3^{S727} in a NOTCH dependent manner (Androutsellis-Theotokis *et al.*, 2006). The association of STAT3 with stem like cells suggests that it may also be involved in promoting drug resistance by its involvement in regulating crucial genes in these cell types. Furthermore,

STAT3 has been shown to function in collaboration with CD44 to induce *cyclin D* (Lee *et al.*, 2009) as well as *MDR1* (Bourguignon *et al.*, 2008b). Although STAT3 may not be directly associated with mediating trastuzumab resistance, it may play a role in sustaining the TICs responsible for drug insensitivity. Therefore, it would be interesting to compare gene expression signatures in the BT474, HR5, and HR6 cell lines following trastuzumab treatment by a ChIP-on-chip analysis to understand genes which remain activated or are induced by STAT3. In addition, it would be interesting to FACS sort BT474 cells for CD44 and assess P-STAT3^{S727} in the high and low populations to determine if it correlated to TICs in the model under study.

4.3 CONCLUSION

We have demonstrated the requirement of YB-1 in HER2 over-expressing breast cancers. This collection of work describes a novel mechanism where YB-1 mediates resistance to trastuzumab by inducing a tumour-initiating phenotype through its regulation of *CD44*. Taken together, our data suggest that YB-1 would be good candidate for therapeutic targeting to improve the clinical efficacy of trastuzumab.

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APPENDICES

Appendix I

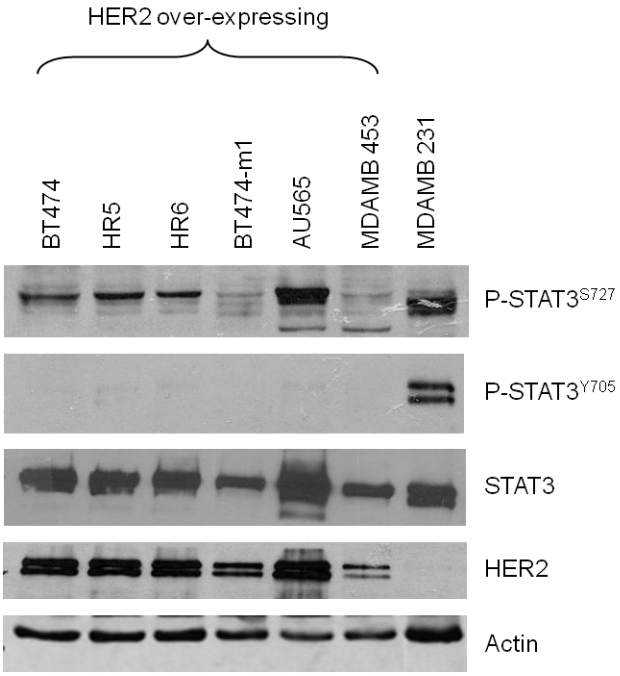

Figure I. Investigation of activated STAT3 in a panel of breast cancer cell lines. Immunoblot analysis of whole cell lysates of six HER2 positive breast cancer cell lines (BT474, HR5, HR6, BT474-m1, AU565, and MDAMB 453) demonstrates expression of P-STAT3^{S727}, while P-STAT3^{Y705} is absent. P-STAT3^{Y705} expression is limited to the MDAMB 231 cell line, which is a model of basal like breast cancer.

Appendix II

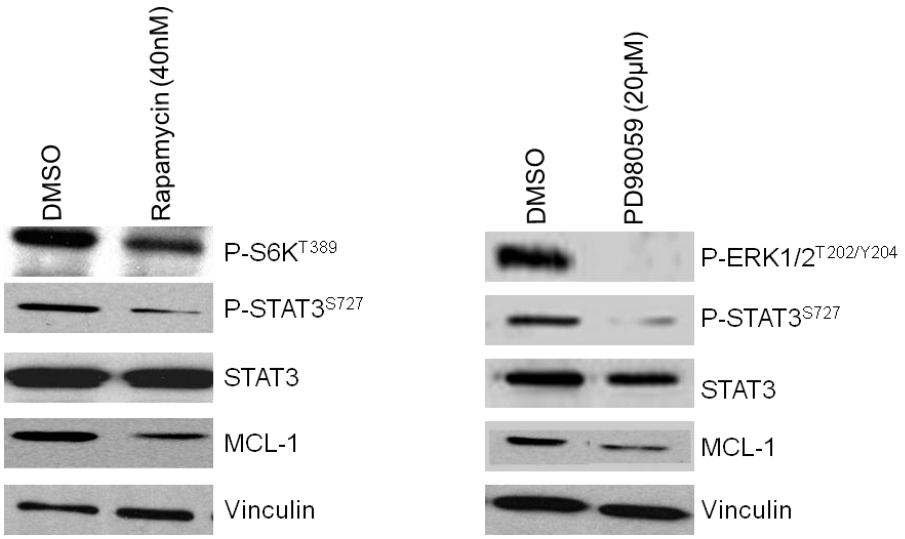

FIGURE II. mTOR and ERK phosphorylate STAT3 at Serine 727. BT474-m1 cells were treated with 40nM of Rapamycin for 24 hours or PD98059μM for 6 hours. Protein levels were then assessed by immunoblotting. P-STAT3^{S727} levels decreased with the inhibition of both mTOR and ERK. Furthermore, MCL-1 protein levels also decreased with a reduction in P-STAT3^{S727}.

Appendix III

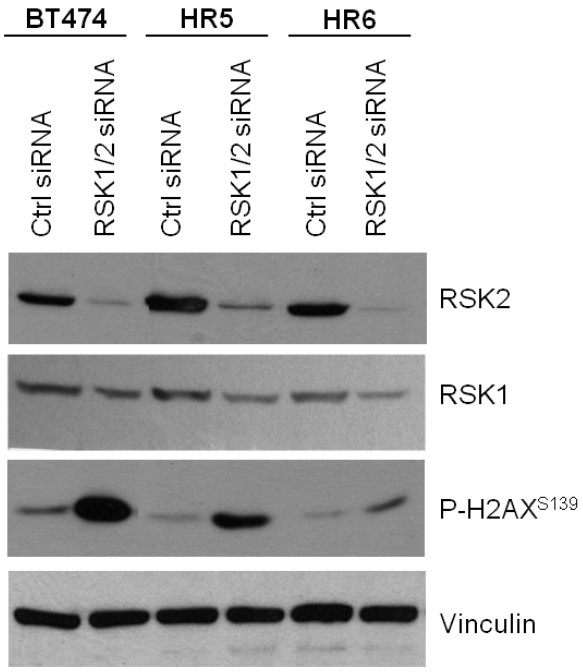

Figure III. RSK inhibition induces apoptosis. BT474, HR5, and HR6 cells were transfected with 20nM of control siRNA or RSK1 and RSK2 siRNA for 96 hours. Immunoblot demonstrates an induction of apoptosis with loss of RSK.

Appendix IV

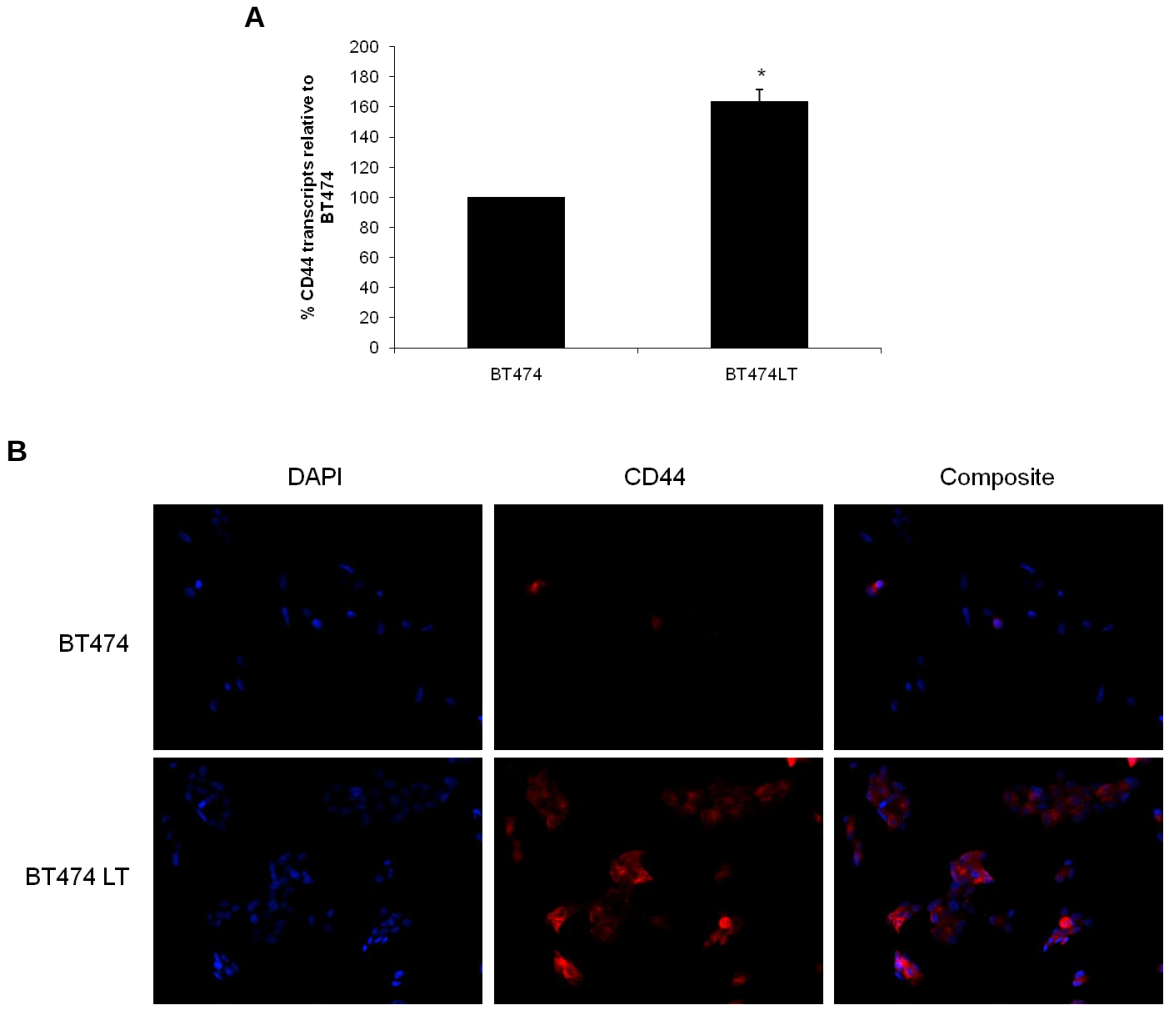

Figure IV. BT474 LT cultures resistant to trastuzumab have elevated levels of CD44. BT474 were continuously treated with trastuzumab (20µg/mL) for over thirty days, cells escaping treatment were labelled BT474 LT. **A.** q-RT-PCR of BT474 and BT474 LT show increased CD44 transcript levels in the resistant variants. **B.** Cells grown on glass coverslips were fixed and stained for CD44. BT474 LT, trastuzumab resistant cell lines demonstrate increased CD44 protein levels.

Appendix V

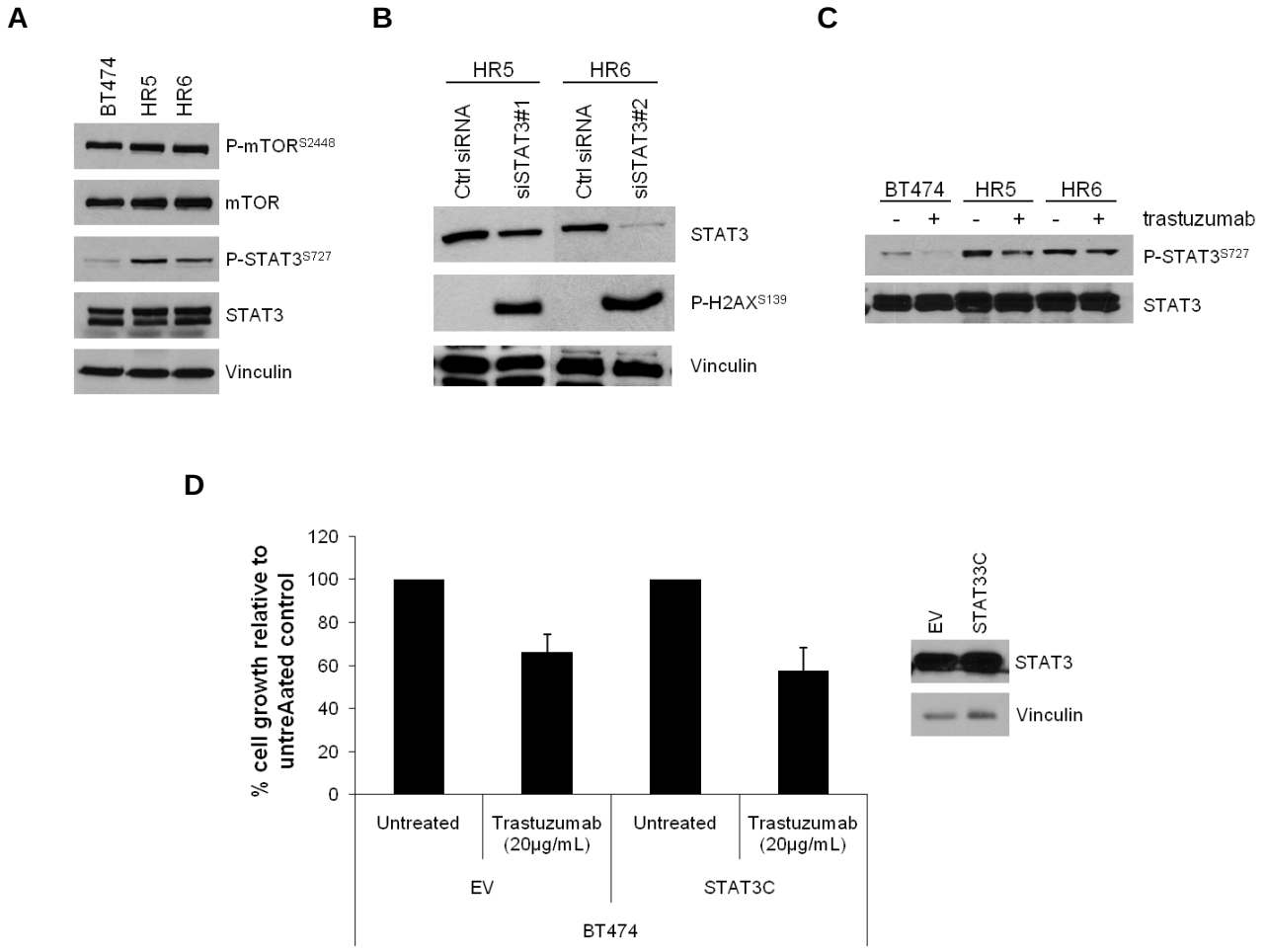

Figure V. Investigation of STAT3 in trastuzumab resistance. A. BT474, HR5, and HR6 cells were evaluated for P-STAT3^{S727} and P-mTOR^{S2448} levels by immunoblotting. **B.** The trastuzumab resistant HR5 and HR6 cell lines were transfected with control siRNA or siSTAT3 and protein levels were assessed 72 hours post transfection. Inhibition of STAT3 induced apoptosis in both resistant cell lines. **C.** BT474, HR5, and HR6 cells were treated with trastuzumab (20µg/mL) for 72 hours. Following, P-STAT3^{S727} levels were investigated by immunoblotting. The drug resistant, HR5 and HR6 cells express higher levels of P-STAT3^{S727} in comparison to the BT474 cell line. Furthermore, activated STAT3 levels are sustained following trastuzumab treatment in the trastuzumab resistant cell lines while levels decrease in the sensitive BT474 cells. **D.** Trastuzumab sensitive BT474 cells were transiently transfected with 2µg of EV or Flag-STAT3C. 24 hours following DNA transfection, growth media was supplemented with 20µg/mL of trastuzumab, and cells were cultured for an additional 72 hours. Next, cells were fixed and stained with Hoechst and analyzed by the Cellomics ArrayScan VTI. Expression of STAT3C was not able to rescue the sensitive BT474 cells from trastuzumab.