Y-BOX BINDING PROTEIN-1 INDUCES *MET*, *CD44*, AND *CD49F* THEREBY PROMOTING THE CAPACITY FOR TUMOUR INITIATION

by

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ABSTRACT

Y-box binding protein-1 (YB-1) is an oncogenic transcription/translation factor expressed in >40% of breast cancers, where its expression correlates with enhanced tumour cell growth and drug resistance. YB-1 is also significantly associated with disease recurrence, suggesting links to tumour-initiating cells (TICs). We recently reported that YB-1 binds to the promoters of genes associated with a stem/progenitor-like phenotype through a chromatin immunoprecipitation (ChIP)-on-chip screen, including genes encoding the MET receptor, CD44, and CD49f. We first confirmed that YB-1 induces oncogene *MET* by activating its promoter. Further, both *YB-1* and *MET* expression were detected in purified populations of primary human mammary progenitor cells. Similarly, we confirmed that YB-1 binds the gene promoters of reported TIC markers CD44 and the stem cell marker CD49f (α6 integrin) using conventional ChIP. Human breast TICs, reportedly $CD44^{+}/CD24^{-/low}$, self-renew, grow in mammospheres, and may evade current drug therapies, leading to increased relapse rates in patients. We observed that $P\text{-}YB\text{-}1^{S102}$ co-localized to a $CD44^{High}$ breast cancer cell subpopulation with increased growth capacities. Silencing YB-1 down-regulated MET, CD44 and CD49f, while overexpression of wild-type ($YB\text{-}1^{WT}$) or a constitutively activated form ($YB\text{-}1^{S102D}$) increased MET, CD44 and CD49f transcripts and proteins. Consistent with these findings, the mammary glands of YB-1 transgenic mice had elevated CD44 and CD49f protein with associated hyperplasia. Moreover, the expression of $YB\text{-}1^{S102D}$ in SUM 149 cells, enhanced mammosphere formation and growth in soft agar. Conversely, silencing either *CD44* or *CD49f* in these cells reversed $YB\text{-}1^{S102D}$ enhanced growth. Consistent with a role for TICs in relapse, Paclitaxel activated YB-1 and this correlated with induced CD44. Further, expression of $YB\text{-}1^{WT}$ enhanced mammosphere growth in the presence of Paclitaxel. Taken together, our data suggests that YB-1 induces *MET*, *CD44*, and *CD49f* expression which may be a part of the mechanism used to enhance tumour cell growth, disease relapse, and drug resistance.

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4-OHT	4-hydroxytamoxifen
7-AAD	7-Amino-actinomycin D
AML	Acute myeloid leukemia
BSA	Bovine serum albumin
ChIP	Chromatin immunoprecipitation
CML	Chronic myeloid leukemia
COC	ChIP-on-chip
CSD	Cold-shock domain
DAPI	4',6-Diamidino-2-phenylindole
DMEM	Dulbecco's modified essential medium
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMSA	Electrophoretic mobility shift assay
EMT	Epithelial mesenchymal transition
ER	Estrogen receptor
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
HGF	Hepatocyte growth factor
HGFR	Hepatocyte growth factor receptor
IGF-1	Insulin-like growth factor-1
JAK	Janus kinases
LCK	Leukocyte-specific protein tyrosine kinase
MAPK	Mitogen-activated protein kinase
MDR-1	Multidrug resistance-1
MET	Mesenchymal epithelial transition
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffer saline
PCNA	Proliferating cell nuclear antigen
PDK-1	Phosphoinositide-dependent kinase-1
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PR	Progesterone receptor
RSK	Ribosomal S6 kinase
SERM	Selective estrogen receptor modulator
STAT3	Signal transducers and activators of transcription
TKI	Tyrosine kinase inhibitors
YB-1	Y-box binding protein-1

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CO-AUTHORSHIP STATEMENT

Chapter 2:

The manuscript in this chapter was published in *Oncogene* in March 2009. The work described was designed by Sandra Dunn, Melanie Finkbeiner, Arezoo Astanehe, and me. I had designed, conducted and analyzed data from the quantitative real-time PCR experiments using isolated primary normal mammary progenitors, the chromatin immunoprecipitation (ChIP) using SUM 149 xenograft tissues, the experiments characterizing the SUM 149 stable shYB-1 cell line, and all experiments involving small molecule compound OSU-03012. Melanie Finkbeiner analyzed the ChIP-on-chip data. Additionally with Arezoo Astanehe, Melanie Finkbeiner designed, performed, analyzed all YB-1 knockdown western blots, YB-1 overexpression western blots, quantitative real-time PCRs, ChIP experiments, EMSAs, luciferase promoter activity experiments, HGF-stimulation experiments, and soft agar experiments. Immuno-fluorescence experiments were performed by Abbas Fotovati. Alastair Davies analyzed the ChIP-on-chip data using the Ingenuity Pathway Analyses software. Yang Lucy Zhao first established the SUM 149 stable shYB-1 cell line. Helen Jiang performed the ChIP for microarray chip analysis. Anna Stratford constructed the plasmid encoding YB-1^{S102D}. Ashleen Shadeo performed the array comparative genomic hybridization. Carla Boccaccio and Paolo Comoglio provided the MET promoter reporter construct. Peter Mertens provided the phospho-YB-1^{S102} antibody. Peter Eirew assisted with establishment of SUM 149 tumours in mice. Afshin Raouf isolated the primary normal mammary progenitor cells. Connie Eaves provided the mice for the establishment of SUM 149 xenografts as well as the primary normal mammary progenitor cell samples. The manuscript was written and prepared by Melanie Finkbeiner, Arezoo Astanehe, Abbas Fotovati, Connie Eaves, and Sandra Dunn.

Chapter 3:

The work described in this chapter is a manuscript just submitted to a peer-reviewed journal. The research program was identified and designed jointly by Sandra Dunn and me. I designed, conducted, and analyzed the ChIP assays, experiments involving cell sorting by FACS, mammosphere assays, soft agar assays, mammosphere passaging self-renewal assays, YB-1 knockdown and overexpression western blots and quantitative real-time PCRs, flow cytometry analyses, immunofluorescence experiments, CD44 and CD49f knockdown experiments, Matrigel assays, and Taxol treatment characterizations. Abbas Fotovati designed and conducted the staining protocol and imaging of all immunofluorescence photographs. Kristen Reipas provided technical assistance to cell culture assays. Jennifer Law and Kaiji Hu performed and analyzed the chemotherapy drug screen. Jing Wang and Isabelle Berquin bred the YB-1 transgenic mice, provided histological sections, and conducted the H&E staining as well as imaging. Hans-Dieter Royer originally established the YB-1 transgenic mice, and gave editorial guidance. Pauline Johnson, Afshin Raouf, Connie Eaves provided intellectual and editorial input. The manuscript was written and prepared jointly by Sandra Dunn and me.

CHAPTER ONE : INTRODUCTION

1.1 BREAST CANCER

Cancer is a disease that arises when the tightly regulated programming governing the life cycle of a cell has been disrupted, leading to uncontrolled cell growth and the disease burden of an accumulating mass. It is a clonal disease where a cell's genetic programming is sufficiently disturbed such that the normal cell's plethora of preventative and repair mechanisms are unable to recover. A cancer cell achieves such genetic aberrations through the combination of accumulated contact with harmful mutagens in its surrounding environment and/or innate inherited mutations in its genome. In some cancers, the malignant cells in growing tumours can acquire new properties due to genomic instability to disseminate from the primary tumour. The cell or group of cells can metastasize to a distant site or sites with hospitable microenvironments and generate tumours at a secondary site. Metastasis is the sole cause of death in breast cancer patients. Breast cancer stems from the epithelial cells of the mammary gland, which are composed of two lineages of cells, luminal and myoepithelial cells[1, 2]. In the past decade, breast cancer has been demonstrated to be a heterogeneous disease. Seminal gene expression studies on large cohorts of breast cancer patients have resulted in a classification of breast cancers into 5 major subtypes based on well characterized breast cancer receptors for which treatment is available [3, 4]. Breast cancer is first dissected into two main branches based on the expression of estrogen receptor (ER). Under the ER positive breast cancers are the "Luminal A" and "Luminal B" subtypes. ER negative breast cancers are sub-divided into the "Normal-like", "Basal-like", and "Her-2+" subtypes. More recently a new subtype termed "Claudin-low" related to the "Basal-like"

group has also been identified[5]. It is hypothesized then that similar to leukemias and lymphomas for which the theory has been validated, the different subtypes of breast cancer represent the diversity of differentiation stage and lineage of the originating cancer clone[6, 7]. Thus, understandably, the current field of breast cancer research is closely linked to the status of mammary gland biology research.

1.2 CURRENT TREATMENT OPTIONS

The American Cancer Society estimates that globally over 1.3 million women will be diagnosed with breast cancer each year. With the current clinically available therapeutics, the 10-year survival rate in the United States was quoted at approximately 80% in 2007 by the National Cancer Institute. The survival rate however drops significantly in the presence of local and distant disease relapse, the 10-year survival rate dropping to 60% and 10% respectively, leaving better treatment options to be desired. An overview of currently available breast cancer treatments will be discussed and a select number of specific drugs will be highlighted.

Mastectomies, lumpectomies, and lymph node dissections, if the cancer has spread, are the primary treatment option for most breast cancer patients. Surgery is accompanied by a combination of radiation therapy, chemotherapy, hormone therapy, and/or targeted therapy. There is currently a movement in the clinic towards "personalized medicine", where diseases like cancers can be classified into subtypes based on clinically relevant parameters innate to the properties of the tumour type, like drug response and overall prognosis[4, 8]. This trend is therefore made possible by much basic, translational, and

clinical research focused on stratifying patient tumours in response to the combinations of currently available therapies.

Solid tumours were once viewed by many as homogeneous cell masses in which all cells divided uncontrollably. Treatments available currently were thus designed to simply target dividing cells. Chemotherapeutic drugs were generated to prevent cell division, where dosing allowed a differential window to kill the fastest dividing cells, believed to be the most aggressive cancer cells, leaving normal cells relatively unscathed. Alkylating agents (mustard gas derivatives, platinum-based), plant alkaloids (vinca alkaloids, taxanes, podophyllotoxins, camptothecan analogs), anti-tumour antibiotics (anthracyclines), anti-metabolites (folic acid antagonist, pyrimidine antagonist), PARP inhibitors, and topoisomerase inhibitors, are all (with the exception of the alkylating agents) cell cycle specific and work to compete with normal molecules in the cell during cell division to prevent the normal mechanisms from being manipulated by a cancer cell. In breast cancers, the approved chemotherapies used in adjuvant and neoadjuvant settings in various combinations include doxorubicin and epirubicin (anthracyclines), paclitaxel and docetaxel (taxanes), cyclophosphamide (alkylating agent), 5-fluorouracil and gemcitabine (anti-metabolite), and vinorelbine (vinca alkaloid). Paclitaxel and docetaxel specifically are taxane compounds that target the metaphase of mitosis by hyper-stabilizing the β subunit of tubulin in microtubules, preventing their dissociation and causing apoptosis[9]. Chemotherapies are generally used in adjuvant settings except for very aggressive late stage breast cancers.

Historically, the mainstay of breast cancer hormone therapy is tamoxifen which targets ER. Thus, tamoxifen though successful in ER+ patients, resulting in very high survival rates for the ER+ breast cancer subtype, precludes the 40% of ER- patients[3, 4]. Tamoxifen itself has little affinity to ER[10]. Its liver metabolite 4-hydroxytamoxifen (4-OHT), however, has much greater affinity to the receptor[10]. The 4-OHT/ER complexes then compete with and inhibit normal estrogen/ER complexes to translocate into the nucleus to transactivate downstream growth enhancing genes[10]. Further, the 4-OHT/ER complexes recruit other co-repressors to the promoters of genes with identified estrogen response elements[10]. As ER is often overexpressed in ER+ cancers[10], the prevention of estrogen to promote cell proliferation is only partially effective. Since the first conception of estrogen, many small molecules have been developed to inhibit the estrogen/ER signaling pathway. Of note, raloxifene, another compound belonging to the family of selective estrogen receptor modulators (SERMs), is FDA-approved for the treatment of some invasive breast cancers, and acts directly on the estrogen receptor[11, 12]. Additionally, aromatase inhibitors, like anastrozole, which prevents the synthesis of estrogen, are approved for the treatment of localized breast cancers[12].

Tyrosine kinase inhibitors (TKIs) have in recent years accrued much achievement in the clinic. The one major example is imatinib, a TKI against BCR-ABL, a fusion constitutively active oncoprotein frequently found in chronic myeloid leukemias (CML)[13]. It has since been shown that imatinib is not BCR-ABL specific but also targets other tyrosine kinases important in cancers including c-Kit, and this multi-target specificity has proven to be potent against these leukemias[14]. In breast cancers, small

molecule tyrosine kinase inhibitors, gefitinib and erlotinib, designed against the active site of EGFR were effective in pre-clinical studies[15] but eventually failed in later clinical trials[16]. Still, there is more recent evidence to show that basal-like breast cancers, over 60% of which overexpress EGFR, may be sensitive to EGFR TKIs in combination with chemotherapy or downstream signaling inhibitors[17, 18]. In contrast, they have been successful in the clinical management of advanced of metastatic non-small cell lung cancer (NSCLC), as well as malignancies of the ovaries, head, and neck[16]. Lapatinib, which is designed against both the tyrosine kinase active sites against EGFR and Her-2, however, showed promise early on in pre-clinical studies[19] and have since been approved to use in combination with capecitabine[20].

Trastuzumab was the success story of targeted therapies for breast cancer in the 1990s. It is a monoclonal antibody that binds to Her-2 (erbB2) of the erbB family of tyrosine kinase receptors, all of which often overexpressed in breast and ovarian cancers[15]. Her-2 specifically is overexpressed in 15 to 25% of all breast cancers and forms heterodimers with other membrane receptors including other erbB family members, EGFR, and Her-3, to signal downstream activating cell growth pathways[15]. Trastuzumab has been shown via various physical mechanisms to decrease Her-2 signaling intracellularly and affect tumour growth in patients[15].

Signal transduction inhibitors also include a class of compounds that inhibit signaling downstream of membrane receptors intracellular. One example is OSU-03012, a small molecule inhibitor to phosphoinositide-dependent kinase-1 (PDK-1), one of two upstream

kinases to Akt[21]. OSU-03012 is a derivative of Celecoxib, designed against COX-2, originally intended for inflammatory diseases but are currently undergoing Phase II clinical trials for the treatment of breast cancers[22]. OSU-03012 inhibits the Akt signaling pathway in breast cancer cells leading to cell growth inhibition and apoptosis[21, 22]. It is also potent in cells of imatinib-resistant CML[23], rhabdomyosarcomas[24], glioblastomas[25], myelomas[26], NSCLCs[27], pancreatic cancers[28], and hepatocellular carinomas[29].

Much research currently lies in further stratifying patients that would respond well to the above mentioned drugs, as well as unique combinations of them. To achieve that, new biomarkers are constantly proposed to better treat the patient groups and minimize over-treatment. For example, overexpression of β3-tubulin isoform[30] and presence of β-tubulin mutations[31] have been associated with paclitaxel resistance, whereas one study showed Her-2 overexpression and ER negativity defined a population of patients with increased sensitivity to paclitaxel[32]. Importantly, activation of the anti-apoptotic PI3k/Akt pathway has been cited to promote resistance to many chemotherapeutic drug classes, including taxanes, as well as targeted therapies such as trastuzumab and tamoxifen[33, 34]. Moreover, IGF-1R and EGFR signaling downstream through the PI3k/Akt and MAPK pathways have also been attributed to tamoxifen resistance[34, 35]. Thus, clinical trials for new compounds that target the PI3k/Akt pathway members, other tyrosine kinase receptors, membrane receptors, kinases, and phosphatases, are currently being developed.

This is especially important for the basal-like subtype of breast cancer patients. As they are ER-, PR-, and Her-2-, prognosis is much poorer for these patients[4]. For the basal-like breast cancers, EGFR and c-Kit, which are both expressed in high proportions of this tumour subtype, have been promising targets in pre-clinical models[18, 36, 37]. Interestingly, dasatinib, the small molecule which has achieved much success in targeting c-Kit activity in BCR-ABL$^+$ CML patients has shown early efficacy against basal-like breast cancer cells[38]. More recently, the Notch signaling pathways have also been implicated in breast cancer cells[39], and in particular, basal-like breast cancers[40, 41]. Additionally, basal-like tumours have been demonstrated to share biological similarities to *BRCA1*-associated tumours[42]. Studies suggest that BRCA1-related defective DNA-repair pathways confer sensitivity to platinum-based and PARP inhibiting chemotherapies[43-46], and thus could be an avenue to explore for the treatment of these cancers. Finally, as it has been alluded before, the development of the current breast cancer therapy options was based on the concept that solid tumours are a homogeneous cell mass. It has since been demonstrated that tumours are heterogeneous within themselves atop of patient genome heterogeneity[47]. Cells derived clonally from a tumour have been shown to have very different growth rates, differentiation status, tumourigenic ability, radiation and drug responsiveness[6, 19, 47, 48]. Therefore, there is much more biology that needs to be unravelled to fully understand the resistance of ER+ cancers to tamoxifen, Her-2+ tumours to trastuzumab, and tumours of all types to currently available chemotherapies.

1.3 TRANSCRIPTION FACTORS IN CANCER

Transcription factors are biological molecules that can alter gene expression. It is one of the largest classes of biological molecules with an estimated 2000 genes encoding transcription factors[49]. Transcription factors have long been implicated in human diseases. Transcriptional regulation is an exquisitely specific and monitored phenomenon that governs every cell phenotype. As such, transcription factors in turn are also tightly monitored by genetic and epigenetic controls. As transcription factors often regulate many genes supporting many different phenotypes, it is conceivable that aberrations in these molecules could be potentially dangerous in any biological setting. In cancer cells, transcription factors that elicit genes governing growth, proliferation, invasion, motility, drug resistance, and/or cell cycle inhibition often become overexpressed or constitutively activated such that cells acquire an abnormal and uncontrolled survival and growth capacity. Some examples of transcription factors that are targets of developing anti-cancer peptides and small molecules include the ETS family, AP-1 family, FoxO family, NFκB, Stat family, c-Myc, and, b-Myb[50]. For example, there exist Stat3-specific peptide inhibitors and Stat3-targeted oligonucleotide decoy inhibitors that reduce the ability of Stat3 homodimers to interact with downstream gene promoters[51, 52]. Similarly, c-Myc peptides have been found to reduce the expression of downstream genes, including p53[53]. Because transcription factors are upstream of their target genes, they make powerful targets for therapy as one molecule frequently regulates many downstream genes, which often work collaboratively. While mutations, amplifications, and activation by phosphorylation are often the cause of oncogene overexpression, transcriptional activation often play an important role. For example, 70

to 80% of all metastatic breast carcinomas overexpress EGFR[54] and its overexpression in these breast cancer cases has been sited to be frequently caused by transcriptional activation[55]. As known EGFR transcription factors include YB-1[18, 56], SP-1[57], and c-Jun[58], they may be good prospective anti-cancer targets. More importantly, many studies have repeatedly shown that the overexpression of EGFR is associated with poorer prognosis in breast cancer patients[15]. It is important that the molecular mechanisms that drive oncogene overexpression in these malignant cells be elucidated. Further, transcription factors are often the convergence of multiple signaling pathways originating from even more membrane receptors and/or receptor tyrosine kinases. Therefore, transcription factors make powerful molecules to better understand in cancer initiation and progression.

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

Y-box binding protein-1 (YB-1) is an oncogenic transcription/translation factor in the Y-box family of proteins. This family of proteins has a well-characterized and higly conserved cold-shock domain (CSD), flanked by a N-terminal domain and a C-terminal domain. The N-terminal domain is alanine and proline rich, believed to be involved in gene transactivation[59], while the C-terminal domain is a set of 4 repeats of alternating approximately 30 acidic and 30 basic residues[60]. The prokaryotic Y-box proteins were first identified in bacteria for their ability to sustain protein synthesis in cold temperatures[61]. It was later discovered that in both prokaryotes and eukaryotes, the CSD has a strong affinity for nucleic acids, both single and double-stranded DNA and RNA[62]. The CSD of YB-1 was later found to be crucial to the protein's affinity for nucleic

acids[60]. The CSD of YB-1 is believed to bind to the inverted CAAT box motif, ATTG[63, 64] and is also responsible for nuclear transport[65]. Expectedly, YB-1 was later discovered to have a major role in the regulation of transcription and translation[59, 66]. Of note, while YB-1 has major roles in translation, this literature review will focus on its transcriptional roles in cancer as it is our research focus and more frequently documented.

YB-1 is an important player in many known cancer related pathways. As a multi-functional regulator of gene expression, YB-1 was first reported to be putatively involved in cell proliferation and transformation in 1995[67]. Since then, YB- 1's speculated roles in cancer have been better characterized in a variety of cell types. YB-1 is known to interact directly with p53 at gene promoters and can negatively modulate its ability to transactivate downstream gene p21[68], suggesting an interplay between oncogenic and tumour suppressive phenotypic regulation. YB-1 is regulated by c-Myc by direct promoter occupancy[69]. YB-1 was identified as a novel downstream Akt substrate by our lab group in 2005[70]. Since then, we have also found it to be a target of the p90 ribosomal S6 kinase (RSK), and demonstrated using a kinase assay that RSK has a higher affinity for YB-1 than Akt does, where both kinases competes for the Serine 102 (Ser102) site of YB-1[71]. Our lab has evidence to show that phosphorylation of YB-1 enhances its movement into the nucleus and increases its affinity for nucleic acids[70, 71], suggesting a mechanism by which its activation leads to altered gene expression profiles and aggressive phenotypes. One mechanism by which this occurs is through its regulation of the STAT3 pathway[72].

YB-1 is consistently associated with increased cell growth rates and drug resistance in cancers of different origins. It correlates positively with the expression of proliferating cell nuclear antigen (PCNA), a highly proliferative cell marker, in colorectal tumors[73] as well as Ki67 in breast cancers[56]. YB-1 is not expressed at high levels in normal breast tissues but is highly expressed in breast tumours[74]. Likewise, YB-1 is overexpressed in cancers of the prostate[75], colon[73], ovary[76], bone[77], lung[78], myelomas[79], and rhabdomyosarcomas[80] but undetectable or detected at low levels in their normal tissue counterparts. In addition, YB-1 knockdown with RNAi in breast, melanoma, adenocarcinoma, hepatoma, fibrosarcoma and colon cancer cells results in cell death[81]. The focus of this review will be on evidence suggesting YB-1 as an oncogene in breast cancers. Transgenic mice with targeted YB-1 expression in their mammary glands developed invasive tumors with 100% penetrance[82]. YB-1 also stimulates the proliferation of pre-neoplastic breast cancer cells by inducing cyclin A and cyclin B mRNA transcripts[65]. More recently, our lab has reported that overexpressing wild-type YB-1 in breast cancer cells of different subtypes enhanced tumor cell growth in monolayer and 3-dimensional assays, while overexpressing a mutant form of YB-1 (S102A) had no effect[70, 83, 84]. Further, we found that the overexpression of wild-type YB-1 leads to the induction of *EGFR*[56], *Her-2*[56], *MET*[83], and *PIK3CA* (the gene encoding the α-subunit of the p110 activating subunit of the PI3k)[84], which are often overexpressed and/or activated in human breast cancers. More specifically, YB-1 activates these genes transcriptionally[18, 21, 56, 83, 84]. YB-1 is expressed at comparable levels in all breast cancer subtypes; however, we find that the activated form of YB-1, phosphorylated by Akt or RSK or PKC at Serine 102, more frequently in the ER- breast cancer subtypes and/or the

basal-like subtype[83]. We have demonstrated YB-1 is essential to all the different subtypes of breast cancers *in vitro*[18, 56, 72, 83, 84], and it is important for xenograft growth *in vivo*[72]. Further, it has been reported that YB-1 is expressed in cancers of the breast at onset and early stages of the disease[82, 85]. There, YB-1 enhances cancer progression through its contribution to mitotic failure and centrosome amplification, leading to chromosomal instability[82].

In addition, YB-1 transcriptionally activates MDR-1 (multi-drug resistance gene-1, also known as p-glycoprotein) which can protect cancer cells from chemotherapeutic agents[74]. YB-1 has been associated with chemoresistance to paclitaxel and other chemotherapeutic agents in breast cancer cells[74, 86, 87], synovial sarcomas[77], head and neck cancer cells[88], ovarian cancer cells[76, 89, 90], rhabdomyosarcomas cells[80], prostate cancer cells[91], glioma cells[91], and myeloma cells[79, 92]. Most recently, in a study of 4000 cases of breast cancers, our group showed a significant correlation between high YB-1 expression and poor prognosis and cancer recurrence across all breast cancer subtypes[93], suggesting a link to chemoresistance. Taken together, YB-1 is a strong oncogenic factor in the breast and other cancers.

While there is clear evidence that YB-1 contributes to cell and tumour growth in human cancers, there has also been reports of its exhibiting growth suppressive effects in chicken embryo fibroblasts[94]. Therefore, it can be concluded that YB-1 is involved in many signaling pathways that may be tissue and cell type-specific. Most recently, we have explored the expression of YB-1 in normal mammary progenitor cells. While YB-1 has

been previously demonstrated to be expressed at very low levels in normal mammary tissue[74], we and others were able to find YB-1 transcripts in this rare primitive population[83, 95], and have further found infrequent but detectable expression of YB-1 localizing to the basal layers of normal mouse mammary glands (unpublished data), suggesting that YB-1 may have a developmental role in the mammary gland as it does in embryogenesis[96-101] and neural development[101, 102].

1.5 MEMBRANE RECEPTORS IN CANCER

Membrane receptors make up approximately 20% of cellular molecules and 60% of current drug targets[103], highlighting their significance in biological outcomes. All cells depend on their environment for signals for every activity, including proliferation, motility, metabolism, and death. Membrane receptors then are extremely important in cell fates and decisions. In cancers, membrane receptors, whether adhesion molecules or receptor tyrosine kinases, are often characterized by heightened signals promoting growth, proliferation, motility, as well as survival, and down-regulating signals for apoptosis, offsetting an equilibrium of controlled cell numbers and cell cycles. As all cells depend on extracellular signals to grow, membrane receptors are crucial to the survival of cancer cells as well. Further, cell surface receptors also adhere to and interact with adjacent epithelial, stromal and immune cells, all of which can contribute to tumour formation. Importantly, membrane receptors are easily accessible by small molecules, and thus make attractive targets for therapy.

1.6 MET RECEPTOR

The MET (mesenchymal-epithelial transition) receptor, also known as c-Met or the hepatocyte growth factor receptor (HGFR), is a membrane receptor that binds strictly binds the hepatocyte growth factor, also known as scatter factor[104]. MET in response to its ligand HGF have important roles in physiological invasive growth observed in embryonic development and would healing[104]. In mouse embryogenesis, HGF and MET are co-expressed in endodermal and mesodermal structures[105]. In organogenesis and matured developmental stages, MET is expressed solely by epithelial cells and myoblast progenitors while HGF is expressed by mesenchymal cells[105-107]. Upon stimulation by HGF, MET induces a scatter phenotype, which has been compared to an epithelial mesenchymal transition[108]. EMT is a biological process that occurs in embryonic development and tissue regeneration, where cells transiently adopt a spindle-shaped morphology and increased motility to regulate ordered cell migration and morphogenesis of new tissues[109]. As demonstrated by knock-out mice, HGF and MET are essential to the development of the placenta and the liver[110]. MET mediates physiological invasive growth upon the binding of ligand HGF, activating downstream signaling pathways to the tyrosine kinase membrane receptor[104]. The MET kinase phosphorylates and forms complexes with integrins, including $\alpha 6\beta 4$[111], and other membrane receptors, like EGFR[112-114] and CD44[115, 116]. The heterodimers signal through adaptor proteins thereby activating downstream cell survival and proliferation effectors, the PI3k/Akt, the MAPK pathways, and the JAK-STAT pathway[104].

Recent studies have drawn similarities between cancer cell survival and proliferation mechanisms and that of primitive cell populations, including embryonic cells[117]. Aberration of MET expression and activation suggests that cancer cells can adopt the invasive properties induced by MET to promote their own growth, migration, and survival. MET mutations are rare in human cancers though germline missense mutations are found in hereditary kidney cancers[118], and activation mutations are observed in non-hereditary tumours including sporadic papillary renal cancers, childhood hepatocellular carcinomas, and gastric cancers[119]. The most frequent type of MET aberration in human cancers however is overexpression of MET in the absence of mutations and increased production of autocrine HGF[120]. Overexpression of MET is found in carcinomas including those of the breast[113, 121], colon[122, 123], prostate[124], lung[125], and ovary[126]. MET overexpression in epithelial cells then favours HGF ligand activation in addition to crosstalk with other membrane receptors, increased signaling downstream, and consequently a heightened invasive and motile phenotype. MET is associated with poor prognosis and a metastatic phenotype[120]. In recent years, not unlike other oncogenes, MET has also been speculated to mediate a more primitive, early embryonic pro-survival, proliferative, and migratory phenotype to cancer cells as it has been reported to function biologically in more stem-like populations of the brain, liver, and skeletal muscles[104]. MET has also been used as a marker for liver stem cells that differentiate into its two lineages, hepatocytes and cholangiocytes[127]. Importantly, MET inhibitors are currently in pre-clinical and clinical trials[128].

1.7 CD44

CD44 is a trans-membrane receptor expressed ubiquitously in human tissues mostly known for its role as a cell adhesion molecule. A major receptor for hyaluronan or hyaluronic acid (HA), CD44 has also been reported to bind other extracellular matrix components of the human basement membrane including fibronectin, laminin, collagen, osteopontin, and serglycin[129]. CD44 pre-mRNA is encoded by 20 exons, ten of which are regulated by alternate splicing mechanisms which results in the 18 CD44 variants encoding for extracellular domains of variable size[129]. In particular, YB-1 is involved in splicing CD44v4 and CD44v5[130-132], both variants reportedly associated with tumourigenesis[133-135]. In addition to this, the extracellular portion of CD44 is glycosylated variably depending on the variant, allowing the receptor to assume many different roles, binding to a wide range of substrates and forming heterodimers with a host of membrane receptors. Intracellularly, CD44 has 2 known serine sites that are regulated by protein kinase C (PKC)[136]. PKC has been demonstrated to de-phosphorylate Ser325 but phosphorylate Ser291[136]. The phosphorylation status of CD44 in relation to its function is mostly unknown but has been associated with its ability to bind its intracellular binding partners, ankyrin, as well as ezrin, radixin, and moesin, also known as the ERM proteins[136]. Cytoplasmic CD44 along with its recruited proteins have functions in altering actin and other cytoskeletal interactions adjacent to the cell membrane[137, 138], which consequently affects the extracellular binding affinities, ultimately changing cell motility, adhesion, and migration. CD44 thus has important roles in tissues where adhesion in hyaluronin-rich areas is important. An example of this is in leukocytes, where CD44 is crucial to the homing of normal hematopoietic and

leukemic stem cells in normal development and in immune responses[139-144]. Elevated CD44 and its variant isoforms have been documented in AML cells[141, 142]. Thus, it is not surprising that targeting CD44 is effective at depleting tumour-initiating cells[140, 145].

While CD44 functions mainly as a cell adhesion molecule, it partners with kinase receptors at the cell membrane to facilitate signaling downstream. Its common co-receptors include EGFR[146], Her-2[147, 148], MET[115, 116], as well as Rhamm[149], another hyaluronan receptor. In forming heterodimers with these known tyrosine kinase receptors, CD44-ECM ligand interactions also mediates extracellular signals to affect major downstream growth and apoptotic pathways such as the MAPK pathway and the PI3k/Akt pathway. Similarly, signaling by ligands of CD44 partnering receptors, EGF, Heregulin, and scatter factor/hepatocyte growth factor (SF/HGF), are mediated by CD44 expression. In addition to signaling with other membrane receptors, it is speculated that CD44 itself can signal without intrinsic catalytic activity in its cytoplasmic tail[129]. CD44 has been reported to recruit Src[138], Rho GTPase[150], Rho kinase[151], PKC[136, 146], and proto-oncogenic kinases, LCK[137, 152] and FYN[152] to its cytoplasmic region. However, while hyaluronin-CD44 interactions and anti-CD44 antibodies have been demonstrated to affect downstream intracellular signaling, no concrete evidence has demonstrated that CD44 can act alone.

In the breast epithelium, CD44 is transcriptionally induced by the presence of insulin-like growth factor-1 (IGF-1) and epidermal growth factor (EGF) in mice[153]. CD44v6 has been associated with proliferation marker Ki67 in normal breast tissues of both luminal

and myoepithelial lineages[154]. The CD44 splice variants with more extensive extracellular amino acid chains have been found frequently in cancer tissues, although they are expressed on normal breast tissue as well[154]. It has been hypothesized that the longer protein structure allow for more opportunities for glycosylation and thus enhanced invasive and motile capabilities, however association between CD44 variants and cancerous tissue has been controversial[154]. CD44 is negatively transcriptionally regulated by p53 in transformed and untransformed breast cells[155]. In these cells, CD44 is reportedly involved in cell proliferation, invasion, motility, metastasis, and chemotherapy resistance[155]. CD44 is also reported to interact with and activate known oncogene Stat3[156, 157]. Further, CD44 is classified as a basal-marker for breast epithelial cells[6, 95, 158]. In most recent years, CD44 has been reported to be a marker for isolating the most tumourigenic cell populations in breast[47], ovarian[159], prostate[160], colon[161], stomach[162], and blood-related cancers[139, 140], termed the tumour-initiating cells. CD44 expression has also been reported to confer radiation[163] and chemotherapy resistance[19, 145, 156, 156] in these cells. Relatedly, independent gene expression profile studies have found that the gene signature of CD44+ primary breast cells normal and cancerous include highly expressed genes overlapping with that of stem cells, genes involved in cell motility and angiogenesis, and genes that correlated with a poorer clinical outcome in patients[6, 164].

1.8 CD49F (ALPHA-6 INTEGRIN)

CD49f, also known as α6 integrin, belong to the integrin family of cell surface receptors with roles mainly in cell polarity, extracellular matrix and cell-cell interactions. Integrins are the main receptors by which the extracellular matrix affects intracellular signaling

pathways. Integrin are heterodimers comprised of 2 trans-membrane receptors, an α and a β subunit[165]. To date, 18 α and 8 β subunits have been identified, and 24 combinations have been reported, allowing cells to bind and respond to all the ligand components in its extracellular microenvironment[165]. Integrins transmit signals both mechanically and chemically, through its interactions with cytokeratin intermediate filaments (by α6β4) and actin cytoskeleton (all others), which consequently regulate downstream cell survival and proliferative pathways, including the JAK-STAT, PI3k/Akt, and MAPK pathways[165].

CD49f in the mammary gland mainly interacts with β4 integrin (CD104) in humans, and β1 integrin (CD29) in mice[166]. The human α6β4 integrin is the receptor for laminin, thereby connecting the basally-located myoepithelial and/or luminal cells to the laminin in extracellular matrix. As such, the α6β4 integrin is important for cell polarity, and breast cells with normal α6β4 forms a spheroid in a laminin-rich Matrigel culture *in vitro*, with the basal ends of the cell outwards in contact with the Matrigel[167]. The loss of α6β4 integrin function disrupts the ability of cells to form polarized structures in the presence of basement membrane proteins[167]. Furthermore, α6β4 integrins not only respond intracellularly to their major ligand, but also to ligands of their binding partners. The α6β4 heterodimer also associates with receptor tyrosine kinases, including EGFR[168-170], Her-2[169, 171-173], Her-3[174], and the MET receptor[111]. Consequently, the interactions of α6β4 to laminin also mediates downstream EGFR, Her-2, and MET receptor signaling, and thus the crosstalk between these receptor tyrosine kinases and α6β4 have important implications for the major signaling pathways modulating cell survival, growth, proliferation, apoptosis, polarity, and migration. In cancer cells, its overexpression up-

regulates these downstream pathways causing increased migration and proliferation[165, 175]. In breast cancers, it has been demonstrated that the overexpression of α6 is correlated to poor survival[176].

In recent years, CD49f has been published as a marker for human and mouse mammary stem/progenitor cells[177-179]. Intriguingly, mammary tissue deficient in the gene encoding for α6 integrin develop and function normally, with both lineages present[180]. Still, CD49f has also been implicated in tumour-initiating cells[179, 181, 182], allowing this rare population to mediate survival and growth responses to its important niche signals.

1.9 NORMAL AND CANCER STEM CELLS

It has been demonstrated that breast and other solid tumours are a bulk collection of a heterogeneous population of cells, all with varying morphologies, growth kinetics, drug and radiation resistance abilities, and *in vivo* tumourigenic properties when injected into immuno-compromised mice[19, 47, 48, 161, 163, 183-185]. Cancer is a clonal disease, requiring a cell or cell population to be dividing for a duration of time sufficient for the accumulation of mutations at random, It is thought that only a cell population with a longer lifespan can cause disease. And so it has been hypothesized that there exists a cell population, with acquired or innate properties associated traditionally to normal stem cells, which may be the originating clone of the tumour[186]. The term cancer stem cell does not preclude or imply that the cell originates definitively from a stem cell. The cancer stem cell is defined as a cancer cell with related properties to a normal stem cell, including long-term self-renewal and proliferative capabilities[186]. This is measured by phenotypic assays

prospectively where the cell must be able to generate tumours *in vivo* with a small starting number of cells, and be able to generate tumours through several passages[47]. Thus, cancer stem cells are more accurately termed tumour-initiating cells, based on their prospective identification by functional assays. In breast cancers, tumour-initiating cells have been reported to be $CD44^{+}CD24^{low}EpCAM^{+}$[47] and/or $ALDH1^{+}$[187]. Further, *in vitro* mammosphere assays that promote the preservation of normal mammary primitive cell populations have been adapted to imitate the *in vivo* tumourigenicity assay where breast cancer cells are challenged to grow in a non-adherent manner in a scarcely supplemented environment with stem cell-responsive growth factors fibroblast growth factor (FGF) and EGF components[188]. This assay implies a shared phenotype between normal and cancerous primitive populations. Similar to *in vivo* tumour passaging, to demonstrate the ability to self-renew over time, a cell must be able to produce new mammospheres through passaging several times. The mammosphere-forming efficiency of cells in this *in vitro* assay has in recent years proven to correlate well with the outcome of *in vivo* tumourigenicity assays[155, 185, 189], as well as patient response to chemotherapy where the mammosphere forming efficiency negatively correlates to the likelihood of chemotherapy response in patients[19]. On top of using this assay in attempts to enrich for tumour-initiating cells, groups have also tried to use the mammosphere assay to decipher the active signaling pathways of tumour-initiating cells, and to determine drug response of tumour-initiating cells[39], though its actual efficacy is uncertain. The cancer stem cell model has emerged as an increasingly accepted hypothesis that at least complements the existing clonal evolution model for cancer initiation, progression, metastasis, and recurrence in a growing list of leukemias, sarcomas, and carcinomas[186, 190, 191].

The hypothesis of the cancer stem cell originates in the field of the hematopoietic system, where the hierarchy of normal stem cell development and differentiation is well characterized and ahead of research in other human tissues and organs[186]. There, in the blood, John Dick and his group first identified a cancer stem cell population in acute myeloid leukemia patients capable of initiating leukemia in immuno-compromised mice and expresses the same primitive hematopoietic stem cell marker profile $CD34^{+}CD38^{-}$ [,192]. As technological advances and the development of functional assays with increased sophistication in the past decades has allowed for the identification and isolation of primitive populations of tumour cells with self-renewal abilities, this has enabled initial observations of the molecular underpinnings and gene signatures of these unique and deadly cells in recent years. For example, the Akt pathway has been nominated as an important target for cancer-initiating cells[193-197]. In systems with historically more robust wealths of knowledge in their stem cell research fields, like leukemias, proteins for targeting the primitive genetic programs have been proposed[14, 193, 196, 198-201]. There is a need for breast cancer and other solid tumours, with more recent isolations of cancer stem cells, to also elucidate the driving molecules of cancer origins. The limitation to understanding breast cancer stem cells is tied to understanding the normal development of the mammary gland. The mammary stem cell has only recently been isolated and assayed in the human and in the mouse[177, 178] but evidence for this distinct subpopulation of cells in the mammary gland arose over a decade earlier[202, 203]. Finally, epigenetic patterning has most recently been demonstrated to also underlie the differences between more primitive and differentiated subsets of the mammary epithelial cell compartments[95] and so it is likely to play a role in the malignant counterparts as well. Further

understanding the molecules underlying differentiation into the two mammary epithelial lineages and finding markers for the stages of differentiation would be crucial to elucidating the origin of tumour growths.

It is likely that the cancer stem cell and clonal evolution theories are over-simplified. Some have hypothesized that tumour progression is probably a combination of the two hypotheses at an unknown ratio. A cell with the capacity to divide long term over time, or have acquired the mutation to divide extensively over time, may likely have acquired other mutations to arm itself against chemotherapies and radiation. Chemotherapies[19] and radiation therapies[163] may in turn may select for its own resistant clones within the tumour-initiating cell population. In addition, acquired genetic instability over the time required for tumour burden to arise may have resulted in a varied tumour cell population possibly too different from the original clone for comparison. Taken together, like leukemias, it is possible that breast cancers arise from primitive cell populations in the mammary gland, and better molecular characterization of this population may be key to finding better treatments to breast cancers.

1.10 THESIS HYPOTHESIS AND OBJECTIVES

Hypothesis:

YB-1 confers an enhanced growth and survival phenotype in breast cancers by inducing stem cell-associated receptors, MET, CD44 and CD49f.

Objectives:

I. To query an association between YB-1 and stem cell-associated genes.
II. To validate a relationship between YB-1 and the MET receptor in normal and cancerous breast epithelial cells.
III. To assess the relationship between YB-1 and two receptors associated with the tumour-initiating cell phenotype, CD44 and CD49f.
IV. To evaluate the functional importance of CD44 and CD49f in three-dimensional culture systems *in vitro*.
V. To determine YB-1's role in the drug-resistance of $CD44^{+}/CD24^{-/low}$ breast cancer cells.

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CHAPTER TWO : PROFILING YB-1 TARGET GENES UNCOVERS A NEW MECHANISM FOR MET RECEPTOR REGULATION IN NORMAL AND MALIGNANT HUMAN MAMMARY CELLS*

This chapter presents work which introduces the novel finding of YB-1 expression in a normal primitive mammary cell population. We validate a chromatin immunoprecipitation-on-chip screen which suggests that YB-1 occupies the promoter of a cassette of genes associated with stem cells by elucidating the molecular biology behind a new relationship between YB-1 and MET, one of the resulting genes from the ChIP-on-chip screen. Further, we were able to demonstrate that YB-1 transcript levels are detected in a purified population of normal human mammary progenitor cells that correlated to Met transcript levels. This study verified that YB-1 may possibly be a regulator of other stem cell-associated genes on the ChIP-on-chip screen list.

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2.1 INTRODUCTION

Basal-like breast cancers (BLBCs) remain one of the greatest challenges given that the probability of survival beyond 2 years is only 30%[1]. Because BLBCs do not express estrogen receptors (ERs), progesterone receptors (PRs) or HER-2, they are unresponsive to anti-estrogens[2] or Herceptin that target these gene products[3]. Therefore, much effort is now focused on identifying new molecular targets in BLBCs. One target of current interest is the transcription/translation factor Y-box binding protein (YB-1) because of its documented high expression in approximately 70% of BLBCs[4] and its association with poor survival[5]. YB-1 is consistently associated with high rates of relapse in virtually all breast cancer subtypes including BLBC[5]. Complimenting the strength of YB-1 as a biomarker of aggressive cancer we reported that inhibiting with siRNAs attenuates the growth of BLBC cell lines[6]. YB-1 is known to stimulate cell growth by binding to inverse CAAT boxes of many genes, thereby inducing their expression. Examples include *EGFR* (epidermal growth factor receptor)[7], *HER-2*[7], and *TOPOII* (topoisomerase 2)[8]. It has been suggested that such YB-1-mediated activation of *EGFR* transcription also promotes the growth of BLBCs[4]. The induction of *EGFR* expression is dependent on phosphorylation of YB-1 at S102[4, 7] which is achieved by serine/threonine kinases Akt[9] or RSK[10] leading to nuclear translocation. Importantly, nuclear YB-1 has been reported in many instances to be a universal biomarker for drug resistance thus understanding how it regulates gene expression is essential[11]. Given this, it is of particular interest to understand the specific target genes responsible for the growth dysregulation of YB-1 over-expressing BLBC cells.

Another gene associated with poor outcomes and a greater risk of metastasis in BLBCs is *MET*[12]. The *MET* gene encodes a cell surface tyrosine kinase receptor for hepatoctye growth factor (HGF, also known as scatter factor). HGF is a pleiotropic cytokine with pro-migratory, anti-apoptotic, and mitogenic activities[13-15]. Once activated, MET can promote cancer cells to proliferate and migrate and it has thus become another potential therapeutic target[16].

To understand how YB-1 regulates the growth of BLBC at the transcriptional level, we performed genome-wide chromatin immunoprecipitation (ChIP) on chip (COC) analyses using promoter arrays. These revealed a subset of YB-1 target genes associated with the basal-like signature including *MET* and other genes expressed by normal progenitor and malignant mammary cells such as *CD44*, *CD49f* and several members of the *NOTCH* and *WNT* families. Further studies were then undertaken to investigate how YB-1 regulates *MET* expression.

2.2 MATERIALS AND METHODS

Cell lines

SUM149 cells were obtained from Asterand (Ann Arbor, MI) and grown according to the supplier's recommendations. HCC1937 cells were obtained from Dr. W.D. Foulkes (McGill University, Montreal, Quebec) and were cultured in RPMI-1640 (Invitrogen) supplemented with 10 mM HEPES pH 7.6, 4.5 g/L glucose (Sigma), 1 mM sodium pyruvate (Sigma) and 10% FBS. BT474-M1 cells were obtained from Dr. Mien-Chie Hung (University of Texas, M.D. Anderson Cancer Center, Houston, TX). All other breast cancer cell lines were purchased from the American Tissue culture collection (Rockwood MD) and cultured as recommended.

ChIP on Chip (COC)

SUM149 cells were grown to 80% confluency on 15-cm diameter plates (8×10^6 cells). Chromatin immunoprecipitation (ChIP) was performed on the pooled lysates from 12 plates of cells. YB-1:DNA complexes were isolated as previously described[7] using a anti-chicken antibody (Dr. Isabella Berquin, Wake Forest University, North Carolina). Following elution of the DNA from the beads, the DNA was amplified using the protocol provided by NimbleGen (additional details can be found in the supplemental Materials and Methods). From the ChIP on chip hybridization, a list of accession numbers of genes with promoters to which YB-1 potentially binds was generated. Using the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/), the accession numbers were decoded and all genes with a greater than log 2-fold change

were identified. Data was analyzed using Ingenuity Pathway Analysis software (Redwood City, CA).

Immunoblotting

Breast cancer cell lines were harvested and immunoblotting was performed as described previously[7] by probing the blots with anti-MET (Santa Cruz Biotechnology clone C-12, Santa Cruz, CA), P-YB-1^{S102} (Cell Signaling Technologies (CST), Danvers, MA) and total YB-1 (Abcam, Cambridge, MA). Transient transfections were performed with 2 μg of empty vector, Flag:YB-1(WT), Flag:YB-1(D102) or Flag:YB-1(A102) into SUM149 or HCC1937 cells as previously described[9] and 72 hours later MET receptor levels were evaluated by immunoblotting. Flag YB-1 was detected with an anti-Flag antibody (Sigma) and total YB-1 levels were determined. Actin (CST) was used as a loading control. YB-1 knockdowns were performed as described below, proteins harvested after 96 hours, immunoblotted, and MET and YB-1 levels determined using Vinculin (Sigma clone Vin 11-5, V4505 antibody) or actin as loading controls. Intensity of the signals was quantified using ImageJ 1.38X image analyzing software.

Immunocytochemistry for P-YB-1$^{(S102)}$

SUM149 and MDA-MB-231 cells ($1.0x10^5$) were seeded on glass coverslips, washed with phosphate-buffered saline (PBS), fixed with 2% formaldehyde for 20 min, rinsed twice with PBS, and then incubated with PBS containing 0.1% Triton X-100 (Sigma) for 30 min. Next, the coverslips were washed with PBS, incubated with rabbit anti-Met (Santa Cruz Biotechnology clone C-12, Santa Cruz, CA) P-YB-1$^{(S102)}$ (CST) antibodies

dissolved in buffer containing 10% BSA and 2% goat serum for 1 hour at room temperature in a humidified container. After washing three times with PBS, glass slides were incubated with Alexa 488 anti- rabbit antibody for 1 hour, washed three times and then mounted using Vectashield mounting medium (Vector Laboratories, CA). DAPI was used for nuclear staining.

Isolation of primary progenitor cells

Bipotent progenitor-enriched fractions were isolated from freshly thawed vials of three reduction mammoplasty samples, as previously described[17] (see Supplemental Materials and methods for more detail). We also isolated also bipotent progenitor-enriched fractions from 3 different reduction mammoplasty samples, as previously described[18]. To assess the transcript levels of *YB-1* and *MET*, 40 to 60 ng of RNA from each sample were reverse transcribed into cDNA as described[19]. For comparison, RNA was isolated from MDA-MB-231, MDA-MB-468, HCC1937, and SUM149 cells grown in log phase using the Qiagen RNeasy Mini Kit (Qiagen) and its prescribed protocol. 1 μg of extracted RNA was reverse-transcribed using Superscript III Reverse Transcriptase and its prescribed protocol (Invitrogen). QRT-PCR (7000 Sequence Detection System, Applied Biosystems) was performed using TaqMan Gene Expression Assays designed against human *YB-1* (Custom TAMRA probe, sequence: 6FAM-AAGCCCGGCACTACGGGCAGC-TAMRA, Applied Biosystems), human *MET* (Assay ID: Hs00179845_m1, Applied Biosystems), and human TATA-box binding protein (TBP) as an endogenous control (Part No. 4326322E, Applied Biosystems). Relative expression of *YB-1* and *MET* transcript levels was determined by normalizing to TBP.

Traditional Chromatin Immunoprecipitation (ChIP)

SUM149 and HCC1937 cells were grown until 90% confluent in a 15-cm dish (1×10^7 cells) and ChIP was performed as previously described[7] to isolate the YB-1-promoter complexes. Fifteen potential YB-1 binding sites were identified on the first 2 kb of the *MET* promoter. Therefore we designed three sets of primers to narrow down where YB-1 binds to the *MET* promoter using ChIP. PCR was carried out using 6 μl of purified DNA. Primer sequences were as follows. *MET 1*: TTGACCTTCACACACCCAGAT (forward), TTCTGAGTTTGAGTGCCATGA (reverse); *MET 2*: TCATGGCACTCAAACTCAGAA (forward), CCAGTCAGGTGTCCTTCACA (reverse); *MET 3*: GCAAAATGGTTCAATGCAAG (forward), GGGCCTCGGTGAACTCTATT (reverse). PCR conditions were optimized for the primer sets as follows: 94°C for 3 minutes, 40 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 70°C for 30 seconds, followed by 70°C for 10 minutes. PCR products were visualized by agarose gel electrophoresis (2% gel) and DNA stained with ethidium bromide (Invitrogen, Burlington, Ontario, Canada). The YB-1:*MET* promoter complex was also chromatin immunoprecipitated from a SUM149 xenograft. Briefly, NOD/SCID mice were injected with $5x10^5$ cells into the mammary fat pad and the tumors were allowed to develop over a 14-week period. Endogenous YB-1 was subjected to ChIP from the SUM149 xenografts as previously described by us[20] and the DNA was amplified for MET promoter binding using the primers indicated above**.**

Electrophoretic mobility shift assay (EMSA)

Nuclear proteins were isolated from SUM149 and MDA-MB-231 cells using NE-PER extraction kit (Pierce Biotechnology, Rockford, Illinois, USA) according to the manufacturer's protocol. The Lightshift Chemiluminescent EMSA kit (Pierce Biotechnology) was used to perform EMSAs using oligonucleotides corresponding to potential YB-1 binding sites according to our previously published methods[4]. Briefly, binding reactions contained 1x binding buffer, 50 ng/μl poly dIdC, 20 fmol biotin-labeled double stranded DNA and 6 μg nuclear extract. Unlabelled oligonucleotide (16 pmol) was used as competition. Chicken anti-YB-1 antibody (1 μg) was used to determine YB-1 involvement and CREB antibody (1 μg) acted as a negative control. Oligo1:TAACCCATGACTTTCAATAACGAAGATATC , Oligo 1 mutant:TAACCCATGACTTTCCCCAACGAAGATATC, Oligo 4: ACTCTTGTAGGTGCCAATTTTTATAGCGAA, Oligo 4 mutant: ACTCTTGTAGGTGCCCCCTTTTATAGCGAA.

Inhibition of YB-1and MET using siRNA or OSU-03012

The siRNAs targeting *YB-1* or *MET* were diluted in 500 μl serum-free OPTI-MEM (Invitrogen) to achieve a final concentration of 20 nM and 6 μl Lipofectamine RNAiMAX (Invitrogen) was then added. The mixtures were incubated at room temperature for 20 minutes in the wells of a 6-well plate to each of which 4 x 10^5 cells were then added in 2 ml of media. After 96 hours, cells were harvested. Small inhibitory RNAs targeting *YB-1*, referred to as siYB-1#2, and siYB-1#3, were obtained from Dharmacon as previously described[4], and Qiagen (Catalog number SI03019191)

respectively. The si*MET* oligonucleotide was obtained from Qiagen (Catalog number SI00604821). Stable knockdowns were performed using shYB-1 previously described[21] in the SUM149 cells.

SUM149 cells were seeded at 85% confluence into 6-well plates 24-hours before OSU-03012 treatment. OSU-03012 was added into the media to achieve final concentrations of 10 μM. Equal volumes of DMSO were added as vehicle control treatments. SUM149 cells were harvested at 6 hours of OSU-03012 treatment. Western blotting and RT-PCR analyses were performed as previously described[20]. At later time points, OSU-03012 killed the cells; therefore we were not able to collect the cells after longer exposure times.

Luciferase assays

To determine the effect of *YB-1* expression on *MET* promoter activity –3.1kB portion of the receptor was cloned into a pGL2 reporter[22]. Forty-eight hours (MDA-MB-231 cells) or 72 hours (SUM149 cells) after siRNA treatment, cells were transfected with this *MET* promoter construct, a renilla expression vector, pRL-TK (Promega, Madison, Wisconsin, USA), or an empty vector. After an additional 48 hours (MDA-MB-231 cells) or 24 hours (SUM149 cells), cells were harvested in 1x Passive Lysis buffer (Promega) and luciferase activity was measured using the Dual Luciferase kit (Promega). Additional cells were lysed in ELB buffer with protease inhibitor cocktail to check for evidence of YB-1 knockdown. Experiments were performed in triplicate on two separate occasions and the results were averaged.

YB-1 over-expression and site-directed mutagenesis

Over-expression of wild type and mutated YB-1^{A102} was performed as previously described[9]. The YB-1 S102D was generated by site-directed mutagenesis (Stratagene, Windsor, ON, USA) of the WT-YB-1 construct using the following primer: 5'-ccaggaagtaccttcgcgatgtaggagatggagagactgtgg-3'. These three constructs have the 3x FLAG tag. The plasmid transfections were performed with 2 μg of DNA and Lipofectamine 2000 (Invitrogen), and maintained for 72-96 hours.

Effect of siYB-1 on MET receptor signaling and tumor cell growth in soft agar

In order to understand the impact of silencing *YB-1* expression on HGF-dependent stimulation of MET receptor signaling, MDA-MB-231 cells were exposed to siYB-1#2 or siYB-1#3 for 96 hours. Cells were then serum starved over night and then stimulated for 15 minutes with HGF (20 ng/ml). Proteins were isolated and evaluated for changes in the MET receptor adaptor protein P-Gab-1$^{(Y307)}$ (CST, cat number 3234)[23]. Total Gab-1 was used as a loading control (CST, Cat 3232). To study anchorage independent growth, cells were plated at a density of 1 x 10^4 cells/well (SUM149 cells) and 2.5 x 10^3 cells/well (MDA-MB-231 cells) in a 24-well plate in 0.6% agar plus 40 ng/ml HGF (R&D Systems, Minneapolis, MN) on top of a 1.2% cell-free agar. After 28 days, colonies were counted. Experiments were performed in replicates of four on two separate occasions and results averaged.

2.3 RESULTS

COC suggests YB-1 regulates MET

COC was undertaken to systematically identify a full list of potential targets of YB-1 in SUM149 BLBC cells. The results revealed that YB-1 potentially interacts with >6000 candidate promoters. A high proportion of these encode enzymes, kinases, transporters and known or potential growth factor receptors (Figure 2.1, A) (for a complete list, see Supplementary Table 2.1). Notable amongst the latter was *MET* (Table 2.1). *CD44*[24] and *CD49f*[25] were also found to be potential YB-1 target genes and notably both are previously reported to bind to and enhance MET activation[26]**.** Several members of the NOTCH and WNT pathways were also identified (Table 2.1). Their potential functional relationships to one another in terms of signal transduction activation are illustrated using Ingenuity Pathway Analysis software (Figure 2.1B). We highlight that two major signaling nodes are created using this approach; one that involves the MET/CD44/CD49f signaling complex and the other that includes WNT/NOTCH interactions. While it is beyond the scope of this work to validate all of the YB-1 target genes associated with these signaling networks, this preliminary analysis does suggest that there are common features of YB-1 responsive genes that could have an important bearing on cell growth control.

P-YB-1$^{(S102)}$ and MET are coordinately expressed in BLBC cell lines

To investigate if there is a relationship between levels of MET and YB-1 protein, a panel of breast cancer cell lines was screened. Levels of total YB-1 protein were consistent across all cell lines, but P-YB-1$^{(S102)}$ levels were higher in the BLBC cells. Strikingly,

MET was expressed exclusively in the BLBC cell lines. Like many transcription factors, YB-1's access to the genome is governed by phosphorylation[9]. Herein we show that P-YB-1$^{(S102)}$ was intensely expressed in the nucleus of SUM149 and MDA-MB-231 cells. MET was also expressed at considerable levels in both SUM149 and MDA-MB-231 based on immunofluorescence (Figure 2.2B). This was further confirmed by immunoblotting (data not shown).

Total YB-1 and MET are co-expressed in normal human mammary progenitor cells

We asked whether YB-1 transcripts are present in populations of the most primitive normal human mammary progenitors that can be reproducibly isolated at high purities (30-50%)[18]; i.e., bipotential progenitors that produce mixed colonies containing both mature luminal and myoepithelial cell progeny. Suspension cultures containing bipotent progenitors were isolated from freshly thawed aliquots of reduction mammoplasty cells taken from three different individuals. The mRNA from these samples was amplified for *YB-1* and *MET* by qRT-PCR. In addition, we evaluated the expression of *YB-1* and *MET* in mammoplasty cells that were isolated and subsequently cultured for three days in media that specifically enriches for progenitors where the proportion of progenitors increased from ~5% in the freshly thawed isolates to 30-50% in the selective media. *YB-1* and *MET* mRNA was expressed in the bipotential progenitor populations regardless of the culturing method (Figure 2.2C). However, the levels of *YB-1* transcripts in these cells were up to 2000 times lower than in the cancer cell lines and the levels of *MET* mRNA were as much as 300 times lower (data not shown). Nevertheless, there was a strong correlation (r^2=0.8785) between the levels of *YB-1* and *MET* mRNA in the normal

progenitor-enriched samples (Figure 2.2D). This indicates for the first time that *YB-1* and *MET* transcripts are both detectable in a very primitive normal mammary subpopulation albeit at very low levels compared to cancer cell lines.

Verification that YB-1 binds to the promoter of MET

To obtain more direct evidence that YB-1 binds to the *MET* promoter in BLBC cells, traditional ChIP experiments were carried out on extracts of three BLBC (SUM149, MDA-MB-213 and MDA-MB-468) cell lines, as well as, from a primary tumor that developed from SUM149 cells injected into a NOD/SCID mouse. PCR amplifications were performed using 3 primer sets designed to flank potential YB-1 binding sites on the *MET* promoter referred to as *MET 1*, *MET 2* and *MET 3* (Figure 2.3A, *top*). YB-1:*MET* promoter binding was validated using *MET 1* primers and this was evident in each of the BLBC cell lines and the xenograft cells (Figure 2.3A, *bottom, left*). Yet the putative YB-1-responsive elements (YREs) that would have been amplified by the *MET 2* and *MET 3* primers were not authentic binding sites based on a lack of amplification (Figure 2.3A, *bottom, right*). Using this strategy, we localized YB-1 binding sites to the *MET 1* region that contains six potential binding sites. To determine which of the potential YB-1 binding sites was most important, five oligonucleotides (oligo 2 contained two YREs in close proximity to one another) were designed to the putative binding sites in the *MET 1* region and EMSA was then performed (data not shown). The results of these assays revealed that oligo 1 (-1151 to -1180) and 4 (-1006 to -1035) elicited the strongest shifts in the presence of a nuclear extract from SUM149 cells (Figure 2.3B). This was confirmed with MDA-MB-231 nuclear extracts (data not shown). Both oligos showed a

strong supershift with the addition of the YB-1 antibody but not a CREB antibody, indicating the specificity of the YB-1 binding. Furthering this observation, mutation of the YRE in oligo 4 resulted in loss of binding, whereas this was not the case with oligo1 (Figure 2.3C). Therefore, it can be concluded that a bona fide YRE on the *MET* promoter resides -1018 bp upstream of the translational start site while YB-1 binding to the oligo 1 region must be indirect.

To investigate this apparent association further, the introduction of either Flag:YB-1 or constitutively active Flag:YB-1(D102) increased MET protein levels in SUM149 and HCC1937 cells compared to the empty vector yet the inactive A102 mutant did not (Figure 2.4A). The introduction of siRNA designed to silence YB-1 reduced MET protein and mRNA expression by 40-60% in SUM149 and MDA-MB-231 cells (Figure 2.4B-C). Similarly, stable inhibition of YB-1 using a shYB-1 approach suppressed the expression of MET protein and mRNA (Figure 2.4B-C). The same effect was observed when the cells were infected with a lentiviral vector expressing shYB-1 (Figure 2.1 supplemental). In all of these studies, MET mRNA decreased by ~50%. Consistent with this finding, the treatment of the cells with siYB-1 #2 caused a 54% reduction in *MET* promoter activity (Figure 2.4D). In contrast, wild type YB-1 and Flag:YB-1(D102) increased *MET* promoter activity Flag:YB-1(A102) did not (Figure 2.4E). Likewise, treating SUM149 cells with the PDK-1 inhibitor OSU-03012 for 6 hrs decreased P-Akt^{S473}, P-YB-1^{S102} and attenuated MET protein (Figure 2.4F*, left*) and mRNA (Figure 2.4F, *right*). Thus, inhibiting a major signal transduction pathway known to activate YB-

1 also perturbs MET protein expression[20]. These data provide evidence that YB-1 phosphorylation is important for its control of MET expression.

Silencing YB-1 or MET with siRNA inhibits HGF-stimulated signaling and anchorage-independent growth

Because HGF is the major stimulus for MET receptor, we expected that inhibiting YB-1 would interfere with this important signaling pathway. In support of this idea, silencing *YB-1* for 96 hrs inhibited HGF-mediated signaling through MET given that Gab-1 phosphorylation was suppressed (Figure 2.5A). Silencing *YB-1* with siYB-1#3 remarkably suppressed the expression of the MET receptor and therefore HGF-induced signaling through Gab-1. We also silenced *MET* itself as a positive control to show that HGF must use this receptor to elicit signaling through Gab-1 (Figure 2.5A). Of note, the partial suppression of MET with siYB-1#2 was not enough to fully block HGF-induced signaling. This is likely because there was enough residual MET receptor present to transmit the signal. Taken together, these results indicate that the expression and activity of MET in BLBC cells is at least partly dependent on YB-1. To study the effect of YB-1 and MET knockdown on the proliferative activity of BLBC cell lines, the clonogenic ability of siRNA-treated SUM149 and MDA-MB-231 cells in soft agar assays was analyzed in cultures enriched with HGF. Both cell lines showed greater sensitivity to the loss of YB-1 than MET interference, with up to a 90% reduction in colony-forming ability by siYB-1 #2 –treated SUM149 cells (Figure 2.5B). However, a reduction of up to 57% in clonogenic ability of these cells was seen when MET was directly targeted. MDA-MB-231 cells were slightly less sensitive to either of these treatments (72%

reduction with siYB-1 #2 and 34% with siMET, Figure 2.5C). These data indicate that disruption of YB-1 leads to a remarkable suppression of anchorage-independent growth, some of which is attributable to loss of *MET* expression.

Our data collectively supports the idea that MET is transcriptionally up-regulated by YB-1 in BLBC. One could argue that high levels of MET may be due to gene amplification. To begin to address this, we performed comparative genomic hybridization on MDA-MB-231 and reported that amplifications were not found in the 7q31.1 where the *MET* gene resides[27]. Similarly, MET is not commonly amplified in primary BLBC based on the analysis of ten tumors (Figure 2.2, supplemental). These findings do not support a role of gene amplification causing the increased *MET* expression that is characteristic of these cells and reinforce the potential importance of other mechanisms such as YB-1-mediated up-regulation.

2.4 DISCUSSION

In this study, we present results of the first global COC analysis of genes potentially regulated by YB-1 in BLBC cells. This analysis revealed approximately 6000 candidate targets including many genes encoding growth factor receptors and their signaling intermediates. One of these was *MET,* which encodes the tyrosine kinase cell surface receptor for HGF and has been previously implicated in primary human BLBCs[28]. In addition, forced over-expression of *MET* together with *Myc* in murine mammary cells has been found to produce BLBC in mice[12]. While many aspects of MET activity have been well studied[29], surprisingly little has been uncovered about its transcriptional regulation with the exception of evidence for a role of ETS[22], hypoxia inducible factor-1 a[30] and b-catenin[31]. In the studies described herein, using traditional ChIP and EMSA, we now demonstrate that *MET* expression is transcriptionally up-regulated in BLBC cells by direct binding of YB-1 to the *MET* promoter at –1018 bp from the transcriptional start site. In addition, we showed that inhibition of YB-1 in these cells decreased *MET* mRNA and protein levels, and attenuated HGF-induced signaling. Interestingly, examination of *MET* expression in different breast cancer cell lines showed *MET* to be up-regulated in four BLBC cell lines, but not in the three representative luminal and HER-2 expressing cell lines tested. Further, our data suggest that MET signaling is important to the growth of BLBC cells, based on the level of suppression of BLBC colony formation obtained from siRNA-treated cells. These results provide strong support for an important role of MET as a downstream effecter of YB-1-mediated transformation of BLBC cells. Our present finding indicates that primary samples of this subgroup of breast cancer do not show amplification of the *MET* gene, in spite of their up-regulated expression of MET

expression[12, 28]. This observation underscores the likelihood that this is a downstream consequence of the enhanced expression and trans-regulatory activity of YB-1 on the *MET* promoter.

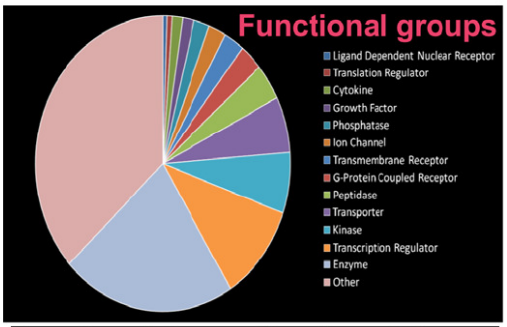
It should be noted that inhibiting YB-1 consistently had a greater effect on attenuating MET protein than it did on modulating *MET* mRNA levels. For example, when *YB-1* expression was stably inhibited with shRNA, the levels of MET protein became virtually undetectable, yet *MET* mRNA was reduced by only ~40-50%. This observation was confirmed using two different MET receptor antibodies. Consistent with these results, silencing *YB-1* with a lentiviral shRNA vector yielded identical findings; the levels of MET protein were inhibited to a greater extent than could be explained by changes in mRNA. While we demonstrate herein that the MET promoter is a direct target of YB-1, it may also influence its translation. Thus, additional regulatory avenues need to be explored to more fully understand the relationship between YB-1 and MET.

As predicted by such a model, inhibiting YB-1 also markedly suppressed the growth of the BLBC cells under anchorage-independent conditions. Indeed, the inhibition obtained was even greater than that achieved by inhibiting MET alone, consistent with the COC findings indicating likely effects of YB-1 on other genes important to the growth of BLBC cells. These data suggest that targeting MET, as well as, YB-1 could be useful therapeutic strategies for improving the treatment of BLBC in patients. In this regard, it is interesting to note that inhibitors of MET are already available and in clinical trials in other types of patients[29]. Our group is actively developing inhibitors to YB-1.

Some of the other genes that YB-1 bound reportedly have stem/progenitor associations; e.g., CD44, CD49f, *c-KIT*, *BMI-1*, and both *NOTCH* and *WNT* pathway elements[18, 32-34]. It is interesting to note that high expression of many of these genes has also been associated with human breast cancer cells that have an ability to initiate tumor formation in immunodeficient mice[35-37]. It might be speculated, therefore, that heightened expression of YB-1 is a key mechanism used by normal mammary stem cells to maintain their primitive status and one that is used and/or high jacked by breast cancer stem cells. Further validation of the genes that YB-1 regulates in normal and malignant breast cells should therefore be of interest to understand why YB-1 expression correlates so strongly with breast cancer recurrence.

2.5 FIGURES AND TABLES

A



Functional Group	Number of Genes
Ligand Dependent Nuclear Receptor	19
Translation Regulator	24
Growth Factor	65
Cytokine	68
Phosphatase	86
Transmembrane Receptor	101
Ion Channel	144
Peptidase	189
G-Protein Coupled Receptor	225
Kinase	267
Transporter	391
Transcription Regulator	530
Enzyme	892
Other	3551
Total Genes	**6552**

B

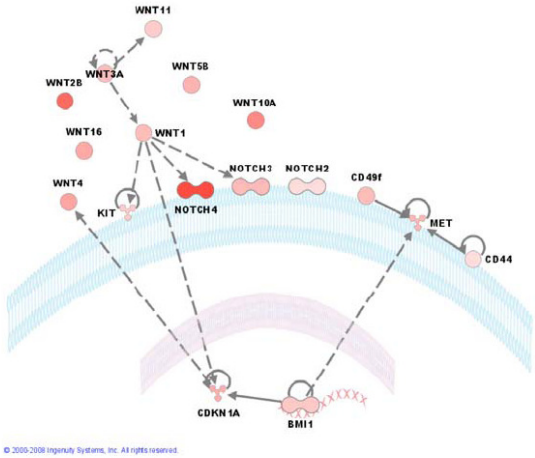


Figure 2.1. Categorization of YB-1 COC data into functional groups and schematic presentation of the possible interactions among the select genes that are linked to primitive cells and tumor initiation. A) Genes were grouped into 15 functional categories. The largest known group was represented by genes that encode enzymes using Ingenuity Pathway Analyses. Enumeration of the genes in each category is found in the list. **B**) From the ~6000 potential YB-1 targets identified by COC, a group of genes involved in self-renewal and tumor initiation were identified. The way in which the protein products of these genes interact with one another and thereby influence cell signaling is depicted. From this analysis, it is clear that there are several members of two common signaling nodes that are potentially regulated by YB-1. These nodes involve MET/CD44/CD49f and the WNT/NOTCH networks. Solid arrows indicate direct interactions whereas broken arrows represent indirect relationships.

Gene name	*Accession number*	*Fold change*
Bmi-1	NM_005180	3.5
CD44	NM_000610, NM_001001389, NM_001001390, NM_001001391, NM_001001392	2.6
CD49f (ITGA6)	NM_000210	5.3
C-Kit	NM_000222	3.8
Met	NM_000245	6.6
Notch2	NM_024408	2.4
Notch3	NM_000435	8.3
Notch4	NM_004557	11.9
P21/CDKN1A/Cip1	NM_000389, NM_078467	7.1
WNT1	NM_005430	4.7
WNT2B	NM_024494	9.4
WNT4	NM_030761	6.9
WNT3A	NM_033131	4.9
WNT5B	NM_032642	5.5
WNT10A	NM_025216	7.2
WNT11	NM_004626	4.3
WNT16	NM_057168	6.4

Table 2.1. Select putative YB-1 target genes identified in the COC screen that are common to one another in that they each are associated with a stem cell signature. From this list of genes, the MET receptor was selected for validation because it forms a network with other proteins in this list such as CD44 and CD49f.

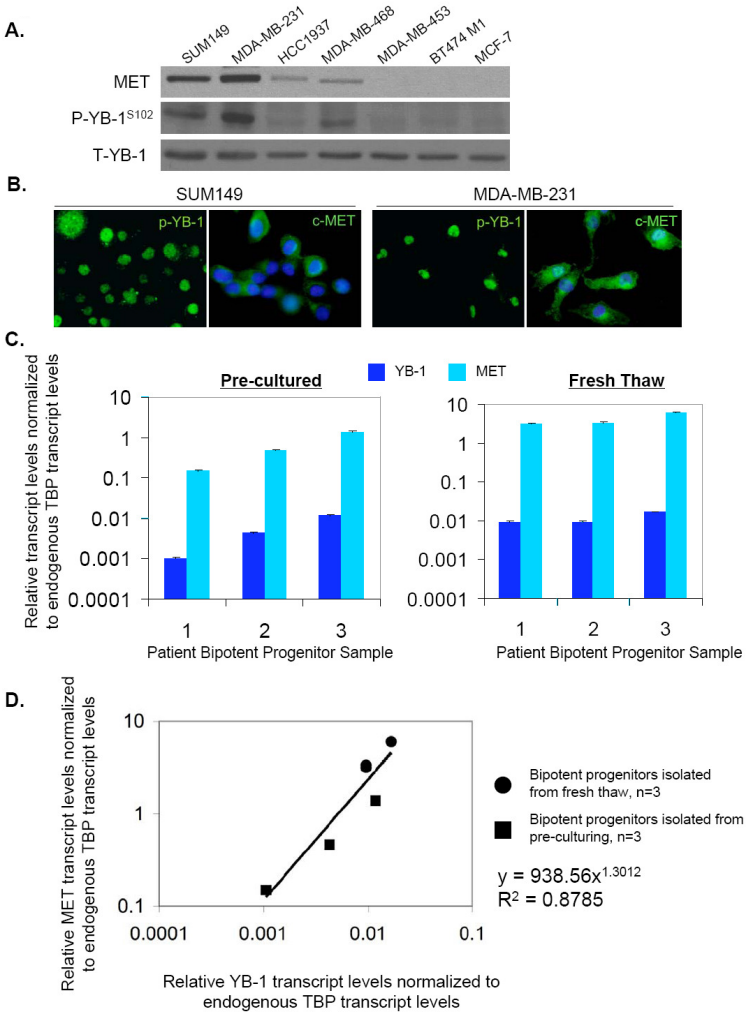


Figure 2.2. YB-1 and MET receptor expression levels are positively correlated in basal-like breast cancer cell lines and primary normal mammary progenitor cells. **A)** Investigation of MET and YB-1 protein levels in a panel of breast cancer cell lines corresponding to BLBC, HER-2 over-expressing, and luminal subtypes. This comparison included four BLBC cell lines (SUM149, MDA-MB-231, HCC1937 and MDA-MB-468), as well as three additional lines representing other breast cancer subtypes; i.e., MDA-MB-453 (a model for the HER-2 over-expressing subtype), BT474-M1 (a model of the luminal B subtype), and MCF-7 (a model of the luminal A subtype). MET expression was restricted to the BLBC cells lines, subtypes where P-YB-1$^{(S102)}$ levels were also higher. **B)** P-YB-1$^{(S102)}$ was localized to the nucleus of the SUM149 and MDA-MB-231 cells. Levels of Met receptor protein were also visualized in the cell lines by immunofluorescence. DAPI was used for nuclear staining. **C-D)** *YB-1* and *MET* receptor transcript levels in normal mammary progenitor cells isolated from three different reduction mammoplasties. Cells were either isolated from freshly thawed tissues or from cells cultured in media that enriches for mammary progenitors. Samples were analyzed by qRT-PCR assays for *YB-1* and *MET* mRNA and normalized to endogenous TBP. *YB-1* and *MET* mRNA tightly correlated across these samples (R^2=0.8785).

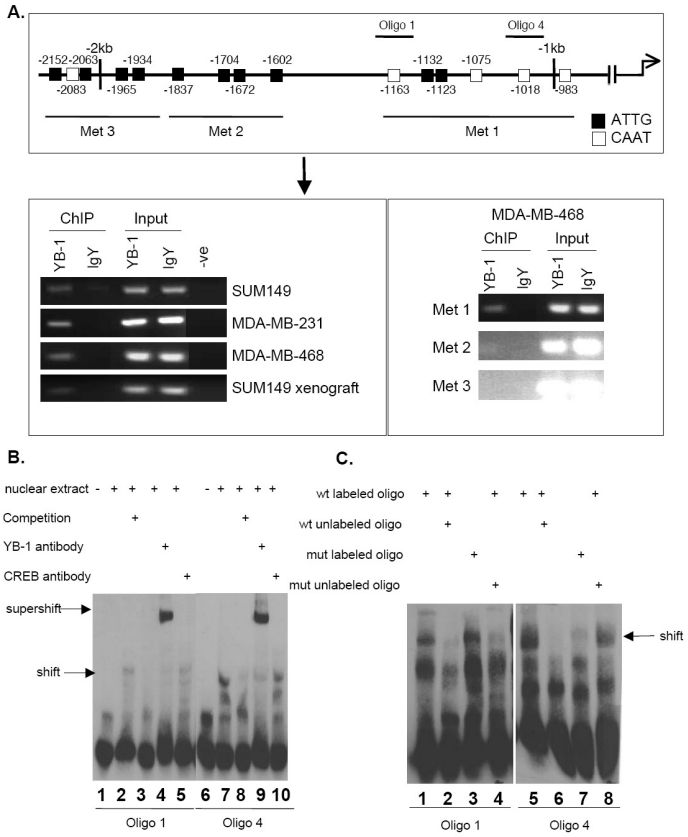


Figure 2.3. Traditional ChIP and EMSA localize YB-1 binding to the -1018 YRE. **A)** Analysis of the *MET* promoter indicated 15 putative YB-1 binding sites where the black boxes indicate 5'-ATTG-3' sequences and white boxes indicates 5'-CAAT-3' sites. Primers were designed to amplify portions of the MET receptor that captured several non-overlapping YRE's referred to as *MET 1*, *MET 2*, and *MET 3*. Solid and open boxes refer to ATTG and CAAT binding regions, respectively. In addition, the location of confirmatory oligonucleotides used for subsequent gel shift assays described below referred to as oligo1 and 4 is also illustrated. Traditional ChIP analysis showed strong binding of YB-1 in the *MET 1* region in both SUM149 and MDA-MB-468 extracts. In addition, ChIP from a SUM149 xenograft showed YB-1 binding *in vivo* at this site. Binding was not observed using primers to the MET2 or MET3 regions, for example in MDA-MB-468 cells. **B)** EMSA analysis with *MET* oligos 1 and 4. The regions corresponding to these oligos were -1151 to -1180 for oligo1 and -1006 to -1035 for oligo 4. In the absence of nuclear protein (lane 1&6) no binding was observed. In contrast, the addition of nuclear extracts (lane 2&7) resulted in binding which could be competed with cold oligonucleotide (lane 3&8) or by incubating the nuclear extracts with YB-1 antibody (lane 4&9). Addition of YB-1 antibody causes a supershift (lane 4&9), while addition of a CREB antibody did not (lane 5&10) indicating site specificity. **C)** Mutant oligonucleotides were next introduced to oligos 1 (-1151 to -1180) and 4 (-1006 to -1035) where the YB-1 binding site was changed to 5'-CCCC-3'. Loss of the putative YB-1 response element causes a reduction in YB-1 binding in oligo 4 (lane 7) but not oligo 1 (lane 3).

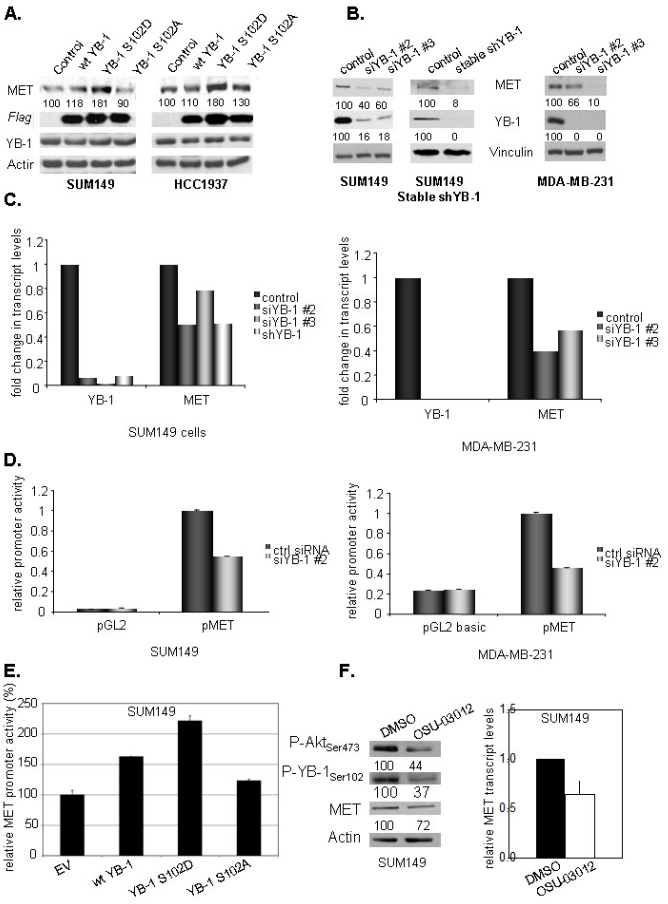


Figure 2.4. ***MET*** **mRNA and protein levels and reporter activity can be modulated via YB-1. A)** SUM149 and HCC1937 cells were transiently transfected for 72 hrs with empty vector, Flag:YB-1(WT), Flag:YB-1(D102) or Flag:YB-1(A102). MET levels increased following the expression of Flag:YB-1(WT) and more so with Flag:YB-1(D102), while Flag:YB-1(A102) was unable to cause the same effect. **B)** YB-1 knockdown with either siYB-1#2 or siYB-1#3 reduced MET protein expression in the SUM149 and MDA-MB-231 cell lines. Vinculin was used as a loading control. Similarly, shYB-1 expression lead to decreased MET receptor protein in SUM149 cells. **C)** QRT-PCR revealed that transcript levels of *MET* were also reduced in YB-1 knockdown samples compared to cells treated with control siRNA for all cell lines. **D)** SUM149 and MDA-MB-231 cells treated with siYB-1#2 or siYB-1#3 decreased *MET* reporter activity by 45% and 54%, respectively. Inhibition of YB-1 had no effect on the activity of empty vector, pGL2 basic, as expected. **E)** In contrast, over-expression of wild type YB-1 increased *MET* promoter activity which again was more so with Flag:YB-1(D102). Similar to transcription level, Flag:YB-1(A102) was unable to increase the promoter activity **F)** OSU-03012 attenuated Akt activation resulting in reduced P-YB-1^{S102} and eventually reduction of MET protein expression in SUM149 cells. Inhibition of PDK-1 signalling via OSU-03012 treatment also significantly inhibited MET mRNA levels based on qRT-PCR. SUM149 cells were treated with DMSO (black) or OSU-03012 (white) for 6 hours. *MET* transcripts were significantly reduced by OSU-03012 compared to the DMSO treated cells ($p < 0.05$).

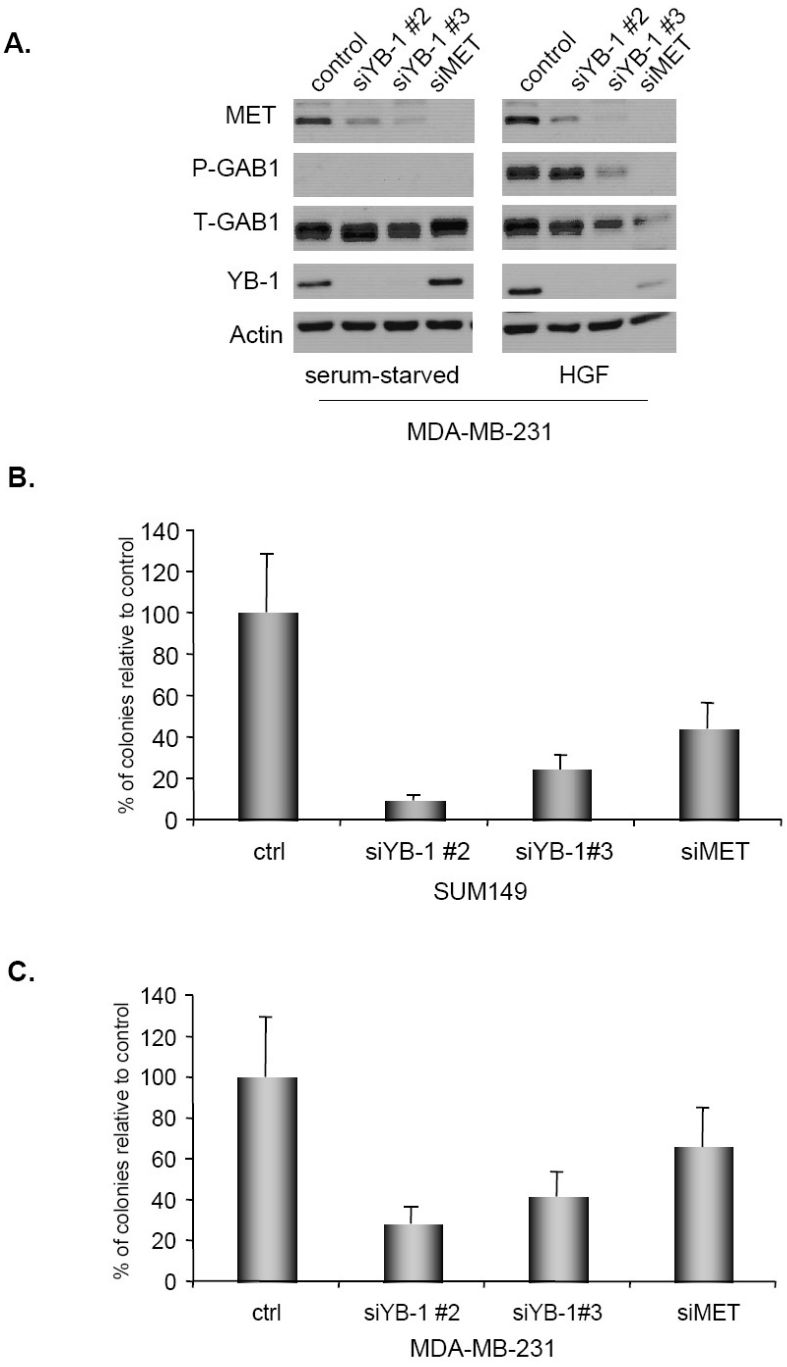


Figure 2.5. Inhibition of YB-1 disrupts HGF/MET receptor signaling and anchorage-independent growth. A) MDA-MB-231 cells were transfected with siYB-1 for 96 hours then stimulated with HGF for 15 minutes. The impact on signal transduction was evaluated via decreased P-Gab-1^{Y307} levels. **B-C)** Soft agar assays with YB-1 knockdown show that the loss of either protein results in a marked reduction in anchorage-independent growth in both SUM149 and MDA-MB-231 cells by 80-90%. Decreased MET receptor expression by siRNA suppressed anchorage-independent growth of both cell lines by approximately 50%.

2.6 SUPPLEMENTARY MATERIALS AND METHODS

Lentiviral down-regulation of YB-1

A pSuper plasmid harboring an established sh-YB-1 sequence[21], a gift from Dr. Peter Mertens, Aachen, Germany, was digested with EcoRI and HindIII to eject and isolate the approximately 200 bp H1 RNA promoter and YB-1-specific RNAi sequence. The 200 bp fragment was cloned into a pSuper shuttle vector, and further digested with BamHI and NheI. The RNAi sequence and corresponding promoter was finally inserted at position 2376 into the KA391 lentivector[38] with a modified yellow fluorescence protein (YFP). A Lenti-EV plasmid was also constructed by inserting the H1 promoter with no sh-RNA sequence into the KA391 lentivector at the same location. Lenti-EV and Lenti-shYB-1 viruses were produced, purified, and titred as previously described[38]. SUM149 cells were grown in log-phase in their described medium and infected with either the purified Lenti-EV or Lenti-sh-YB-1 virus as previously described[38]. Successful transfectants were selected based on fluorescence analysis by the BD FACSVantage flow cytometry system, and the top 20% of YFP+ cells were isolated and propagated in culture. The pSuper shuttle vector, KA391 lentivector with modified YFP, virus production reagents, virus purification reagents, and selection of successfully infected SUM149 cells materials were gifts from Dr. Connie Eaves, Vancouver, Canada. Western blotting and quantitative PCR for MET and YB-1 were performed as it described in Materials and Methods.

Acknowledgement: Dr. Yun Zhao, PhD, Vancouver, Canada, for lentivector cloning advice.

Array CGH analysis

Ten primary tumors were characterized as being basal-like by immunohistochemistry (negative for ER, PR and Her-2 while positive for EGFR) and then DNA was extracted from the tumors and analyzed for gain or loss of copy number by SMRT tiling arrays as previously described[4].

Detailed description of primary progenitor isolations

Bipotent progenitor-enriched fractions were isolated from freshly thawed vials of three reduction mammoplasty samples, as previously described[17]. In this case, an aliquot of the initially obtained single cell suspension were fractionated immediately by FACS after staining with antibodies against human EpCAM, CD49f, CD31 and CD45. The basal fraction ($CD49f^{+}EpCAM^{-/low}CD31^{-}CD45^{-}$) isolated by this method comprises mainly mature myoepithelial cells, and also bipotent and myoepithelial-restricted progenitors. The luminal fraction ($CD49f^{+}EpCAM^{+}CD31^{-}CD45^{-}$) comprises mainly mature luminal epithelial cells, and also luminal-restricted progenitors. An average of 5% of cells in fractions isolated using this methodology are progenitors[17]. We also isolated bipotent progenitor-enriched fractions from 3 different reduction mammoplasty samples, as previously described[18]. In this case, the culture condition enrich for the expansion of progenitors. Briefly, cryopreserved organoid-enriched human mammary cells were defrosted, enzymatically dissociated to generate a single cell suspension and cultured for 3 days in EGF-containing medium. An Epithelial Cell Adhesion Molecule (EpCAM)+ fraction was isolated immunomagnetically, and the cells were further fractionated by fluorescence activated cell sorting (FACS) after staining with antibodies against human

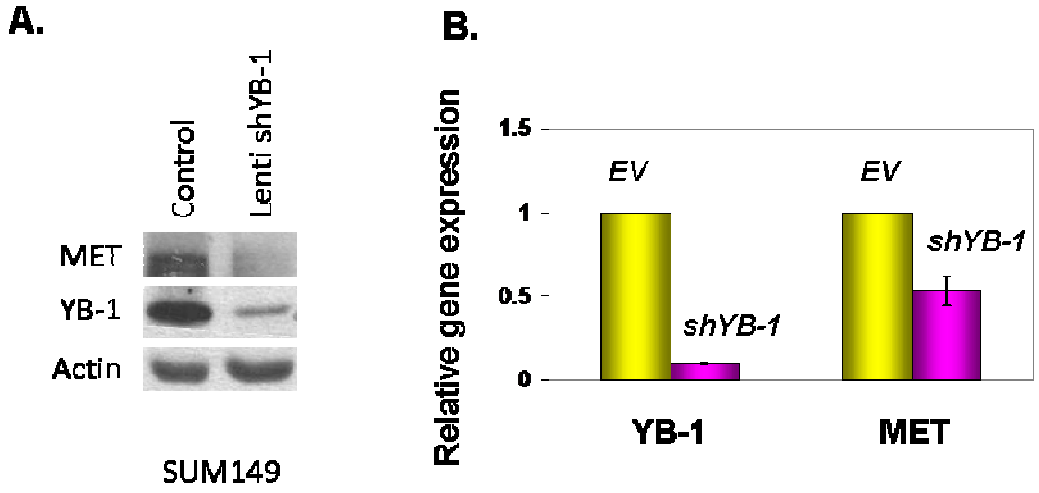
CD49f, CD10, Thy1, Mucin 1 (MUC1) and AC133. This method yields progenitor purities of 30-50% in the $EpCAM^{+}CD49f^{+}(CD10/Thy1)^{+}$ (bipotent progenitor enriched) isolates[18]. To assess the transcript levels of *YB-1* and *MET*, 40 to 60 ng of RNA from each sample were reverse transcribed into cDNA as described[19]. For comparison, RNA was isolated from MDA-MB-231, MDA-MB-468, HCC1937, and SUM149 cells grown in log phase using the Qiagen RNeasy Mini Kit (Qiagen) and its prescribed protocol. 1 μg of extracted RNA was reverse-transcribed using Superscript III Reverse Transcriptase and its prescribed protocol (Invitrogen). QRT-PCR (7000 Sequence Detection System, Applied Biosystems) was performed using TaqMan Gene Expression Assays designed against human *YB-1* (Custom TAMRA probe, sequence: 6FAM-AAGCCCGGCACTACGGGCAGC-TAMRA, Applied Biosystems), human *MET* (Assay ID: Hs00179845_m1, Applied Biosystems), and human TATA-box binding protein (TBP) as an endogenous control (Part No. 4326322E, Applied Biosystems). Relative expression of *YB-1* and *MET* transcript levels was determined by normalizing to TBP.

COC

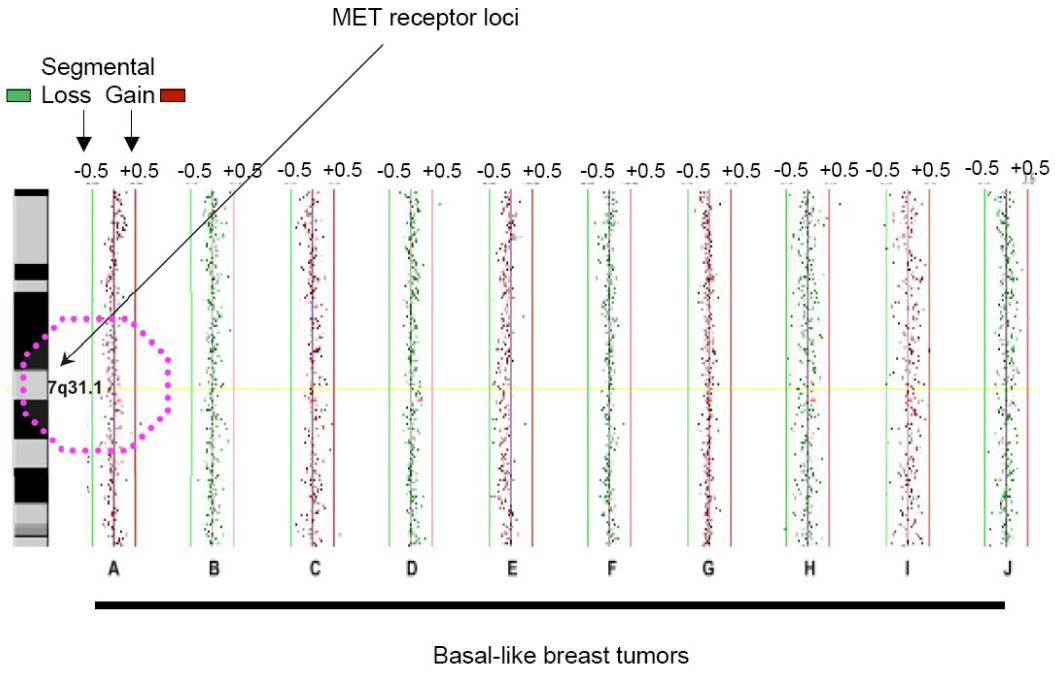
SUM149 cells were grown to 80% confluency on 15-cm diameter plates (8×10^6 cells). Chromatin immunoprecipitation (ChIP) was performed on the pooled lysates from 12 plates of cells. YB-1:DNA complexes were isolated as previously described[7] using a anti-chicken antibody (Dr. Isabella Berquin, Wake Forest University, North Carolina). Following elution of the DNA from the beads, the DNA was amplified using the protocol provided by NimbleGen. Briefly, DNA end blunting was performed by T4 DNA polymerase (NEB, #203L) for 20 minutes at 12°C on eluates and input controls. 3 M

NaOAc, 20 mg/ml glycogen and phenol/chloroform was added and samples were vortexed for one minute. Aqueous supernatants were removed, DNA precipitated in ethanol and the pellet suspended in 25 µl water. T4 DNA ligase (NEB, #202L) ligated the blunted DNA to 15 µmol/L of annealed linkers oJW102 (5' – GCGGTGACCCGGGAGATCTGAATTC) and oJW103 (5' – GAATTCAGATC). The samples were incubated at 16°C overnight and precipitated in ethanol. The oJW102 primer was used to perform ligation-mediated PCR (LM-PCR). PCR conditions were 55°C for 2 minutes, 72°C for 5 minutes, and 95°C for 2 minutes, then 22 cycles of 95°C for 1 minute, 60°C for 1 minute and 72°C for 5 minutes. QIAQuick PCR Purification Kit (#28104) was used to purify the samples and NanoDrop® ND-1000 Spectrophotometer was used to quantify the DNA. From the ChIP on chip hybridization, a list of accession numbers of genes with promoters to which YB-1 potentially binds was generated. Using the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/), the accession numbers were decoded and all genes with a greater than log 2-fold change were identified. Data was analyzed using Ingenuity Pathway Analysis software (Redwood City, CA).

2.7 SUPPLEMENTARY FIGURES



Supplementary Figure 2.1. Stable Lentiviral down-regulation of YB-1 (Lenti-shYB-1) in SUM149 and its effect on MET expression. Silencing YB-1 using a lentiviral shYB-1 approach reduced MET receptor **A)** protein and **B)** mRNA levels. The protein was reduced by ~90% while the mRNA was decreased by approximately 50%. This was consistent with the effect observed by stable shYB-1 silencing using plasmid DNA and siRNA targeting approaches.



Supplementary Figure 2.2. CGH analysis using SMRT high resolution tiling arrays on primary BLBC. Array CGH analysis indicating that MET is not commonly amplified in primary BLBC. Gain or loss of copy number was considered significant if the levels were – or + 0.5 from the norm. A small segmental gain was observed only in sample J, yet not considerable increases were observed in the other nine primary tumors.

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CHAPTER THREE : YB-1 INDUCES EXPRESSION OF *CD44* AND *CD49F* LEADING TO ENHANCED SELF-RENEWAL, MAMMOSPHERE GROWTH, AND DRUG RESISTANCE **

This chapter draws on a manuscript that further validates the findings of Chapter 2, where we first introduced YB-1 as a regulator of genes associated with primitive stem cell populations. CD44 and CD49f were two of the candidate genes that were suggested by the ChIP-on-chip screen to be putatively regulated by YB-1. In Chapter 3, we discover two novel relationships between YB-1 and CD44, as well as YB-1 and CD49f. We characterize their interactions and find a regulatory relationship. Further, we demonstrate functional roles for these relationships in the context of tumour cell growth in 3-dimensional assays. Finally, we show for the first time the important role of YB-1, an oncogene, in breast cancer cells cultured in a mammary stem cell associated mammosphere non-adherent assay and a related self-renewal assay.

**A version of this chapter has been submitted for publication. To K, Fotovati A, Reipas KM, Law JH, Hu K, Lee L, Johnson P, Wang J, Berquin IM, Royer HD, Raouf A, Eaves CJ, and Dunn SE. YB-1 induces CD44 and CD49f thereby promoting the capacity for tumour initiation.

3.1 INTRODUCTION

Breast cancer relapse confers a poor prognosis, decreasing survival rates from 80% to 60% when the recurrence is local, and from 80% to 10% for metastatic disease. Relapse is postulated to be mediated by the persistence of cancer stem cells that have survived an initial treatment regimen with radiation[1] and/or chemotherapy[2-4] possibly due to a selective resistance of the cancer stem cells to these agents. Human breast cancer stem cells have been identified as a phenotypically restricted subset of $CD44^{+}CD24^{-/low}$ cells that form tumours in immuno-compromised mice in limiting dilution transplant assays[5]. The same $CD44^{+}CD24^{-/low}$ subset has also been associated with breast tumour mammosphere generation *in vitro*[3]. However, the nature of the essential molecular properties of breast cancer stem cells has remained poorly defined. Recently, Weinberg's group showed that p53 inhibits CD44 expression in untransformed and transformed mammary epithelial cells[2] suggesting that higher levels of CD44 would also be an expected consequence of p53 inactivation.

YB-1 (Y-box binding protein-1) is a transcription/translation factor that is commonly overexpressed in many cancers, including human breast cancer (40%)[6-8]. This elevated expression of YB-1 in human breast cancer correlates with high rates of relapse[6]. Targeted overexpression of YB-1 in the mouse mammary gland leads to the development of mammary tumours[9], confirming a role of YB-1 as an oncogene in that tissue. YB-1 is directly phosphorylated, and therefore activated, on its serine 102 site by Akt[10] and even more potently by RSK[11], a major component of the MAP kinase pathway. Thus, YB-1 is positioned as a key player in both the PI3K/Akt and MAPK pathways. YB-1 regulates

genes that promote breast cancer cell growth and survival including *EGFR*[7], *Her-2*[7], *PIK3CA*[12], and *MET*[13] and biologically, is essential for breast cancer cell growth *in vitro*[7, 10, 11, 14] and *in vivo*[15]. YB-1 expression has also been shown to be associated with drug resistance via the induction of genes such as *MDR-1*[16, 17]. Consistent with this, inhibition of YB-1 was found to sensitize breast cancer cells to Paclitaxel[16], a chemotherapeutic agent commonly used in the clinic to treat advanced breast cancer. As a first step towards defining the transcriptional programming activity of YB-1, we performed chromatin immunoprecipitation-on-chip (ChIP-on-chip) assays[13]. This screen revealed a subset of genes known to be active in and important to a number of stem cell populations, including *c-KIT*, *BMI-1*, members of the *WNT* and *NOTCH* signaling pathways, as well as *CD44* and *CD49f* (also known as *α-6 integrin*)[13]. We also found that *YB-1* transcripts were present in purified $CD44^{+}CD49f^{+}$ subpopulations of primitive human mammary progenitor cells populations isolated from normal reduction mammoplasties[13]. Taken together, this led us to design a series of experiments to test the hypothesis that *YB-1* plays a key role as an oncogene by transactivating genes associated with a cancer stem cell phenotype.

3.2 MATERIALS AND METHODS

Cell lines and culturing

Human breast cancer cell lines, MDA-MB-231 and MDA-MB-468, were purchased from the American Tissue Culture Collection (ATCC, Rockwood, MD), and were grown in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Invitrogen). SUM 149 cells were purchased from

Astrand (Ann Arbor, MI) and were grown in Ham's F12 medium (Invitrogen) supplemented with 5 µg/mL insulin (Sigma Aldrich, St. Louis, MO), 1 µg/mL hydrocortisone (Sigma Aldrich), 10 mM pH 7.6 Hepes (Sigma Aldrich), and 5% fetal bovine serum (FBS). SUM 149 cells are a heterogeneous basal-like breast cancer cell line that are maintained by a subpopulation of $CD44^{+}CD24^{-}EpCAM^{+}$ cells[3]. For all cell lines, the medium was changed twice a week, and cells were maintained in log-phase growth at 37ºC, 5% CO_2.

Chromatin immunoprecipitation (ChIP)

Cells were maintained in log-phase until 90% confluency was reached in a 15 cm tissue culture dish. ChIP using a polyclonal chicken antibody to precipitate endogenous YB-1 was performed as previously described[7]. Three primer sets were designed to flank seven putative YB-1 binding sites (ATTG) in the first two kilobases of the *CD44* promoter, and similarly, two primer sets flanking eight putative YB-1 binding sites in the first two kilobases of the *CD49f* promoter were also designed. The sequences, annealing positions, and optimized annealing temperatures (T_a) of the 4 sets of primer sequences were as follows: CD44 (a), -721 to -1067, Forward (F) – TGCGTTTGATTTCCAAACAT, Reverse (R) – TCCACCATCCTCTTCTCCAC, T_a = 57ºC; CD44 (b), -1645 to -2022, F – CCCCATACCTGTAACCTCATGT, R – CCAAACCCTATTATGGCTGCT, T_a = 59ºC; CD44 (c), -1657 to -1900, F – TCAAACACAATTTTGCTTTTAGTAATG, R – TGGCTGCTTCTTAGTTGTGTG, T_a = 59ºC; CD49f (a), -151 to -389, F – CCCACTTTCACACTGATGTTCT, R – ATTCCCCAAAGTGGCACTAA, T_a = 59ºC; CD49f (b), -615 to -863, F –

GAAGGGATAGCAAAGAAAAGAGG, R – TGAATGGCTAACCTCCATGT, T_a = 57ºC.

Immunofluorescence assay

MDA-MB-231 and SUM 149 cells were seeded atop of glass cover slips in 6-well plate wells to achieve 70% confluence 24 hours later. The next day, the glass slides were incubated with phosphate buffered saline (PBS) for 5 minutes, fixed with 2% formaldehyde for 20 minutes, and rinsed twice further with PBS. The slides were then blocked with 2% goat serum in PBS and incubated with either rat anti-human and anti-mouse fluorescein isothiocyanate (FITC)-conjugated anti-CD49f antibody (1:25, Clone GoH3, #555735, BD Pharmingen), rat anti-human phycoerythrin (PE)-conjugated anti-CD44 antibody (1:100, Clone 515, #550989, BD Pharmingen), or unconjugated rabbit anti-human phospho-YB-1^{S102} antibody (P-YB-1^{S102}, 1:100, Clone C34A2, #2900, Cell Signaling Technology) diluted in 1% bovine serum albumin (BSA) in phosphate buffer saline (PBS) for 1 hour at room temperature. Unconjugated anti-P-YB-1^{S102} was followed by a PBS-diluted secondary anti-rabbit FITC antibody incubation (1:200, #711-096-152, Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) for 1 hour at room temperature. All slides were further washed three times with PBS, stained with Hoechst33342 (Sigma Aldrich) or 4',6-diamidino-2-phenylindole (DAPI) (Sigma Aldrich) at 1 μg/mL in PBS, and mounted with cover slips using ProLong Gold mounting medium (Molecular Probes, Invitrogen). Slides were visualized using an Olympus BX61 Fluorescent Microscope and photographed using an Olympus DP71 digital camera (Olympus, Japan).

Fluorescence-activated cell sorting (FACS) and analysis

Cells were harvested by scraping and digested with a dispase solution (Stem Cell Technologies, Vancouver, BC) and DNase (Sigma Aldrich) at 37ºC for 10 minutes. Cells were then diluted into a 2% FBS PBS buffer and passed through a 40 μm cell strainer to achieve a single cell suspension. Cells were subsequently stained with anti-CD44 conjugated to PE (BD Pharmingen, same as in drug screen). 7-aminoactinomycin D (7-AAD) viability dye (AnnexinV:PE Apoptosis Detection Kit I, BD Pharmingen) was added to a final 2% FBS PBS resuspension buffer. Data acquisition was performed on a BD FACS Calibur using Cell Quest Pro software and further analyzed using FlowJo software. For cell sorting, the top and bottom 10% of cells with the highest and lowest CD44-PE fluorescence were collected into 50% FBS PBS buffer and seeded subsequently into a 96-well plate for immunofluorescence staining or into growth assays. In all cases, cell events that were negative for 7-AAD, with high forward and side light scatters were gated, and analyzed.

Mammosphere assay

MDA-MB-231 and SUM 149 cells were made into a single cell suspension by enzymatic dissociation with dispase digestion and mechanical dissociation by repeated pipetting. Cells were seeded at 5×10^3 and 2×10^4 per well into Ultra-Low Attachment coated 6-well culture plates (Corning, Lowell, MA) in a 1:1 DMEM/F12 (Invitrogen) basal media freshly supplemented with 20 ng/mL human basic fibroblast growth factor (bFGF, Invitrogen), 20 ng/mL epidermal growth factor (EGF, Invitrogen), 10 μg/mL heparin (Sigma Aldrich), 1:50 B27 supplement without Vitamin A (Sigma Aldrich). Spheres

containing at least 15 cells were counted 7 days after seeding. In the self-renewal serial passaging experiments, all cells from primary mammosphere cultures were collected, centrifuged at 350 *g* for 5 minutes and dissociated into a single cell suspension with 0.25% trypsin for 5 minutes at 37ºC and counted. The single cells were then seeded into new cultures under the same conditions at 2 x 10^4 cells/well and the number of spheres present counted after another 7 days of incubation.

Soft agar anchorage-independent growth assay

MDA-MB-231 and SUM 149 cells were mixed with 0.3% agar at densities of 5×10^3 and 1.5×10^4 cells/well, respectively, and placed on top of a preformed layer of 0.6% agar in a 24-well plate. Agar solutions were made with the same media used for cell line propagation. Colonies formed were counted between Day 21 and 28 post-seeding.

Small-interfering RNA (siRNA) transfection

siRNAs were transfected into cells using the Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's protocol. Cells were seeded into a 6-well plate 24 hours before transfection at 3×10^5 cells/well for MDA-MB-231 and MDA-MB-468 cells, and 4×10^5 cells/well for SUM 149 cells. For all experiments, 20 nM of either a scrambled control (AllStars Negative Control siRNA, #1207820, Qiagen, Hilden, Germany), YB-1 siRNA oligonucleotides #1 and #2 (custom designed as previously described[12]), YB-1 siRNA oligonucleotide #3 (Hs_YBX1_1_HP Validated siRNA, #SI03019191, Qiagen), CD44 siRNA (Hs_CD44_5_HP Validated siRNA, #SI00299705, Qiagen), and CD49f siRNA (Hs_ITGA6_6_HP Validated siRNA,

#SI02654078, Qiagen) were incubated with the cells for 96 hours before assessment of biochemical or biological effects.

Quantitative real-time PCR

RNA isolation, cDNA synthesis, and real-time PCR experiments were performed as described[13]. TaqMan Gene Expression Assays designed for CD44 (Hs00153304_m1, Applied Biosystems, Foster City, CA), CD49f (Hs00173952_m1, Applied Biosystems), and the human TATA-box binding protein (TBP) endogenous control (Part No. 4326322E, Applied Biosystems) were used.

Immunoblotting

Log-phase growing cells were harvested by scraping, resuspended in Egg Lysis Buffer (ELB) and extracts used in immunoblotting analyses as described previously[7]. Primary immunoblotting antibodies used were as follows: anti-total YB-1 (T-YB-1; 1:2000, #ab12148, Abcam, Cambridge, MA; 1:1000, #2749, Cell Signaling Technology, Danvers, MA; 1:2000, a polyclonal antibody designed against the C-terminus of YB-1, produced by and a generous gift from Dr. Colleen Nelson, University of British Columbia, Vancouver, BC), anti-P-YB-1^{S102} (1:1000, Clone C34A2, #2900, Cell Signaling Technology), anti-CD44 (1:500, Clone EPR1013Y, #ab51037, Abcam), anti-CD49f (1:1000, #3750, Cell Signaling Technology), anti-Flag (1:2000, Clone M2, #F3165, Sigma Aldrich), anti-total Akt (T-Akt; 1:1000, #9272, Cell Signaling Technology), anti-pan-Actin (1:1000, #4968, Cell Signaling Technology), and anti-vinculin (1:1000, Clone hVIN-1, #V9131, Sigma Aldrich).

Matrigel semi-fluid basement membrane growth assay

Matrigel basement membrane (BD Biosciences) were added at 40 μL per well in a 96-well plate and incubated briefly at 37ºC for the gel to solidify. MDA-MB-231 and SUM 149 cells in single cell suspension were added in their respective media at 2 x 10^3 and 5 x 10^3 cells/well. Growth was assessed in photographed fields 7 days after seeding.

DNA plasmid transfection

In a 6-well plate format, 2 or 4 μg of a Flag:EV, Flag:YB-1^{WT}, or Flag:YB-1^{S102D} plasmid construct as described previously[17, 21] were transfected into cells at 80 to 90% confluence with 5 or 10 μL of Lipofectamine 2000 (Invitrogen) respectively as specified by the recommended manufacturer's protocol. Transfection reagent and DNA complexes were replaced with fresh media 5 hours post-transfection, and cells were harvested 96 hours post-transfection.

Stable transfectant cell lines

Stable EV, shYB-1, Flag:EV, Flag:YB-1^{WT}, and Flag:YB-1^{S102D} cell lines were established by transfecting 4 μg of DNA constructs with 10 μL of Lipofectamine 2000 into cells in a 6-well plate, and expanding the transfectant cell lines to a 80% confluence in a 10 cm tissue culture dish. Then, 400 μg/mL of G418 (Calbiochem, EMD Chemicals, San Diego, CA) was introduced to the culture media and replaced every 3 to 4 days. Cells were continually split at low density to allow for optimal selection of transfectants with acquired G418 resistance.

YB-1 transgenic mouse and mammary gland sectioning

YB-1 transgenic (Tg 2 line) and wild-type (WT) mice were revived from cryopreserved embryos generated previously[9]. YB-1 transgenic mice have previously been shown to develop hyperplastic nodules in the mammary glands by 6-8 months of age followed by the appearance of diverse breast carcinomas in all mice by 52 weeks[9]. For our study, histological sections were obtained from the mammary glands of 6 to 8-month old female mice post-lactation after two cycles of mating and nursing. The mice were mated twice and nursed their pups for 3 weeks each time. They were euthanized at the end of their second lactation (day 20 to 22 after the birth of the pups) and mammary gland tissues were then collected. The mammary glands were dipped into Tissue-Tek O.C.T. Compound (Sakura Finetek USA, Torrance, CA), frozen, and kept at -80ºC. OCT-embedded tissues were sectioned (6 μM) using a Cryostat and placed on Fisherbrand Superfrost Plus glass slides (Thermo Fisher Scientific, Waltham, MA) and kept at -80ºC. The sections were defrosted at room temperature and incubated with phosphate-buffered saline (PBS) for 5 minutes, fixed with 2% formaldehyde for 20 minutes, rinsed twice with PBS. Subsequent immunofluorescence staining and imaging protocol was performed as described above. The only exception was the primary purified rat anti-mouse CD44 antibody (1:100, Clone IM7, #550538, BD Pharmingen), and the CD44 slides were stained with a secondary anti-rat Alexa 546 antibody (Molecular Probes, Invitrogen) diluted in 1% BSA PBS for 1 hour at room temperature.

Anti-cancer drug screen and further evaluation of Paclitaxel treatment

SUM 149 cells were seeded onto collagen-coated 96-well plates at 5000 cells/well. An initial screen was conducted using commonly employed chemotherapeutic agents at a fixed concentration of 10 μM and cell viability was assessed using Hoechst33342 (Sigma Aldrich) staining 72-hour later. The cells were also stained with anti-CD44 conjugated to PE (1:5, Clone G44-26, #555479, BD Pharmingen). Plates were read on a Cellomics ArrayScan instrument (Cellomics, Pittsburgh, PA). Following the screen, we questioned whether lower, more physiologically relevant doses of Paclitaxel would have a reproducible effect on CD44 induction. Thus, SUM 149 cells were treated in monolayer with 10 nM of Paclitaxel dissolved in DMSO (Paclitaxel, Sigma Aldrich) for 24 hours and harvested for immunoblotting. MDA-MB-231 Flag:EV and Flag:YB-1^{WT} cells were treated in monolayer or in the mammosphere assay in the presence of 0 nM or 10 nM of Paclitaxel with DMSO as vehicle control for 7 days. Cells grown in monolayer had the media changed at Day 4 and cells were harvested for immunoblotting on Day 7.

Statistical Analysis

All quantitative data are represented as mean ± standard error of the mean (s.e.m.) of 3 independent experiments. P-values were generated using the paired Student's T-test where * denotes $p < 0.05$, ** denotes $p < 0.01$, and *** denotes $p < 0.001$.

3.3 RESULTS

YB-1 binds to upstream regulatory regions of the genes encoding CD44 and CD49f, and regulates their expression

Several putative YB-1 binding sites were identified 2 kilobases upstream of the transcriptional start site of the *CD44* and *CD49f* genes initially identified in a ChIP-on-Chip screen[13] (Figure 3.1A, upper panel). To validate these results, we performed conventional ChIP analyses and provide direct evidence of the ability of YB-1 to bind to the promoters of both genes in two human breast cancer cell lines (MDA-MB-468 and SUM 149, Figure 3.1A, lower panel). To investigate whether YB-1 plays a role in controlling CD44 and CD49f expression in these cells, we first asked whether the expression of CD44 would fluctuate concordantly with changes in P-YB-1^{S102}. Immunohistochemical analysis of SUM 149 cells grown either in monolayer cultures or as xenografts illustrated coincident staining of P-YB-1^{S102} and CD44 protein in individual cells (Figure 3.1B). Quantification of this association of CD44 expression with the presence of P-YB-1^{S102} was demonstrated by isolating the highest and lowest 10% of CD44-expressing viable cells by FACS sorting and co-staining with CD44 and P-YB-1^{S102} antibody. There, we observed a >2-fold difference in P-YB-1^{S102} levels between the subsets expressing the highest and lowest levels of CD44 (Figure 3.1C, upper panels), corresponding to enhanced growth in mammosphere cultures and soft agar (Figure 3.1C, lower panels).

We next asked whether YB-1 is a determining regulator of *CD44* and *CD49f* expression in breast cancer cells by assessing the immediate effect of knocking down YB-1

expression in MDA-MB-231 and SUM 149 cells using three separate siRNA oligonucleotides designed to target *YB-1* transcripts specifically. The result was a consistent and significant decrease in both transcript and protein levels of *CD44* and *CD49f* (Figure 3.2A and 3.2B, respectively). To obtain stable inhibition of YB-1 expression, SUM 149 cells were transfected with anti *YB-1* shRNAs. This produced sustained decreases in *CD44* and *CD49f* transcript and protein levels as compared to control cells transfected with an empty vector (EV, Figure 3.2C). Stable knockdown of *YB-1* transcripts also resulted in a decreased expression of CD104 (β4 integrin), a beta chain partner of CD49f (Figures 3.2B and 3.2C).

Conversely, when we forced a transient elevation in the expression of either Flag:YB-1^{WT} or a constitutively active Flag:YB-1^{S102D}, increases in both *CD44* and *CD49f* transcript levels were obtained in both cases, with the largest increases resulting from the constitutively active *YB-1* construct (Figure 3.3A). A similar result was seen when these constructs were stably expressed in the same cells (Figure 3.3B and data not shown). Importantly, we confirmed that a heightened expression of CD44 and CD49f was concomitantly induced in the same transfected YB-1-overexpressing cells (Figure 3.3C).

To determine whether elevated YB-1 expression would have a similar effect on the expression of CD44 and CD49f in primary mammary epithelial cells *in vivo*, we evaluated the mammary glands of 6 to 8-month old YB-1 transgenic mice (9). The mammary cells in these transgenic mice expressed markedly higher levels of CD49f and CD44 protein (Figure 3.3D and Supplementary Figure 3.1A) as compared to mammary

cells from wild-type animals (n=4/group). The increased expression of CD49f in the YB-1 transgenic mice was further validated with a second antibody (Supplementary Figure 3.1B). The specificity of the CD44 antibody staining was confirmed by the lack of staining obtained on B6/129 CD44$^{-/-}$ mouse kidney cells (Supplementary Figure 3.2). Interestingly, we also noted that the cells in the mammary glands from the YB-1 transgenic mice were hyperplastic and displayed atypical nuclei by comparison to those in the wild-type controls (Figure 3.3D). Taken together, these findings indicate that YB-1 regulates the expression of CD44 and CD49f both in human transformed mammary cells *in vitro* and in primary mouse mammary cells generated *in vivo*.

YB-1-regulated expression of CD44 and CD49f controls the anchorage-independent growth of transformed breast cells

We have previously demonstrated that YB-1 is essential for cancer cells to grow in soft agar assays *in vitro*[10, 14, 15] and for tumourigenesis *in vivo*[15]. To test whether the ability of YB-1 to regulate CD44 and/or CD49f expression was important for this altered growth activity, we next examined the effect of treating MDA-MB-231 cells with anti-CD44 and anti-CD49f siRNAs on mammosphere formation (Figure 3.4A). SiRNAs directed against transcripts for YB-1 as well as CD44 and CD49f all inhibited primary mammosphere formation by >50%. Conversely, forced increased expression of Flag:YB-1^{WT} or Flag:YB-1^{S102D} increased the yield of mammospheres by 2 and 8-fold, respectively (Figure 3.4B). Moreover, this effect was inhibited if the Flag:YB-1^{S102D} expressing cells were treated with either CD44 or CD49f siRNAs (Figure 3.4B). The ability of the siRNA treatments to induce specific decreases in CD44 and CD49f protein expression in these

experiments was confirmed by immunoblotting (Figure 3.4B). Further, this effect is perpetuated over time through passaging as SUM 149 cells transduced to obtain stable high expression of either Flag:YB-1^{WT} or Flag:YB-1^{S102D} also produced a higher number of cells in mammospheres in secondary non-adherent cultures than control cells transduced with an empty vector plasmid (Flag:EV) (Figure 3.4B). Consistent with the mammosphere assays, analogous experiments with siRNA-treated MDA-MB-231 and SUM 149 cells, showed both CD44 and CD49f to be required for the clonal anchorage-independent growth of both of these cell lines in soft agar (Figure 3.4C). Conversely, forced expression of Flag:YB-1^{S102D} increased the ability of transfected cells to grow in soft agar and this was again blocked when the expression of either CD44 or CD49f was silenced (Figure 3.4C). The ability of MDA-MB-231 cells to proliferate and form extensive structures in Matrigel cultures was also inhibited when the cells were pre-treated with CD44 or CD49f or YB-1 siRNAs (Figure 3.4D). These findings demonstrate a critical role of YB-1 in enabling breast cancer cells to grow in 3-dimensional assays that is dependent on the downstream up-regulation of CD44 and CD49f.

Paclitaxel-treated SUM 149 cells show increased expression of YB-1 and CD44

The preceding findings suggested that the increased expression of YB-1 associated with breast cancer relapse in patients might be due to a selection of cells showing an increased expression of CD44. To investigate this possibility, we first screened a panel of commonly used anti-cancer drugs (all at 10 μM) for their ability to kill SUM 149 cells *in vitro* in parallel with an assessment of the proportion of $CD44^{High}$ cells present 72 hours later. All compounds killed >80% of the input cells and the percentage of $CD44^{High}$ cells

amongst the survivors was consistently higher than in the control treatment cells (Table 3.1). The most marked effect in this regard was obtained with paclitaxel treatment which produced an almost 10-fold increase in the proportion of viable $CD44^{High}$ cells (Table 3.1). Next, we determined that paclitaxel at a much lower and more clinically relevant concentration of 10 nM was able to increase YB-1 binding to the CD44 promoter after 48 hrs (Figure 3.5A). Consistent with this, exposing the cells to paclitaxel (10 nM) for 48 hours induced P-$YB\text{-}1^{S102}$ which correlated with activation of the RSK pathway (Figure 3.5A). To further confirm RSK activation, a second substrate, P-$GSK\beta^{S9}$ was detected (Figure 3.5A). The induction of this pathway then led to increased CD44 after 72 hours (Figure 3.5B). When Flag:$YB\text{-}1^{WT}$ was stably expressed in MDA-MB-231 cells (pooled clones) there was a further increase in CD44 expression after 10 nM paclitaxel treatment (Figure 3.5C). We also noted that following paclitaxel treatment, there was a greater amount of Flag:$YB\text{-}1^{WT}$ detected per cell on the whole and this is thought to be because the drug positively selected for cells with more YB-1 because it provides a significant protective advantage. Finally, the Flag:$YB\text{-}1^{WT}$-transfected MDA-MB-231 cells formed more mammospheres in the presence of paclitaxel (10 nM) as compared to control transduced cells (Figure 3.5D).

3.4 DISCUSSION

Here we present evidence that YB-1 utilizes CD44 and CD49f to promote self-renewal, mammosphere growth and colony formation in soft agar. In addition we demonstrate that these downstream mediators play an important role in controlling resistance of breast cancer cells to conventional chemotherapeutic agents. CD44 is a widely recognized marker of many cells with cancer-initiating[5, 18-20] and, in some cases, metastatic activity[21-23]. Furthermore, both CD44 and CD49f are selectively expressed on normal stem cells in the breast and other tissues[24-27]. However, little is known about their regulation in breast cancer or the role they play in this disease. YB-1 is the first oncogene identified which induces both CD44 and CD49f. It is foreseeable that it induces other genes associated with cancer stem cells as well.

We evaluated of over 4,000 cases of primary invasive breast cancer and reported that elevated YB-1 expression in the primary tumour is consistently associated with high rates of recurrence[6]. However, the role of YB-1 in causing relapses and its mechanism of action remains unclear. One possibility is that the regulation of CD44 and CD49f by YB-1 is a feature of normal mammary epithelial cells that is retained by or reactivated in cells that acquire cancer-propagating activity. An alternative, but not mutually exclusive, possibility is that YB-1 expression and/or activation is the result of a preceding oncogenic event. This may then lead to a perturbation of its effects on CD44 and CD49f regulation as part of a broader program of epithelial-mesenchymal transition (EMT) characteristic of many advanced carcinomas. Snail, Twist and TGFβ are all examples of EMT-inducing factors[8, 28]. Transfection of immortalized human mammary epithelial

cells or transformed mammary cells with all of these resulted in the expression of higher levels of CD44, enhanced ability to form mammospheres in liquid suspension cultures, colonies in soft agar, and tumours *in vivo*[29]. Here we provide new and direct evidence that these altered biological properties are regulated by YB-1 expression through its ability to control expression of requisite levels of CD44 and CD49f. The fact that YB-1 has been reported to be regulated by Twist[30, 31], implicates YB-1 activation as the mechanism by which CD44 is up-regulated in Twist-induced EMT.

Novel mechanisms to suppress the growth of cancers have emerged from the idea that they are propagated by a small subset of cells that are innately less drug sensitive than the bulk of the tumour. YB-1 expression has already been reported to be associated with chemoresistance[16, 17] perhaps through the regulation of MDR-1. Here we show that treatment of a breast cancer cell line with Paclitaxel (and other chemotherapeutic drugs commonly used to treat invasive breast cancer) selects for cells expressing high levels of CD44 and that this is enhanced in stably YB-1-transfected cells. These findings are consistent with previous reports showing that YB-1 is found in the nucleus of tumour cells taken from breast cancer patients treated with Paclitaxel and that the drug stimulates the nuclear translocation of YB-1 in breast cancer cells *in vitro*[32]. Together, this supports previous claims that activated YB-1 is a relevant target to overcome multi-drug resistance.

YB-1 is also a direct target of Akt[10] and RSK[11], and hence these pathways are also likely to have roles in mediating drug resistance mechanisms involving both MDR-1 and CD44.

Our lab and others have reported the role of YB-1 in the responsiveness of cancer cells to Gefitinib[14], Paclitaxel[16], and an experimental therapeutic, OSU-03012[33]. Here we demonstrated that YB-1 overexpressed in the murine mammary gland was able to increase the number of cells expressing high levels of CD44 and CD49f, suggesting that the molecular relationships between YB-1/CD44/CD49f and their biological outcomes are well conserved between human and mouse. Thus, in the future, YB-1 transgenic mice may be extremely useful to assess potential agents that have the ability to suppress the transforming properties mediated by increased expression of CD44 and CD49f. We conclude that YB-1 inhibition may be a new approach to eliminating tumour-initiating cells and by extension possibly cancer recurrence.

3.5 FIGURES AND TABLES

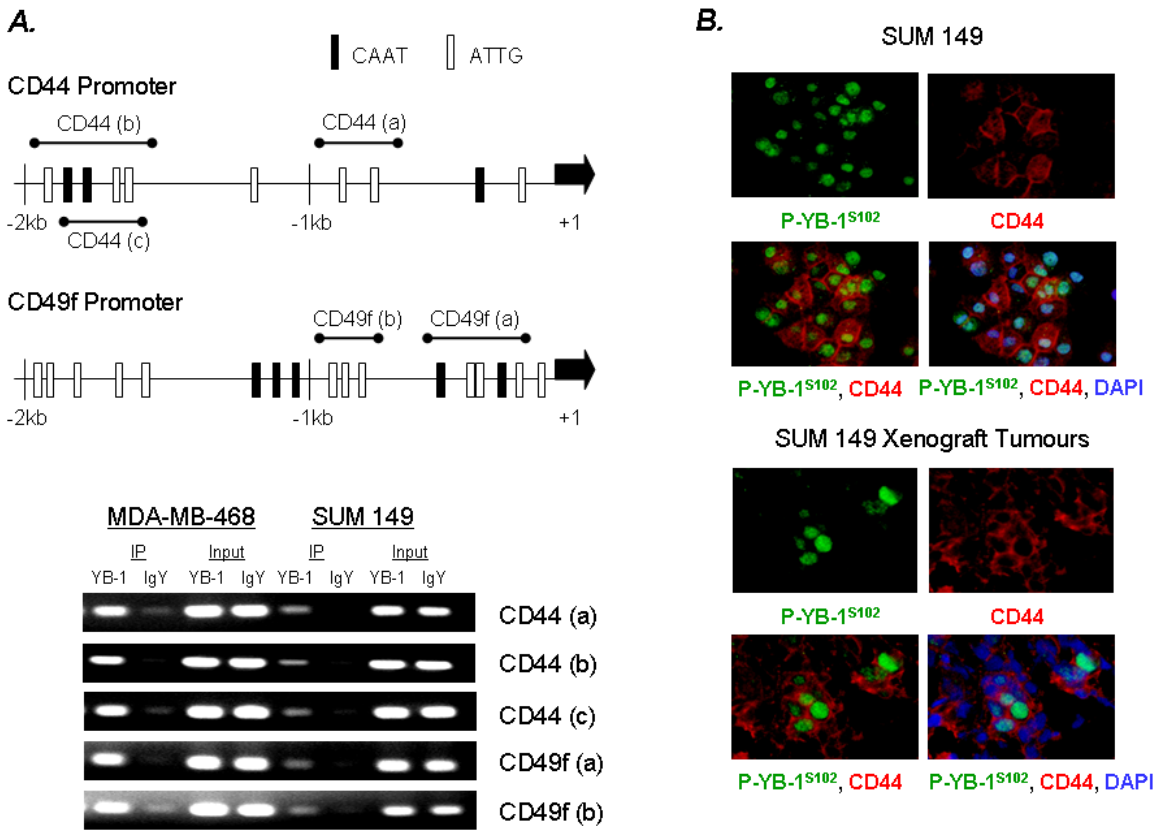
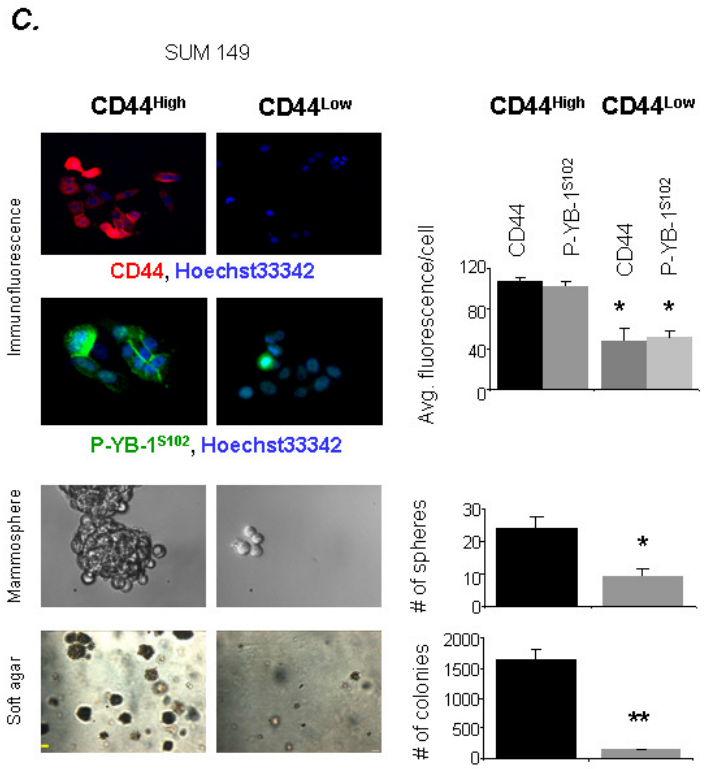


Figure 3.1. YB-1 binds to the promoters of *CD44* as well as *CD49f* and expression of CD44 correlates with the presence of P-YB-1^{S102}. A. Schematic diagrams of the human *CD44* and *CD49f* promoters displaying putative YB-1 binding sites, an inverted CAAT box, ATTG, and the amplification regions of ChIP primers used (upper panel). Representative ethidium bromide-stained agarose gel pictures of PCR reactions amplifying DNA templates from ChIP experiments in MDA-MB-468 and SUM 149 cells (lower panel). DNA templates were pulled down with either a YB-1 or non-immune IgY antibody, and amplified using primers flanking regions of the human CD44 or CD49f promoters. Input designates isolated DNA from input cross-linked DNA/protein complexes that were used for the immuno-precipitations. **B.** SUM 149 cells in monolayer *in vitro* (upper panels) and in fixed tumour xenograft (lower panels) preparations stained with antibodies against P-YB-1^{S102} (FITC, green) and CD44 (PE, red) and then with DAPI to identify all nuclei.



C. Analysis of subsets of SUM 149 cells isolated by FACS according to their expression of CD44. The highest and lowest 10% of CD44-expressing viable ($7AAD^-$) cells were collected and mean fluorescence intensities determined for the staining of CD44 (PE, red) and P-YB-1^{S102} (FITC, green) in both subsets. The abilities of the same isolates to generate mammospheres in non-adherent cultures (7 days) and soft agar (28 days) were also assayed.

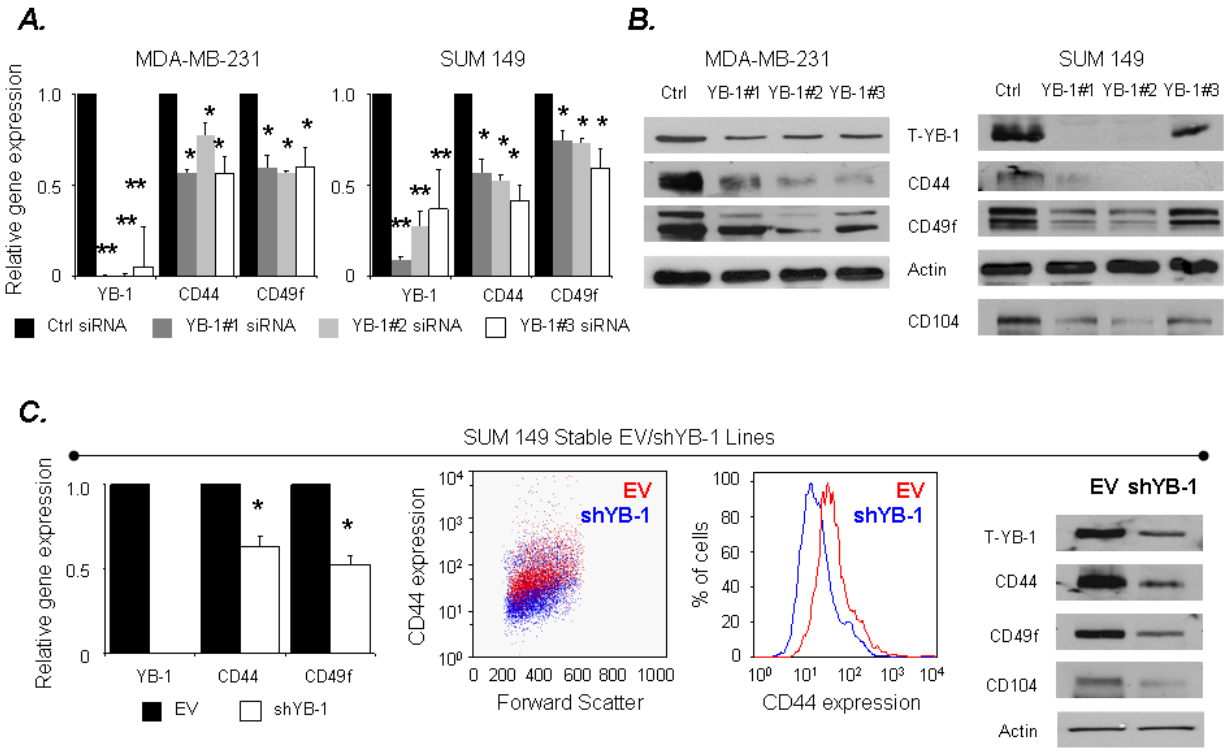


Figure 3.2. Silencing YB-1 down-regulates the expression of CD44 and CD49f. A-B. Quantitative real-time PCR and immunoblotting results of MDA-MB-231 and SUM 149 cells harvested after 96-hour treatment with 20 nM of either a control or one of 3 unique YB-1 siRNA oligonucleotides. Data shows altered YB-1, CD44, and CD49f mRNA transcripts and protein levels. T-YB-1 denotes total YB-1 protein. Actin served as loading control. **C.** CD44 and CD49f expression analyses of an established SUM 149 shYB-1 cell line and its empty vector (EV) counterpart using quantitative real-time PCR, FACS, and immunoblotting techniques. Single cell suspensions of EV and shYB-1 lines were stained with a CD44-PE antibody for FACS. FACS analyses are represented in a dot plot and a histogram. CD104 (β4 integrin), the partnering β chain for CD49f (α6 integrin), was also probed in the same preparations. Actin served as loading control.

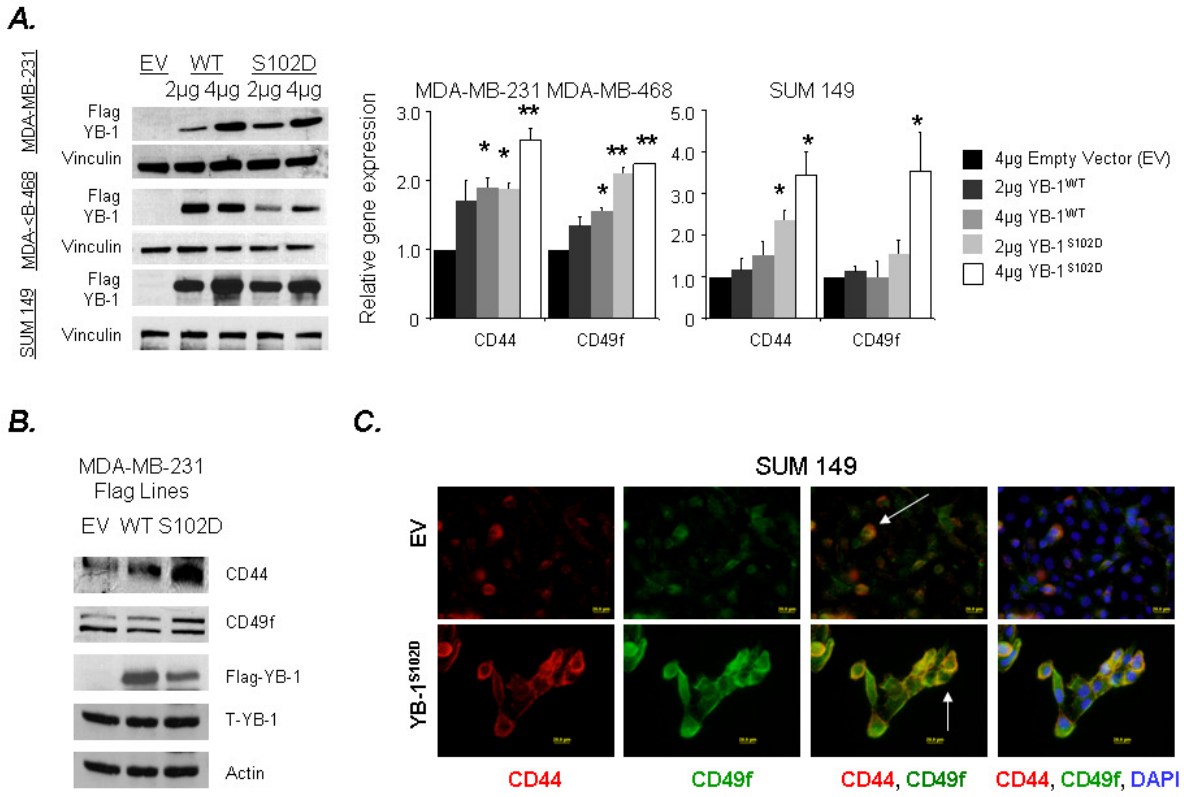
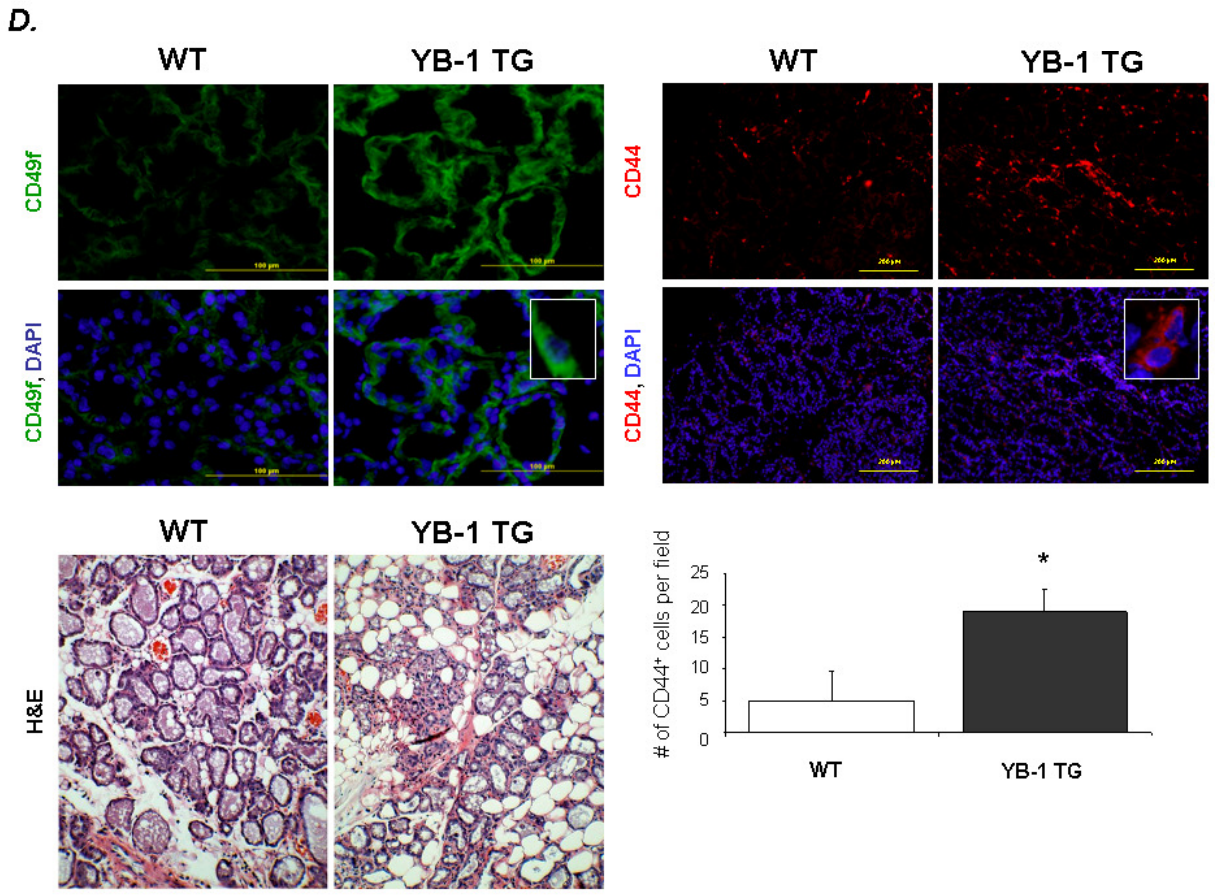


Figure 3.3. YB-1 overexpression induces CD44 and CD49f expression. A. Transient, incremental exogenous Flag:YB-1^{WT} and Flag:YB-1^{S102D} expression in MDA-MB-231, MDA-MB-468, and SUM 149 cells was achieved via transfection of 2 and 4 µg of plasmid, visualized by immunoblotting with a Flag antibody, and affected CD44 and CD49f transcript levels measured by quantitative real-time PCR experiments. Vinculin served as loading control. **B.** MDA-MB-231 cells stably expressing Flag:EV, Flag:YB-1^{WT}, Flag:YB-1^{S102D} were analyzed for long term Flag, CD44, and CD49f expression by immunoblotting. Actin served as loading control. **C.** CD44 and CD49f protein expression was induced by Flag:YB-1^{S102D} in SUM 149 cells.



D. BGL-YB-1/HA and WT counterpart mammary glands were collected from 6 to 8-month old female mice post-lactation after two cycles of mating and nursing, frozen in O.C.T. compound, and sectioned. Slides were stained with primary CD49f-FITC (green) or unconjugated CD44 with secondary Alexa 546 fluor antibody (red) and DAPI. Representative images of cells from 4 mice are shown. Insets show enlarged photographs of the CD44 and CD49f membrane-localized staining. The number of $CD44^{+}$ cells per field was counted.

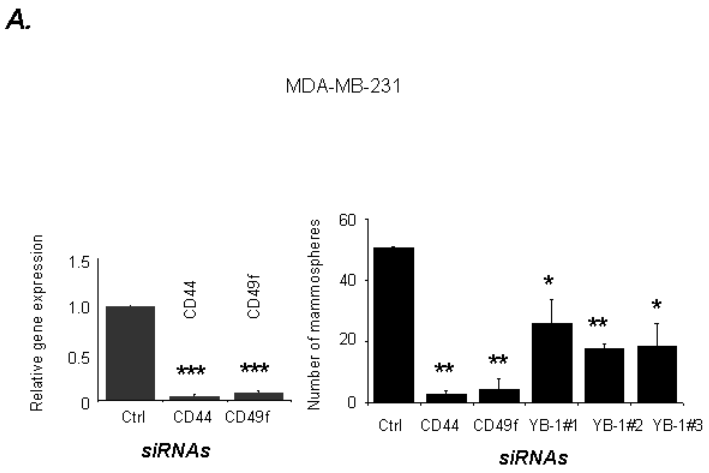
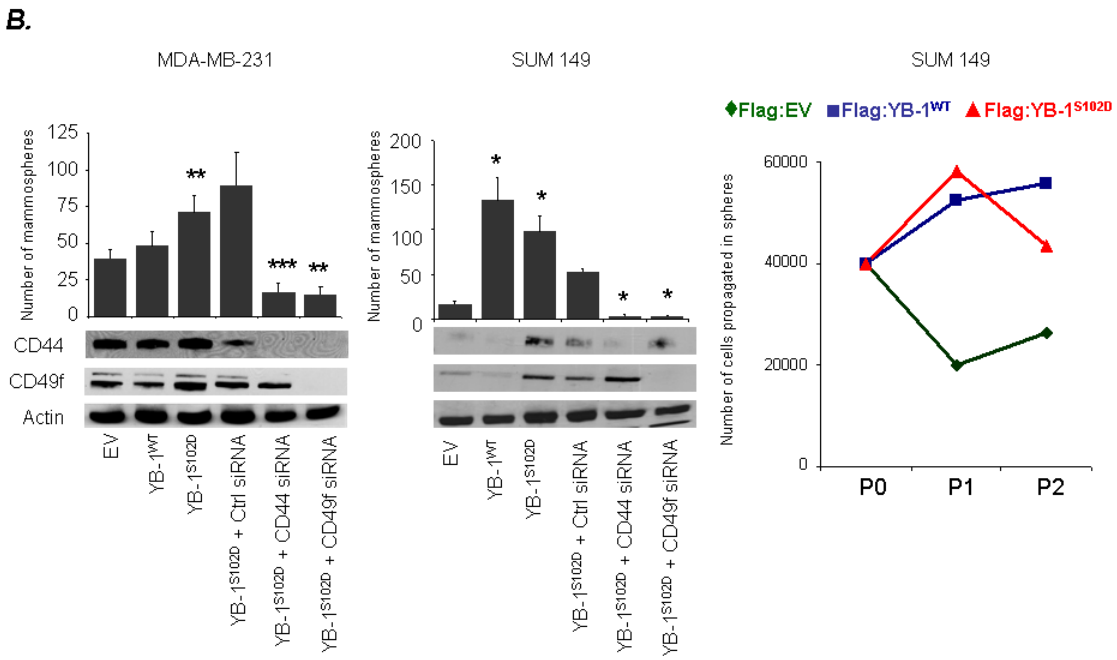
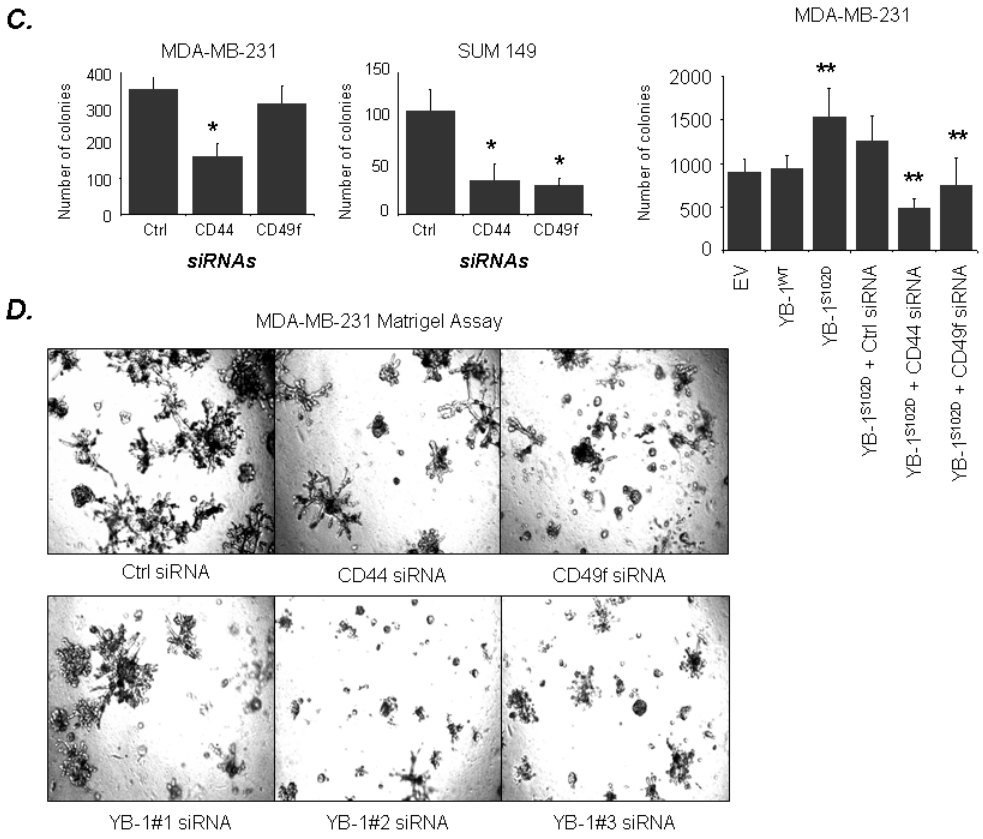


Figure 3.4. CD44 and CD49f are essential for breast cancer cell growth in 3-dimensional *in vitro* assays. A. MDA-MB-231 (and SUM 149 cells, data not shown) were incubated with 20 nM of CD44 and CD49f siRNA oligonucleotides for 96 hours and their respective transcript expression levels were monitored. The ability of MDA-MB-231 cells to form mammospheres was challenged by knocking down CD44, CD49f, and YB-1 individually with their corresponding siRNA oligonucleotides at 20 nM for 96 hours prior to seeding into the assay.



B. Stable G418-selected lines of MDA-MB-231 and SUM 149 cells expressing Flag:EV, Flag:YB-1^{WT}, Flag:YB-1^{S102D} were assayed for their abilities to form mammospheres. The Flag:YB-1^{S102D} lines of both cell lines were first subjected to 96-hour treatment of 20 nM of CD44 and CD49f siRNA gene knockdowns and their mammosphere-forming abilities quantified. Gene overexpression and subsequent knockdowns in the stable YB-1-overexpressing lines were confirmed by immunoblotting for CD44 and CD49f as shown. Actin served as loading control. Further, SUM 149 Flag:EV, Flag:YB-1^{WT}, Flag:YB-1^{S102D} cells were placed in mammosphere cultures. After 7 days, the spheres were dissociated into single cells, counted and the calculated yield of cells obtained after 7 days in secondary non-adherent cultures.



C. The functional roles of both receptors in anchorage-independent growth were analyzed in a soft agar assay by single CD44 or CD49f gene knockdowns (20 nM for 96 hours). MDA-MB-231 Flag:EV, Flag:YB-1^{WT}, Flag:YB-1^{S102D} cells, as well as Flag:YB-1^{S102D} cells treated with Control, CD44 or CD49f siRNAs (20 nM for 96 hours), were examined for their ability to form colonies in soft agar subsequent to the gene knockdowns. **D.** MDA-MB-231 cell growth in Matrigel was challenged by pre-treating the cells with 20 nM of YB-1, CD44, and CD49f siRNA oligonucleotides for 96 hours. Photographs were taken on Day 7 of the assay post-seeding into Matrigel.

Class	Drug	% of viable $CD44^+$ cells	% of viable $CD44^+$ cells normalized to control treatment	% of viable cells normalized to control treatment
	Control	8.0	100.0	100.0
Anticancer	Daunorubicin hydrochloride	30.0	375.0	1.7
Anticancer	Azacytidine-5	30.3	378.1	18.0
Anticancer	Etoposide	35.0	437.8	19.2
Anticancer	Nocodazole	59.6	744.4	10.3
Anticancer	Doxorubicin hydrochloride	78.9	986.3	6.3
Anticancer	Paclitaxel	82.0	1025.3	5.3

Table 3.1. Treatment of SUM 149 cells with chemotherapeutic agents enriches for a $CD44^{High}$ subset. A. A panel of cancer chemotherapeutic agents was screened against SUM 149, a heterogeneous CD44-expressing cell line for 72 hours at 10 μM. Cell viability and CD44 expression were monitored. Cells were stained with CD44-PE antibody and Hoechst33342 and compared to DMSO vehicle control.

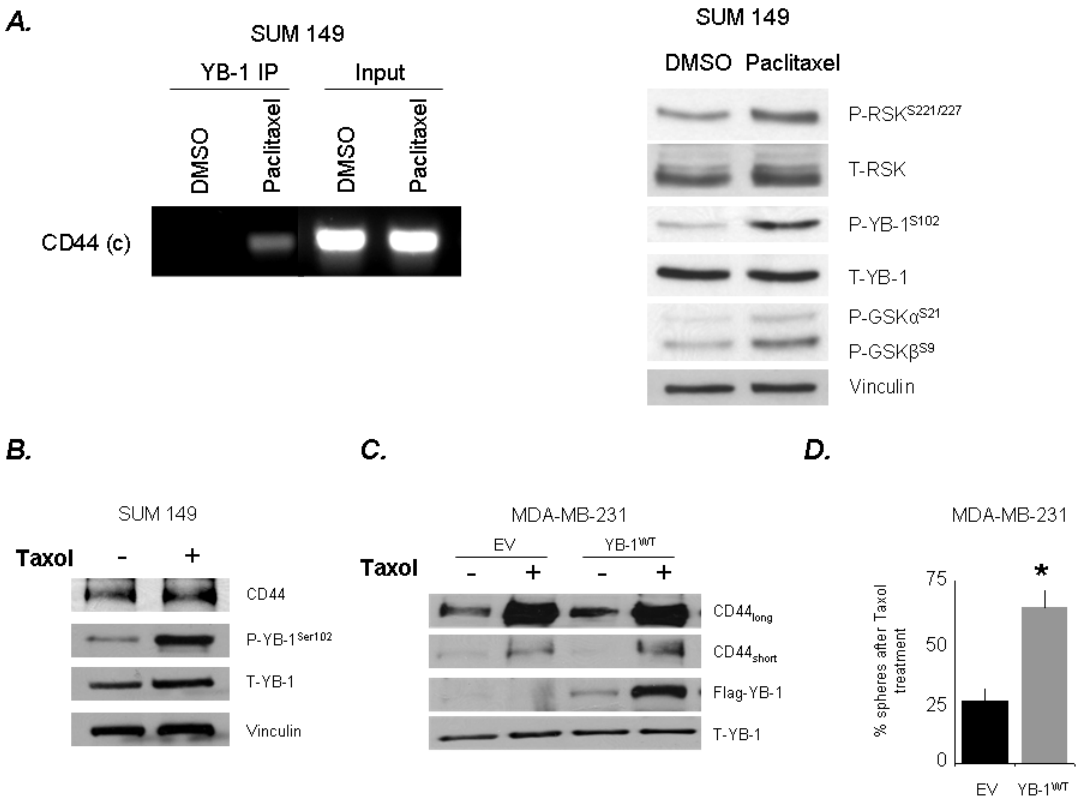


Figure 3.5. Paclitaxel treatment preferentially selects for breast cancer cells with elevated P-YB-1^{S102} expression. **A.** Treatment of SUM 149 cells with paclitaxel for 48 hours increased YB-1 binding to the CD44 promoter based on ChIP. The same cells were harvested for immunoblotting to detect P-RSK$^{S221/S227}$, P-YB-1^{S102}, total YB-1, and P-GSK3β^{S9}. Vinculin served as loading control. **B.** SUM 149 cells were treated with 10 nM paclitaxel for 72 hours to examine CD44 levels. **C.** In MDA-MB-231 cells, where empty vector (EV) and YB-1 expression vectors were stably overexpressed, cells were treated with 10 nM paclitaxel for 7 days and harvested for immunoblotting to assess CD44, exogenous Flag:YB-1, and endogenous total YB-1 levels. Endogenous total YB-1 served as loading control. **D.** MDA-MB-231 EV and YB-1-overexpressing cells were grown in mammosphere cultures in the presence of DMSO or 10 nM paclitaxel for 7 days. Bar graph represents the percent of spheres obtained in the paclitaxel treated cultures as compared to DMSO for each cell type.

3.6 SUPPLEMENTARY MATERIALS AND METHODS

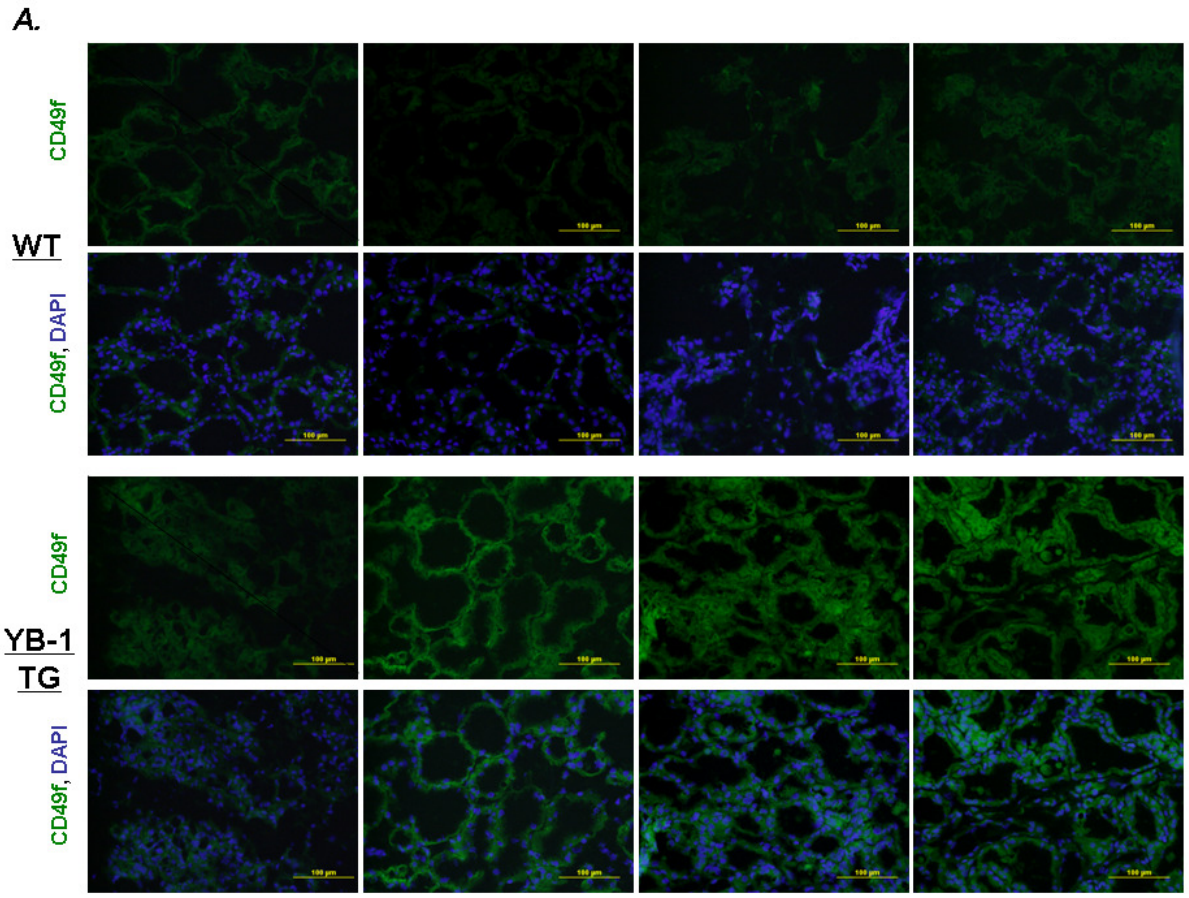
Immunofluorescence

A second CD49f antibody, unconjugated anti-CD49f (1:200, #3750, Cell Signaling Technology, same as immunoblotting), was used for validation of the YB-1 transgenic mouse mammary gland staining results.

Immunohistochemistry

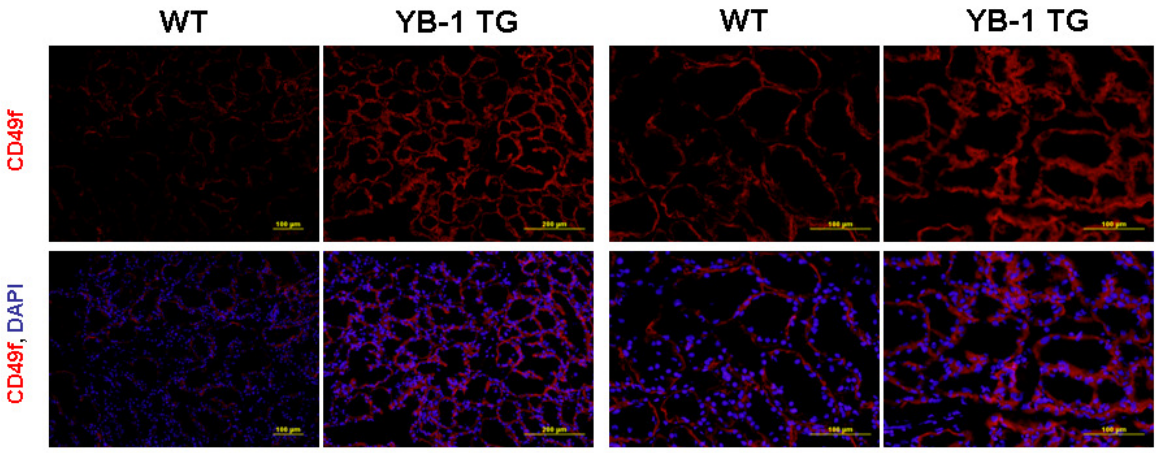
B6/129 WT and $CD44^{-/-}$ mice were previously described and supplied by Dr. Pauline Johnson[35]. For immunohistochemical analysis, kidney samples excised from wild type and mutant mice were fixed with 10% buffered formalin and embedded in paraffin. Sections (5 µm thick) were cut and mounted on Fisherbrand Superfrost Plus glass slides (Thermo Fisher Scientific, Waltham, MA). After routine deparaffinization and rehydration through gradient ethanol immersions, the slides were steam-heated for 10 minutes for antigen retrieval. Endogenous peroxidase activity was quenched using 3% (v/v) H_2O_2 followed by three 5 minute washes in PBS containing 0.2% (v/v) Triton X-100. The sections were blocked with 10% (v/v) normal goat serum in PBS. Specimens were incubated for 1 hour with purified rat anti-mouse CD44 antibody (1:100, Clone IM7, #550538, BD Pharmingen) diluted in PBS containing 0.3% (v/v) Triton X-100 and 0.1% (w/v) bovine serum albumin. Signal detection was performed using Dako LSAB 2 System-HRP (Dako, Glostrup, Denmark) according to manufacturer protocol. Specimens were counterstained with haematoxylin for 30 seconds and washed with tap water. The sections were then mounted with Glycergel Mounting Medium (Dako) and cover slips. Slides were visualized using an Olympus BX61 Fluorescent Microscope and photographed using an Olympus DP71 digital camera (Olympus, Japan).

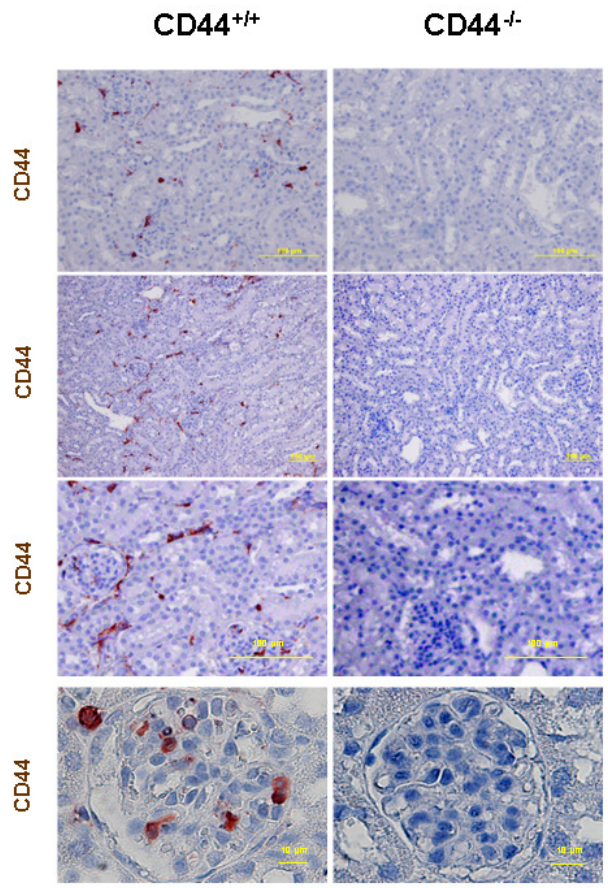
3.7 SUPPLEMENTARY FIGURES



Supplemental Figure 3.1. Targeted induction of YB-1 in the mammary gland leads to elevations in CD49f. A-B. BGL-YB-1/HA and the WT counterpart mammary glands were collected from 6 to 8-month old female mice post-lactation after two cycles of mating and nursing, frozen in O.C.T. compound, and sectioned. Slides were stained with primary CD49f-FITC (green) or unconjugated CD49f with secondary Alexa 546 fluor antibody (red) and DAPI. Staining for 4 sets of WT and transgenic mice are shown in Supplemental Figure 3.1A.

B.





Supplemental Figure 3.2. Validation of purified rat anti-mouse CD44 antibody. B6/129 WT and CD44$^{-/-}$ mouse kidneys were sectioned and stained with CD44 and visualized by immunohistochemistry techniques. CD44$^{-/-}$ knockout mice were characterized previously[35].

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CHAPTER FOUR : DISCUSSION AND FUTURE DIRECTIONS

4.1 CONTRIBUTIONS AND SIGNIFICANCE

Basal cells in the mammary gland directly interact and cooperate with components of the extracellular matrix through the expression of cell surface membrane receptors. The breast epithelium is surrounded by a very stromal-enriched environment, with which it mutually exchanges information and materials. It is conceivable that as membrane receptors relay extracellular signals into the cell, cell fates and decisions in normal, cancer, or transition tissues, undifferentiated or specialized, depend on the functional status of these cell surface proteins. The bulk of this work primarily investigates the contributions of transcription/translation factor YB-1 to the orchestration of these receptors on aptly located basal breast cancer cells. The first chapter of this thesis relates YB-1 to MET, which we report also for the first time as both a novel basal cancer cell interaction and a normal progenitor cell interaction. There we also narrate the importance of MET in basal breast cancer cell growth. Coupled to these findings are our other data showing that YB-1 putatively binds to the promoters of stem cell associated genes (including CD44 and CD49f) in breast cancer cells. This work importantly relates YB-1 directly to a primitive cell population in the normal mammary gland whereas it has only previously been detected in cancer tissues, implying a role in normal development that may contribution to perturbation in breast cancers. The third chapter of this thesis validates the idea that YB-1 maintains the expression of CD44 and CD49f, two known basal cell markers. This work is a description of a novel interaction showing the induction of the two genes important to normal breast development by an oncogene *in vitro* and *in vivo*, in both a human and mouse model. Further, we reveal for the first time

the heterogeneous expression of phosphorylated YB-1 and downstream gene *CD44* in a breast cancer cell line and their association to distinct phenotypes. Next, we correlated YB-1, CD44, and CD49f, expression with the ability for cells to grow in non-adherent mammosphere cultures long term *in vitro*, a property of tumour-initiating cells in patients. Finally, the roles of CD44 and CD49f in cell growth and CD44 in drug resistance in basal breast cancer cells were also investigated. Here we have presented evidence that a single transcription factor YB-1 can regulate a panel of proteins that all individually mediate a tumourigenic phenotype in breast cancer tissues that could potentially be related to primitive cell properties. Together, this thesis provides a detailed possible mechanism by which YB-1 is associated to poor prognosis and recurrence.

4.2 IMPLICATIONS AND FUTURE DIRECTIONS

YB-1 regulates partnering signal transducing receptors

We have shown that YB-1 individually induces *CD44*, *CD49f*, and *MET*. Previous reports by our group has also shown YB-1 inducing *EGFR* and *Her-2* receptors in breast cancer cells[1]. While we have demonstrated that breast cancer cells are dependent on each surface receptor for growth irrespective of the other receptors, there is substantial evidence that these receptors do not act alone. In our work, while it was not surprising that the expressions of CD49f (α6 integrin) and its β chain partner, CD104 (β4 integrin), were associated, we noticed that CD44 and CD49f expression were also at least partially dependent on one another. For example, when we incubated cells with CD44 siRNA, decline in CD49f expression was seen, and conversely, CD44 was down-regulated with CD49f siRNA treatment. Moreover, by overexpressing YB-1 in our cell line models, we

noticed that *CD44* and *CD49f* induction frequently occurred in the same cells, further suggesting an associated expression mechanism. Relatedly, a CD44 variant and α4β1 have been shown to interact in chronic leukemic B cells[2]. In the literature, CD44 has been shown to directly interact and cooperatively signal with EGFR in melanoma cells[3], as well as, Her-2 in ovarian tumour cells[4]. Additionally, MET forms heterodimers with CD44[5, 6], and EGFR[7] in breast cancer cell models. And finally, the α6β4 integrin (as well as the α3β1 and α2β1 integrins) have also been reported to cross-talk with EGFR[8], Her-2[9-12], and MET[13]. In addition to cooperative signaling, these receptors also compensate for the loss of their partners where it has been shown that inhibition of more than one of these surface proteins had an additive effect on managing cancer cell growth. For example, both CD44 and CD49f are reported to activate the focal adhesion kinase (FAK)[3, 14, 15] and possibly the integrin-linked kinase (ILK)[16], thus perhaps the cross-talk between the two receptors contributes to the regulation of this activation and consequently downstream pathways leading to an invasive phenotype. Interestingly, in the work presented in this thesis, upon overexpression of a constitutively activated mutant of YB-1 (YB-1$^{S102D)}$, where we've previously shown that other YB-1 downstream genes are all up-regulated, including *EGFR*[1], *Her-2*[1], *PIK3CA*[17], and *MET*[18], singly knocking down individual CD44 and CD49f was able to abrogate the enhanced cell growth significantly. While we do not know the expression status of the other YB-1 downstream genes, it is possible that the expressions of the said surface proteins are all co-dependent at least partially. It remains to be determined whether this is a cancer cell model or basal-like breast cancer subtype dependent effect, or whether targeting the other genes alone would also achieve the same level of growth inhibition. Taken together, we

hypothesize that YB-1-regulated important cancer growth receptors EGFR, Her-2, MET, CD44, and CD49f are coordinated to seamlessly form interchangeable signaling partners depending on the presentation of extracellular signals, allowing for the adapting nature of aggressive cancer cells. Therefore, cancer cell growth is likely manageable by using a combination of inhibitory agents against these receptors. The challenge of course lies in the heterogeneity within and between tumours and the fluidity of the unstable genome. Perhaps transcription factors like YB-1 which direct the expression of many such genes unique to cancers would be a good therapeutic target.

p53/CD44 interactions and basal phenotype

Robert Weinberg's group recently reported that p53 regulates CD44[19]. They demonstrated that CD44 mediates tumour growth and Doxorubicin-resistance in transformed breast cells, supporting our findings. Further, they show that p53 directly negatively regulates *CD44* by binding to a promoter region within 500 bp of the promoter region in our experiments. As p53 and YB-1 have been reported to directly interact to modulate gene expression[20], the presence of two opposing transcription factors at the *CD44* promoter is intriguing. In our studies, we have focused on triple-negative breast cancer cell lines, which primarily reside in the basal-like breast cancer subtype classification of breast cancer[21-23]. As *p53* mutation is frequent in this subtype[23, 24], it appears then that the absence of wild-type p53 allows for YB-1 to transactivate *CD44*, suggesting a mechanism by which CD44 is not only mediating a possible primitive phenotype but also associated with the basal-like subtype. In fact, the MDA-MB-231 and SUM 149 cell lines both harbour *p53* mutations[25, 26]. Another possibility is that YB-1

prevents the binding of wild-type p53 to its downstream gene promoters, as it has been reported that YB-1 reduced the transactivation of *p21* by p53[20], suggesting that YB-1 overexpression in cancers likely affects wild-type p53 function.

Other mechanisms of MET, CD44, and CD49f regulation

Besides our report of YB-1 and *MET*, the latter has been shown to be regulated by the ETS1 transcription factor[27], a regulator of basal-like breast cancer poor prognosis marker alphaB-crystallin[28], further associating MET to a basal phenotype. In addition to the report of p53 negatively regulating CD44, there is one other publication that shows the transcriptional induction of mouse *CD44* by IGF-1 and EGF[29]. As IGF-1 and EGF are activators of the Akt and MAPK pathway, both of which phosphorylate YB-1, this mechanism implicates YB-1's role in the up-regulation of mouse CD44 expression, complementing our findings in the mouse *in vivo* model. To our knowledge, we are the first to show the transcriptional activation of *CD44* by an oncogene and the regulation of *CD49f* by any transcription factor. While we focus on the transcriptional role of YB-1, it also translationally regulates many genes in cancer cells[30, 30, 31]. In fact, it has been reported that *CD44* mRNA is also bound more frequently by ribosomes with the overexpression of YB-1[31]. Thus, while CD44, CD49f , MET, expression appears to be sensitive to YB-1 levels in the basal breast cancer cell models in our work, it remains to be seen whether YB-1 has other co-factors with which it interacts or other mechanisms like epigenetics also have a role. Finally, because YB-1 activation by phosphorylation is essential to its function as a transcription factor, manipulating upstream kinases Akt or

RSK, could also be an important avenue to regulating the roles of YB-1 in promoting MET, CD44, and CD49f.

Cancer stem cells

As an important extension, we also wish to propose a working hypothesis that YB-1 putatively manages a protein expression profile that resembles that of a cell of a more primitive origin in normal development, and thus YB-1 also has a putative role in normal stem cells. Tumours have now been demonstrated to be heterogeneous populations that are driven by cells with properties traditionally characterizing stem cells. In addition to this, using mouse models of tumour initiation and progression, many reports also suggest that the clonal origin of cancers lie in a normal stem/progenitor cell or a cell that has accumulated enough mutations to acquire properties of those cells. Therefore, it has become more apparent in recent years that tumour-initiating cells though perhaps with an unstable genome could still bare resemblance to the genotypic and phenotypic composition of its precursor cell. Very recently, we reported that *YB-1* expression was detected in purified populations of normal human mammary progenitor cells[18], and it was positively correlated to that of *MET* in those cells. YB-1 expression in primitive progenitor cell subsets in the mammary gland has also been verified by another group[32]. Importantly, this suggests that our studies of YB-1's roles in cancer cells were also found in normal primitive subsets. Further, MET, CD44, CD49f, and EGFR, have all been directly detected on isolated stem cells. Taking into consideration our previous and current reports of YB-1 in normal and cancerous breast cells, we hypothesize that YB-1 governs an undifferentiated cell state, which includes cells that co-express EGFR, MET,

CD44 and CD49f, on their cell surface, with said receptors cooperatively signaling in the cell[3, 6, 33]. This cell population then putatively offers a mechanism for and translates into our findings of YB-1's correlation to poor prognosis and disease recurrence. It would be intriguing to determine whether YB-1 or phospho-YB-1^{S102} is expressed and assume a functional role in the $CD44^+/CD24^-/EpCAM^+$ cell populations of breast cancer patients. In addition, as an extension, detecting YB-1 protein in purified primary mammary stem/progenitor populations upon isolation and through the differentiation process would be very interesting.

YB-1, EMT, and the stem cell-associated phenotype

We evaluated of over 4,000 cases of primary invasive breast cancer and reported that elevated YB-1 expression in the primary tumour is consistently associated with high rates of recurrence[34]. However, the role of YB-1 in causing relapses and its mechanism of action remains unclear. One possibility is that the regulation of CD44 and CD49f by YB-1 is a feature of normal mammary epithelial cells that is retained by or reactivated in cells that acquire cancer-propagating activity. An alternative, but not mutually exclusive, possibility is that YB-1 expression and/or activation is the result of a preceding oncogenic event. This may then lead to a perturbation of its effects on CD44 and CD49f regulation as part of a broader program of epithelial-mesenchymal transition (EMT) characteristic of many advanced carcinomas. Snail, Twist and TGFβ are all examples of EMT-inducing factors[31, 35, 35]. Transfection of immortalized human mammary epithelial cells or transformed mammary cells with all of these resulted in the expression of higher levels of CD44, enhanced ability to form mammospheres in liquid suspension cultures,

colonies in soft agar, and tumours *in vivo*[36]. Here we provide new and direct evidence that these altered biological properties are regulated by YB-1 expression through its ability to control expression of requisite levels of CD44 and CD49f. The fact that YB-1 has been reported to be regulated by Twist[37, 38], implicates YB-1 activation as the mechanism by which CD44 is up-regulated in Twist-induced EMT. Finally, as HGF is capable of inducing EMT[39], YB-1-regulated MET may also be an important player in this phenomenon of breast carcinoma progression.

Using cell line models of tumour cell heterogeneity

In primary tumours, there exists a subpopulation of cells that have the capacity for self-renewal over time to maintain the tumour cell mass. In breast tumour, these have been identified to exhibit a $CD44^{+}/CD24^{-}/EpCAM^{+}$ surface profile[40]. These cells have been demonstrated to be able to form non-adherent spheres *in vitro*[41], to be resistant to current chemotherapies[41], to survive radiation therapy[42], and to form tumours in immuno-compromised mice in low numbers at injection[40]. While cell lines are traditionally clonal in origin and are treated as a bulk homogeneous population, there is accumulating evidence that cell lines are propagated *in vitro* as a heterogeneous population with a subpopulation that have distinct stem-like properties[43, 44]. In our work, we demonstrate that in the SUM 149 cells, there is a $CD44^{High}$ population that are resistant to chemotherapies, can form more and larger mammospheres, soft agar colonies, and Matrigel growths *in vitro* than the $CD44^{Low}$ population. We have also shown that phospho-YB-1^{S102} is higher in that $CD44^{High}$ population, suggesting there are cell subsets with distinct signaling features within a cell line, like a tumour. Paclitaxel has been

shown to cause the nuclear localization of YB-1[45], and thus allowing it to up-regulate P-glycoprotein[45], MDR-1[46], and CD44. Perhaps YB-1 mediates its drug resistance phenotype through CD44, as its expression has been linked to chemo-resistance and specifically paclitaxel[14, 47]. Cell lines are artificial in nature as they have often been cultured in a simulated environment for much longer than the lifespan of a tumour. They have accumulated many more mutations than in a human cancer, and their likeliness to tumour heterogeneity still needs further elucidation. Still, they offer an alternative model to the scarcity and limited *in vitro* longevity of primary tumour samples.

Animal model implications

As the cancer research field faces the emergence and acceptance of the cancer stem cell model, it will be important to see if patients will benefit from the development of novel therapeutics for new targets in these TICs. As we demonstrated that YB-1 induced in the murine mammary gland was also able to overexpress downstream CD44 and CD49f, this proposes that the relationship between YB-1/CD44/CD49f could be conserved between human and mouse. As CD49f have been used as markers for human and mouse mammary stem cells[48-50], the TICs in both models may also retain similar surface marker profiles to their normal precursors. In the future, the YB-1 transgenic mice could be used to assess potential agents that have the ability to suppress the $CD44^{+}/CD49f^{+}$ TICs.

Global applications

Though we have focused on breast cancer cells of the basal-like subtype, which express higher levels of CD44 and CD49f, YB-1 overexpression in breast cancer is not subtype-

specific. It also regulates *EGFR*, *Her-2*, *MET*, and *PIK3CA*, all key players of breast cancer progression which have been implicated in stem cell and cancer stem cell populations[33, 51-55]. Furthermore, YB-1, a downstream gene product of c-Myc and p73[56], has been associated with poor prognosis in cancers of the prostate[57], colon[58], blood[59], lung[60], skin[61], ovarian[62, 63], and brain[64] as well as drug resistance globally[37, 45, 46, 59, 61, 65-72]. Thus its function in cancers is not tissue specific, implicating its putative mechanistic role in mediating a poorly differentiated cell population or tumourigenic phenotype could be globally validated.

Clinical and therapeutic implications

As YB-1 is overexpressed in 40% of all breast cancer patients and is associated to poor prognosis and disease recurrence[34], there is an obvious need to better understand the mechanisms of this transcription/translation factor. Here we show a mechanism by which YB-1 regulates cell membrane receptors that are all individually and synergistically important for the growth of carcinoma cells. Importantly, we offer a new concept whereby with sufficient validation and further studies, YB-1 could be a target in cancer stem cells, which are traditionally resistant to chemotherapies. We had found a dramatic difference between the YB-1 transcript levels in cancer cells and normal progenitor cells, offering an opportunistic therapeutic window.

4.3 LIMITATIONS OF STUDIES

Improvements that could be made to the described studies include:

- Verifying YB-1's regulatory relationships in primary human breast cancer cell samples. This may require transient immortalization of these cells using lentiviral

delivery of the YB-1 expression plasmids and shYB-1 expression plasmids for gene overexpression and knockdown experiments respectively.

- Constructing *CD44* and *CD49f* promoter reporter plasmids to verify the transactivating role of YB-1 using luciferase assays.

- Repeating our experiments in the human breast cancer models with mouse mammary cancer models to determine if the relationship is conserved across species. This could include further investigating the mammary cells from the YB-1 transgenic mice, constructing a conditional knockout YB-1 transgenic mouse, and/or the use of mouse mammary cancer cell lines derived from various transgenic mice.

- Characterizing the *in vivo* YB-1 transgenic mouse mammary gland cells further by dissociating the YB-1 transgenic mammary glands to analyze activated signaling pathways. Further injecting at limiting dilutions the YB-1 transgenic mammary epithelial cells along with the wild-type counterparts into cleared mammary fat pads of immuno-compromised mice to assess tumourigenic potential of individual tumour-initiating cell populations.

- Passaging the mammospheres after YB-1, CD44, and CD49f knockdown may have addressed the stem cell-like capacities of the cancer cells in question. Although the gene knockdown of the surviving cells would be questionable, lentiviral delivery of shRNA expression plasmids could circumvent that problem.

- Tracking YB-1 translocation into the nucleus of SUM 149 cells with paclitaxel treatment could be accomplished with a fluorescently-tagged YB-1 protein and real-time fluorescent microscope monitoring.

- Determining an enrichment of chemotherapy-resistant tumourigenic population by injecting paclitaxel-resistant SUM 149 cells into mice along side DMSO-resistant SUM 149 cells

- Validating the effects of another chemotherapy agent on SUM 149 cells other than paclitaxel.

4.4 CONCLUDING REMARKS

We have accumulated a body of evidence to suggest that *YB-1* is an oncogene that could be mediating a tumourigenic phenotype to breast cancers by overexpressing genes that are also shared by normal primitive cell populations for propagation and survival. We nominate YB-1 as a good target for cancer therapy.

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